International Journal of
Molecular Sciences

Molecular Research of Endometrial Pathophysiology

Edited by
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Printed Edition of the Special Issue Published in
International Journal of Molecular Sciences

www.mdpi.com/journal/ijms

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This is a reprint of articles from the Special Issue published online in the open access journal *International Journal of Molecular Sciences* (ISSN 1422-0067) from 2018 to 2019 (available at: https://www.mdpi.com/journal/ijms/special_issues/endometr_pathophysiol)

For citation purposes, cite each article independently as indicated on the article page online and as indicated below:

LastName, A.A.; LastName, B.B.; LastName, C.C. Article Title. *Journal Name* **Year**, *Article Number*, Page Range.

ISBN 978-3-03921-495-2 (Pbk)
ISBN 978-3-03921-496-9 (PDF)

Cover image courtesy of Andrea Romano.

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About the Special Issue Editors

Paola Viganò obtained her biological degree at the University of Milan and a post-graduate residency in Experimental Endocrinology at the School of Pharmacy of the same University. She obtained her Ph.D. in Prenatal Medicine at the University of Siena in 2003 and conducted a postdoctoral fellowship at the Department of Reproductive Medicine of the San Raffaele Scientific Institute in Milan. Currently, Dr. Viganò is the Coordinator of the Assisted Reproductive Technology Laboratory at the San Raffaele Scientific Institute and Group Leader of the Reproductive Sciences Laboratory, Division of Genetics and Cell Biology at the same institute. She is President of the Italian Society of Human Reproduction (SIRU) and in the Board of Directors of the World Endometriosis Society.

She has published over 200 peer-reviewed papers in the area of human reproduction, with a more specific scientific production in the field of endometrium and endometriosis basic research.

Andrea Romano graduated in Biology (1995) at the University of Bologna (Italy) and subsequently moved to the Netherlands, University of Wageningen, where he obtained his Ph.D. degree in 2002. He continued his research activity as a postdoc at the Maastricht University (2002–2007), where he is currently Group Leader and Associate Professor (Faculty of Health, Medicine and Life Sciences). Andrea Romano's research focuses on steroid hormone signaling and local steroid metabolism in the context of endometrial pathophysiology. Andrea Romano served as associate editor for *Human Reproduction* (Oxford Press, 2013–2016). He is currently chair-elect of the European Network for Individualised Treatment of Endometrial Cancer (ENITEC, under the umbrella of the European Society Gynaecologic Oncology), coordinator of the Special Interest Group for Endometriosis & Endometrial Disorders (under the umbrella of the European Society Human Reproduction & Embryology), and ambassador for the World Endometriosis Society.

Preface to "Molecular Research of Endometrial Pathophysiology"

The human endometrium is a highly dynamic organ undergoing cycles of shedding and regeneration, a process that is unique to humans and higher-order primates. Throughout each cycle, seven phenotypic differentiation states are achieved by endometrial cells and other cells residing in the endometrium: proliferation, decidualization, implantation, migration, breakdown, regeneration, and angiogenesis. This ultimately creates the correct environment for embryo attachment, implantation, nidation, and growth.

Such complexity in functions is only possible because of highly coordinated interactions between a plethora of biological processes: the delicate balance and interaction between distinct cell types, stroma, epithelial, stem cells, endothelial cells, resident macrophages, and other immune cells; and concerted intracellular signaling pathways, response to steroid hormones and other signaling molecules.

Recent technological progresses in molecular and biochemical analyses, like deep sequencing technologies, mass spectrometry to profile steroids and metabolites, and the availability of more sophisticated in vivo models that—better than past models—mimic human diseases, have allowed important improvements in many fields of medicine, including in endometrial pathophysiology. Here, we witnessed an important expansion of our understanding of the molecular and intracellular pathways regulating cell functions, stem-cell niche and final differentiation, cell–cell interaction, gene regulation via noncoding RNAs, local steroid metabolism, hormone and vitamin D action.

The present book is a collection of 14 reviews and six original articles that aim at portraying the state of the art of our knowledge, technologies, and models used to investigate the pathophysiology of the endometrium. This book is based on articles published in the Special Issue entitled 'Molecular Research of Endometrial Pathophysiology' that we had the pleasure and the privilege to edit for the *International Journal of Molecular Sciences*. For the present book, we coherently grouped the articles into three sections: The first section is dedicated to endometrial physiology, where six review articles focus on the relevant signaling cascades operating during the endometrial cycles, the endometrial steroid metabolism and intracrinology, the new insights in highly specialized cell–cell interactions, the role of stem cells, Vitamin D, and of noncoding RNA molecules; A second part describes, in two reviews and three original articles, the novel insights in the pathogenesis of endometriosis; and the third section consists of six reviews and three original articles that describe new developments in endometrial cancer biology, diagnosis, treatment, and in vivo research tools.

We hope this book can be a guide and inspiration, or source for further readings for all those professionals working in the field of endometrial pathophysiology, for those wishing to get an introduction to it, or simply for people interested in the scientific aspects of the endometrium.

We would like to thank all experts and authors who contributed with reviews and original articles to compile this extraordinary collection of scientific papers, the very helpful staff from the editorial office, and MDPI, who made all this possible.

Paola Viganò, Andrea Romano
Special Issue Editors

International Journal of
Molecular Sciences

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Review

Inside the Endometrial Cell Signaling Subway: Mind the Gap(s)

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Received: 4 July 2018; Accepted: 4 August 2018; Published: 21 August 2018

Abstract: Endometrial cells perceive and respond to their microenvironment forming the basis of endometrial homeostasis. Errors in endometrial cell signaling are responsible for a wide spectrum of endometrial pathologies ranging from infertility to cancer. Intensive research over the years has been decoding the sophisticated molecular means by which endometrial cells communicate to each other and with the embryo. The objective of this review is to provide the scientific community with the first overview of key endometrial cell signaling pathways operating throughout the menstrual cycle. On this basis, a comprehensive and critical assessment of the literature was performed to provide the tools for the authorship of this narrative review summarizing the pivotal components and signaling cascades operating during seven endometrial cell fate "routes": proliferation, decidualization, implantation, migration, breakdown, regeneration, and angiogenesis. Albeit schematically presented as separate transit routes in a subway network and narrated in a distinct fashion, the majority of the time these routes overlap or occur simultaneously within endometrial cells. This review facilitates identification of novel trajectories of research in endometrial cellular communication and signaling. The meticulous study of endometrial signaling pathways potentiates both the discovery of novel therapeutic targets to tackle disease and vanguard fertility approaches.

Keywords: endometrial cell; pathway; proliferation; decidualization; migration; angiogenesis; regeneration; breakdown; implantation

1. Entrance

The compound adjective "highly dynamic" is a cliché when it comes to portraying the endometrium. Nonetheless, it perfectly recapitulates a tissue that quite uniquely executes a remarkable loop of proliferation, differentiation, shedding, and regeneration 400 times in its lifetime. A fine-tuned interplay between ovarian hormones and numerous cell types, including stem and immune cells, governs the orchestration of endometrial cell functions [1]. The tissue itself is stratified into two layers: the functional, a superficial transient layer adjacent to the uterine cavity, and the basal, a deeper permanent layer adjacent to the myometrium. The functional layer consists of a single strand of luminal epithelium, the stroma and the superficial glands (glandular epithelium) whereas the terminal part of the glands is embedded in the basal layer. The thickness of the tissue is determined by its functional layer, which changes throughout the menstrual cycle according to hormonal influences [2]. The phases of the menstrual cycle are defined on the basis of phenomena occurring during the ovarian cycle as the follicular phase (day 0 to day 13), the ovulation (day 14) and the luteal phase (day 15 to day 28). Considering the endometrial cycle phenomena this time round, these phases would rather be the menses (day 0 to day 5), the proliferative phase (day 6 to day 13) and the secretory phase (day 15 to day 28). At the end of menstruation, and until the end of follicular phase (day 6–day

13 of cycle), the rapid construction of the functional layer is governed by **proliferation** of endometrial cells, which grow under estrogenic influence [3]. During this proliferative phase, when estrogen levels are high, the tissue is extensively repaired from the damage caused by menses, the innate immunity is suppressed and growth factor molecules lead cell proliferation. Following ovulation and for the duration of the secretory phase (day 14 to day 28), pituitary hormones and ovarian progesterone (P4) take the estrogen-primed functional layer through extensive differentiation towards **decidualization** [4]. The decidualized endometrium is ready to provide the optimum environment for the **implantation** (day 20 to day 25) of the blastocyst and early growth of the embryo [5]. During this period, a number of signaling cascades stemming from both the blastocyst and the endometrium operate to facilitate apposition, attachment and invasion of the blastocyst but also **migration** of the endometrial stromal cells that move towards the site of implantation to counterbalance the blastocyst-induced tissue remodeling [6]. In the absence of implantation, the corpus luteum absorbs and ceases P4 release. In response to P4 withdrawal, the arteries supplying blood to the functional layer constrict, so that cells in that layer become ischaemic and die. The functional layer undergoes **breakdown** and completely sheds to signify menstruation (day 28–day 5), which is characterized by activation of tissue damage and destruction pathways, vasoconstriction, ischemia, and the high abundance of free radicals and immune cells [7,8]. At the final days of menstruation, simultaneous breakdown and repair will cooperate to allow the endometrium to **regenerate** a new functional layer. The process implicates a number of repair mechanisms, including cell transformation and migration to repopulate the endometrial epithelium, early form of vascular remodeling and progenitor stem cells that reside at the basalis layer, the fountain of youth for regeneration [9,10]. The rise in estradiol (E2) enrolls the surface-regenerated functionalis into continual growth during the phase of proliferation, which is facilitated by intense **angiogenesis** aiming to construct a new vascular network. The newly build vascular network further matures under the influence of P4 during the secretory phase. The aforementioned seven functional "routes" of endometrial cell signaling are depicted in a transit map (Figure 1) with a primary purpose to help "passengers" familiar with endometrial research, or newcomers to the field, to decide on the direction in their research, allow overview of the impressive network of activities occurring inside a unique tissue and, plausibly, identify gaps pending narrowing. Below, each route is elaborated to narrate the key mediators participating endometrial cell signaling.

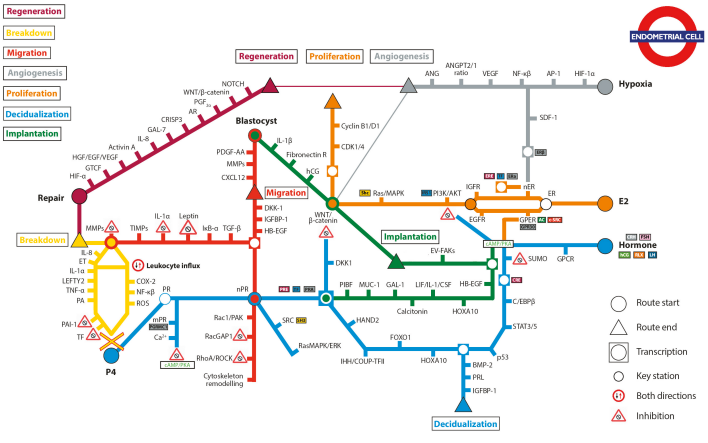

Figure 1. Endometrial cell signaling network illustrated as a subway map showing the seven routes operated by different molecules, narrated in the review. TF in blue boxes denotes transcription factors. All abbreviations are expanded in the main text. The X mark in the red circle indicates progesterone withdrawal.

2. Proliferation Route: Building the Functionalis

The increasing mitotic activity seen throughout the endometrial surface/glandular epithelium and stroma, governed by E2, intends to thicken the functional layer in preparation for implantation. The concentration of E2 ranges between 40 pg/mL (end of menses) and 250 pg/mL (before ovulation) [11,12]. A minimum of five days is enough to build a thick layer, however, the proliferative phase is not characterized by a uniform period of endometrial growth. The general consensus is that estrogens exert their effect by modifying gene expression through activation of their nuclear receptors or contributing to growth cascades via nongenomic pathways, which can be receptor-dependent or -independent. Proliferative pathways are active in all cellular types and compartments. Elegant human xenograph experiments in mice have introduced the concept of "interactive proliferation" between the stroma and the epithelium [13]. According to this model, the proliferative response originates in the stroma and feedbacks growth pathways via paracrine signaling in the endometrial epithelium. The predominant estrogen receptor (ER) involved in the transduction of proliferative signals is estrogen receptor alpha (ERα) [14], which is expressed in all endometrial cell types during the proliferative phase and in much higher abundance compared to estrogen receptor beta (ERβ) [15]. Expression of ERβ is higher in the secretory phase of the cycle as a consequence of ERα inhibition by P4, a critical step in itself for the establishment of implantation [15,16]. E2 may also bind to transmembrane G protein-coupled estrogen receptor 1 (GPER), which mediates rapid signaling and is reviewed elsewhere [17]. The diversion of the proliferation route at the ER point, illustrated in Figure 1 at the start of the orange line, is a first critical step upstream all proliferative cascades.

E2-dependent transcription leading up to the synthesis of mitogens is mostly active in the stroma, which communicates in a paracrine manner the response to the epithelial cells [18,19]. Indeed, conditional mutagenesis studies established that stromal-derived ERα is fundamental for directing epithelial cell proliferation, while epithelial ERα is expendable [20]. In a genomic ligand dependent manner, E2 binds nuclear ER (nER) in the cytoplasm and following dimerization, allows for its translocation to the nucleus [21]. The dimer acts as a transcription factor by binding directly estrogen responsive element (ERE) on estrogen responsive genes. Alternatively, E2-nER dimers regulate gene expression independent of ERE but through tethering different transcription factors on mitogen-promoting genes [22]. The result of E2-nER transcription is upregulation of genes involved in the G1 to S progression of cell cycle-Cyclin D1, Cyclin D3, CDK1 and CDK3 are amongst those genes [3,23]. Moreover, E2-nER transcription induces insulin-like growth factor 1 (IGF-1) and mitogen-activated protein kinase (MAPK) pathway related genes [24–26]. In a positive feedback, IGF-1 and MAPK cascades are involved in the nongenomic ER-dependent and -independent regulation of E2-driven proliferation [27,28]. In this context, the most well characterized nongenomic model of ER action is mediated through the activation of IGF-1 receptor (IGF-1R). According to the model, cytosolic E2-ERα complexes bind the transmembrane part of IGFR resulting in a bidirectional phosphorylation: IGF-1R phosphorylates ER, which phosphorylates IGF-1R to activate two downstream nongenomic mitogenic signaling pathways: Ras/MAPK and PI3K/Akt [23,29,30]. The first involves the phosphorylation of the adaptor protein Src collagen homologue (Shc) followed by the activation of Ras [31]. The Ras/MAPK pathway contains an elaborate kinase cascade that ultimately enhances the activity of the available transcription factors. The pathway can also induce phosphorylation of nER, which upon dimerization and translocation to the nucleus will initiate transcription of MAPK related genes, notably in an E2-independent manner [32]. ER, total and activated ERK1/2 kinase levels are seemingly comparable in stroma and epithelium of the proliferative endometrium, suggesting pathway activity in both compartments [28]. The PI3K/Akt pathway, on the other hand, results from phosphorylation of the endocytic regulator insulin receptor substrate 1 (IRS-1). Activated IRS-1 interacts with the phosphoinositide 3-kinase (PI3K), to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3). Once generated, the phospholipid PIP3 recruits certain kinases to the plasma membrane including the protein kinase B (PKB)/Akt family of kinases [33]. Activation of Akt

in the endometrium phosphorylates a number of downstream targets, which play key roles in cell survival in normal but also in pathological conditions in the endometrium [34,35].

The aforementioned alternative for the E2-initiated proliferation route is to bind the membrane-associated ER to set off nongenomic cascades. The GPER, formerly known as G protein receptor 30 (GPR30), mediates rapid responses in several types including endometrial cells [36,37]. It is located on both the plasma and the endoplasmic reticulum membrane and is in high abundance as expected during the proliferative phase [38]. It is assumed that GPER functions from its location in the plasma membrane. Ligand-activated GPER can trigger two different pathways. The first involves the stimulation of the enzyme adenylate cyclase (AC) to produce cyclic adenosine monophosphate (cAMP), which in turns activates the protein kinase A (PKA) pathway ultimately inducing the recruitment of transcription factors to the promoter of genes with a CRE (cyclic-AMP responsive element) [17,39]. The PKA pathway plays an important role in balancing the proliferative activity of endometrial cells. Specifically, the abundance of cAMP defines whether the transcription will be in favor of proliferation, thus inducing cyclin D/E, or not, in which case the expression of p27Kip1 is instead induced [23]. The endometrial tube map (Figure 1) allows for the observation of the pleiotropic properties of the cAMP/PKA pathway. Indeed, the pathway resembles an interchange subway station serving additionally the decidualization and the implantation routes. One of the important functions of the pathway is to successfully inhibit Akt signaling during decidualization [40]. Indeed, recent studies on infertile women have reported that impaired Akt signaling during proliferation might contribute to endometrial ineptitude to promote relevant cascades en route to decidualization [41].

Besides cAMP/PKA, GPER activates the epidermal growth factor (EGF) receptor (EGFR) to induce a consequent downstream signaling of MAPKs and PI3K. The cascade initiates when the ligand activated-GPER recruits tyrosine-protein kinase c-Src that triggers the release of EGF from the membrane. The latter results in transactivation of EGFR and activation of MAPK and PI3K pathways, as described for the nER-IGFR pathway with induction of proliferation-associated gene expression [42,43].

Another critical operator of endometrial proliferation and growth is the canonical WNT/β-catenin pathway. The pathway functions in endometrial cells in a delicate order, whereby early response to E2 through signaling pathways described above provides the transcriptomic supply for molecules that contribute to the regulation of WNT/β-catenin-mediated late endometrial growth [44]. The cascade involves a destruction complex, which is a complex of proteins consisting of AXIN1-2, β-catenin, adenomatosis polyposis coli (APC), casein kinase (CK1) and glycogen synthase kinase 3 beta (GSK3β) [43]. When no WNT ligands bind the receptor frizzled, the complex assembles and both CK1 and GSK3β phosphorylate β-catenin, which undergoes ubiquitination and proteasomal degradation. However, upon binding of WNT ligands, the activation of disheveled blocks the destruction of the complex and β-catenin accumulates in the cytoplasm and can translocate to the nucleus to interact with members of the TCF/LEF transcription factor family, to regulate the expression of genes associated with proliferation and survival such as cyclin D1 and c-MYC [45,46]. It is believed that the WNT/β-catenin signaling operates with greater intensity in the stroma compared to epithelium, which corresponds to higher abundance of nuclear β-catenin in that cellular compartment [47]. Early proliferative ERα signaling induces the expression of the receptor Frizzled, numerous ligands including WNT4/WNT5a/WNT7a and β-catenin, hence, promotes nuclear localization of β-catenin in epithelium and stroma [48–52]. On the contrary, the pathway inhibitor Dickkopf-related protein 1 (DKK1) is downregulated by ER signaling in the endometrium [53]. ER-mediated PI3K/Akt and Ras/MAPK pathways additionally positively regulate the WNT/β-catenin pathway via inhibition of GSK-3β, which enhances the intracellular stabilization of β-catenin [54]. There is some evidence that the canonical WNT/β-catenin pathway in the mouse endometrium can be activated by E2 in an ER-independent manner. Specifically, E2 can induce the expression of WNT/β-catenin targets in endometrial epithelial cells lacking ER [55]. The authors confirmed this observation in vivo in ERα-lacking mice [56]. Although understanding the mechanism of the ER-independent activation

of WNT/β-catenin could help scrutinize endometrial cancer, where the expression of the pathway components is markedly impaired, this area remains unexplored in humans. The subway analogy allows appreciating the importance of WNT/β-catenin system in decidualization, implantation and angiogenesis with some operations in the route towards regeneration. The research into WNT/β-catenin serving migration is also emerging.

A decade ago, the field was introduced to the microRNAs (miRNAs), small noncoding RNAs with posttranscriptional regulation properties. These RNA binding molecules can either degrade mRNAs or suppress their translation. Since their discovery in the physiological and pathological endometrium, miRNAs have been mostly investigated in luteal phase [57,58]. Recently, the first global characterization of miRNAs in the proliferative endometrium emerged to back up individual studies suggesting miRNAs as important players in the fine-tuning of endometrial growth [59]. How different miRNAs regulate components, targets and even transcriptional outcomes of ER-driven signaling in the proliferative endometrium is yet to be fully understood and consolidated but is expected to shape the future of research in the field.

A detailed transcriptomic regulation emanating from ER-mediated E2 operation in the proliferative human endometrium has been systematically reviewed in human and mouse [25]. Better characterization of the operative pathways that induce this transcriptomic signature will generate new targets to circumvent aberrant proliferation that will most definitely lead to failed differentiation [60] and to numerous pathologies including endometrial hyperplasia, cancer, endometriosis and infertility [61,62].

At the end of the proliferative phase after ovulation, the locally rising P4 shifts the endometrium towards a state of endometrial receptivity, a tightly regulated phase in which the endometrium is receptive to embryo implantation.

3. Decidualization Route: Priming the Endometrium for Implantation

Decidualization is the process by which P4 induces endometrial stromal cell differentiation into decidual cells to form a new tissue termed decidua. The decidua provides a source of growth factors and cytokines that regulate embryo invasion, support embryo development, modulate immune responses, and support angiogenesis [63]. Priming of the endometrium to become receptive is initiated by E2 but requires the intricately coordinated signaling of E2 and P4 between the luminal and glandular epithelia and the stroma [64]. Each endometrial compartment has a distinct agenda. Stromal cells follow simultaneous proliferation and differentiation. In contrast, epithelial cells cease to proliferate and only differentiate. The stromal cells will stop proliferation and only undergo differentiation into decidual cells at the end of the receptive phase, when already introduced to a blastocyst. From mid-secretory phase, differentiation of stromal cells predominates over proliferation. Usually cellular differentiation follows cell cycle arrest and inhibition of proliferation, however during the secretory phase these functions are temporal. The mechanisms controlling the interconnection of P4 and E2 in the regulation of cell cycle in endometrial cells are surprisingly poorly comprehended, highlighting a major gap in endometrial physiology.

The molecular protagonists in the decidualization route are P4 and cAMP. Because cAMP is involved in routes other than that of decidualization, Figure 1 does not exemplify its cardinal role. A separate branch in the route stemming from cAMP and arriving to the endpoint of decidualization aims, therefore, to signify the independent action of cAMP. Indeed, a spike of LH induces cAMP to elicit an initial and rapid response in endometrial cells while P4 action is independent, slower but persistent. In vitro, the response of endometrial cells to P4 is downstream cAMP activation but this is not believed to be the case in vivo [65]. Nevertheless, it is well established that P4 and cAMP act synergistically to drive endometrial cells through successful decidualization [66]. However, the hierarchy in their responses is still not clear.

At the end of ovulation the endometrium is exposed to high levels of hormones and other endocrine factors such as follicle-stimulating hormone (FSH), relaxin (RLX), corticotropin-releasing

hormone (CRH), LH, cyclooxygenase-2 (COX-2) and, in case of pregnancy, human chorionic gonadotropin (hCG) [67,68]. These bind to their respective G protein-coupled receptors (GPCRs) on endometrial stromal cell membrane and stimulate the production of cAMP [69]. The latter will activate the PKA pathway, resulting in phosphorylation of cAMP-response element modulator (CREB), binding to the cAMP-response element (CRE) and initiation of decidualization-specific gene transcription [70]. The genes induced through this pathway include a number of transcription factors capable of interacting with the progesterone receptor (PR) such as forkhead box protein O1 (FOXO1), signal transducer and activator of transcription 5 (STAT5), STAT3 and CCAAT-enhancer-binding protein β (C/EBP β) [67,71–73]. In this manner the fast acting cAMP sensitizes stromal cells to the slow-acting P4, which will act through PR in a genomic or nongenomic manner to inhibit epithelial cell proliferation and stimulate differentiation of stromal cells. cAMP is additionally contributing to the cell cycle regulation by inducing the transcription of p53, a tumor suppressor protein, arresting endometrial cells at G2/M checkpoint [74]. Transrepression of p53 from C/EBP β has been observed in endometrial stromal cells with C/EBP β being considered a stabilizer of G2/M inducing factors such as cyclin B2 and CDK1 [75]. Conversely, the other cAMP-induced factor, FOXO1, suppresses cyclin B1/2 and CDK1 [76]. Considering that the cAMP/PKA pathway is an inhibitor of the PI3K/Akt proliferative pathway, the complexity of cell cycle regulation during decidualization is highlighted [40]. An important role of cAMP in sensitizing endometrial cells to P4 is to prevent sumoylation of the PR by altering the expression of numerous small ubiquitin-like modifier (SUMO) enzymes [77]. These downstream targets of cAMP are part of the route branch leading up to decidualization (Figure 1). Recently this branch was reinforced by an interesting study allocating roles for long noncoding RNAs (lncRNAs) in the endometrium [78]. In that work, human decidualization was highly dependent on the expression of the lncRNA LINC473, which was under the positive control of the cAMP/PKA pathway. The downstream targets of LINC473 have yet to be established before its definite roles in decidualization can be confirmed. In light of the recent aspirations to characterize the global lncRNA profile in the endometrium in relation to physiology and pathology, it is envisaged that the gap in our understanding of the RNA binding molecules actions will be eventually filled [79–81].

Looking at the tube map illustration, the role of P4 signaling stands strong in the journey towards decidualization. P4, acting in a similar molecular fashion to E2, exerts transcription-dependent and -independent effects in the endometrium. The genomic actions are mediated via the two nuclear progesterone receptors (nPR) subtypes PRA and PRB, upon which P4 binding translocate to the nucleus and associate with progesterone response elements (PRE) in the promoter region of target genes or with other transcription factors and coactivators. PR expression is stimulated by ERα-mediated transcription in endometrial cells and, consequently, E2 is required for P4 responsiveness throughout the luteal phase [82]. Conversely, ERα expression is inhibited by P4 via nPRs [83]. This functional feedback interaction between the two hormonal systems is important for balancing their often-opposing actions. Epithelial cells mostly express PRB, suggesting that PRB is perhaps involved in the control of glandular secretion, whereas PRA is the predominant type in stromal cells and the lack of its expression results in impaired decidualization reflecting the need for prolonged stromal cell PRA-mediated action of P4 in the establishment of pregnancy [84,85]. Different signaling routes have been established for the two receptors. For example, PRB activates rapid cytoplasmic signaling events via interaction with the Src-homology 3 (SH3) domain of the Src tyrosine kinase (SRC) at the plasma membrane, which triggers the Ras/Raf1/MAPK pathway critical for decidualization [86,87]. PRA, on the other hand, is a known transcriptional inducer of differentiation and decidualization. PRA-signaling induces the expression of the basic helix-loop-helix transcription factor (HAND2) in the stroma to suppress the production of fibroblast growth factors (FGFs) and, consequently, their mitogenic action on epithelial cells [88]. In the epithelium, P4 induces the Indian hedgehog (IHH) to activate COUP transcription factor 2 (COUP-TFII) in the stroma [89,90]. Rodent studies showed that COUP-TFII suppresses E2-mediated effects in the epithelium via inhibition of both SRC-1 and ERα phosphorylation [91]. COUP-TFII

activates the bone morphogenetic protein 2 (BMP2), which will drive decidualization via activation of the key molecules WNT4 and COX-2.

PR-mediated transcription has profound effects on the WNT/β-catenin pathway [48]. Although the activation of the pathway is critical for implantation, as described later in this review, P4 dramatically upregulates its inhibitor DKK1 in the differentiating stroma and evidently induces blockade of WNT/β-catenin [92]. Albeit repression of the pathway is seemingly essential for proper decidualization, opposing reports add a layer of complexity. For example, WNT4, a potent ligand of the pathway, is increased in the stroma during decidualization in response to the nPR-mediated upregulation of BMP-2 and FOXO1 [93,94]. It is fair to speculate that due to the complexity of the endometrial signaling agenda during decidualization, the WNT/β catenin operates distinctly to meet the needs of each one of the cell functions: proliferation, differentiation, migration and decidualization. It is possible that the pathway is not inhibited during decidualization but reduced to prevent aberrant expression. Indeed, it is thought that embryonic signals stimulate activation of the pathway whereas maternal P4 via DKK1 prevents its hyperactivity to allow for differentiation in the presence of marginal proliferation. Recently, research in the role of miRNAs in the secretory endometrium has identified a novel regulatory pathway by which WNT/β catenin is controlled [95]. The authors of the study observed that P4 induces the expression of miRNA-152, which via direct binding suppresses WNT ligands in endometrial epithelial cells. That study contributed towards delineating the P4-induced suppression of endometrial proliferation in the epithelium. However, more studies are needed to acquire a better understanding of the possible diverse roles of WNT/β catenin pathway en route to decidualization.

The cAMP-induced transcription factor FOXO1 engages in transcriptional cross-talk with the nPR resulting in upregulation not only of the aforementioned WNT4 and BMP-2 but also established markers of decidualization such as the IGFR, IGF binding protein 1 (IGFBP1), prolactin (PRL) and p57 [94]. The nPR-induced transcription factor homeobox protein Hox-A10 (HOXA10) in epithelial cells also contributes to decidualization by elevating stromal expression of IGFBP1, COX-2 and prostaglandin receptors EP3 and EP4 [96]. FOXO1 and HOX10 transcription factors reportedly interact with the nPR on the IGFBP1 promoter [97]. Another cAMP-induced transcription factor, STAT5, which is predominantly expressed in the glandular epithelium with some selective expression in stromal cells, additionally interacts with nPR on the promoter of PRL [98]. The known coactivators promoting the initiation of IGFBP1 and PRL transcription are CBP/p300 and SRC-1/p160, which enhance the activities of the transcription factors in complex with nPR [99,100]. Collectively, PR signaling provides the platform for the formation of a decidua-specific transcriptional complex composed of diverse transcription factors and coactivators leading to the expression of cell cycle regulators (e.g., cyclins, CDKs, p21, p27, p53, p57) or essential decidualizing factors (e.g., BMP-2, PRL, IGFBP1).

Membrane PR (mPR) initiated responses have also been observed with progesterone receptor membrane component 1 (PGRMC1) being the mostly studied in this context [101,102]. Because PGRMC1 was predominantly found expressed in stromal cells as opposed to epithelial cells in the mid-secretory phase, it was initially presumed that it was a critical regulator of decidualization. In the past year, a more convincing study demonstrated that overexpression of PGRMC1 in stromal cells compromised in-vitro-induced decidualization as manifested by attenuated PRL synthesis and absence of typical morphological features [103]. Notably, the authors have previously discovered the PGRMC1 protein to be one of the few differentially expressed between receptive and nonreceptive endometrium [104]. The precise mechanism upstream and downstream mPR activation is yet to be established. However, several studies demonstrated that mPR-induced mobilization of intracellular Ca^{2+} in endometrial cells is known to activate MAPK cascades and inhibit cAMP synthesis [105,106]. The latter could explain how overexpression of PGRMC1 inhibits decidualization. These studies have set the seed and expected to stimulate considerable research to fill our gaps in the understanding of membrane-initiated responses to P4 during the process of decidualization.

Upon arrival of the blastocyst to the uterine cavity, the endometrium starts a cascade reaction to accommodate the needs of the blastocyst during the window of implantation.

4. Implantation Route: Accepting the Blastocyst

Implantation-associated signaling pathways are largely influenced by maternal P4 and signals emanating from the blastocyst [107]. PR is expressed throughout the endometrial epithelium before blastocyst implantation but reportedly decreases during implantation; hence the role of PR signaling is to establish endometrial receptivity prior to implantation [108]. For this purpose, P4 blocks E2-driven proliferation in epithelial cells and induces genes that allow the endometrium to respond to the embryo and permit its attachment [109]. Apposition and adhesion of the blastocyst occurs in a chemokine and cytokine enriched microenvironment that is integrin-dependent. Implantation-associated cytokines including leukemia inhibitory factor (LIF), interleukin 1 (IL-1) and colony stimulating factor (CSF) as well as EGFs such as the heparin-binding EGF (HB-EGF) and amphiregulin are under P4 transcriptional control [109,110]. It has been recently demonstrated in mice that upregulation of LIF expression requires the downregulation of PRA in endometrial epithelial cells at the time of receptivity [111]. Surprisingly, this mechanism is yet to be explored in humans.

The hallmark of decidualization is polyploidization and some research has informed on the events underlying the increase in the genome DNA content in decidua cells. For example, HB-EGF binds to the EGFR, the synthesis of which is also maintained by P4, to promote decidual growth and establish polyploidization in the stroma through upregulation of cyclin D3 [112]. Death effector domain-containing protein (DEDD) is essential for polyploidization and is highly expressed in stromal cells during decidualization to arrest the proliferating cell at the G2/M checkpoint [113]. DEDD forms a complex with cyclin D3 to stabilize the cyclin D3/CDK4 and cyclin D3/CDK6 complex to allow further growth [114]. Considering the central role of polyploidization in decidualization, we currently know little about the mechanisms that control it although lively mitochondrial activity is reportedly paramount to allow polyploidization [115].

The blastocyst remains for 72 h in the uterine cavity prior implantation. One of the mechanisms by which P4 prevents premature attachment of the blastocyst, is by a PRA-mediated upregulation of mucin 1 (MUC-1) antiadhesive glycoprotein [116]. P4-induced HOXA10 also plays roles during the window of implantation. Increase in epithelial HOXA10 promotes the expression of $\alpha v\beta 3$ and $\alpha 4\beta 1$ integrins and induces formation of apical epithelial projections termed pinopodes critical determinant of blastocyst implantation [109,117]. Integrin $\alpha v\beta 3$ is further stimulated by IL-1α and IL-1β secreted by the blastocyst, suggesting an active reciprocal mechanisms between mother and embryo. The importance of these embryo-derived interleukins in the implantation-related cascades in the endometrium has been proposed in the late 1990s, but the notion has been challenged in the recent years [118,119]. Hence, more evidence is needed to understand whether their contribution is pivotal. HOXA10-driven induction of EP3/EP4 and COX-2 is also relevant to implantation and P4-guided secretion of chemokines such as IL-8, membrane cofactor protein 1 (MCP-1), chemokine (C-X-C motif) ligand 1 (CXCL1) and C-X-C chemokine receptor type 4 (CXCR-4) is prerequisite for embryo-endometrial cross-talk during the receptive phase [120]. Another example of this cross-talk is the induction of fibronectin receptor in the blastocyst, which is driven by the PR-regulated secretion of calcitonin from the endometrial stroma [121]. Adhesion and invasion of the semiallogenic implanting blastocyst will introduce an immune challenge to the endometrium. P4 signaling negates the challenge and establishes immunotolerance via the expression of progesterone-induced blocking factor (PIBF) in endometrial cells, which alters the arachidonic acid metabolism, inhibits NK cell activity and promotes a Th2 cytokine response in the stroma-infiltrating leukocytes [122–124]. Immunotolerance is further fostered by the induction of IL-10 from tolerogenic dentritic cells, recruited by the P4-driven secretion of galectin-1 (GAL-1) from endometrial cells [125].

As mentioned above, active WNT/β-catenin signaling is needed in the process of implantation [126]. Mouse implantation sites are rich in various WNT ligands and receptors and

the activity of the pathway itself is greatly increased during the window of implantation in specific endometrial regions close to the invading blastocyst [127,128]. The importance of the pathway is clarified by the impact of its inhibition; pre-treatment of mouse blastocysts with a WNT/β-catenin inhibitors Sfrp2 or Dkk1 results in dramatic decrease in implantation rate [127,129]. The mechanisms underlying the dependence of implantation from WNT/β-catenin are not understood. Theories regarding the possible influence of the pathway on migratory cascades in endometrial cells can be postulated and are discussed later in this review. To date, only one study in mouse has proposed a role for WNT/β-catenin in polyploidization of decidua cells [130]. This notion is both interesting and credible considering the novel discovery that WNT signaling can influence the position and orientation of the mitotic spindle during cell division in other systems [131]. This line of investigation undoubtedly deserves elaboration.

The window of endometrial receptivity has been extensively studied in order to establish a transcriptomic signature compatible with successful implantation and unravel the signaling pathways pursuing it. A recent analysis defined a meta-signature of endometrial receptivity involving 57 transcripts as putative receptivity markers [132]. The meta-signature genes highlighted the importance of signaling with regard to immune responses, the complement cascade pathway and extracellular vesicle (EV)-mediated communication in mid-secretory endometrial functions. These genes and the involved pathways will generate new hypotheses and direct future research to delineate further endometrial cell signaling events during the window of implantation. Some research has already shed light into the utilization of EV trafficking by endometrial cells at the time of implantation. Human endometrial-derived EVs are rapidly internalized by trophoblast cells and enhance their adhesive capacity [133]. The mechanism underlying this functional effect of endometrial EVs is believed to involve the delivery of a cargo rich in adhesion molecules. These include the integrin-binding fibronectin and numerous members of the Focal adhesion kinase (FAK) pathway, all of which increase in trophoblasts following endometrial-EV uptake [133]. The invasion of the blastocyst into the decidua will send the endometrial cells onto a migratory route whereby differentiating stromal cells actively promote implantation by moving around and encapsulating the blastocyst.

5. Migration Route: Promotion of Blastocyst Invasion

A function largely neglected by the literature is the migration of endometrial stromal cells during implantation, which is regulated by both the invading blastocyst and the stroma. The embryo itself has a crucial function in modulating stromal gene expression and function to allow for its invasion. An in vitro implantation model whereby human blastocysts were placed on a monolayer of decidualizing endometrial stromal cells showed that within a period of 48 h highly motile cells surrounded the blastocyst [134]. When the motility of cells was suppressed, trophoblast invasion was inhibited. In another model using spheroids instead of blastocysts, decidualizing stromal cells aligned around the spheroid in a different manner compared to nondecidualizing cells, highlighting that cell migration was directed by decidualization [135]. Indeed, in vitro motility was enhanced in decidualizing compared with undifferentiated endometrial stromal cells and both invasion and chemotactic migration largely increased when decidualizing cells were in contact with trophoblasts [136,137]. A recent study refined these observations by exploring how migration is impacted following co-incubation of decidualized and not decidualized cells with secretome of human embryos with different qualitative features [138]. Their classical migration assays confirmed that only good quality embryos stimulate migration of decidualized cells, but notably not of not decidualized cells. A molecular mechanism to account for this observation was not discussed by the authors. However, it is not unlikely that the WNT signaling is partly involved due to its putative role in cell migration in different tissues (reviewed in [131]).

The pleiotropic functions of WNT pathway activation in the endometrial cells makes it extremely difficult to study isolated events, such as migration, and interpret the generated findings. The different modes of WNT signaling—canonical or noncanonical—add an additional layer of complexity. It needs

to be emphasized that the research in the field of noncanonical WNT pathway operating in the endometrial cell has barely scratched the surface. Especially of the WNT/planar cell polarity (PCP) signaling pathway that controls tissue polarity and cell movement through the activation of Rho GTPases. Rho GTPases are putative targets of nPR signaling in the endometrium during the window of implantation being a family of proteins that modulate cytoskeleton dynamics, myosin activity and cell adhesion. Rac-1 is a member of the Rho family of GTPases that acts through interaction with p21-activating kinase (PAK). Rac-1-induces promotion of lamellipodial protrusion at the front of migrating cells to provide integrin-mediated adhesion while RhoA induces retraction at the rear [139]. ROCK1 activation by the RhoA generates contractile forces through actin-myosin interactions. Contraction and detachment of trailing edges allows for the promotion of the cell body. Rac-1 reduces RhoA activation, and the RhoA target Rho-kinase (ROCK) can inhibit Rac-1 [140]. P4 sets off rapid nongenomic activation of RhoA/ROCK and Rac-1/PAK cascades that help migration of cells through regulation of cytoskeletal fluidity and continuous destabilization and stabilization of cortical actin stress fibers. Silencing of Rac-1 in human endometrial stroma leads to inhibition of implantation whereas silencing of RhoA results in outgrowth of blastocysts [134,141]. In line, migration of endometrial stromal cells can be directly inhibited by decreasing the activity of ROCK [30]. It is, therefore, well-understood that enhanced endometrial stromal cell motility occurs in the presence of ROCK inhibition, downstream of RhoA.

The link between WNT pathway and RhoA/ROCK has never been explored in the endometrium in this context. However, the ligand mostly associated with noncanonical activation of the WNT receptors is WNT4, which is highly secreted by trophoblasts during implantation [93]. The production and synthesis of WNTs from the invading embryo at a first glance does not fit well with the established inhibition of WNT/β-catenin by P4 signaling in endometrial cells during implantation. Especially because the P4-induced expression of DKK1 inhibitor of the WNT pathway in endometrial cells has been demonstrated to diminish trophoblast invasion [142]. This inconsistency could perhaps be explained by the great gap in the understanding of dynamics in the operation of WNT canonical and noncanonical pathways in endometrium during the window of implantation. It is possible that a canonical-transcription dependent pathway is blocked by P4 to decrease mitosis in the epithelium and at the same time noncanonical-transcription independent pathway, facilitated by Rac1 and ROCK, is underway to promote endometrial cell motility. The plausibility of this notion requires deliberation and has the potential to inform on novel unprecedented aspects of WNT signaling in the endometrium. Due to its unconfirmed status, the migration route in Figure 1 is not crossing the interchange station denoting the WNT pathway.

Endometrial P4-induced IGFBP-1 also contributes to trophoblast migration by activation of α5β1 integrins on the surface of trophoblasts leading to activation of FAK and MAPK cascades [143]. On the blastocyst side, the platelet-derived growth factor AA homodimer (PDGF-AA) is an important putative signal that mobilizes stromal cells at the implantation site [137]. A transcriptomic analysis performed to identify factors with key roles in orchestration of migration during implantation found PDGF-AA to be expressed in competent blastocysts with their corresponding receptor being expressed in the receptive endometrium [144]. PDGF-BB homodimer is also a stimulus of chemokinesis and chemotaxis in undifferentiated and decidualizing cells [137]. PDGF-BB homodimer binding can activate Rac-1 in stromal cells and indirectly inhibit ROCK contributing to enhanced motility [145]. RacGAP1, a GTPase-activating protein that exerts its GAP activity on RhoA and on Rac-1, is downregulated in endometrial stromal cells in response to blastocyst signals and current knowledge suggest that RacGAP1 is upstream of Rac-1 [141]. However, the specific embryo-derived factors mediating the observed reduction in RacGAP1 levels remain to be characterized. Trophoblast cell-derived CXCL12 may be another important factor to stimulate migration and was shown to up-regulate CXCR4 (the receptor for CXCL12) in first-trimester decidual cells and to promote their invasiveness [146]. Additionally, HB-EGF, a multifunctional mediator of embryo-endometrial communication during implantation, is an important chemoattractant for stromal cells acting through EGFR to facilitate

endometrial cell migration [137]. CD82, a metastasis suppressor that is specifically induced in the decidual stroma, may have a key role in trophoblast invasion as CD82-positive decidualized stromal cells are highly responsive to trophoblast signals in migration and invasion assays [136,147]. Silencing of CD82 in decidualizing stromal cells results in attenuation of chemotactic migration [148].

Active remodeling of the extracellular matrix (ECM) is a known contributor to the regulation of decidua migration and deep trophoblast invasion leading up to the formation of a haemochorial placenta. Proteolytic enzymes such as matrix metalloproteinases (MMPs) control the invasive growth of trophoblasts and their activity is under negative regulation by P4. The role of P4 demonstrates that the process of ECM remodeling during migration involves both signals that promote it and those that restrict it. In particular, it is believed that P4 functions to prevent excessive invasion [121]. Matrix metalloproteinase-2 and -9 that digest the main component of basal membranes collagen IV are highly secreted by invasive trophoblast cells [121]. P4 blocks the secretion of MMP-9 from trophoblasts and inhibits the activities of MMP-1, -2, -3, -7 and -9 in human endometrial explants where it increases the MMP tissue inhibitor (TIMP)-3 [149,150]. The mechanisms by which P4 affects these factors involve direct transcriptional modulation. P4 inhibits the binding of transcription factor SP4 to the promoter of MMP-2 by directing SP4 degradation and the binding of NF-κB to the promoter of MMP-1, -3 and -9 by upregulating its inhibitor IkBα [151,152]. These events result in overall decrease in MMPs activity. P4 evidently also inhibits IL-1α-induced MMP-3 activation and stimulates TGF-β in stromal cells [153], which activates TIMPs and inhibits MMP-7 expression in the epithelium [154]. The expression of leptin, a P4-regulated gene, is suppressed in endometrium during migration, additionally impacting the availability of MMP-2 and MMP-9 [155].

Vanguard research in the field is slowly introducing a new concept in the regulation of endometrial cell migration: vesicle-mediated communication between endometrial cells and trophoblasts to promote cell motility. Endometrial epithelial cells release EVs containing the glycosylated transmembrane protein extracellular matrix metalloproteinase inducer (EMMPRIN), and this release is increased when cells are stimulated with a GPER ligand [156]. EMMPRIN mediates cell invasion and can induce the release of MMP-9 from endometrial fibroblast [157]. Whether EV-EMMPRIN can act on trophectoderm cells or on neighboring endometrial epithelial cells to contribute to invasion and migration has yet to be explored. In support of the role of EVs in the mechanisms regulating migration, endometrial stromal cell Rac-1 pathway seems to elevate vesicular trafficking [158]. Considering the recent meta-analysis pointing out that numerous genes contained within the human uterine fluid during the secretory phase are involved in vesicle trafficking, the concept of EV-mediated migration of endometrial cells during implantation deserves attention and is set to create a new research trajectory [132]. Decoding the players involved in migration potentiates discovery of candidate therapeutic targets for the management of implantation pathologies.

In the absence of implantation at the late secretory phase, the availability of both steroids falls due to corpus luteum regression. The latter triggers infiltration of leukocytes, proteolytic breakdown, shedding of the endometrium, and consequently menstrual bleeding.

6. Breakdown Route: Shedding the Functionalis

Menstrual breakdown is limited to humans, primates and a few mammals including some bats. It results from P4 withdrawal in decidualized stromal cells, which in the absence of PR signaling undergo functionalis-specific tissue degradation to demolish the decidualization-induced assembly of pericellular structures. Complex cascades involving endocrine and paracrine signaling within the endometrium govern the process of shedding. The PR withdrawal-initiated breakdown route (Figure 1) can enhance inflammatory reactive oxygen species (ROS) via inhibition of superoxide dismutase activity, which in turn upregulates NF-κB and COX-2 signaling and results in the production of inflammatory factors, including prostaglandin F2α (PGF2α) [159,160]. PGF2α induces myometrial contractions and vasoconstriction of the spiral arteries both of which are critical events in the menstruation process. However, ROS-mediated activation of NF-κB alone may result in the production

of the inflammatory factors such as MCP-1, IL-6, TNF, and IL-1 [161]. These can stimulate influx of neutrophils in the stroma, which also represent a major source of ROS [162]. Stromal cells is an additional source of ROS, which are generated as byproducts of normal metabolism. Perhaps the best-characterized function of infiltrating neutrophils at the time of menstruation is to provide the matrix with proteases such as MMPs [163]. MMPs play a leading role in the breakdown of the ECM during menstruation, which can be reversed by synthetic inhibitors of MMPs [164,165]. Most MMPs are expressed in the human endometrium where their activity is tightly regulated both spatially and temporally to ensure that extensive tissue breakdown is restrained to the functionalis while allowing ECM remodeling during blastocyst implantation. The regulation of MMPs occurs at the levels of transcription, activation, membrane recruitment, TIMP-induced inhibition and endocytic clearance.

Several cytokines/growth factors and other molecules regulate the expression and activity of MMPs in the human endometrium. The most important and well-established of these have been illustrated as stations in a second branch stemming from P4 withdrawal in the breakdown route of Figure 1. One of them is plasmin, detected in high amounts in the menstrual material and generated by plasminogen activators (PAs), which are produced in the endometrium [166]. Plasmin can degrade numerous connective tissue proteins, for example fibronectin, laminin, proteoglycans, and collagen type IV , I.V. Plasmin also activates cytokines in the TGF-β family, which are highly expressed in the human endometrium during menstruation and localized within the stromal cells, glandular cells and macrophages, and found in the shed endometrial tissue [167,168]. Plasmin expression is regulated by P4. During the secretory phase of the cycle, P4 stimulates the expression of the PA inhibitor (PAI)-1 by endometrial stromal cells, leading to an increase in the number of urokinase (uPA) receptors and enhancing internalization of uPA/PAI-1 complexes [169]. At the end of the secretory phase, the low availability of P4 removes the repression of PA activity, enhances the fibrinolytic activity of the menstrual fluid and promotes the degradation of ECM [170]. Another molecule able to stimulate the expression of MMP-1 and MMP-3 by stromal cells is IL-1α [171]. Expression of IL-1α by stromal and epithelial cells is differentially modulated by P4, which inhibits IL-1α in stromal cells via an unknown mechanism but has no effect on epithelial IL-1α [171]. LEFTY-2 (endometrial bleeding associated factor), a member of the TGF-β superfamily, is selectively expressed in the menstruating endometrium [172]. Its expression is strongly repressed by P4 and recombinant LEFTY-2 stimulates the expression of MMP-3, -7 and -9 [173,174]. It is the most potent inducer of MMPs in endometrial cells upon P4 withdrawal at menstruation.

The expression of the potent vasoconstrictor endothelin (ET) reaches a peak in glandular cells during the perimenstrual phase and both TGF-β1 and IL-1α induce its expression [175]. ET receptor B is also upregulated in the stromal and glandular cells at menstruation and its stimulation increases MMP-1 and MMP-3 [175,176]. TNF-α, which is expressed in the wall of the spiral arterioles and in glands at menses, also induces MMP-1, -3, and -9 and mediates apoptosis, cell-cell dissociation in endometrial epithelial cells and compromises vascular integrity leading to haemorrhage [177]. EMMPRIN, EGF, PDGF-BB, IGF-II, CCL-16, CCL-21, IL-8, and IL-6 all contribute to the abundance of MMPs in the stroma [178,179].

The decline in circulating P4 additionally triggers reduction in tissue factor (TF) to create a pro-hemorrhagic and fibrinolytic milieu [180]. TF gene promoter lacks a PRE site, hence its induction by PR in human endometrial stromal cells occurs via enhanced expression of the transcription factor, SP1 and requires the presence of EGF [181]. P4-stimulation of TF expression continues in stromal cells throughout pregnancy to protect against bleeding and possibly contributes to peripartum hemostasis [182].

Although P4 withdrawal is the primary trigger for endometrial breakdown and shedding, the downstream regulators of this signaling are vaguely understood. Scrutinizing the molecular mechanisms has the potential to inform on the pathophysiology of many disorders including heavy menstrual bleeding and postpartum hemorrhage, and therein aid the development of therapeutics for their management.

Menstruation is followed by restoration of vascular integrity, angiogenesis, and efficient endometrial repair [7].

7. Regeneration: Repairing the Functionalis

Regeneration of the functionalis occurs simultaneously with degeneration. As early as day 2 of the cycle, during active shedding, stumps of residual glands in the basalis protrude from the stroma forming glandular cones. Glandular epithelial cells proliferate and migrate laterally to repopulate the luminal epithelium in a process termed re-epithelialization [9]. Furthermore, the luminal epithelium in the cornua and isthmus regions escape desquamation and additionally contribute to re-epithelialization. By day 4, two-thirds of the endometrium lining is covered by epithelium and re-epithelialization is completed by day 6 [183].

Endometrial regeneration essentially includes four important events: (i) proliferation and migration of residual glandular and luminal epithelial cells with the aim to re-epithelialize the lumen during the process of repair; (ii) cellular transdifferentiation of stromal cells into epithelial cells, an event called mesenchymal to epithelial (MET) transition; (iii) engraftment of bone marrow cells into the endometrium and (iv) contribution of progenitor stem cells to a more differentiated progeny [184,185]. The repair of endometrium occurs when circulating E2 levels are still low and epithelial cells lack ER-α in a rapid scar-free process, complete within 48 h, highlighting the conserved wound healing mechanism in the endometrium [186].

It is a mystery how residual glandular epithelial cells proliferate in the absence of hormones while the mechanism underlying their migration to the luminal epithelium is also poorly understood. A role of growth factors including EGF and hepatocyte growth factor (HGF) in the mediation of glandular cell migration has been hypothesized [187,188]. Other "wound-healing" factors such as Activin A, VEGF, cysteine-rich secretory protein 3 (CRISP3), and galectin-7 (GAL-7), as well as the activation of development pathways including WNT/β-catenin and NOTCH are thought to contribute to re-epithelialization and endometrial wound repair [189–194]. However, these studies require further consolidation. Androgen receptor (AR) signaling has been recently proposed as a potential regulator of endometrial wound repair in mice and further studies are underway to address the underlying mechanism [195,196].

Although endometrial inflammation results in tissue breakdown, it is also likely to form a fundamental component of the repair process. Indeed, the recruited leukocytes at the time of menstruation have an active role in the endometrial repair whereas depletion of neutrophils in mice results in a profound impairment of this process [197]. In addition to inflammatory cells, increased chemokine production in the perimenstrual endometrium may itself contribute to the endometrial repair process. IL-8 increases during the late secretory phase under the control of hypoxia inducible factor (HIF)-1α [198,199]. Endometrial expression of connective tissue growth factor (CTGF) is also increased in the repairing endometrium and at sites of connective tissue formation under the influence of PGF2a [200–202]. Lastly, platelet-rich plasma (PRP) was recently documented to facilitate endometrial repair [203]]. Platelets contain granules rich in growth factors and cytokines including VEGF, TGFβ, PDGF, FGF, IGF1, EFG, HGF, CXCL12, and CCL5. These are released in response to platelet activation at the site of inflammation, in this case endometrial wound, where they activate stromal cells and recruit leukocytes to promote angiogenesis and induce repair mediated by cell proliferation and migration. These platelet-derived factors are pivotal to endometrial progenitor cell activity [204]. Several types of endometrial stem/progenitor cells are present in the endometrium including mesenchymal stem cells (eMSCs), epithelial progenitor cells (eEPs), and side population (SP) cells [186]. Although a number of markers have been identified for the recognition and isolation of these populations, their exact roles in endometrial regeneration is unclear. It is suggested that eEPs are located in the base of the glands and are the source of the proliferative cells for re-epithelialization [205]. A recent study has proposed that endometrial stem cells can promote the repair of stromal cells by activating the p38 MAPK and Akt signaling pathways [206].

Deep sequencing and epigenetic profiling of endometrial stem/progenitor cells and their differentiated progeny will shed new light on their regulations and functions. It would be interesting to examine whether these stem cells participate in the process of MET during regeneration [207]. EVs have been proposed to mediate endometrial and progenitor cell deposition to the endometrial surface to contribute to re-epithelization. In this hypothesis, after endometrial shedding, the platelets released in the uterine cavity initially secrete soluble factors to mobilize cells towards the surface and then export vesicles to commit cells to re-epithelization [208]. Characterization of the EV-cargo and the mechanism underlying their internalization from endometrial cells will consolidate the aforementioned newly developed hypothesis.

Following re-epithelization, local E2 availability increases and orchestrates endometrial epithelial and stromal cell growth and proliferation, which is associated with profound angiogenesis.

8. Angiogenesis Route: Building the Endometrial Vascular Network

Formation of new blood vessels from already existing capillaries defines angiogenesis, a two-step process essential for endometrial function [209]. Blood vessels must be repaired during the end of the menses and then capillaries need to grow, mature, and coil during the proliferative and secretory phase. It is accepted that vessel growth in human endometrium occurs by a nonsprouting elongation in response to metabolic demands of surrounding cells and intense hypoxia in the luminal portion of the endometrium [210,211]. The absence of sprouting elongation is in line with the lack of ERα endothelial receptor and, hence, no active proliferation cascades [212]. Endothelial cells forming the capillary bed are under the influence of factors produced by surrounding tissue and angiogenic factors that circulate in the blood during the menstrual cycle. Vascular repair and angiogenesis in the endometrium are dominated by local hypoxia and nER signaling during the follicular phase of the cycle, but vascular maturation occurs during the secretory phase under P4 influence. While hypoxia is a major regulator of endometrial remodeling during menstruation, E2 plays an important role in the reconstruction of a new vascular network and rapid vessel growth [213]. VEGF governs human angiogenesis with the help of two tyrosine kinase receptors, VEGFR-1 and VEGFR-2 [214,215]. Most biological effects of VEGF are mediated by VEGFR-2 [180]. The expression of VEGF in the human endometrium is well described and its involvement in endothelial cell proliferation, migration and assembly of capillary tubes is well documented [216]. However, VEGF is also an essential factor for the first wave of angiogenesis occurring during repair and possibly plays an important role in re-epithelialization [217]. Hypoxia is a known inducer of VEGF via activation of HIF-1α in human endometrial stromal cell, which is suppressed under normoxic conditions [218]. In the glandular and stromal endometrial cells, HIF-1α is abundant during the late secretory phase and menstruation, thus appearing to be related to the process of menstruation [219]. HIF-1α binds directly to the hypoxia-response elements (HRE) in the promoters of the genes encoding VEGF [220]. Activation of nER can also induce VEGF in cultured endometrial stromal cells while nPR signaling inhibits its transcription [221]. The P4-inhibition of VEGF is potentially indirectly mediated by the nPR-induced downregulation of the nER in the human endometrium. Angiopoietins (ANGPT) comprise a second key group facilitating angiogenesis with roles in the regulation of vessel growth, maturation and regression with interesting interactions with VEGF [222]. ANGPT1 promotes the association of endothelial cells with pericytes and vascular smooth muscle cells, which contributes to the maturation of newly formed blood [223]. In the presence of VEGF, a natural antagonist of ANGPT1, ANGPT2 initiates neovascularization. The balance in the availability of ANGPT1 and ANGPT2 is critical for angiogenesis [224]. Concurrently with VEGF induction, hypoxia increases ANGPT2/ANGPT1 ratio, which is associated with new blood vessel formation [180]. E2 also increases ANGPT2/ANGPT1 ratio by decreasing the expression of ANGPT1 [225]. The increase in the ANGPT2/ANGPT1 ratio and VEGF creates an optimum environment for the development, reconstruction and remodeling of the endometrial blood vessels. A lower ANGPT2/ANGPT1 ratio, following exposure of stromal cells to P4, appears to favor the maturation and stabilization of the newly developed vessels in the endometrium, which may underlie angiogenic actions during secretory

phase. Another commonly known mediator of angiogenesis in the endometrium is the stromal cell-derived factor 1 (SDF-1), a member of the CXC chemokine family, that signals through its only receptor CXCR4 [226]. SDF-1 and VEGF interact to promote changes in gene expression in relation to angiogenesis [227]. Surprisingly, hypoxia decreases the expression and synthesis of SDF-1, in contrast to nER signaling [228]. The mechanism of this inhibition may be facilitated by two hypoxia-induced transcription factors: the activator protein 1 (AP-1) and NF-κB [229]. Expectedly, P4 antagonizes the E2-stimulation of SDF-1, hence steroid hormones rather than hypoxia may be the main regulator for SDF-1 [230]. Angiogenin (ANG) is another potent inducer of angiogenesis operating under the influence of hypoxia, which induces its expression in stromal and epithelial endometrial cells [231]. However, the major suppliers of ANG in the endometrium undergoing angiogenesis are thought to be infiltrating leukocytes such as natural killer cells and decidual macrophages during postpartum involution [232,233]. Considering that imbalanced expression of ANG has been associated with multiple pathologies including endometriosis, more research is pending to understand the mechanism of its regulation in the endometrium [234].

The previously described effects of E2 are exerted via ERα either directly or indirectly, acting on endometrial epithelial and stromal cells to secrete angiogenic growth factors [235]. Instead, E2 signaling is believed to be mediated by ERβ in endometrial endothelial cells [212]. Still some controversy exists as to which ER operates the angiogenesis specific activities in the endometrial cell subtypes throughout the cycle [236]. There are also conflicting reports regarding the presence of PR in the vascular endothelium [237,238].

Participation of WNT proteins in the process of vasculogenesis and angiogenesis is described [44]. A sustained WNT pathway activation can be utilized to generate endothelial progenitors from mesodermal lineage of embryonic stem cells. The WNT5A ligand is a potential protagonist in endothelium recovery resulting in angiogenesis, as it takes part in the healing of the damaged endothelium, but not in proliferation and migration of the endothelial cells nor elongation [239,240]. WNT7A of epithelial origin might be a chemoattractant for endothelial cells in the process of physiological endometrial angiogenesis and it is upregulated during the proliferative phase [193]. Finally, β-catenin can function in the endometrium either directly on endothelial cells or indirectly through its action on endometrial cells where it promotes the expression of VEGF [237]. WNT/β-catenin pathway in the process of endometrial angiogenesis is still largely unexplored.

9. Exit

The ability of endometrial cells to perceive and correctly respond to their microenvironment forms the basis of homeostasis. Errors in endometrial cell signaling interactions and cellular information processing are responsible for endometrial disease that can span in severity from poor endometrial receptivity to cancer. Analysis of endometrial cell signaling networks with a combination of experimental and theoretical approaches, including modeling and simulation, has been informing the scientific community over the years. Yet, persistent gaps do not allow for the synthesis of the complete physiological endometrial signaling landscape. An in-depth appreciation of the hitherto literature identifies the WNT pathway as a contributor to the gaps responsible for the incomprehension of various signaling "routes" of the endometrial cell "tube map". The all-pervasive WNT signaling pathway exploits various molecular regulators of endometrial cycling, standing up to its sobriquet as the "chameleon" of the physiological endometrial signaling. Novel trajectories of endometrial cell signaling should be explored considering recent discoveries of diverse embryo-endometrial communication mechanisms utilizing EVs [241] and the contribution of stem cells to endometrial pathophysiology [186]. However, revisiting previously performed global expression studies and utilizing meta-analyses to screen out inconsistencies and clarify interpretations is additionally a valuable strategy to contribute to the state-of-the art.

The meticulous study of endometrial signaling pathways potentiates both the discovery of novel therapeutic targets to tackle disease and the development of artificial endometrium, a staple tissue for futuristic in vitro gestations.

Author Contributions: S.M. and, P.V. authored the manuscript. The coauthors provided suggestions, comments and participated equally in the critical assessment. All authors agreed to the final version.

Funding: S.M. has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie Grant Agreement number: 796206-CERVINO-H2020-MSCA-IF-2017.

Acknowledgments: The authors would like to thank Eufemia Putortì for her kind assistance with the graphic design.

Conflicts of Interest: The authors declare no conflict of interest.

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International Journal of
Molecular Sciences

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Review

Endometrial Intracrinology: Oestrogens, Androgens and Endometrial Disorders

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Received: 30 August 2018; Accepted: 15 October 2018; Published: 22 October 2018

Abstract: Peripheral tissue metabolism of steroids (intracrinology) is now accepted as a key way in which tissues, such as the endometrium, can utilise inactive steroids present in the blood to respond to local physiological demands and 'fine-tune' the activation or inhibition of steroid hormone receptor-dependent processes. Expression of enzymes that play a critical role in the activation and inactivation of bioactive oestrogens (E1, E2) and androgens (A4, T, DHT), as well as expression of steroid hormone receptors, has been detected in endometrial tissues and cells recovered during the menstrual cycle. There is robust evidence that increased expression of aromatase is important for creating a local microenvironment that can support a pregnancy. Measurement of intra-tissue concentrations of steroids using liquid chromatography–tandem mass spectrometry has been important in advancing our understanding of a role for androgens in the endometrium, acting both as active ligands for the androgen receptor and as substrates for oestrogen biosynthesis. The emergence of intracrinology, associated with disordered expression of key enzymes such as aromatase, in the aetiology of common women's health disorders such as endometriosis and endometrial cancer has prompted renewed interest in the development of drugs targeting these pathways, opening up new opportunities for targeted therapies and precision medicine.

Keywords: decidualisation; oestradiol; aromatase; testosterone; dehydroepiandrosterone (DHEA); endometriosis; endometrial cancer; sulfatase

1. What Do We Mean by 'Intracrinology'?

The term 'intracrine' emerged in the 1980s as a new concept in endocrinology based on the ability of cells within non-gonadal tissues to both produce (synthesise) a hormone (peptide, protein or steroid) and to respond to that same factor [1,2]. For many researchers working in the field of sex steroid hormones, the 'At the cutting edge' review by Fernand Labrie published in 1991 and simply titled 'Intracrinology' was the paper that first made them expand their horizons beyond thinking of gonad-derived sex steroids as the only regulators of steroid target tissues such as the endometrium [1]. That review made what at the time appeared to be a bold claim, that the 'best estimate of the intracrine formation of oestrogens in peripheral tissues in women is in the order of 75% before menopause, and close to 100% after menopause'. In recent years, particularly following increasing use of liquid chromatography–tandem mass spectrometry (LC-MS/MS), there has been an increase in the number of studies providing evidence for changes in tissue-specific concentrations of steroids that did not necessarily parallel those in blood, as well as increased understanding in the range and number of enzymes and pathways under consideration [3–6]. Peripheral tissue metabolism of steroids is now accepted as a key way in which tissues such as the endometrium can respond to local physiological demands and 'fine-tune' the activation or inhibition of steroid hormone receptor-dependent processes. The original concept of 'intracrine' regulation was defined as involving both biosynthesis and response within the same cell (see Figure 1 in [1]) to distinguish it

from autocrine or paracrine regulation. However, in more recent studies and reviews, 'intracrinology' is now usually discussed on the basis that it is tissue-specific, local production (and inactivation) of sex steroids without significant release of active sex steroids into the peripheral circulation [1,5,6], with less attention being paid to the source and site of action being in the same cell and a greater emphasis on the tissue micro-environment. In this regard, a strong case has been made that the 'inactive' adrenal steroid dehydroepiandrosterone (DHEA) is the most important precursor of bioactive androgens in women [7]. There has also been a rapid increase in the number of studies considering the role of locally produced (intracrine) steroids in the aetiology of pathologies including cancers of the breast [8,9] and endometrium [10–12], as well as in regulation of fertility [13] and the aetiology of the oestrogen-dependent disorder endometriosis [14]. In the current review, we have based our discussion on the evidence for 'local' production and/or activation of steroids that may act in an intracrine, autocrine or paracrine manner within the endometrium or associated disorders.

In the following sections we will provide a brief overview of the structure of the endometrium, its regulation by ovarian-derived steroids and expression of steroid receptors as the prelude for a review of the evidence supporting a role for local tissue activation/biosynthesis of bioactive oestrogens (oestrone, E1: oestradiol, E2) and androgens (testosterone, T: dihydrotestosterone, DHT) in the normal endometrium and in some disorders associated with endometrial malfunction. We will also briefly review the evidence that supplementation with inactive steroids or administration of drugs targeting intracrine steroid biosynthesis may offer a new therapeutic opportunity to treat a range of disorders, including infertility.

2. Endometrium—A Sex Hormone-Dependent Multicellular Tissue

2.1. Endometrial Tissue Structure and Response to Ovarian-Derived Hormones

The human endometrium is located within the central area of the uterus, surrounded by the muscular layers of myometrium, with a layer of epithelial cells providing an interface between the tissue and the luminal compartment (Figure 1) [15]. Histologically, the human endometrium has two distinct layers: an outer basal compartment and an inner functional compartment. Both layers contain glands bounded by epithelial cells embedded in a multicellular stroma consisting of fibroblasts, resident immune cells and an extensive vascular compartment (endothelial cells, pericytes and vascular smooth muscle). In response to fluctuating changes in the concentrations of oestrogen and progesterone circulating in the blood as a result of changes in ovarian function, the tissue also experiences cyclical episodes of proliferation (oestrogen-dominated proliferative phase), differentiation of stromal fibroblasts (progesterone-dominated secretory phase), and in the absence of a pregnancy, breakdown of the inner (functional) layer, shedding and scarless healing (menstruation).

We, and others, have shown that changes in endometrial tissue function are characterised by changes in the architecture of the glands and differentiation of stromal cells (decidualisation) (Figure 1B) [16]. These are accompanied by changes in both the number and population of resident immune cells that play key roles in regulating differentiation of the tissue vasculature in preparation for implantation and in endometrial tissue repair at the time of menstruation [17–20]. It is notable that many authors have classified menstruation as an 'inflammatory event' highlighting increased concentrations of prostaglandins, which may also be generated by intracrine mechanisms, involving local enzyme expression, as well as increased synthesis of pro-inflammatory cytokines, chemokines and matrix metalloproteinases [21,22].

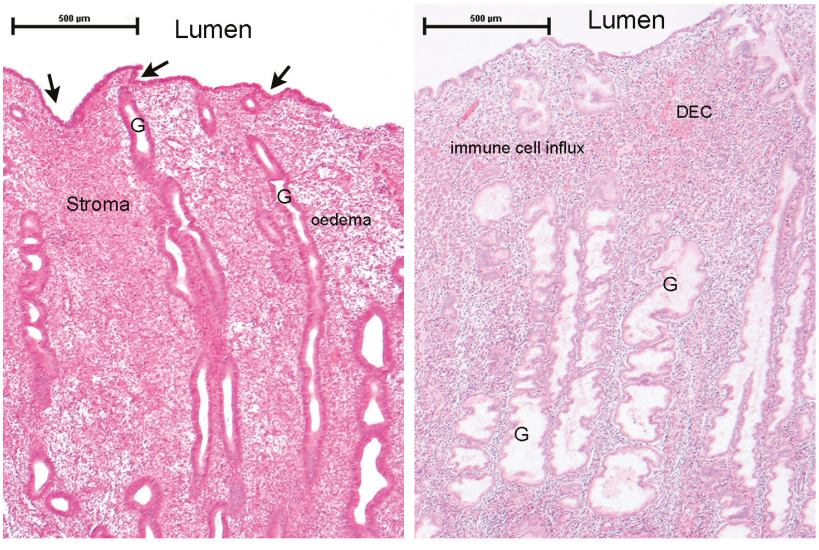

Figure 1. Histology of the human endometrium during the normal cycle. Full-thickness endometrial biopsies from hysterectomy specimens stained with haematoxylin and eosin. (**A**) Proliferative phase: note the presence of long curving glands (G) and some stromal oedema [23]. (**B**) Secretory phase: note the prominent glands (G), which have a dilated lumen and an irregular outer border stretching down into the basal compartment [24]. In the luminal (functional layer) immune cells are readily detected (most of these are likely to be macrophages and uterine natural killer (uNK) cells [17,19]), as are areas of decidualised fibroblasts (DEC) close to arterioles.

2.2. Expression of Androgen and Oestrogen Receptors in the Endometrium, Endometriosis and Endometrial Cancer

Steroid hormone action is classically mediated by intracellular proteins encoded by members of the nuclear receptor subfamily NR3: there is a single androgen receptor gene *NR3C4* (*AR*) located on the X-chromosome and two oestrogen receptor genes *NR3A1* (*ESR1*, oestrogen receptor alpha) and *NR3A2* (*ESR2*, oestrogen receptor beta) located on chromosome 6 and chromosome 14, respectively, in women (https://www.nursa.org/nursa/index.jsf). These receptors may act within the nucleus as ligand-activated transcription factors by several different mechanisms: (1) binding directly to hormone 'responsive elements' on target genes for example DNA sequences shown to have specificity for the androgen receptor (androgen response element, ARE) or either of the oestrogen receptors (estrogen response element, ERE); (2) in association with the binding of other transcription factors (AP-1, Sp-1) or (3) acting outside the nucleus via rapid, 'non-genomic' pathways—the evidence for all of these pathways has recently been extensively reviewed and will not be discussed further [25,26]. Detailed immunohistochemical studies using fixed full-thickness endometrial tissues in combination with validated antibodies [27] have identified nuclear staining for AR, ERalpha and ERbeta in the human endometrium during the normal menstrual cycle [16,18,28,29]. Notably, the tight spatial and temporal localisation of these receptors can provide insights into the cells that may be influenced by the actions of intracrine-derived steroids. Notably, key target cells for androgens are endometrial fibroblasts, which are AR-positive in the functional layer during the proliferative phase and in the basal compartment throughout the cycle. AR is downregulated in stromal cells in the functional layer during the secretory phase and upregulated in epithelial cells when progesterone levels decline (functional

withdrawal with demise of the corpus luteum) [28] or in response to administration of progesterone receptor antagonists/selective modulators [30].

In the human endometrium, ERalpha and ERbeta exhibit cell-specific patterns of expression during the menstrual cycle [29]. Notably, ERalpha is present in the epithelial cells lining the glands and lumen during the proliferative phase, at a time when circulating oestrogens are rising rapidly due to growth of antral follicles containing granulosa cells expressing aromatase [31], but is downregulated during the secretory phase [29]. Immunolocalisation of ERbeta protein suggests it does not mirror the dynamic change in expression in stromal or epithelial cells seen with ERalpha and that the protein is present in endothelial cells and multiple populations of immune cells that are ERalpha-negative [17,18,29]. As discussed below, the identification of ERbeta in endothelial and immune cells is consistent with evidence for direct actions of E2 on these cell types. Studies using targeted deletion of *Esr1* and *Esr2* in mice have reported E2-dependent signalling via ERalpha is critically important for stromal-epithelial interactions in the endometrium and epithelial cell proliferation. Uterine epithelial ERalpha is dispensable for proliferation but essential for complete biological and biochemical responses; [32]. Global ablation of *Esr2* resulted in a predominant ovarian phenotype [33]. There is also evidence for the expression of variant isoforms of both subtypes in the human endometrium formed by translation of mRNAs generated by alternative splicing of the *ESR1* and *ESR2* genes [25,29,34]: these variants are not present in mice and their function is poorly understood.

Comparison between the patterns of expression of receptor proteins in normal endometrium with samples of endometrial cancer and endometriosis lesions has revealed evidence of aberrant expression of both AR and ERs, which may result in novel disease-specific targets for the action of steroids generated by intracrine activation/metabolism discussed below. Examples include epithelial cell expression of AR in endometrial cancers [26] and upregulation of ERbeta in endometriosis [35].

3. Methodology

On 4 May 2018 searches were conducted of the PUBMED (https://www.ncbi.nlm.nih.gov/pubmed/) and SCOPUS (https://www.scopus.com/search/form.uri?display=basic) databases using a range of terms. A variable number of references, some of which overlapped, were identified: 'intracrinology' (167 references Scopus); intracrinology[and]endometrium (11 references Pubmed/21 references Scopus). These basic searches were expanded by considering individual enzymes known to be implicated in intracrine biosynthesis and pathologies: this yielded a larger number of references, probably indicating that local expression of enzymes in tissues is not always tagged as being indicative of 'intracrinology'. Examples from Pubmed searches include aromatase[and]endometrium = 398, aromatase[and]endometrium[and]cancer[and human] = 135; sulfatase[and]endometrium = 84: HSD[and]endometrium = 131.

4. Intracrine Steroid Biosynthesis in the Normal Endometrium

Classically, the endometrium was considered a target for endocrine hormones, with early studies focusing on the capacity for the tissue to metabolise (inactivate) steroids. Subsequent more detailed investigations have demonstrated that the endometrium expresses enzymes capable of biosynthesis, as well as metabolism of steroids, with endometrial cells having the capacity to enzymatically convert androgens into oestrogens, as well as to utilise adrenal and sulphated steroid precursors [36]. Notably, the capacity to convert different substrates was found to vary with menstrual cycle phase, characterised by increased conversion of DHEA, and formation of T during the secretory phase [36]. Although biosynthesis of active steroids was known to be a feature of endometrial disorders such as endometriosis and endometrial cancer (see Section 5), it has only become apparent in the last 10 years that intracrine steroid biosynthesis and metabolism plays an important role in the regulation of normal endometrial function and fertility.

4.1. Insights Gained from Measurement of Steroid Precursors and Metabolites in Endometrial Tissue

Aided by improvements in sensitivity of LC/MS-MS for measurement of oestrogens, the Poutanen group and their collaborators compared concentrations of E1 and E2 in blood and in matched endometrial tissue biopsies from individual women. Strikingly, they found that concentrations of oestrogens were higher in endometrial tissue than in the circulation and that concentrations of E2 were increased in secretory phase compared to proliferative phase tissues [3,37], with the latter finding being consistent with increased expression of enzymes such as aromatase or 17β-HSD in response to a decidualisation stimulus (see Section 4.3). In complementary studies, the concentrations of androgens and progestins were also measured in blood and in matched endometrial tissue. Notably, intra-tissue concentrations of the androgen precursor DHEA were significantly increased compared to serum concentrations in samples collected from the secretory phase. In contrast, they found that concentrations of androstenedione (A4) and T were significantly lower in endometrial tissue homogenates than in the serum and that these were not cycle phase-dependent [3].

4.2. Androgen Activation and Metabolism

Androgen biosynthesis within tissues can arise from de novo steroidogenesis, such as in the ovary, or via conversion of androgen precursors in extragonadal tissues. De novo steroid biosynthesis requires cholesterol, which is shuttled to the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR), where it undergoes side-chain cleavage by CYP11A1 (Cholesterol side chain-cleavage enzyme) to yield pregnenolone (P5). P5 undergoes two enzymatic conversions mediated via CYP17A1, first to 17α-hydroxypregnenolone (17α-hydroxylase activity) and then following 17,20 lyase action to yield DHEA [38]. DHEA is produced by the adrenal glands and by the ovary and is abundant in the circulation. Expression of StAR, CYP11A1 and CYP17A1 has been reported in the endometrium [39]; however, to the best of our knowledge no study has identified definitive de novo steroidogenesis from normal endometrial tissue.

DHEA and its sulphated form dehydroepiandrosterone sulphate (DHEAS) are abundant in the circulation and can be utilised as precursors by endometrial cells. We and others have reported that 3β-hydroxysteroid dehydrogenase (3βHSD) is expressed in endometrial stromal cells [13] and that during decidualisation DHEA can be utilised as an androgen precursor yielding both A4 and T via 3βHSD [3,40,41]. Following conversion of DHEA to A4 by 3βHSD, activation of androgen agonists T and DHT is tightly controlled via the action of aldo-keto reductase family 1 member C3 (AKR1C3). Assessment of endometrial tissue samples by immunohistochemistry and qRT-PCR of whole-tissue homogenates performed by Catalano et al. demonstrated that AKR1C3 expression is increased in the secretory phase relative to proliferative phase with peak expression reported in the early- to mid-secretory phase [42]. Our studies using an in vitro time course of decidualisation paralleled this expression pattern with peak expression detected between day 2 and 4 of an eight-day decidualisation protocol [43] (Figure 2). In contrast to AKR1C3, expression of 5α-reductase (SRD5A1), which converts T to the more potent and non-aromatisable androgen DHT, is decreased in endometrial cells as decidualisation progresses [43]. Thus, time-dependent conversion of A4 to T determines substrate availability and hence production of DHT.

Interconversion of active/inactive androgens is mediated via 17β-hydroxysteroid dehydrogenase isozymes. Expression of several 17β-hydroxysteroid dehydrogenase (HSD17B) isozymes have been reported in endometrial tissues/cells [44]. In addition to AKR1C3 (also known as HSD17B5), HSD17B7 and 12 are reported to have reductive 17β-HSD activity, mediating conversion of A4 to T, and mRNA expression of *HSD17B7* and *HSD17B12* has been reported in total tissue homogenates of normal human endometrium but with no significant cycle-dependent change in expression [4]. The predominant isoform with oxidative 17β-HSD activity in the endometrium is HSD17B2, which converts active T to A4. Expression of HSD17B2 is increased by progesterone and elevated in the secretory phase [4,45]. HSD17B14 also has oxidative 17β-HSD activity and has been immunolocalised to endometrial glandular epithelial cells [46] although relative efficiency for oxidation of E2 or T was much lower than

that of HSD17B2 in cell metabolism assays. Although HSD17B2 has oxidative action on androgens and oestrogens, our own metabolism studies suggest that this activity is decreased in endometrial stromal cells during decidualisation, even when *HSD17B2* mRNA expression is increased [13,43]. This may reflect alternative activity of HSD17B2 during the secretory phase, such as by activating 20α-hydroxyprogesterone to increase bioavailability of P4 [47].

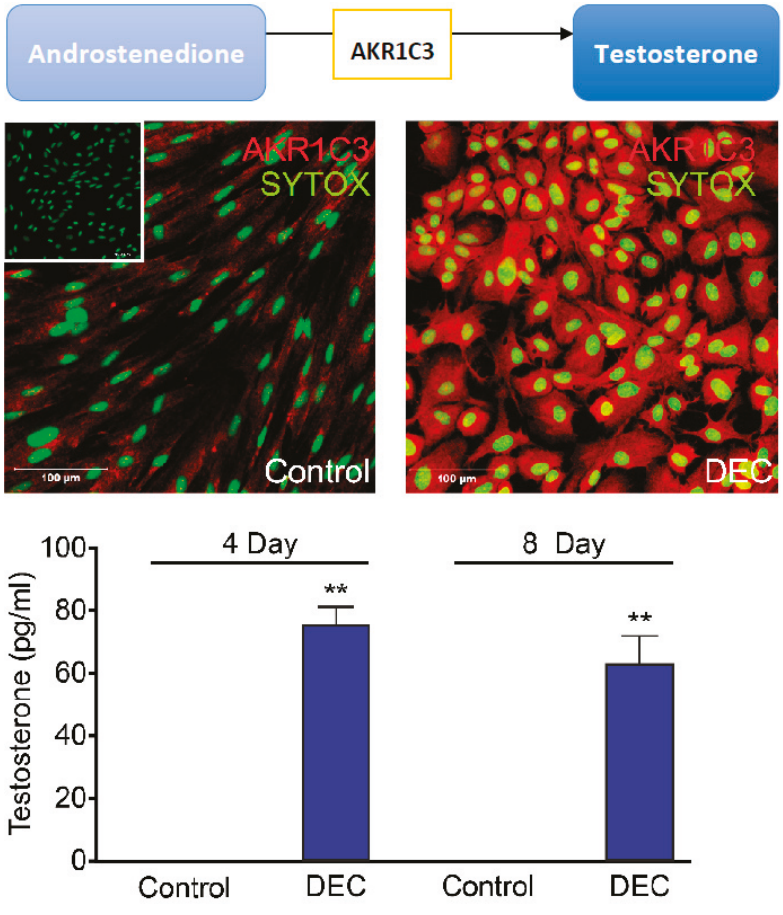

Figure 2. Expression of AKR1C3 in human endometrial stromal fibroblasts is increased in response to a decidualisation stimulus resulting in increased biosynthesis and secretion of testosterone. Based on a figure published in [43] under a CC-BY licence: concentrations of T were determined using an ELISA on days 4 or 8 of the experiment. Immunohistochemistry was determined on day 4 of experiment; AKR1C3 (red stain) Sytox Green nuclear stain (green), no primary antibody negative control (inset) (** $p < 0.01$).

4.3. Oestrogen Biosynthesis and Metabolism

There are two main pathways by which oestrogens are synthesised within endometrial cells: (1) conversion of androgens such as A4 and T to active oestrogens, E1 and E2, respectively, via the action of the aromatase enzyme complex, the key component of which is the aromatase protein encoded by the *CYP19A1* gene; (2) conversion of sulphated oestrogens oestradiol sulphate (E2S) or oestrone sulphate (E1S) into their bioactive metabolites (E2 and E1, respectively) via the action of steroid sulphatase encoded by *STS* (discussed below) [13,48].

Landmark studies in mice by the Bagchi group demonstrated that aromatase activity within the endometrium was essential for establishment of pregnancy in that species [49,50]. In a number of elegant experiments they showed that decidualisation and vascular remodelling were impaired if an aromatase inhibitor was administered in vivo [49]. In women, when assessed as whole tissue homogenates, expression of aromatase is low/undetectable in endometrial samples recovered during the normal cycle. However, using primary human endometrial stromal cells we discovered that aromatase protein and enzyme activity were increased following decidualisation resulting in increased synthesis of E1 and E2 [13] (Figure 3). Critically, these studies demonstrated the temporal regulation of this process, whereby expression of aromatase and secretion of E2 increased in a time-dependent manner, while metabolism (inactivation) of E2 to E1 decreased [13], resulting in an increased E2:E1 ratio. We speculate that the failure to detect altered expression in tissue homogenates may reflect tight temporal and/or spatial regulation, which will be missed unless samples are recovered from areas of active decidualisation in the functional layer of the tissue.

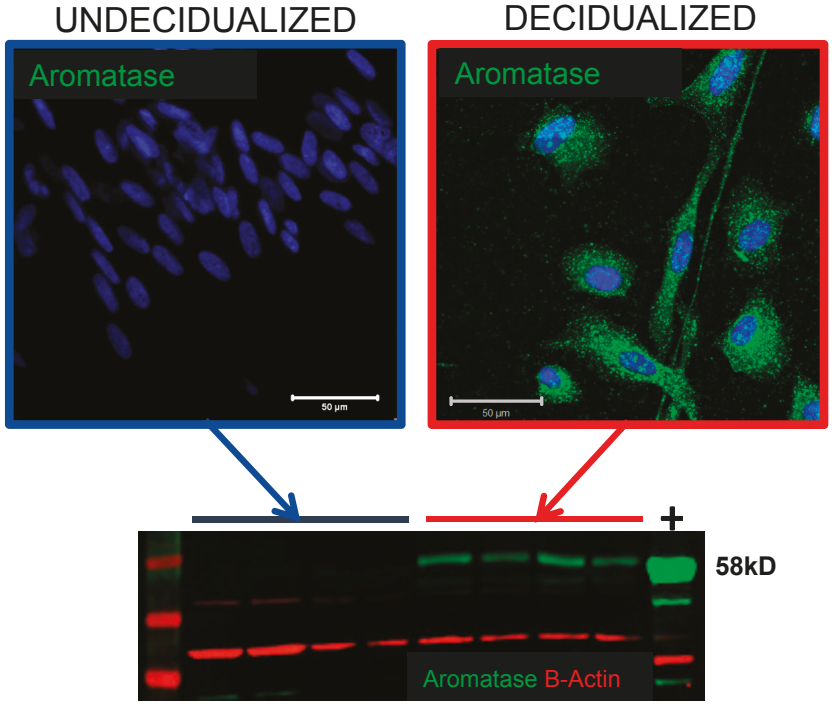

Figure 3. In vitro decidualisation of endometrial stromal fibroblasts results in upregulation of aromatase protein expression (green). Based on data reported in [13]. ESC—primary endometrial stromal fibroblasts; positive control (+) was a protein extract from human placenta, aromatase protein 58kDa. Nuclear counterstain DAPI (blue); Aromatase (green); B-Actin loading control (red).

Expression of oxidative and reductive 17BHSD isoforms may also contribute to generation of E2 from E1: although both E1 and E2 can activate ERs, E2 is generally considered to be a more potent agonist. Activation of E1 to E2 via reductive 17β-HSD activity is primarily mediated by HSD17B1 but also HSD17B4 and 7 in the uterus. Expression of these enzymes has been reported in normal endometrium [4], and as elevated in endometrial disorders (see Section 5).

4.4. Role of Sulphated Steroids as a Source of Endometrial Androgens and Oestrogens

Androgens and oestrogens can be metabolised from the common precursor DHEAS via the action of steroid sulfatase (STS), which de-sulphates DHEAS to DHEA. Additionally, STS can metabolise E1S and E2S sulphate to bioactive E1 and E2 respectively. STS is expressed in normal endometrial tissues and in endometrial cancers [51,52]. STS expression and activity is increased during decidualisation of endometrial stromal cells in vitro, consistent with a detectable increase in oestrogens detected in tissue samples recovered during the secretory phase [3,48].

Sulfation is a key mechanism for deactivating steroids and is essential for appropriate intracrine regulation within the tissue environment. Sulfation is mediated by sulfotransferase enzymes and requires the action of 3′-phospho-adenosine-5′-phosphosulphate synthase (PAPSS) enzymes to provide a sulphate moiety for conjugation [53]. There is little evidence for expression of DHEA sulphating enzymes/activity in the endometrium, although expression of *SULT1A1* and *SULT2B1a* were reported in endometrial stromal cells together with *PAPSS1* and *PAPSS2* [48]. The main oestrogen sulphotransferase (SULT1E1) is expressed in endometrial epithelial cells and expression is increased in response to progesterone [54] highlighting a potentially complex balance between activation and inactivation of steroids during the fertile phase of the cycle that may involve more than one cell type.

4.5. Intracrine and Paracrine Impact of Tissue Biosynthesis of Androgens and Oestrogens in Endometrium

Perivascular decidualisation is associated with accumulation of specialised immune cells known as uterine natural killer cells (uNK) that are critical mediators of vascular remodelling in early pregnancy. In common with other immune cells found in the endometrium (macrophages, mast cells [55,56]), uNK cells express ERbeta [18]. Strikingly, we have demonstrated that conditioned media from decidualised stromal cells increases uNK migration in an oestrogen-dependent manner. In addition, treatment of uNK cells with E2 resulted in increased secretion of angiogenic factors, including CCL2, which had a significant impact on endometrial endothelial cell network formation [57]. The impact of a local oestrogen-rich microenvironment on the function of endometrial macrophages or mast cells has yet to be elucidated. Recent evidence suggests that sulphated oestrogen precursors are also utilised by endometrial stromal cells as intracrine/paracrine regulators, as secretion of E1 and IGFBP1, a prominent decidualisation marker, are decreased in the presence of the STS inhibitor STX64 (Irosustat) [48]. Other studies that have highlighted oestrogen-dependent changes in endometrial endothelial cell gene expression [58] that will also be influenced by local (intracrine) metabolism/biosynthesis of bioactive oestrogens. In summary, the evidence presented in the studies described above, together with those in mice [49], all support the hypothesis that tight temporal-spatial regulation of tissue function by intracrine oestrogens plays a previously underappreciated role in modulating the function of cells (endothelial, uNK, decidual fibroblasts) that play key role(s) in the formation of a receptive endometrium capable of supporting a viable pregnancy.

Endometrial biosynthesis of androgens has also emerged as a regulator of fertility and endometrial function. Specifically, human primary stromal cells are AR-positive and treatment of cells from proliferative phase endometrium with DHT has identified a number of androgen-regulated genes [28]. Complementary studies by Gibson et al. [43], in which primary cells were stimulated to decidualise, showed local intracrine biosynthesis of androgens may play a critical role in maintenance of a decidual phenotype, with addition of flutamide reducing biosynthesis of the decidualisation marker IGFBP1 [43,59]. These studies complement and extend those of Cloke et al. [60,61], who showed that AR and PR regulated distinct genomic pathways during decidualisation, consistent with a role for androgens (either local or peripheral) in regulation of fertility. Studies in mice have also identified a potential role for androgens in endometrial repair at the time of menstruation [62] at a time when peripheral oestrogens are low, which merits further investigation.

The identification of aromatase expression and production of oestrogens in both mouse tissue and human endometrial cells has highlighted the importance of locally produced androgens acting as precursor steroids for oestrogen biosynthesis. Results from studies reporting that intra-tissue

concentrations of androgens T and A4 were lower than serum [3] would be consistent with this hypothesis. However, one caveat is that these studies assessed whole endometrial tissue homogenates, which do not allow for the contribution of specific cellular compartments to be quantified. We therefore investigated the temporal dynamics of androgen metabolism using an in vitro model of isolated primary human endometrial stromal cells. In these studies, treatment of cells with the AR antagonist flutamide, reduced secretion of both decidualisation and endometrial receptivity markers [43]. We further demonstrated that supplementation with the androgen precursor DHEA increases biosynthesis of T and DHT and is associated with dose-dependent increases in expression of the decidualisation markers IGFBP1 and prolactin, as well as the endometrial receptivity marker SPP1 [40]. Taken together, these studies suggest both local activation and metabolism of androgens occur during decidualisation and that temporal regulation of intracrine androgen bioavailability is a critical mediator of endometrial competence during remodelling required for establishment of pregnancy [39,59]. These studies are of particular relevance to the impact of aging on fertility, as circulating concentrations of androgens precursors, such as DHEA and A4 as well as T and DHT decline with age [38,63].

5. Evidence for the Importance of Intracrinology in Endometrial Disorders

5.1. Endometriosis

Endometriosis is a chronic oestrogen-dependent disorder that is characterised by growth of endometrial cells/tissue fragments (lesions) outside the uterus [64]: there are three broad classifications based on location of the lesions, peritoneal, ovarian (endometriomata) and deep infiltrating lesions in the rectovaginal area. A recent review provides an excellent overview of the features of endometriosis as determined by magnetic resonance imaging [65]. Common histologic features of all three manifestations include the presence of endometrial-like cells (either stromal and/or glandular, vascular cells and nerves) as well as evidence of inflammation (immune cell populations). Endometriosis can be associated with debilitating pelvic pain and in most but not all women, symptoms regress after menopause, with many of the drug regimens used to treat symptomatic endometriosis being based on suppression of ovarian cyclicity [64]. Examination of ectopic lesions revealed that they are characterised by high aromatase expression levels, together with a deficiency in 17β-HSD2, the enzyme responsible for the inactivation of E2 to E1 [66]. A recent Cochrane review concluded that, 'for women with pain and endometriosis, suppression of menstrual cycles with gonadotrophin-releasing hormone (GnRH) analogues, the levonorgestrel-releasing intrauterine system (LNG-IUD) or Danazol were beneficial interventions' [67]. However, it is notable that for many women, particularly those wishing to conceive, ovarian suppression is not desirable as the associated menopausal side effects can be severe and intracrine mechanisms are not suppressed. Whilst our literature search terms 'intracrine and endometriosis' only identified five papers on PUBMED it is notable that the alternative search 'aromatase and endometriosis' identified more than 300, reflecting widespread interest in the role of local (intracrine) biosynthesis in the aetiology of this complex disorder and the potential that this might provide a novel therapeutic opportunity [68].

Local oestrogen production, accompanied by intracrine and paracrine signalling via ERbeta in endometriotic tissues is believed to contribute to a feed-forward signalling cascade that maintains an inflammatory state and cell proliferation within the endometriotic lesions (for a comprehensive review on the role of oestrogen production and action in endometriosis see [69]). Peritoneal fluid from women with endometriosis contains high concentrations of pro-inflammatory cytokines, such as TNFα and IL-1β [70,71], which have the capacity to stimulate expression of the prostaglandin synthesis enzyme COX-2 and increase secretion of prostaglandin E2 (PGE_2) by endometriotic cells and peritoneal macrophages [72,73]. PGE_2 in turn stimulates the production of cyclic AMP (cAMP), which, together with steroidogenic factor 1 (SF1), whose expression is aberrantly upregulated in endometriotic tissue compared to endometrial tissue, induces expression of mRNAs that encode enzymes that play a critical

role in the steroidogenic enzyme machinery, including STAR, 17-hydroxylase/17,20-lyase, 3β-HSD and aromatase, which may be consistent with the synthesis of E2 from cholesterol within lesions [72,74].

The significance of intracrine signalling in the aetiology of endometriosis has been supported by Huhtinen et al., who used LC/MS-MS to interrogate the concentrations of steroids in matched endometrial, endometriotic (lesions) and serum samples from women with or without endometriosis [3,4]. A striking finding from these studies was that the concentrations of testosterone in endometriotic lesions (both ovarian and extra-ovarian) far exceeded those in the blood regardless of menstrual cycle stage [3]. This increase was mirrored by elevated expression of *CYP11A1*, *CYP17A1* and *HSD3B2* in endometriotic lesions, especially those associated with the ovary (endometriomas), compared to intrauterine endometrium. It was also accompanied by significant changes in expression of androgen-regulated genes (*PRUNE2*, *HGD*, *PDGFRL*) [3] providing a readout of the action of androgens binding to AR expressed in the lesions. Thus, increased intra-tissue testosterone synthesis in endometriotic lesions, as well as providing a substrate for aromatase and biosynthesis of E2, may promote the activation of an AR transcriptional network within the lesions.

Apart from the discrepancy between intra-tissue steroid concentrations and those in the circulation, there are also significant variations in the levels of endometrial and circulating steroid hormones that are menstrual cycle-dependent. Using the same experimental approach described above, Huhtinen et al. reported that while endometrial and endometriotic intra-tissue concentrations of E2 were significantly higher compared to those in the serum of women in the proliferative phase of the menstrual cycle, the opposite is the case for the secretory phase [4]. Moreover, expression of *HSD17B2* was significantly lower within lesions compared to endometrial tissue while expression of *HSD17B6* and *CYP19A1* was significantly higher [4]. It must be noted that there is a distinct difference both in the local steroid concentrations and the expression of steroid metabolising enzymes within different types of lesions. For example, in the proliferative phase, E2 concentrations in ovarian lesions (endometrioma) were approximately 3430 pg/mL, while in peritoneal lesions, E2 concentrations were 238 pg/mL [4]. This demonstrates a heterogeneity in intracrine steroid action within different types of lesions, which in the case of ovarian lesions could derive from the proximity of the endometriotic cells to ovarian follicles and the constant supply of steroids from the follicles within the ovaries. Further studies are required to explore this possibility.

5.2. Endometrial Cancer

Endometrial cancer (EC) is the fourth most common cancer in women, with the majority of women being diagnosed at early stages of the disease following a uterine bleed after menopause [cancerresearchuk.org]. Established risk factors for development of EC include obesity and the presence of premalignant lesions associated with endometrial hyperplasia [75,76], with oestrogen exposure considered a key driver of both endometrial hyperplasia and Type I EC that make up 75% of EC cases [76]. Rizner and colleagues have recently provided a comprehensive overview of the different studies contributing to our current understanding of the mechanisms that contribute to increased bioavailability of oestrogens in EC [52,77]. A few key studies are described below.

Expression of the *CYP19A1* gene is regulated by tissue-specific promoters, distributed over a 93kb regulatory region, which have been the subject of extensive investigation [78]. Notably, Bulun and colleagues have found that in cancers of breast, endometrium and ovary, expression is primarily regulated by increased activity of the I.3/II promoter region, which can be upregulated by prostaglandins such as PGE2, providing a link between the overexpression of PGs that has been reported to occur in EC [79] and intracrine oestrogen biosynthesis [80]. Sasano and collaborators have developed novel antibodies and reported evidence of increased immunoexpression of aromatase [81], STS [82] and 17βHSD enzymes [83] in endometrial hyperplasia and EC. These findings are all consistent with increased bioavailability of E1/E2 in association with malignant transformation. Notably, expression of aromatase and 17βHSD 1 in EC have both been correlated with poor prognosis [84,85].

We and others have shown that AR are widely expressed in EC and also in EC cell lines such as Ishikawa, which have been extensively studied (reviewed in [26]). In a recent study, Kamal et al. [86] reported that the expression of AR was downregulated in high-grade EC but elevated in metastases, raising the possibility that they might be a target for therapy. In a recent review, Ito et al. summarised the epidemiological data supporting an association between elevated androgens in the circulation and the risk of developing EC [11]. Whilst there has been less interest in the intracrine generation of androgens other than as substrates for aromatase, it is notable that a study by the same group revealed that EC tissues had an 8-fold elevation in DHT compared with that in normal endometrial tissues [87]. The same study compared AR expression with that of 5 alpha reductase enzymes 1 and 2 (5αR1 and 5αR2), concluding that expression of 5αR1 (65% of samples) was positively correlated with histological grade (but not clinical grade). They found that women immunonegative for both AR and 5αR1 had a poorer prognosis [87], consistent with other studies that have suggested androgens can be anti-proliferative for EC cells.

6. Intracrinology and Metabolism

Whilst this review has focused on endometrial tissue and its pathologies, there is a growing body of evidence showing the importance of intracrine metabolism in non-reproductive tissues. For example, in their recent comprehensive review of intracrine androgen biosynthesis and metabolism, Schiffer and colleagues highlighted the importance of peripheral metabolism of steroids in metabolic target tissues including adipose and skeletal muscle [88]. Studies on the role of AR in skeletal muscle conducted using transgenic mouse models have shown it is expressed in multiple cell types in muscle [89,90]. The pharmaceutical industries have developed a number of selective androgen receptor modulators to target AR in muscle as a therapy for age-related or cancer-related loss of muscle function [91,92].

7. New Therapeutic Approaches for Treatment of Endometrial Disorders Based on Intracrine Targets

Labrie and colleagues have conducted several studies and clinical trials to analyse the impact of intravaginal administration of DHEA (Prasterone; brand name in USA Intrarosa) on adverse symptoms resulting from postmenopausal steroid deprivation including vaginal atrophy and pain during sexual intercourse [93]. They have demonstrated positive impacts on vaginal dryness and other steroid hormone-dependent parameters without any evidence of peripheral changes in serum E2 [94,95]. In other studies, significant benefit for vaginal health has been demonstrated using estriol gel [96]: these data have collectively provided powerful evidence for intracrine steroid modulation playing a key role in regulation of the vaginal microenvironment, which can be harnessed for therapeutic benefit after ovarian secretion of oestrogens stops at menopause [97]. A recent study reported supplementation of culture media with DHEA enhanced expression of receptivity genes by decidualised stromal cells from women with a mean age of 44. Thus there is the potential that administration of DHEA during the secretory phase alone may also assist fertility in older women, although this clearly requires further evaluation [40].

In other studies the emphasis has been on inhibition of enzymes that appear dysregulated in disease. For example, there are a number of reports that aromatase is expressed in endometriosis lesions with evidence of a positive feed-forward loop involving local biosynthesis of both E2 and the pro-inflammatory regulator prostaglandin E2 [69,98]. These have been complemented by several studies reporting on the positive impact, or lack thereof, of aromatase inhibitors (AI) including letrozole and anastrozole on symptoms of endometriosis in both pre- and post-menopausal women [99,100]. The current consensus is that AI should be considered as a treatment for endometriosis-associated pain in women who are postmenopausal but still symptomatic as it will target intracrine oestrogen biosynthesis, which is thought to play an important role in this age group [99].

To target intracrine biosynthesis of steroids and prostaglandins, Bayer Pharma has developed an AKR1C3 inhibitor as a therapy for endometriosis (BAY 1128688). A phase I trial was completed in

2016 and a phase II trial is listed as underway in Spain (EudraCT number 2017-000244-18) with results awaited as to efficacy. Insulin stimulates AKR1C3 expression in the adipose tissue of women with PCOS and its inhibition has been suggested as offering a therapeutic target to reduce the hyperandrogenism that is a feature of this disorder [101].

A number of 17βHSD inhibitors have been developed to target intracrine biosynthesis of oestrogens in hormone-dependent disorders, including cancer and endometriosis [102]. Promising results have been reported using a 17βHSD1 inhibitor to suppress conversion of E1 to E2 in endometriosis tissue homogenates [103]. Forendo Pharma, based in Turku, Finland (http://www.finlandhealth.fi/-/forendo-pharma), has developed a specific 17βHSD1 inhibitor (FOR-6219) for which a phase Ia trial was initiated in July 2018. High expression of 17βHSD1 is associated with poor prognosis in EC [84]. In a recent study, Konings et al. [104] reported detection of 17βHSD1 in EC metastatic lesions and had promising results demonstrating the inhibition of enzyme activity using the FP4643 type 1 inhibitor in both in vitro and ex vivo models.

The evidence that STS is expressed in endometrial cancers [52,105] and endometriosis [106] has prompted the development of specific STS inhibitors as novel therapies. Several potent STS inhibitors have been developed [107], including STX64, which was effective in blocking synthesis in endometrial cancer cells in vitro. STX64 has been renamed Irosustat (Ipsen) and was tested in a phase 2 open-label trial in women with advanced/metastatic or recurrent oestrogen receptor-positive endometrial cancer, but did not result in better survival rates and so has not been developed further.

Another inhibitor, estradiol-3-*O*-sufamate (E2MATE), used on human endometrial explants and in a mouse model of endometriosis, has shown some promising results [108]. Currently under the name PGL2, this compound is listed on the web as being part of a phase II clinical trial for endometriosis (Jenapharm: https://adisinsight.springer.com/drugs/800026648).

Whilst monotherapies are still under testing, drugs that have dual action have also been developed to target both aromatase and STS (DASI), as well as STS and 17βHSD1. A dual STS/17βHSD1 inhibitor has been shown to block the proliferation of cancer cells treated with E1S/E1 but not those treated with E2 alone [109], but this awaits further testing in vivo. A range of DASI drugs have been developed and tested by Barry Potter and his colleagues, with promising results in cell and animal models (reviewed in [110]), but have yet to be tested as treatment for women with EC or endometriosis.

8. Conclusions

Intracrine regulation of oestrogens and androgens has emerged as a key regulator of endometrial function, both during the normal cycle and in endometrial disorders such as cancer and endometriosis. Further studies are needed to better define their role in the complex cross-talk between different cell types and the interplay between metabolic and inflammatory processes. To date, regulation of normal and abnormal endometrial tissue resulting from intracrine biosynthesis of steroids has focused on regulation of gene expression; however, with new evidence that a wide range of non-coding RNAs are also likely to play a role in endometrial tissues, studies need to be devised to explore whether their availability is also regulated by steroids [111]. Importantly, the manipulation of intracrine sex steroid metabolism has emerged as a therapeutic target for the treatment of endometriosis and endometrial cancer and the scope of these studies is likely to broaden as we gain a greater understanding of their roles in fertility.

Author Contributions: All authors contributed to writing the review and revising the text.

Funding: Research activity and staff salaries in the Saunders Group are supported by Medical Research Council grants MR/N024524/1 and MR/P00265X/1.

Conflicts of Interest: The authors declare no conflict of interest.

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Review

Direct Cell–Cell Interactions in the Endometrium and in Endometrial Pathophysiology

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Received: 14 June 2018; Accepted: 23 July 2018; Published: 30 July 2018

Abstract: Cell contacts exhibit a considerable influence on tissue physiology and homeostasis by controlling paracellular and intercellular transport processes, as well as by affecting signaling pathways. Since they maintain cell polarity, they play an important role in cell plasticity. The knowledge about the junctional protein families and their interactions has increased considerably during recent years. In contrast to most other tissues, the endometrium undergoes extensive physiological changes and reveals an extraordinary plasticity due to its crucial role in the establishment and maintenance of pregnancy. These complex changes are accompanied by changes in direct cell–cell contacts to meet the various requirements in the respective developmental stage. Impairment of this sophisticated differentiation process may lead to failure of implantation and embryo development and may be involved in the pathogenesis of endometrial diseases. In this article, we focus on the knowledge about the distribution and regulation of the different junctional proteins in the endometrium during cycling and pregnancy, as well as in pathologic conditions such as endometriosis and cancer. Decoding these sophisticated interactions should improve our understanding of endometrial physiology as well as of the mechanisms involved in pathological conditions.

Keywords: cell contacts; tight junction; adherens junction; gap junction; endometrium; implantation; decidualization; endometriosis; endometrial cancer

1. Introduction

Direct cell–cell contacts connect cells to each other to maintain cell polarity, stability and integrity. Moreover, they mediate selective paracellular as well as intercellular transport of molecules [1]. Due to these functions, they exhibit a considerable influence on tissue physiology, homeostasis, and tissue remodeling. In this regard, the endometrium is a special tissue, because in contrast to most other tissues, it undergoes considerable physiological changes and reveals an extraordinary plasticity due to its crucial role in the establishment and maintenance of pregnancy. Hormonally regulated cyclic changes in the tissue enable it to be transformed to a receptive state, which allows embryo implantation, attachment and invasion through the epithelium into the underlying stromal compartment [2,3]. During pregnancy, the endometrial stroma regulates trophoblast invasion and provides the blood supply for nutrition of the developing organism [4]. During these processes, the luminal endometrial epithelial cells undergo an epithelial-to-mesenchymal transition, whereas a mesenchymal-epithelial transition can be observed in the endometrial stromal cells [3,5,6]. These complex changes are accompanied by alterations in cell morphology, physiology and function concomitant with changes in direct cell–cell contacts to meet the various requirements in the respective developmental stage. Impairment of this sophisticated differentiation process may lead to failure of implantation and embryo development and may be involved in the pathogenesis of endometrial diseases.

In this review, we focus on the knowledge about the distribution of the different direct cell–cell contacts in the endometrium during cycling and pregnancy, as well as in pathologic conditions such as endometriosis and cancer. Results from human endometrium are correlated with findings from research with human endometrial cell lines and animal models.

2. Intercellular Junctions

Tight junctions, adherens junctions, desmosomes and gap junctions were originally identified by their morphological appearance in electron microscopy [7] and are localized mainly at the lateral membranes of polarized epithelial cells (Figure 1).

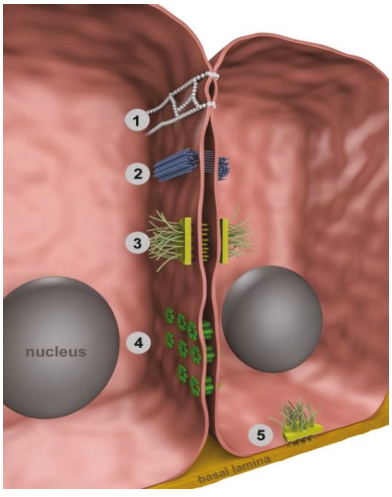

Figure 1. Localization of intercellular junctions. Tight junctions (**1**) are located at the uppermost part of the lateral cell membrane of two adjacent cells thus regulating paracellular transport between cells (gate function) and maintaining apicobasal polarity (fence function). Adherens junctions (**2**) and desmosomes (**3**) connect adjoining cells to each other. Meanwhile, adherens junctions are linked to intracellular actin bundles, desmosomal plaques are linked to intermediate filaments. Gap junctions (**4**) are intercellular membrane channels connecting directly the cytoplasm of adjacent cells, thus allowing the exchange of ions, second messengers and small metabolites. A gap junction channel is composed of two hemi-channels (connexons), each of which is composed of six protein subunits (connexins). Hemidesmosomes (**5**) connect intracellular filaments to the basal lamina.

In recent years, there has been a considerable gain in knowledge about the molecules that contribute to the structure, function and regulation of those cell–cell junctions. Tight junctions are located at the uppermost part of the lateral cell membrane. They build up a selective barrier between the adjacent cells thus regulating and restricting paracellular transport between these cells (gate function). In addition, they maintain the strict organization of the plasma membrane of epithelial cells in an apical and a basolateral compartment (fence function) [8]. Meanwhile, a large number of proteins have been identified in this junctional complex. The key players in building up the barrier and fence functions are two types of transmembrane tetraspanins—claudins and MARVEL domain proteins like occludin—which form the core of the tight junction and are associated with cytoplasmic plaque proteins including ZO-1, -2, and -3 and MUPP1 linking tight junctions to the actin-cytoskeleton, as well as to the other members of the junctional complex. Of these, claudin proteins constitute the largest protein family, with 27 members identified in the mouse and 26 in humans [8,9]. In addition, claudins are able to recruit occludin to tight junctions [10]. Occludin is also a tetraspanin, acting as

a paracellular barrier and maintaining cell polarity through interaction with ZO-1 [11] and ZO-2 [12]. The zona occludens proteins ZO-1, ZO-2, and ZO-3 can cross-link actin to the claudins and occludins, as well as to other cell–cell contacts such as adhesion junctions and gap junctions [13]. Moreover, they may recruit signaling components, thus playing a role in the regulation of gene expression [14]. In addition, the membrane associated junctional adhesion molecules (JAMs), which comprise three members, are discussed to be involved in junction assembly and paracellular barrier formation [8].

Adherens junctions and desmosomes are located just below tight junctions at the lateral plasma membrane. They keep neighboring cells together, thereby maintaining cell and tissue polarity. These adhesions are mediated by the transmembrane protein family of cadherins which include 20 classical members in the human [15], and which form homodimers in the intercellular space in a calcium-dependent manner [1]. The classical cadherins comprise E-cadherin, which is most abundant in the adherens junctions of epithelial cells, the neuronal N-cadherin, the vascular VE-cadherin, but also the desmosomal components desmogleins and desmocollins [16]. Besides their cell–cell connecting function, cadherins also bind directly and indirectly to many cytoplasmic proteins, particularly to members of the catenin family, like p120-catenin, beta-catenin, alpha-catenin, and to ZO-1, which in turn binds to actin filaments and microtubules [15], as well as to plakoglobin, plakophilins and desmoplakin in desmosomes [16]. ZO-1 also recruits ZO-2 into adherens junctions [12], thus establishing a connection to the tight junctional complex. In addition, it has been well documented that cell–cell adhesion is necessary for the formation of functional gap junctions [17], and that changes in the expression of adhesion proteins such as E-cadherin might contribute to an impaired localization of gap junctional proteins in tumor cells [18]. In epithelia, especially the association of E-cadherin and beta-catenin which may activate the Wnt-signaling pathway, seems to play a crucial role not only in cell adhesion, but also in a variety of other cellular processes and intracellular signaling pathways that control gene transcription [19,20].

In contrast to tight junctions and adherens junctions, gap junctions are intercellular membrane channels connecting directly the cytoplasm of adjacent cells. This intercellular communication allows the exchange of ions, second messengers and small metabolites, and thus influences cell differentiation and proliferation, as well as tissue development, homeostasis and morphogenesis [21]. A gap junction channel is composed of two hemi-channels (connexons), each of which is composed of six protein subunits named connexins (Cx) arranged around a pore. Up to now, 20 members of the connexin gene family have been identified in the human and 21 in the mouse genome. All connexins have four membrane-spanning domains forming the channel, two extracellular loops, a cytoplasmic loop and cytoplasmic N- and C-terminal tail segments which are involved in the regulation of channel function [22]. Gap junction channels comprising different connexins can exhibit different properties with regard to ionic conductance or intercellular metabolic coupling [21,23]. In addition, connexins may interact with cytoskeletal components thus regulating signal transduction processes [24,25]. For example, it has been shown that the cytoplasmic tail of Cx43 binds to ZO-1, ZO-2 and ZO-3, components of the membrane-cytoskeletal complex also associated with adherens and tight junctions [13,26]. Moreover, it is known that connexins may be located in the plasma membrane as undocked connexons allowing an exchange of molecules between the cytoplasma and the environment [27].

Summarizing these aspects, although the different cell–cell junctions comprise different proteins, there are similarities in the roles of specialized transmembrane proteins in forming extracellular adhesive contacts between cells, and intracellular links to the cytoskeleton and signaling pathways which may regulate gene transcription. Thus, all junctions, in addition to their classical function, may also be involved in processes such as proliferation and cell differentiation. Moreover, knowledge about the interaction between the different cell–cell contacts is continuously expanding and may have an impact also on endometrial physiology.

3. Cell–Cell Junctions in the Cyclic Human Endometrium

In the course of the menstrual cycle, both compartments of the endometrium, the epithelial and the stromal cells, undergo considerable hormonally regulated changes in preparation for embryo implantation. The luminal epithelium is transformed from a non-receptive to a receptive state, which allows adhesion and invasion of the trophoblast [3,28], while the glandular epithelium produces the components of the uterine fluid indispensable for the survival of the early embryo [29]. In parallel, the stromal cells differentiate to pre-decidual cells in preparation for trophoblast invasion [4]. Cell–cell contacts mainly have been described in the endometrial epithelial cells, providing and maintaining the polarity of the cells. In addition, they regulate the permeability of the epithelial cells controlling the exchange between the uterine lumen and the endometrium to create an environment which is appropriate for embryo implantation, and they are involved in the timely regulation to transform the luminal epithelium into a receptive state in which this physical barrier can be overcome by the blastocyst.

Already in 1982, freeze-fracture electron microscopy had revealed tight junctions in the luminal epithelial cells of the human uterus showing a decrease in junctional complexity from the late proliferative/early luteal phase to the late luteal phase of the menstrual cycle [30,31]. In parallel, the amount of desmosomes is reduced during this phase [32]. Using electron microscopy, Pan and co-workers also described tight junctions in the glandular epithelium of the secretory phase endometrium located apically at the basolateral membrane [33]. Moreover, the density of tight junctions was also shown to be reduced in the microvascular endothelium of the secretory phase endometrium compared to the proliferative phase [34].

With increasing knowledge about the composition of cell-to-cell junctions and the discovery of the high variety of proteins involved in these complex structures, knowledge about the changes in cell junction composition became more differentiated and complex in recent years. As members of the tight junction complex, up to now, claudin-1, -2, -3, -4, -5 and -7 have been described in human endometrial epithelial cells. Immunohistochemical staining revealed a localization of the claudins apically in the glandular epithelial cells while weak or no staining has been described in luminal epithelial cells [33,35–37]. In contrast, no staining for claudins was found in the endometrial stromal cells. These observations have also been described in vitro, since claudin-1, -3, -4 and -7 were detected in primary culture of isolated human endometrial epithelial but not in stromal cells [38]. Different results have been presented regarding the regulation of claudin expression throughout the phases of the menstrual cycle. While Gaetje and co-workers found no cyclic regulation of claudin-1 and -5 on transcript level by microarray analysis, the expression of claudin-3, -4 and -7 increased in the mid secretory phase compared to the proliferative/early secretory phase. However, this regulation could not be verified by immunohistochemical staining [36]. In contrast, the group of Sobel described a significant upregulation only of claudin-4 transcripts and an increase in claudin-1 and -5 protein in the secretory phase [35]. In contrast to that, other groups did not observe any regulation of claudin-3 and -4 throughout the menstrual cycle [33,37,39]. Interestingly, in women undergoing IVF treatment, the absence of claudin-4 in the presence of leukemia inhibitory factor (LIF) in the endometrium could be correlated with a 6-fold higher probability of successful establishment of pregnancy compared to samples that exhibited a strong claudin-4 and a weak LIF expression [40].

In addition to claudins, the tight junctional transmembrane protein JAM-1 was found at the basolateral membranes of the luminal and glandular epithelium [41] and also in a cytoplasmatic location in the glandular epithelium in both proliferative and secretory phase human endometrium [42]. As part of the cytoplasmatic component of the tight junctions, ZO-1 showed a distinct staining at the most apical part of the basolateral cell membrane of the endometrial epithelial as well as in endothelial cells [37]. For both proteins, JAM-1 as well as ZO-1, no change in the location or expression level could be observed throughout the different phases of the menstrual cycle [37,41,42].

Analyzing adherens junctions and desmosomes, a change in the localization of the cytoplasmatic plaque proteins desmoplakin 1 and 2, and the transmembrane cadherin desmoglein 2 from the

apical pole of the lateral cell membrane in the proliferative phase to an evenly distributed pattern along the lateral cell membranes in the mid- to late luteal phase of the menstrual cycle has been described in the glandular epithelium of human endometrial tissue, while mRNA levels stayed constant. This redistribution was limited to the functionalis layer of the endometrium [37].

Immunostaining for E-cadherin and beta-catenin revealed a subapical localization at the lateral membrane of the glandular epithelial cells during the late proliferative and early luteal phase which disappeared during the mid-to late-luteal phase [37]. Also here, no significant changes in mRNA levels in could be detected throughout the cycle [37,43].

Gap junctions in the human endometrial epithelial cells were shown to consist of Cx26 and Cx32 [44–46]. An increasing intensity of Cx26 staining was observed in the uterine epithelial cells during the course of the proliferative phase, but it could hardly be detected in the secretory phase, including the receptive window. For Cx32, a weak expression could be observed at the basal portion of the epithelial cells which decreased during the receptive phase [44], whereas other studies demonstrated an increase of Cx32 in the early secretory and a decrease in the late secretory phase [46]. In contrast to tight and adhesion junctions, gap junctions are also present in the endometrial stromal cells. These channels are composed of Cx43. Like the endometrial connexins, the level of the Cx43 protein in the endometrial stromal cells has also been shown to decrease during the secretory phase [44]. However, while these publications point to a strong reduction of stromal intercellular communication during the receptive phase, others described an upregulation of Cx43 protein in the secretory phase [47].

Summarizing these findings, the amount and localization of the different junctional proteins change throughout the cycle, especially in the epithelial compartment, due to the hormonally regulated transformation of endometrial function. These changes are summarized in Table 1. Controlling the permeability of the uterine epithelium to establish an optimal milieu for the developing embryo and regulating endometrial receptivity to allow or prevent embryo implantation might be crucial functions of junctional components in the uterus. Though the morphology of the tight junction network gives an indication of their function in the epithelium, the claudin content is the parameter which ultimately determines the permeability characteristics [35,48]. Thus, the combination and ratio of the different claudins may be a key factor controlling embryo implantation. In contrast, up to now, only few junctional proteins have been described in the endometrial stromal cells. It is known that these cells express Cx43, but the findings about the cyclic changes are contradictory. However, the stromal cells undergo complete decidualization only during pregnancy. Here, the decidua plays an important role in embryo implantation and development. Thus, the amount and distribution of junctional proteins may considerably change during the implantation process.

Table 1. Distribution of junctional proteins in cycling human endometrium.

Junctional Component	Analyzed Parameter	Localization	Regulation	Reference
Claudin-1	mRNA		Not regulated	[36]
	Protein	GE	Upregulated in SP	[35]
Claudin-3	mRNA		Upregulated in mid SP	[36]
	Protein	GE	Not regulated	[33,39]
Claudin-4	mRNA		Upregulated in mid SP	[35,36]
	Protein	GE	Not regulated	[33,35,37,39]
Claudin-5	mRNA		Not regulated	[36]
	Protein	GE	Upregulated in SP	[35]
Claudin-7	mRNA		Upregulated in mid SP	[36]
ZO-1	mRNA		Not regulated	[37]
	Protein	GE	Not regulated	[37]
JAM-1	mRNA		Not regulated	[41]
	Protein	GE	Not regulated	[41,42]
Desmoplakin 1/2	Protein	GE (functionalis)	Change of localization	[37]
Desmoglein 2	Protein	GE (functionalis)	Change of localization	[37]

Table 1. *Cont.*

Junctional Component	Analyzed Parameter	Localization	Regulation	Reference
E-cadherin	mRNA	GE	Not regulated	[37]
	Protein		Downregulated in SP	[37]
β-Catenin	Protein	GE	Downregulated in SP	[37]
Cx26	Protein	LE/GE	Downregulated in SP	[44]
Cx32	Protein	GE	Downregulated in mid SP	[44]
			Upregulated in early SP/ Downregulated in late SP	[46]
Cx43	Protein	Stromal cells	Downregulated in SP	[44]
			Upregulated in SP	[47]

GE = glandular epithelium; LE = luminal epithelium; SP = secretory phase.

4. Hormonal Regulation of Endometrial Junctional Proteins

The findings described above indicate that some of the junctional proteins are regulated throughout the menstrual cycle. To gain more detailed insight into this hormonal regulation, experiments in animals as well as in vitro have been performed.

A direct influence of hormonal stimulation on the structure of tight junctions has been described in a freeze fraction study in ovariectomized rats. Application of estrogen resulted in an apical shift of these junctions at the lateral epithelial cell membrane, whereas additional application of progesterone led to their extension down the lateral membrane [49]. Looking at the junctional components, ZO-1 was present nearly throughout the course of the cycle in the rat uterine epithelial cells, but was co-localized with occludin only during the estrogen-dominated proestrous phase, while occludin was absent in tight junction structures during the other phases of the estrous cycle. In parallel, a change in localization of claudin-1, -3, -5 and -7 has been described throughout the estrous cycle in the rat [50]. Similar to the rat, a differential regulation of claudins 1-4, occludin, as well as the adherens junction proteins E-cadherin and beta-catenin has also been shown in the endometrium of ewes throughout cycling [51]. It has been shown that claudin-3 protein is upregulated by progesterone in uterine epithelial cells of ovariectomized mice [52] and claudin-5 is downregulated by estrogens in murine endometrial endothelial cells [53]. In the latter study, it was supposed that claudin-5 regulation may play a role in the development of uterine edema, possibly mediated by estrogen-induced expression of the vascular endothelial growth factor (VEGF). An estradiol-induced VEGF-effect on tight junctions has also been shown in the baboon uterine endometrium by increasing the microvascular paracellular cleft width [54].

Using primary culture of human endometrial epithelial cells, an upregulation of claudin-1, -3, -4 and -7 protein content by progesterone and an inhibition of this upregulation by estradiol was observed. Furthermore, the barrier function of the tight junction as measured by the transepithelial electrical resistance decreased under the influence of progesterone, but not estradiol, while the treatment did not affect the fence function as determined by BODIPY-sphingomyelin diffusion in the membrane [38]. From these studies it has been concluded that the barrier function regulating paracellular permeability of the tight junctions can be varied by hormonal changes to provide an adequate environment for successful fertilization and early embryo development. However, in another study claudin-3 expression was upregulated by both progesterone and estradiol in the human endometrial epithelial cell line ECC-1 [52], indicating a regulation of the protein which differs slightly from the regulation in primary human endometrial epithelial cells and rodents.

The hormonal regulation of endometrial gap junction connexins has been studied in a variety of species. Rodents revealed a similar expression pattern like humans showing Cx26 in the uterine epithelium and Cx43 in the stromal cells [45]. Here, it could be proven that Cx26 and Cx43 protein expression are induced by estrogen and suppressed by progesterone [55–57], the latter leading to a complete suppression of gap junction protein expression during the receptive phase of the

endometrium [45]. Moreover, it was shown that Cx26 in the endometrial epithelium reveals a high sensitivity to the ratio of progesterone to estradiol, since Cx26 can be re-induced in the uterine epithelium by increasing estradiol levels despite high progesterone concentrations [57]. Experiments in estrogen receptor-alpha and -beta knockout mice indicated that this upregulation was mediated via the estrogen receptor-alpha [58]. The endometrial Cx26 shows not only a high sensitivity to estradiol, but also to the strong estrogen agonist diethylstilbestrol (DES) and to selective estrogen receptor modulators like tamoxifen or raloxifene, as well as to the phytoestrogen genistein. These compounds also act via the estrogen receptor since their effect was inhibited by simultaneous application of an antiestrogen [59]. In this study, it was proven that phytoestrogens are able to shift the endometrial gene program even at a relatively low dose, and that the Cx26 gene expression in the rat endometrium can serve as a model for biological activity of estrogens. It is known that phytoestrogens reveal multiple biological effects, including beneficial effects on osteoporosis, on the cardiovascular system and on menopausal symptoms, but it has to be taken into account that they may also shift the hormonal homeostasis, and thus cause severe reproductive tract disorders, including impaired fertility.

To sum up, the distribution and function of the junctional protein complexes are partly sensitive to hormonal regulation. These results are summarized in Table 2. Although the precise mechanisms underlying junctional regulation in the human endometrium are not fully understood yet, a precise regulation of the different junctional components seems to be important for cyclic remodeling of the endometrium and as a consequence for its function during pregnancy.

Table 2. Hormonal regulation of junctional proteins in the endometrium.

Junctional Component	Species	Localization	Regulation	Reference
Claudin-1	Human Rat	Primary hEEC Epithelial cells	Upregulated by P/inhibited by E Change in localization	[38] [50]
Claudin-3	Human Rat	Primary hEEC	Upregulated by P/inhibited by E Change in localization	[38] [50]
Claudin-4	Human	Primary hEEC	Upregulated by P/inhibited by E	[38]
Claudin-5	Mouse Rat	Endothelial cells Epithelial cells	Downregulated by E Change in localization	[53] [50]
Claudin-7	Human Rat	Primary hEEC Epithelial cells	Upregulated by P/inhibited by E Change in localization	[38] [50]
Zo-1	Rat	Epithelial cells	Not regulated	[50]
Occludin	Rat	Epithelial cells	Upregulated by E	[50]
Cx26	Rat	Epithelial cells	Upregulated by E/inhibited by P	[55–57]
Cx43	Rat	Stromal cells	Upregulated by E/inhibited by P	[55–57]

hEEC = human endometrial epithelial cells; P = progesterone; E = estrogen.

5. Cell–Cell Junctions during Implantation and Decidualization

During the luteal phase of the menstrual cycle, the human endometrium is transformed to the receptive state to allow adhesion and invasion of the trophoblast. For successful implantation, however, gene expression in the endometrium in addition is regulated by the implanting blastocyst by precisely synchronized embryo-maternal interactions [60–62]. Both compartments of the endometrium are involved in this process: on the one hand, the epithelium has to allow adhesion of the embryo and invasion through the epithelium, on the other hand the stromal cells must be transformed to decidua cells which regulate trophoblast invasion and provide the placental blood supply necessary for embryo nutrition. In humans, the distribution and function of the various junctional proteins are not fully understood yet, although their importance in the process of implantation and pregnancy is eminent. Since these early embryo-maternal interactions cannot be investigated in humans, numerous studies have been conducted in various animal species, most of them in mouse models.

5.1. Changes in Epithelial Junctions during Embryo Implantation

In the rodent, the blastocyst reaches the uterine lumen on 4.5 dpc in the mouse and on 5 dpc in the rat. Degradation of the luminal epithelium and start of trophoblast invasion is observed from 5.5 dpc (mouse) or 6 dpc (rat) onwards [61,63]. Freeze fraction studies revealed that strands of tight junctions expand during the preimplantation phase of pregnancy on the lateral membrane of the uterine epithelial cells in pregnant rats [64] and in pseudopregnant rabbits [65]. In the rat, ZO-1 has been localized along the apical region of lateral plasma membranes of uterine epithelial cells from day 1 to 6 pc. In these stages of pregnancy, the claudin-1 protein was co-localized with ZO-1 in the apical region of the lateral plasma membrane and revealed a strong increase on day 6 pc. In parallel, occludin expression, which was absent on day 1–3 pc, was strongly induced at the time of uterine receptivity on 6 dpc in this apical region [66]. It has been discussed that occludin in uterine luminal epithelium may interact with claudins to form tight junction connections that control the volume and composition of uterine luminal fluid at the time of implantation to facilitate embryo implantation [67]. This is supported by the finding that besides claudin-1 also claudin-4 proteins increased from day 1 to day 6 pc in the luminal epithelium of the rat endometrium [66,67]. While claudin-3 revealed a consistent strong staining during this early phase of pregnancy both in glandular and luminal epithelial cells in the rat endometrium [67], it was shown in mice that the subcellular localization of claudin-3 and -7 switched from an apical and basal distribution to a strongly apically localization on day 4.5 pc in the luminal epithelium [52,68]. In contrast, high amounts of claudin-10 protein were present in the glandular epithelium in mice, but this claudin was absent in the luminal epithelium during the preimplantation period [68]. In these studies, however, it was not proven that these changes are dependent on the presence of a blastocyst.

Desmosomes [69], hemidesmosomes [70] and adherens junctions [71,72] were described to decline in the preimplantation period, presumably facilitating trophoblast invasion through the epithelial barrier. However, when E-cadherin was conditionally knocked out in the uterus, those mice revealed an implantation failure supposable due to the impairment of blastocyst adhesion to the luminal epithelium [73].

Similar to the situation in humans, gap junction intercellular communication is suppressed in the rodent uterine epithelium during the receptive phase. Prior to implantation, however, Cx26 is induced locally restricted to the luminal epithelium of the implantation chamber [45,56]. This has been shown to be due to a local effect of the blastocyst, since this antigen as well as the corresponding transcripts were neither detected in the inter-blastocyst segments nor in pseudopregnant animals [45,56,74]. This specific blastocyst-mediated induction of Cx26 was shown to act via an estrogen receptor-independent pathway and could also be induced by a mechanical stimulus in the hormonally primed receptive endometrium [58]. Experiments with pseudo-pregnant uteri in organ culture revealed that an inflammatory cascade may be involved in this blastocyst-mediated up-regulation of Cx26 in the uterine epithelium [58]. Such a locally restricted induction of gap junction proteins by the blastocyst in the non-coupled receptive uterine epithelium has also been described in other mammalian species like the rabbit (Cx32) [75] and the ewe (Cx26) [76]. It has been proposed that the restricted expression of Cx26 in the epithelium of the implantation chamber regulates the controlled cell death of the uterine epithelium accompanying the implantation process in the rodents [77]. However, the specific role of this precisely spatially and timely regulated connexin suppression and induction for implantation still remains to be elucidated. It was shown that embryo implantation was impaired by injection of a non-specific gap junction channel blocker [78], however, since this compound not only blocked epithelial but also stromal gap junction channels, a specific role for the epithelial induction of gap junctional communication could not be proven in this study.

The crucial function of junctional components in the uterine epithelium might be to control epithelial permeability and thus the uterine milieu as well as regulating trophoblast adhesion to and penetration through the epithelial lining, whereby the various junctional proteins may exhibit different functions. There is evidence that tight junctions are the only junctional complexes that are maintained during the implantation window. The tight connection of the epithelial cells may maintain an optimal uterine micro milieu for the developing blastocyst during the sensitive phase of implantation [79,80].

In contrast, components of adhesion and gap junctions decrease during preimplantation, possibly to facilitate trophoblast invasion through the epithelial barrier, though some of them, like E-cadherin, may be necessary for successful blastocyst attachment.

5.2. Changes in Stromal Junctions during Decidualization

In preparation for embryo implantation, not only does the uterine epithelium have to differentiate into a receptive state to allow adhesion and invasion of the trophoblast, but the endometrial stromal cells also undergo a complex differentiation process. They transform to decidual cells, which regulate trophoblast invasion, may be involved in the selection of competent embryos, and, moreover, support angiogenesis to build up an extensive vascular network, which is essential for placental blood supply and successful embryonic development [4,81]. Thus, an adequate decidualization process is indispensable for successful embryo implantation and development [82]. During this process, the endometrial stromal cells undergo phenotypic changes reminiscent of mesenchymal-epithelial transition leading to epitheloid cells [83–85], accompanied by changes in expression and localization of numerous cell–cell contact proteins.

In the pre-decidual cells of the human luteal phase endometrium, up to now, only the gap junction protein Cx43 has been described, while there is no knowledge about junctional proteins in the human decidual cells during the early stages of pregnancy. When decidualizing human endometrial stromal cells in vitro, it has been shown that the expression of originally epithelial proteins, including beta-catenin, E-cadherin and ZO-1, is redistributed to the decidualized stromal cells [86], supporting the mesenchymal-epithelial transition. An important role of this induction of E-cadherin for the decidualization process has been demonstrated in mice that lack uterine E-cadherin. In these mice, no decidual response could be observed when decidualization was artificially induced [73].

In rodents, decidualization of endometrial stromal cells is induced with the start of the implantation process. First, the stromal cells surrounding the implantation chamber differentiate to the avascular primary decidual zone, encapsulating the implanting embryo [87]. Here, it has been shown that tight junction proteins are induced during this mesenchymal-epithelial transition of the endometrial stromal cells. However, freeze-fracture studies revealed incomplete tight junctions in the primary decidual zone which were supposed to function as semipermeable barriers to allow the transport of large molecules paracellularly through this compact zone to the developing embryo [88]. Meanwhile, the tight junction proteins occludin, ZO-1, ZO-2 and claudin-1 were demonstrated to form associated complexes in these decidual cells of the primary decidual zone on day 6 pc, forming a barrier surrounding the embryo concurrently with the loss of the adjacent luminal epithelium [87]. Moreover, a strong induction of claudin-10 was observed in the primary decidual cells already on day 4.5 pc—thus, prior to trophoblast invasion—and expanded to the secondary decidua thereafter [68]. From 6.5 dpc onwards, additionally, an intense staining for the claudin-3 protein appeared in the cells of the secondary decidua [52,68], which was co-localized with the endothelial cell marker CD31 towards the mesometrial part of the implantation site [68]. Since Claudin-3 has been described as taking part in building up the blood-brain barrier in endothelia of the central nervous system [89], this protein distribution also could be involved in protecting the implantation site from immunoreactive substances originating from the maternal circulation.

To determine which component of a blastocyst is necessary to induce expression of tight junctional proteins in the decidua, Wang and colleagues examined the expression of various proteins of the tight junction complex in the presence of either a normal blastocyst, trophoblast vesicles or isolated inner cell mass [87]. While blastocysts and trophoblast vesicles induced a similar expression of tight junctional proteins, isolated inner cell mass failed to initiate such a reaction. From these findings, the authors concluded that the trophectoderm appears to be the stimulus for the establishment of the barrier surrounding the embryo.

Besides the induction of these junctional proteins, the decidual cells are also extensively connected by gap junctions. In human, baboon and rodent endometrium, Cx43 is the dominantly expressed gap junction subunit in the stromal compartment [90]. In rodents, stromal Cx43 is suppressed during the

receptive phase and increases considerably during decidualization starting in the primary decidual zone and then spreads out throughout the implantation chamber with ongoing decidualization [45,56]. In rats, but not in mice, in parallel Cx26 is induced in the decidual cells [45]. The presence of Cx43 in the decidua is important for the transformation of stromal cells into the compact decidua, as well as for the formation of new maternal blood vessels in the stromal compartment, which is critical for the establishment and maintenance of pregnancy. This has been proven in mice displaying a conditional deletion of Cx43 in the endometrial stromal cells. This deletion inhibited the transformation of the endometrial stromal cells to decidual cells, concomitant with induction of apoptosis [91], and impaired decidual angiogenesis, resulting in the arrest of embryo growth and early pregnancy loss [92]. Decidual angiogenesis may also be influenced by Cx43 in the uterine vascular endothelium which is involved in cell signaling regulation of uterine blood flow [93]. The important role of this intercellular communication for the decidualization process has been confirmed in human endometrial stromal cells in vitro. Here, knockdown of Cx43 or pharmacological disruption of gap junctional communication impaired decidualization as substantiated by inhibition of secretion of prolactin and VEGF as well as of the expression of markers for mesenchymal-epithelial transition [86,94]. In contrast, overexpression of Cx43 in human endometrial stromal cells led to an upregulation of markers for mesenchymal-epithelial transition as well as of VEGF and ZO-1. In parallel, the expression of N-cadherin as an indicator of epithelial-mesenchymal transition was inhibited [86].

Summing up these findings, there is a considerable induction of various junctional proteins during decidualization which build a selective barrier towards the embryo after breakdown of the epithelial barrier. These proteins play an important role in paracrine signaling within the decidua to sustain differentiation and to support angiogenesis in the maternal compartment as a prerequisite for nutrition of the growing embryo. The clinical significance of these observations is supported by findings that Cx43 levels are reduced in the decidua of women with recurrent early pregnancy loss [95] and by the fact that the anti-malarial medication mefloquine, which blocks Cx43 gap junctions, is associated with an increased risk of spontaneous abortion [96]. Moreover, impaired endometrial decidualization is increasingly attributed to pathophysiological conditions associated with reduced fecundity and pregnancy complications. These include endometriosis, polycystic ovary syndrome, recurrent miscarriage, pre-eclampsia, and preterm birth [81,97–101].

In conclusion, there is a precise temporal and spatial regulation of various junctional proteins in the epithelial, as well as the stromal, compartment of the endometrium during the implantation process (summarized in Table 3). Though the definite role of these proteins in this context has not been deciphered in detail yet, its precise regulation assumes a considerable role in endometrial function, and disruption of these patterns, were shown to lead to impairment of the implantation process or of placental and fetal development.

Table 3. Regulation of junctional proteins during implantation and decidualization.

Junctional Component	Species	Localization	Regulation	Reference
Claudin-1	Rat	Epithelial cells	Increased on 6 dpc	[66]
Claudin-3	Mouse	Decidual cells	Induced on 6.5 dpc Change of localization on 4.5 dpc	[52,68] [68]
Claudin-4	Rat	Epithelial cells	Increase from 1–6 dpc	[67]
Claudin-10	Mouse	Decidual cells	Induced on 4.5 dpc	[68]
Occludin	Rat	Epithelial cells	Induced on 6 dpc	[66]
Cx26	Rat	Epithelial cells Stromal cells	Induced on 5 dpc Induced on 6 dpc	[45] [45]
	Mouse	Epithelial cells	Induced on 4.5 dpc	[58]
Cx43	Rat	Decidual cells	Increased during decidualization	[45]
	Mouse	Decidual cells	Increased during decidualization	[58]

Dpc = days *post coitum*.

6. Direct Cell–Cell Interactions in Endometrial Pathophysiology

6.1. Endometriosis

Endometriosis is characterized by endometriotic tissue growing outside the uterine cavity, affecting 10–15% of women of reproductive age and even up to 50% of women seeking infertility treatment. Although it is a benign endometrial disease, it leads to severe clinical symptoms such as abdominal pain and subfertility [102]. One cause for the ectopic colonization and growth of endometrial tissue may origin in an inappropriate differentiation of the endometrial cells leading to an increase in adhesiveness and invasiveness of the endometriotic tissue. This impairment of differentiation may affect the epithelial-to-mesenchymal or mesenchymal-to-epithelial transition in the endometrial tissue [103], which physiologically is accompanied by a highly regulated expression pattern of intercellular junctional complexes. This has been supported by a morphometric study showing that tight junctions were missing or disrupted in endometrioma compared to eutopic endometrium [39]. In a more recent study, microarray analyses revealed an upregulation of transcripts of JAM-B and JAM-C and of claudin-1, -5 and -11 and a downregulation of ZO-3, occludin and claudin-3, -4 and -7 in peritoneal endometriotic lesions compared to the corresponding eutopic endometrium [104]. However, these observations were not validated by PCR or immunohistochemical staining. In accordance with these findings, Pan and colleagues noted a significantly lower expression of claudin-3 and -4 in ovarian endometrioma compared to eutopic endometrium originating from patients with endometriosis and from healthy controls on mRNA and protein level [39]. In contrast, immunohistochemical analysis showed a decreased staining for claudins-1 and -5 in epithelial cells of peritoneal endometriotic lesions [36].

One hallmark of epithelial-to-mesenchymal transition is the functional loss of E-cadherin expression in epithelial cells. A reduction of E-cadherin, as well as alpha- and beta-catenin, expression in peritoneal [105–107] and ovarian endometriosis [108] compared with the eutopic endometrium has been described. This is supported by studies demonstrating that E-cadherin-negative epithelial cells were increased in peritoneal endometriosis compared with eutopic endometrium and that in vitro, E-cadherin-negative, but not E-cadherin-positive epithelial cells, showed invasive growth [109]. Thus, the loss of E-cadherin expression could constitute a crucial mechanism in the pathogenesis of endometriosis by increasing the invasiveness of endometriotic cells.

Moreover, the inappropriate differentiation of endometrial tissue in endometriosis patients is correlated with an aberrant expression of gap junction connexins. In the eutopic endometrium of women with endometriosis a significant decrease in Cx43 has been described, which correlated with a decrease in physiological cell–cell coupling, while no changes in Cx26 were observed [110]. In parallel to the decrease in Cx43 expression and cell coupling, in vitro decidualization was impaired in these cells, supporting a role of impaired decidualization in the pathogenesis of endometriosis. An aberrant allocation of connexin proteins has also been described in ectopic endometrial lesions. Here, Cx43 expression was enhanced in the endometriotic glands, whereas the number of patients exhibiting Cx26, typical for human uterine epithelium cells, was strongly reduced, and Cx32 was not detectable [111]. Moreover, Cx43, which is located in the stromal cells of healthy patients, was not present in this tissue compartment in endometriotic lesions. Similar results were obtained in the eutopic endometrium of baboons in which endometriosis had been experimentally induced. Here, a loss of Cx26 and Cx32 in the epithelium and an up-regulation of Cx26 in the stromal cells have been observed [112].

Taken together, the above-described alterations in direct cell–cell interaction may contribute to a change in the differentiation program of both the epithelial and stromal compartment of the endometrium. These alterations are summarized in Table 4. Although endometriosis is considered a 'benign' disease, it resembles the biologic behavior of malignant cells [113,114], and a change in the expression of various junctional proteins may support the invasive properties of this tissue and may facilitate its growth at ectopic localizations. Moreover, these impairments may also contribute to endometriosis-associated infertility.

Table 4. Regulation of junctional proteins in endometriosis.

Junctional Component	Analyzed Parameter	Regulation	Reference
Claudin-1	mRNA	Upregulated in peritoneal lesions	[104]
	Protein	Downregulated in peritoneal lesions	[38]
Claudin-3	mRNA	Downregulated in peritoneal lesions	[104]
		Downregulated in ovarian endometriomata	[39]
	Protein	Downregulated in ovarian endometriomata	[39]
Claudin-4	mRNA	Downregulated in peritoneal lesions	[104]
		Downregulated in ovarian endometriomata	[39]
	Protein	Downregulated in ovarian endometriomata	[39]
Claudin-5	mRNA	Upregulated in peritoneal lesions	[104]
	Protein	Downregulated in peritoneal lesions	[38]
Claudin-7	mRNA	Downregulated in peritoneal lesions	[104]
Claudin-11	mRNA	Upregulated in peritoneal lesions	[104]
Jam-B	mRNA	Upregulated in peritoneal lesions	[104]
Jam-C	mRNA	Upregulated in peritoneal lesions	[104]
Zo-3	mRNA	Downregulated in peritoneal lesions	[104]
E-Cadherin	Protein	Downregulated in peritoneal lesions	[105,106]
		Not regulated in endometriosis	[107]
	mRNA	Downregulated in ovarian endometriomata	[108]
α-Catenin	Protein	Downregulated in peritoneal lesions	[105]
	mRNA	Downregulated in ovarian endometriomata	[108]
β-Catenin	Protein	Downregulated in peritoneal lesions	[105,107]
	mRNA	Downregulated in ovarian endometriomata	[108]
Cx26	Protein	No regulation in eutopic endometrium *	[110]
		Downregulated in peritoneal lesions	[111]
Cx43	Protein	Downregulated in eutopic endometrium *	[110]
		Downregulated in peritoneal lesions	[111]

* = of endometriosis patients.

6.2. Endometrial Carcinoma

Endometrial carcinoma is one of the most frequently diagnosed gynecological malignancies [115]. Based on clinical and histopathological criteria, it is classified in two subtypes. Endometrioid adenocarcinoma (Type I), which accounts for about 80% of cases, is low-grade, estrogen-dependent and usually associated with complex and atypical endometrial hyperplasia, whereas type II endometrial carcinoma include serous papillary and clear cell types and is more aggressive and estrogen-independent [116,117]. For the general pathogenesis and progression of cancer, changes in cell–cell contacts have been described to play an essential role [118–120]. They may act via their intercellular communication functions, but may also exhibit their effect independently from these roles since they may be involved in signal transduction regulating gene expression [121,122].

Also, in the pathogenesis and progression of endometrial cancer, changes in cell–cell junctions have been described. A morphological disruption of tight junctions was observed in endometrial adenocarcinoma, but not in atypical hyperplastic endometrium [33]. In parallel, altered claudin expression has been described in the malignant endometrial tissues in this study. In endometrial adenocarcinoma claudin-3 and -4 mRNA and protein increased with the clinicopathologic features of the tissue, progressing from simple to complex and from atypical hyperplasia to endometrioid carcinoma [33]. Since the upregulation of claudins was already visible in atypical hyperplasia but the morphological degeneration of the tight junctions only in endometrioid carcinoma, it has been supposed that the elevated claudin level precedes the disruption of tight junctions. A significant upregulation of claudin-3, -4 and -7 compared to normal endometrial cells has also been found in primary culture of uterine serous papillary tumor cells, the most aggressive kind of

estrogen-independent type II endometrial carcinoma [123]. In contrast, claudin-5 was significantly decreased in these tumor cells. Beyond this, the presence of different claudin subtypes may differ in the different types of endometrial cancer. By evaluating immunohistochemical scores, low claudin-1 and high claudin-2 protein contents were found in hyperplasia and endometrioid adenocarcinoma (type I), whereas in seropapillary adenocarcinoma (type II), high claudin-1 and low claudin-2 levels were detected [35].

Since claudin-4 and, with a lesser affinity, also claudin-3 act as epithelial receptors for *Clostridium perfringens* enterotoxin (CPE) [124–126], probably mediated by binding to the free second extracellular loop of claudins, they may constitute suitable targets for this anti-cancer drug which may be effective also in tumor cells refractory to chemotherapy [127,128]. This is supported by the finding that the cytotoxicity of CPE was even enhanced in an endometrial adenocarcinoma cell line after upregulation of claudin-3 and -4 [38]. The emerging evidence of the involvement of claudins in the pathogenesis of endometrial carcinoma of various subtypes is consistent with findings concerning the pathogenic role of claudins in a variety of other tumors such as in breast, gastric, pancreatic and prostate cancers [129].

In regard to adhesion contacts, the role of the two adhesion molecules E-cadherin and beta-catenin in the carcinogenesis of endometrial carcinoma has been extensively studied, and the expression of these proteins is discussed as a prognostic marker. Although varying in detail, most studies are consistent that low E-cadherin expression correlates with increasing aggressiveness, poor differentiation, and deep myometrial invasion of the carcinoma [130–139]. In accordance, E-cadherin was found to be more often and prominently expressed in endometrioid adenocarcinoma than in serous papillary or clear cell tumors [131,139,140], and a high E-cadherin level has been associated with reduced mortality, disease progression, and disease recurrence rate and thus is associated with a better prognosis [141]. However, a correlation between clinicopathological factors and the score or intensity of E-cadherin immunohistochemical staining of endometrial carcinoma could not be confirmed in another study [142], advising to carefully control the classical clinicopathologic criteria in regard to E-cadherin expression. Further insights on the role of E-cadherin in endometrioid adenocarcinoma are constantly emerging. For example, in endometrioid endometrial carcinoma the expression of the E-cadherin suppressor Snail was found to be negatively correlated with E-cadherin expression [130] and was correlated with abnormal E-cadherin expression in metastases of this tumor [132].

Also for beta-catenin, a decreased level has been demonstrated with increasing grading of endometrial carcinoma [143]. Moreover, β-catenin gene (*CTNNB1*) mutations led to decreased cell–cell adhesion and have been reported in about 15% of endometrioid carcinomas [144,145]. Since beta-catenin is a transcription factor that is involved in the Wnt signal transduction pathway, which in turn is crucial for carcinogenesis, it may exhibit its effect via this signaling pathway [135].

In addition to the roles of tight and adherens junctions described above, there is substantial evidence that an interruption of gap junctional communication or the aberrant expression of connexins constitutes one important step in carcinogenesis [118]. In endometrial hyperplasia and carcinoma, the amount of Cx26 and Cx32 in the uterine epithelium, as well as Cx43 in the endometrial stromal cells, and, as a consequence, gap junctional communication, is reduced and/or aberrantly localized [146,147]. These studies showed that during endometrial carcinogenesis, loss of gap junctional intercellular communication may occur at relatively early stages. A correlation between a reduced connexin expression and the progression of cancer was supported by the observation that activation of the estrogen receptor-alpha by estrogen, which is a primary etiological factor associated with the development of endometrial hyperplasia and adenocarcinoma, reduced gap junctional intercellular communication, and expression of Cx26 and Cx32 in endometrial carcinoma cells [148]. Interestingly, numerous studies have demonstrated that not only the amount of connexins, but also their localization, may effect tumor growth. Connexins may thus act by other mechanisms than by functional coupling of cells. A mutated form of Cx43, revealing a change in protein sequence of the second extracellular region of Cx43 which prevented incorporation of the protein into the plasma membrane, did not decrease its ability to inhibit the growth of tumor cells in vitro [149]. Thus, regulation of cellular growth by Cx43 does not necessarily require well-functioning gap junctions. This has been affirmed

by several reports describing the tumor-suppressing properties of Cx43 and Cx26 in the absence of functionally coupled channels, possibly by regulating key genes involved in tumor growth [150,151].

The junctional proteins regulated in endometrial carcinogenesis are summarized in Table 5.

In conclusion, the different cell–cell contact proteins may exhibit considerable effects on the pathogenesis of endometrial cancer. Besides ensuring cohesion of a healthy cell structure, they may regulate signaling pathways involved in the pathogenesis and progression of endometrial cancer, and thus may represent promising tools for diagnostic and therapeutic approaches in cancer treatment.

Table 5. Regulation of junctional proteins in endometrial cancer.

Junctional Component	Analyzed Parameter	Tumor Staging	Regulation	Reference
Claudin-1	Protein	Type II (USPC)	Upregulated	[35]
Claudin-2	Protein	Type II (USPC)	Downregulated	[35]
Claudin-3	mRNA	Type I	Upregulated	[33]
		Type II (USPC)	Upregulated	[123]
	Protein	Type I	Upregulated	[33]
Claudin-4	mRNA	Type I	Upregulated	[33]
		Type II (USPC)	Upregulated	[123]
	Protein	Type I	Upregulated	[33]
Claudin-5	mRNA	Type II (USPC)	Downregulated	[123]
E-Cadherin	Protein	Type I/Type II	Downregulated during dedifferentiation	[130–139]
β-Catenin	Protein	Type I/Type II	Downregulated during dedifferentiation	[143]
Cx26	mRNA	Type I	Downregulated	[146]
	Protein	Type I	Downregulated	[146,147]
Cx32	mRNA	Type I	Downregulated	[146]
	Protein	Type I	Downregulated	[146,147]
Cx43	mRNA	Type I	Downregulated	[146]
	Protein	Type I	Downregulated	[146,147]

USPC = uterine serous papillary carcinoma.

7. Conclusions

Direct cell–cell junctions are highly specific and precisely regulated during the physiological changes in the endometrium as well as in pathological conditions. Despite their specific function in cell–cell interaction they also may regulate signaling pathways, thereby influencing gene expression in the different compartments of the endometrial tissue. Moreover, besides close interactions of various proteins within the complex structure of the same junctions and with components of the cytoskeleton, insights are increasing about close relationships between the proteins of different junctional complexes. Thus, a close interaction of the components of the different cell–cell junctions might also play an important role in the different physiological conditions of the endometrium. The number of components building involved in these junctions and their interactions has grown considerably in recent years, and up to now, only some of them have been analyzed in the endometrium. An adequate expression of the different junctional proteins in the endometrium is indispensable, since genetic defects and dysregulation of these interactions can cause different diseases, and may impair the implantation reaction and embryonal or placental development resulting in phenomena like preeclampsia or fetal growth restriction. Still, many questions remain concerning the various functions of junctional proteins and their interactions. Extending our knowledge of these essential functions in endometrial physiology and pathogenesis will provide closer insight in female reproductive health.

Funding: This article received no external funding.

Acknowledgments: The authors thank Jürgen Heger for graphical work.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

CPE	*Clostridium perfringens* enterotoxin
Cx	Connexin
dpc	Days *post coitum*
E	Estrogen
GE	Glandular epithelium
hEEC	Human endometrial epithelial cells
IVF	*In vitro* fertilization
JAM	Junction adhesion molecule
LE	Luminal epithelium
LIF	Leukemia inhibitory factor
MUPP	Multi-PDZ domain protein
P	Progesterone
pc	*Post coitum*
PCR	Polymerase chain reaction
SP	Secretory phase
USPC	Uterine serous papillary carcinoma
VEGF	Vascular endothelial growth factor
ZO	Zonula occludens

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International Journal of
Molecular Sciences

MDPI

Review

Endometrial Stem Cell Markers: Current Concepts and Unresolved Questions

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Received: 2 August 2018; Accepted: 10 October 2018; Published: 19 October 2018

Abstract: The human endometrium is a highly regenerative organ undergoing over 400 cycles of shedding and regeneration over a woman's lifetime. Menstrual shedding and the subsequent repair of the functional layer of the endometrium is a process unique to humans and higher-order primates. This massive regenerative capacity is thought to have a stem cell basis, with human endometrial stromal stem cells having already been extensively studied. Studies on endometrial epithelial stem cells are sparse, and the current belief is that the endometrial epithelial stem cells reside in the terminal ends of the basalis glands at the endometrial/myometrial interface. Since almost all endometrial pathologies are thought to originate from aberrations in stem cells that regularly regenerate the functionalis layer, expansion of our current understanding of stem cells is necessary in order for curative treatment strategies to be developed. This review critically appraises the postulated markers in order to identify endometrial stem cells. It also examines the current evidence supporting the existence of epithelial stem cells in the human endometrium that are likely to be involved both in glandular regeneration and in the pathogenesis of endometrial proliferative diseases such as endometriosis and endometrial cancer.

Keywords: endometrium; adult stem cells; endometrial regeneration; stem cell markers; endometriosis; endometrial cancer

1. Introduction

The human endometrium is a highly regenerative organ undergoing over 400 cycles of shedding and regeneration over a woman's life time [1–3]. Menstrual shedding, and the subsequent repair of the endometrial functionalis, is a process unique to humans and higher-order primates [4,5] (Figure 1A,B). The endometrium re-grows from a mere 1–2 mm thickness after menstrual shedding to 14 mm thickness in the secretory phase of the menstrual cycle [6], and is able to completely regenerate after parturition, and in post-menopausal (PM) women when exposed to oestrogen replacement therapy [2,7]. Even after extensive iatrogenic destructive procedures such as ablation [8], the endometrium regrows in some women who continue to bleed (25–75%) [9,10]. This huge regenerative ability suggests that the endometrium has a stem cell basis that supports the tissue maintenance/regrowth.

Prianishnikov was the first to consider the existence of endometrial adult stem cells (ASCs) and, in 1978, he proposed ASCs to reside in the deeper basalis layer, with their differentiation marked by functional changes (acquiring) in hormonal receptivity [11]. He suggested a hierarchical hormone receptiveness in endometrial cells, matching their level of maturity, and therefore, the most primitive hormone-independent ASCs initially differentiate first into oestrogen-dependent cells, and then they may further differentiate into both oestrogen and progesterone-dependent cells.

Terminally-differentiated cells were expected to be only progesterone-dependent, and were postulated to have a limited lifespan [11] (Figure 2).

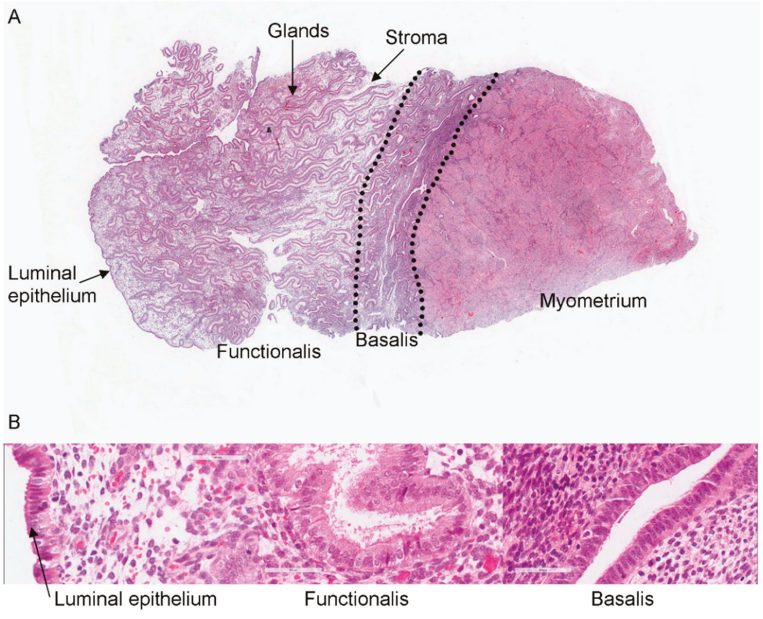

Figure 1. (**A**): Low power (100×) micrograph of full thickness biopsy of human endometrium and sub-endometrial myometrium stained with Hematoxylin and Eosin. (**B**): Representative micrograph depicting the distinct anatomical areas in the human endometrium, luminal epithelium, functionalis, and basalis (magnification 400×).

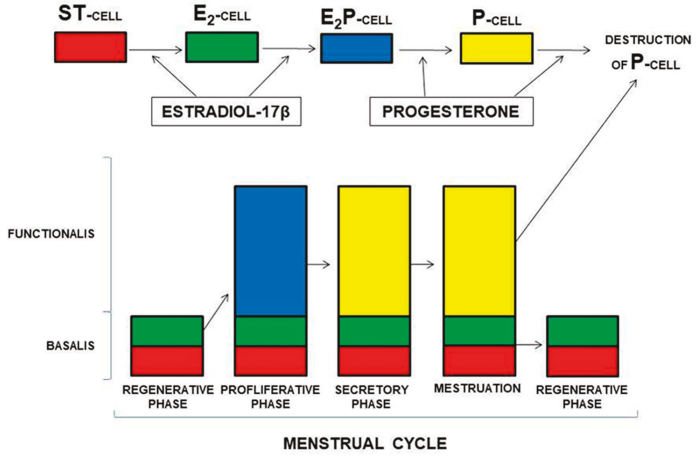

Figure 2. Hormone dependent regulation of the ratio of cell types in the endometrium (Abbreviations ST-cell—stem cell, E2-cell—oestrogen sensitive cell, E2P-cell—oestrogen-progesterone sensitive cell, P-cell—progesterone sensitive cell). Figure adapted from Reference [11].

This hypothesis proposing that the human endometrium regenerates from the deeper basalis layer (which is the proposed germinal compartment that persists after menstruation and is responsible for the regeneration of the new upper/superficial functionalis layer) has been echoed many times [12–15].

Identifying human endometrial epithelial stem cells is problematic due to the lack of specific markers for isolating and examining them for functional properties [16]. Endometrial epithelial cells in particular are difficult to culture in vitro for long durations, with the in vivo phenotype of these cells not maintained using traditional 2D culture methods. For this reason, functional assays that have been developed to examine the stem cell properties in vitro, may not be suitable for endometrial epithelial cells. Furthermore, the true and conclusive confirmation of an endometrial epithelial stem cell requires the demonstration that they are able to produce all of the epithelial cell types that exist in all regions of the endometrium. However, the characterisation of all different endometrial epithelial cell subtypes, to ascertain the mature progeny of the putative stem cell, is not yet complete in the human endometrium.

Early work on endometrial regeneration has been gleaned through scanning electron microscopy (SEM) studies, this includes some evidence for the existence of endometrial epithelial stem cells. SEM studies have confirmed that the total regeneration time for postmenstrual surface re-epithelialisation is approximately 48 h [17] (with the regenerative period beginning between cycle days 2 and 3, and ending between cycle days 4 and 5). This time interval coincides with the maximum area of the denuded basalis [17]. The SEM data also suggests that the surface epithelial repair occurs by simultaneous and progressive outgrowth from the remaining stumps ("mouths") of basal glands, and by outgrowth from the surface epithelium, adjacent to the denuded areas, that has not been lost from the isthmic and cornual regions [17]. A similar observation was reported in rabbit endometrium; the newly restored surface lining was derived from the intact surface epithelium that remained bordering the artificially (rabbits do not menstruate) denuded area [18]. The other important findings of the SEM studies are the descriptions of morphological differences in endometrial epithelial subtypes. For example, ciliated and non-ciliated cells have been observed in the luminal epithelium (LE) [19]. Cells with the same morphology as that of endocrine cells were found in the lower layers of the epithelium at late gestation (clear cells) [20], and are morphologically similar to endocrine cells in other tissues [21]. This further confirms the existence of different epithelial subtypes that are yet to be characterised for the expression of distinct markers or for their possible functional diversity.

2. Scope of This Review

In this review, we will examine the evidence for particular markers to delineate the endometrial stem cell population. We will describe the available evidence under the three main hypotheses that stem cell marker identification in the endometrium has been undertaken, by:

Identifying markers highly expressed in cells with some in vitro stem cell properties (e.g., clonogenic cells, side population (SP) cells).

Identifying markers expressed by cells located in the postulated stem cell niche (basalis and PM glandular epithelium).

Examining endometrium for the expression of putative stem cell markers identified to be expressed in epithelial stem cells (ESCs), or other stem cells of different tissues (e.g., OCT4, Mushashi-1, LGR5, Notch1/Numb).

3. Identifying Markers That Are Highly Expressed in Cells with Some In Vitro Stem Cell Properties

Stromal ASC work was initiated with identifying markers preferentially expressed in the cell populations demonstrating higher clonogenic properties in vitro, by Gargett and colleagues [12]. The in vitro colony-forming efficiency assay they used is thought to be a method that enriches stem cells by inoculating single cell suspensions derived from freshly harvested tissue at low density, in order to generate colonies from individual cells.

Endometrial stromal ASCs were also shown to be capable of multi-lineage differentiation into fat, bone (confirmed with presence of osteopontin, ostenectin and alkaline phosphatase) [22], cartilage, skeletal muscle [23,24], and smooth muscle (expressing specific smooth muscle cell markers including alpha-smooth muscle actin (α-SMA), desmin, vinculin and calponin) [25]. Plasticity has also been shown by the trans-differentiation of endometrial stromal ASCs into neural (neural and glial lineage markers such as Nestin, NF-L, MAP2, PDGFRa, CNP, Olig2, MBP and GFAP) [24,26], Schwann cells (expression of S100 and P75 noted) [27], Oligodendrocytes [28], pancreatic cells (shown by secretion of insulin and markers of β cells such as PDX1, proinsulin and c-peptide) [29,30], urinary bladder epithelial cells (urothelium, as tested by urothelium-specific genes and proteins, uroplakin-Ia/Ib, II, III and cytokeratin 20) [31], hepatocytes (biomarkers albumin and cytokeratin 8, reduced α-fetoprotein and α-SMA expression, synthesised urea, and stored glycogen) [32,33], and megakaryocytes (identified by expression of CD41a and CD42b and reduction of pluripotent transcription factors Oct4 and Sox2, platelets were seen as functional as evidenced by the upregulation of CD62p expression and fibrinogen binding following thrombin stimulation), both in vitro and in animal models [34,35].

Studies employing animal models of Duchenne muscular dystrophy [36], stroke [37], diabetes [38], Parkinson's disease [39,40], and critical limb ischemia [41] have suggested that endometrial stromal ASCs improve outcomes, postulating an in vivo differentiation potential for these cells.

Further in vitro stem cell properties that were utilised to identify endometrial ASCs include examining the side population (SP) cells and label retaining cells (LRC) as detailed below.

3.1. Markers Identified in Clonogenic Cells

3.1.1. CD146 and Platelet Derived Growth Factor–Receptor β Co-Expression

The first markers proposed to identify an endometrial stromal ASC population were CD146 and platelet-derived growth factor–receptor β (PDGF-Rβ) (2 perivascular cell markers) [22]. This is because their co-expression was detected in cells with higher clonogenic ability in vitro. These cells were located in the perivascular area in both the functionalis and the basalis of the intact full thickness human endometrium [42]. In vitro FACS sorted $CD146^+/PDGF\text{-}R\beta^+$ cells had significantly greater colony-forming capabilities than $CD146^-/PDGF\text{-}R\beta^-$ cell populations ($7.7 \pm 1.7\%$ versus $0.7 \pm 0.2\%$ respectively) [42]. $CD146^+/PDGF\text{-}R\beta^+$ cells produced more large colonies with densely packed cells and a high nuclear:cytoplasmic ratio. The $CD146^+PDGF\text{-}R\beta^+$ cells expressed typical mesenchymal stem cell (MSC) surface markers such as CD29, CD44, CD73, CD90 and CD105, and were negative for haematopoietic (CD34, CD45) and endothelial markers (CD31) [42]. When cultured in appropriate induction media, the $CD146^+PDGF\text{-}R\beta^+$ cells underwent multi-lineage mesenchymal differentiation into adipogenic, myogenic, chondrogenic, and osteoblastic lineages [42]. However, these studies used pooled, clonally-derived $CD146^+PDGF\text{-}R\beta^+$ cells (not singly expanded clones) and did not use positive control cells with known multi-lineage differentiation potential (such as human mesenchymal stem cells (hMSCs)) to determine their true differentiation ability. Apart from the colony-forming capacity, and multi-lineage differentiation ability, the authors did not examine other stem cell-related features and functions of the $CD146^+PDGF\text{-}R\beta^+$ cells. Therefore, we are not able to comment on their capacity to produce endometrial stroma either in a more physiologically relevant 3D culture in vitro, or their in vivo tissue reconstitution ability. Therefore, it is difficult to decisively conclude that these cells are an ASC population that regenerates the endometrial stroma. However, it is thought that the $CD146^+PDGF\text{-}R\beta^+$ subpopulation are similar to bone marrow and adipose tissue MSCs in their differentiation potential, and their perivascular location is also shared by MSCs in many other organs [43].

3.1.2. SUSD2

Sushi domain containing-2^+ ($SUSD2^+$) (also known as W5C5) was the next endometrial stromal ASC marker to be reported and it was particularly successful in selecting endometrial MSCs [44].

SUSD2$^+$ cells represented 4.2 ± 0.6% of the freshly sorted endometrial stromal cells using flow cytometry, and assumed a peri-vascular location both in the basalis, and functionalis, with a significantly greater clonogenicity (median 3.6: range, 0.7–6.9) than depleted counterparts (median 0.6: range, 0.1–3.8). W5C5$^+$ cells were able to be differentiated into adipocytes, osteocytes, chondrocytes, myocytes, and endothelial cells (no MSCs were used as an external control), producing endometrial stromal-like tissue in vivo. SUSD2$^+$ cells were transplanted under the kidney capsule of non-obese diabetic, severe combined immunodeficiency mutation and interleukin-2Rγ allelic mutation (NSG) mice, and white growths (small masses) were identified macroscopically on 2 out of 10 kidneys. When the mice were examined by histological and microscopic analyses, stromal-like connective tissue was revealed under all of the kidney capsules. The SUSD2$^+$ cells produced significantly greater numbers of CFUs and the study identified SUSD2$^+$ as a single marker capable of purifying endometrial MSCs [44], thus negating the need to use two markers (CD146$^+$/PDGF-Rβ$^+$) that were proposed in the earlier studies. Although there was a considerable overlap of SUSD2 expression with CD146$^+$PDGF-Rβ$^+$ expression, the co-expression of the markers was not consistent. Moreover, endometrial pericytes are postulated to express CD146$^+$/PDGF-Rβ$^+$ whilst perivascular location is proposed for the SUSD2$^+$ cells suggesting some dissimilarities between the cells identified by these markers [15].

3.2. Side Population Cells

SP analysis distinguishes stem and progenitor cells from other more differentiated, somatic cells based on their ability to extrude DNA-binding fluorescent dyes (such as Hoechst 33342, a fluorescent dye that binds to the minor groove of deoxyribonucleic acid) since they express special ATP-binding cassette–containing pumps (ABC transporters—a type of membrane-bound active transporter, ABCG2). SP cells (0–5%) have been identified in fresh isolates [45–47] and short-term cultures [48] of human endometrial cells. The percentage of SP cells in single cell endometrial suspensions (derived from different patient samples) was reported to be highly variable between individuals. However, higher numbers seemed to be found in the menstrual [48] and proliferative [45,47] stages of the cycle. This variability was also mirrored by CFU activity in human endometrium. There is no consensus yet on whether the absolute number of SP cells are stable throughout the menstrual cycle or not. In agreement with the hypothesis that ASCs constitute a small, quiescent and static resident population [47], the decline of SPs in the secretory phase may result from dilution as the functionalis grows and increases in thickness. When SP cells (freshly sorted) showed little growth in culture, the authors argued that this was secondary to them being quiescent (most of the cells (85%), being in G0 phase of the cell cycle). This is a proposed feature of ASCs, but, in contrast, the SP cells sorted from endometrial short-term cultures were, primarily, in G1 and G1/M/S phases [45]. Endometrial SP cells sorted from short-term cultures did not express endometrial epithelial (CD9), or stromal (CD13) cell differentiation markers to start with, but these markers were re-expressed in subsequent long-term Matrigel cultures, indicating a capacity to differentiate into CD9$^+$E-cadherin$^+$ gland-like organoids (suggesting epithelial differentiation) and CD13$^+$ stromal clusters when cultured for a further 2 months on collagen-coated dishes [48]. However, even FACS, the most efficient sorting method available, does not have 100% efficiency/purity in sorting these cells. Therefore, particularly in long-term culture, a small percentage of both epithelial and stromal cells that are likely to have been contaminating the initial SP (although the authors reported that they do not express measurable levels of epithelial/stromal markers) may have expanded. The other reports examining the endometrial SP cells show SP cells in both the stromal and epithelial populations [1,47,49]. Furthermore, it is known that endometrial cells in 2D culture undergo culture-related changes and loss of cellular phenotypical markers and thus, cultured cells may lose their markers, but they have the capacity to regain them in a more physiologically relevant growth environment, the 3D matrix. Finally, the achievement of a single cell suspension from solid tissue samples (necessary for cell sorting (e.g., enzymatic digestion)) will remove the cell surface proteins, which will later be re-formed in cells with prolonged culture. All these issues need to be considered when interpreting the current literature. The sorted short-term cultured SP cells were able

to be maintained in culture for 3 months, whereas the non-SP cells sorted from the short-term cultured SP cells became senescent within 3 months [48]. This evidence concurs with the longevity associated with ASCs as opposed to differentiated cells.

To demonstrate their functional potential, cultured endometrial SP cells were shown to decidualise after being treated with oestradiol and progesterone, therefore confirming their ability to assume the morphologic, functional, and known terminal differentiation changes characteristic of the secretory endometrium [45].

Additionally, by differentiating into adipocytes and osteoblasts in vitro, endometrial epithelial SP cells have been shown to be multipotent, however, once again the differentiation potential was not compared with a control stem cell type such as hMSCs [1]. After 2 weeks in culture, the authors reported the presence of Oil Red O lipid vacuoles in adipogenic induction media (but the round morphology typical of adipocytes was not seen). Similarly, in osteogenic induction media, positive immuno-reactivity for bone sialoprotein was reported. This evidence is intriguing and needs to be considered with the possibility of contamination with stromal cells (which are known to have the ability to differentiate in the mesenchymal lineages). Further evidence has been produced by Kurita et al., with their elegant set of xenograft experiments demonstrating the adult endometrial epithelium to be lineage specific [50].

Masuda et al., demonstrated unipotency of the endometrial epithelial SP cells in vitro by injecting them under the mouse kidney capsule (mesodermal derivative), and generating endometrial tissue; those cells did not differentiate into kidney parenchyma [44]. This unipotency was echoed with work showing that SSEA-1 positive endometrial cells grown in a chimeric explant model, using kidney tissue isolated from CD1 neonatal mice, injected under the kidney capsule, produced endometrial gland like structures staining positive for endometrial differentiation markers [51].

Serum oestradiol levels have been shown to change in the same manner as the proportion of SP cells in postpartum mice, perhaps indicating that oestrogen is a prerequisite for increasing the size of SP cells populations [52]. This finding of increased oestrogen was confirmed within a mouse endometrial injury model; stromal SP cells significantly increased 6 h after injury, but they were dependent on oestrogen, not progesterone nor a combination of oestrogen and progesterone [53].

A relationship between stromal and epithelial ASCs was seen when stromal SP cells were transplanted under the kidney capsule in mice; only endometrial stroma was formed. When epithelial SP cells were transplanted, only epithelium was formed. Endometrial-like tissue was only generated when both these populations were combined. This observation would support the existence of two distinct ASCs—a stromal and an epithelial ASC [49].

3.3. Markers Identified in Cells with Other MSC Properties

3.3.1. Menstrual Blood-Derived Stem Cells

Another functional assay to identify stem cells is their ability to differentiate in to a variety of cell types, i.e., the differentiation potential. In 2007, it was reported that menstrual blood could be used to obtain endometrial stromal ASCs, and these were capable of differentiation into adipocytes, osteoblasts, chondrocytes, cardiocytes, myocytes, and endothelia [54]. These menstrual blood-derived stromal ASCs (mbdASCs) were also capable of trans-differentiation into endodermal and ectodermal tissue, such as hepatocytes, pulmonary epithelia and neurones. The mbdASCs were mononuclear, and demonstrated positive immuno-reactivity for CD90, CD73, and CD103, but were devoid of CD34 and CD45, suggesting the cells are of mesenchymal, not haematopoietic in origin [54]. The differentiation potential of these cells was compared to cord blood-derived MSCs, by comparing the expression of proteins related to stem cell function. Matrix metalloproteases (MMP-3 and MMP-10), cytokine growth factors (GM-CSF, PDGF-BB) and angiogenic factors (ANG-2) were shown to be expressed by the mbdASCs at higher levels than the cord blood-derived MSCs, suggesting that the mbdASCs have similar phenotypical features to ASCs, but differences in their function exist.

mbdASCs are an attractive target as a treatment for many diseases, due to easy access, non-invasive collection and, if there is a potential therapeutic use, their possible autologous utility will deem them superior to many other ASC types [55]. The use of autologous cells for the subacute phase of stroke offers a practical clinical application [56]. When grown in appropriate conditioned media, the mbdASCs express neuronal phenotypic markers (Nestin, MAP2), and in an in vitro stroke model of oxygen and glucose deprivation it was found that oxygen and glucose deprived-exposed primary rat neurons, (co-cultured with mbdASCs or exposed to the media collected from cultured menstrual blood), exhibited significantly reduced cell death [55]. Transplantation of mbdASCs (either intra-cerebrally, or intravenously, and without immunosuppression) into a rat model of ischaemic stroke, significantly reduced behavioural and histological impairments, compared to vehicle-infused rats, supporting the use of mbdASCs as a stem cell source for cell therapy in stroke [55–58] and other basal ganglia disorders, such as Parkinson's and Huntington's disease [59].

Sepsis (in the cecal ligation and puncture mouse model) has also been shown to have improved outcomes when mbdASCs are utilised in the treatment regime, alongside antibiotics. mbdASCs, in synergy with antibiotics, improved the survival rate (95%) in comparison; with saline (6%); antibiotics alone (73%); and mbdASCs alone (48%); concluding that mbdASCs could constitute a feasible approach for the future clinical treatment of sepsis [60].

A mouse model of premature ovarian failure, treated with mbdASCs, expressed higher levels of ovarian markers (AMH, inhibin α/β and FSH receptor), and the proliferative marker Ki67. In addition, the overall weight, plasma oestrogen level, and number of normal follicles increased overtime compared with controls [61].

mbdASCs have been differentiated into hepatocyte-like cells, and demonstrated in vitro mature hepatocyte functions such as urea synthesis, glycogen storage, and indocyanine green uptake; showing their potential to be used in chronic liver disease management [62–64].

The type 1 diabetes mellitus mouse model was used to show the therapeutic effects of mbdASCs on the mechanism of β-cell regeneration after transplantation [38]. The mbdASCs reversed hyperglycaemia and weight loss, prolonged lifespan, and increased insulin production in the diabetic mice. The mice recovered islet structures and increased their β-cell number, with the majority of the mbdASCs migrating into the damaged pancreas, and being located at the islet, duct, and exocrine tissue. The mbdASCs were found to enhance neurogenin3 expression (represents endocrine progenitors) rather than differentiate into insulin-producing cells, concluding that they stimulated β-cell regeneration through promoting differentiation of endogenous progenitor cells [38].

mbdASCs have also been proposed to be used for bone tissue-engineering purposes (taking advantage of their osteogenic driving potential) [65]; dermatological lesions and diseases [66]; heart muscle repair [67]; limb ischaemia [41]; and muscular dystrophy [68].

More recently, it has been shown that these mbdASCs have the ability to secrete decidualisation markers (prolactin and insulin-like growth factor binding protein-1), and differentiate into decidualised cells, leading to the potential of a therapy for decidualisation insufficiency [69]. When cultured in differentiation-inducing media supplemented with 20% human follicular fluid, the human mbdASCs form oocyte-like cells and express germ cell markers. Cells also expressed FSH and LH receptors, and produced oestrogen and progesterone regulated by gonadotrophin, suggesting a potential to differentiate in to ovarian tissue [70]. The exact origin of these cells (endometrial or bone marrow derived), however, is not known. Another further important consideration is that most diseases for which stem cell therapy has been proposed (e.g., prolapses, strokes, myocardial infarctions) occur in older, PM women who do not menstruate. The regenerative ability of the PM endometrium may allow stem cell harvesting after hormone treatment, yet the effect of such a treatment can be detrimental on the health of an elderly patient with cardiovascular disease and other comorbidities. Therefore, these practical challenges need to be considered when pursuing menstrual blood as a source of stem cells for autologous therapeutic avenues.

3.3.2. Bone Marrow as a Source of Endometrial ASCs

Bone marrow, as a source of endometrial regeneration, is supported by the ability of bone marrow-derived MSC to produce 'decidua-like' stroma, after activation of the protein kinase cAMP-dependent pathway in vitro [71]; together with bone marrow-derived cells being found in the decidua of normal murine pregnancy [72]. Stem cells of bone marrow origin typically express markers such as CD34. Co-culture of bone marrow-derived cells with endometrial stromal cells, and oestrogen stimulation, result in their differentiation into CK^+ endometrial epithelial-like cells [73].

When male bone marrow-derived cells were transplanted into female mice, Fluorescence in situ hybridisation demonstrated the Y-chromosomes to be present only in 0.0002% of CD45-/F4/80- epithelial cells, and 0.0003% of CD45-/F4/80- stromal cells of the endometrium [74]. In another set of experiments, samples from female mice harvested 40 days after a haematological stem cell transplant, showed an average of 6% donor-derived cells in the endometrium, concluding that bone marrow-derived endothelial progenitors contribute to the formation of new blood vessels in the endometrium [75].

In a study of Human leucocyte antigen mismatch transplants, donor-derived endometrial cells were detected in endometrial biopsy samples from all bone marrow recipients, and accounted for a wide range (0.2 to 48%) of epithelial (displaying CD9 marker), and vimentin positive stromal cells (0.3 to 52%) [76–78]. SP cells were not shown to be formed by XY donor-derived cells [77].

Therefore, the evidence presented above may suggest bone marrow to also be a source of stem cells for endometrial regeneration, but its contribution seems to be low. It is likely that bone marrow could be implicated in endometrial repair after times of massive injury, such as ablation, and in the formation of the decidua; when the endometrium requires 'extra-assistance'.

In a more recent study, authors using chimeric mouse endometrial tissue reconstitution with bone marrow derived from transgenic *mTert*-green fluorescent protein (GFP) reporter mice and irradiated recipients have suggested that bone marrow stem cells do not contribute to any of the endometrial cell lineages such as stroma, epithelium or endothelium [79]. All of the cells that were detected in the endometrium were immune cells expressing the pan-leukocyte marker CD45, including $CD3^+$ T cells and $F4/80^+$ macrophages; that immuno-stained weakly for CD45. The macrophages were abundant in the stroma, infiltrating the epithelial and vascular compartments, and it was noted that they could easily be mistaken for bone marrow-derived endometrial cells. The authors concluded (in disagreement with previous studies) that bone marrow cells are unlikely to transdifferentiate into endometrial stroma, epithelium and endothelium. They warned of the massive implications, since bone marrow-derived endometrial stem cells have been anticipated to be useful for numerous treatment strategies discussed previously [79]. Further work to clarify this possibility is urgently needed before their clinical applications.

3.4. Label Retaining Cells

Locating LRC in animals (the use of the LRC technique in humans is not permitted due to BrdU being a recognised health hazard) is a method of identifying somatic stem/progenitor cells and their location in the stem cell niche, when specific markers are unknown. This method relies on the infrequent cell turnover of most ASCs, in comparison to rapidly proliferating TA cells [2,80]. Mouse endometrium was pulse labelled with BrdU and studied after an 8-week chase to identify endometrial LRC.

3.4.1. Epithelial LRCs

Gargett's group reported that 3% of the epithelial nuclei were $BrdU^+$ and were located in the LE. They were shown not to express ERα through dual labelling IF, providing evidence that LE stem/progenitor cells are responsible for the growth of glands during development and in cycling mice [80]. In ovariectomised prepubertal mice, the first cells to proliferate in oestrogen-stimulated

endometrial growth differed from ovariectomised cycling mice, in the first, the epithelial LRC proliferated, suggesting they function as stem/progenitor cells to initiate epithelial regeneration; while in the latter, epithelial LRC and non-LRC rapidly proliferated to regenerate LE and glandular epithelium [80]. Using a mouse model with menstrual breakdown and repair, ERα negative glandular epithelial LRC contributed to the repair of the LE following menstruation, post progesterone withdrawal [81]. Endometrial repair occurred in the absence of oestrogen [81]. $BrdU^+$ epithelia was lost soon into the chase period, leading to the thoughts that the epithelial regeneration could be relying on the self-duplication of a mature epithelial cell type, or that the LRC technique is not sensitive enough to label rare endometrial epithelial cells with an ASC phenotype [82].

3.4.2. Stromal LRCs

Between 6–9% of the stroma were LRCs and they were located just below the LE at the endo-myometrial junctions or near the blood vessels [80,82], with 84% of them being ERα negative. These cells were found to not be leucocytes (by excluding CD45 staining), or endothelia (CD31 staining) [80]. $BrdU^+$ cells surrounding blood vessels were positive for α-SMA, making it probable that these cells represent pericytes. In some studies, 0.6% of stromal LRCs co-expressed OCT4 (a pluripotency marker) and c-kit (a haemopoietic stem cell marker) [82], so, they were potentially in an undifferentiated state; but in others, neither Sca-1 [80] nor c-kit [83] were expressed. However, to date, there are no studies describing cells co-expressing the classical triad of OCT4, NANOG and SOX2 confirming pluripotency, in the human endometrium.

Oestrogen was shown to drive epithelial LRC proliferation in juvenile development, but had a minimal role in epithelial and stromal LRC cyclical regeneration, perhaps indicating that adjacent $BrdU^-/ER\alpha^+$ endometrial cells release paracrine factors to mediate an LRC response [81].

4. Identifying Markers That Are Expressed by Cells Located in the Postulated Stem Cell Niche (Basalis and PM Glandular Epithelium)

Unlike stromal studies, the studies on epithelial stem cell markers considered the hypothesis that the stem cells should reside in the basalis glands, or in the PM epithelium (Figure 1B). The basalis markers described below (SSEA-1, nuclear SOX9, nuclear β-catenin) were the first basalis markers to be presented as epithelial ASC markers in 2013 [13]. Subsequent work has also suggested N-cadherin to be another basalis marker [84], but the very recent work using *LGR5* in situ hybridisation challenges this hypothesis and proposes the existence of more than one epithelial stem cell niche in the human endometrium [85]. These studies, however, are based on the presumption that human endometrial glandular architectural arrangement is a single blunt-ended tube, which is disputed in 3D reconstruction studies [86]. The Nguyen et al., study, in particular, suggests that cells deeper in the glandular base are more likely to be marking the more primitive cell, i.e., a hierarchical arrangement depending on the cellular location within the presumed single tubular, blind-ended glandular structure [84]. Hence, the localisation of these need to be re-examined with the 3D architectural re-modelling of the endometrial glands to fully appreciate the cellular hierarchical arrangement and stem cell organisation of the human endometrial epithelial compartment. In the stroma, only a limited number of stem cell markers have been examined on the basis of their abundance in the postulated stromal stem cell niche, the perivascular region.

4.1. SSEA-1

SSEA-1 is a cell surface glycan, an antigenic epitope, defined as Lewis X carbohydrate, and is expressed by preimplantation mouse embryos, teratocarcinoma stem cells, and mouse ESCs [87–90]. Its presence signifies cells in an undifferentiated state, as expression is lost during stem cell differentiation.

In the human endometrium, immuno-reactivity to SSEA-1 is specific to epithelia and some leucocytes only [13]. Intensity is significantly greater in the epithelium of the proliferative over the

secretory phase, and strongest in the basalis, and basalis-like PM endometrium, when compared with the functionalis epithelium. SSEA-1 enriched cell population has a greater propensity to produce gland-like structures in 3D culture, and also has higher telomerase activity and longer telomere lengths. The function of SSEA-1 in the endometrium remains unknown, but it is postulated to be associated with cell adhesion, migration, and capacity to differentiate [13].

Fibroblast growth factor (FGF) and Wnt-1 are both involved in stem cell maintenance and differentiation; work has shown that SSEA-1 possibly functions to bind and modulate these growth factors [91], and when this is supplemented with the prominent expression of SSEA-1 in the basalis epithelium, it is conceivable that these cells are a component of the endometrial-epithelial stem/progenitor cell niche. However, this study has not demonstrated any other stem cell properties of the SSEA1$^+$ epithelial cells and their in vivo tissue reconstitution ability is also not yet known. More recent work has described some SSEA1$^+$ cells to also be located in the LE, thus the expression is not strictly limited to the basalis [85]. The other criticism of SSEA1$^+$ cells being progenitors is their relative abundance in the basalis and in the PM endometrium. ASCs are expected to be rare cells in a given tissue. However, the huge regenerative requirement of the human endometrium may require a greater number of ASCs. Moreover, the SSEA-1$^+$ cells may be committed progenitors and more primitive ASCs may be a rarer subpopulation of SSEA1$^+$ cells. These possibilities remain to be confirmed in future studies.

4.2. SOX9

SOX9 is a Wnt target transcription factor, and thus is located in the nucleus; it was first discovered in patients with campomelic dysplasia [92]. SOX9 expression differentiates cells derived from all three germ layers into a large variety of specialised tissues and organs, with roles in chondrogenesis [93,94], male gonad development [95], neural crest development [96] and in the lower crypt region of the intestinal epithelium [97]. SOX9 expressing cells detected by immunohistochemistry (IHC) in normal human endometrium were found to be present in significantly larger numbers in the proliferative phase of the menstrual cycle when compared with the secretory phase [98]. Following this work, another IHC study described SOX9 expression to be largely confined to the basal epithelial cells throughout the cycle, with significantly greater numbers of epithelial cells expressing nuclear SOX9 in the basalis (46.2–52.3%) over the functionalis (8–12.1%) glands. The PM endometrium demonstrated the highest SOX9 immunostaining out of the pre/PM endometrial samples, with over 75% of PM epithelial cells expressing nuclear SOX9 [13].

This paper concluded that nuclear SOX9 co-localised with SSEA-1 and nuclear β-catenin, suggesting an activated Wnt pathway in the basal glands of premenopausal endometrium, could function by maintaining the SSEA-1$^+$ cells in a less-differentiated state, representing the endometrial stem/progenitor cell compartment of the stem cell niche, playing an important role in homing stem cells [13]. It was also concluded that high levels of nuclear SOX9 observed in the PM endometrial epithelial cells may function as a checkpoint to prevent hyperplasia, as loss of SOX9 in the intestinal epithelium leads to hyperplasia [99]. Although nuclear SOX9 may mark a potential primitive cell population in the endometrium, due to the nuclear location, SOX9 is obviously not a suitable marker to isolate these cells for further functional studies. The authors further concluded that nuclear SOX9, containing basalis endometrial epithelial cells, can be isolated for functional studies using the surface marker SSEA-1 [13], and their subsequent in vitro experiments demonstrated these epithelial cells to have high telomerase activity and superior ability to generate endometrial gland-like organoids in 3D culture [13]. As mentioned above for SSEA-1, the number of cells containing nuclear SOX9 in the endometrial epithelium is greater than the expected abundance for an ASC population.

4.3. Nuclear β-Catenin

The canonical Wnt/β-catenin signalling pathway is involved in cell fate determination. Wnt signalling pathway activation causes β-catenin to enter the nucleus, where it regulates the

transcription of target genes [100]. In the absence of Wnt signalling, β-catenin is dislocated from the nucleus. In the highly regenerative intestinal epithelium, nuclear β-catenin is highly expressed in the stem cell region of the intestinal crypt [97,101]. It functions to maintain organisation of the intestinal epithelial cells and is crucial for maintaining an undifferentiated state, evidenced by diminished activity in differentiated cells. Loss of nuclear β-catenin in the intestinal crypt results in rapid degeneration of epithelial cells [101,102].

In the endometrium, the Wnt/β-catenin pathway has been shown to be active in pre and PM endometrial cells [92] and is implicated in regulating the menstrual cycle, with increased nuclear β-catenin expression seen in proliferating endometrial epithelial cells [103–105].

Nuclear β-catenin has been identified in a sub population of epithelial cells in the basalis layer of the endometrium co-expressed with SSEA-1 and SOX9, where the stem cell niche is postulated to reside [13]. In this study, nuclear β-catenin expression was restricted to occasional basal glandular epithelial cells, and a similar expression pattern to co-localised SOX9 and β-catenin expression in the intestine was also demonstrated in the endometrium.

Although the published literature on nuclear β-catenin in the endometrium is limited, it does suggest that the endometrial epithelial cells in the basalis which express it could have stem cell properties. This data is in keeping with the current understanding of stem cell activation in the intestinal epithelium.

4.4. N-Cadherin

Data from a Wnt-associated gene profiling study of the endometrium [92] identified N-cadherin (gene *CHD2*) to be enriched in the postulated stem/progenitor cell rich postmenopausal endometrium. Given the known importance of Wnt signalling in stem cell biology, the authors investigated the cell surface marker N-cadherin to determine if it could be a potential marker of the endometrial epithelial progenitors [84]. N-cadherin protein was shown to be expressed in 16.7% (range 3.7–36.7%) of epithelial cell adhesion molecule (EpCAM)$^+$ endometrial epithelial cells sorted with FACS, and in 20.2% (range 8–35.5%) of the epithelial cells sorted with magnetic beads. When colony forming assays were utilised to assess the enrichment of epithelial progenitors, larger clones and significantly higher median cloning efficiency were observed in the N-cadherin$^+$ cells. These clones were large and densely packed, with small cytokeratin-positive cells and a high nuclear:cytoplasmic ratio. When serial cloning was undertaken, N-cadherin$^+$ cells generated clones from freshly isolated suspensions and samples underwent up to three rounds of serial cloning, and were also differentiated into cytokeratin$^+$ gland-like epithelial structures with a lumen in 3D Matrigel. N-cadherin was found by IF to be strongest in the basalis glands adjacent to the myometrium, and rarely co-localised with Ki-67, indicating a quiescent phenotype. Although some overlap of expression was seen, SSEA-1$^+$ cells were described to be phenotypically distinct from N-cadherin$^+$ cells, suggesting a potential epithelial hierarchy [84]. Importantly, the authors reported no N-cadherin expression in the LE. Two further IHC studies were also published in the same year. The first compared infertile patients with fibroids, to fertile controls, using IHC and qRT-PCR [106,107]. This showed that N-cadherin was lower in the LE in the mid secretory endometrium of infertile women when compared to fertile controls, but no significant change was demonstrated in either the immuno-expression or the mRNA. The IHC staining demonstrated by Makker et al (LE expresses strongest levels) [106] is in stark contrast to the IF staining presented in the Nguyen et al study (basalis adjacent to the myometrium expresses strongest levels) [84].

Therefore, the published studies on endometrial N-cadherin seem to report major conflicting differences. However, these studies have reported on N-cadherin expression in a variety of patient populations (healthy and pathological) using different techniques; the exact clones identified by different anti-N-cadherin antibodies were inconsistent and this makes it difficult to draw conclusions from the available endometrial N-cadherin data. However, the cells expressing the N-cadherin epitope described by Nguyen et al may have some progenitor activity, and their exact position in the human endometrial epithelial differentiation hierarchy is yet to be confirmed in a functional study and in

the context of the recently proposed endometrial epithelial 3D architecture [86]. Finally, similar to SSEA1, the N-cadherin expressing cells are also present in a greater number than what is expected for an ASC population.

5. Examining Endometrium for the Expression of Putative Stem Cell Markers That Were Identified to be Expressed in the Epithelial Stem Cells (ESCs), or Stem Cells of Different Tissues (e.g., *OCT4, Mushashi-1, LGR5,* Notch1/numb)

5.1. OCT-4

OCT-4 has been proposed as a marker of pluripotent human ESCs and some ASCs. Although the expression of OCT-4 has regularly been associated with primitive cell types by many authors, it is important to appreciate that the synergistic expression of the classical pluripotency gene triad, *OCT-4*, *NANOG* and *SOX2* is required to maintain pluripotency. OCT-4 was seen in some endometrial samples by IHC, and in all endometrial samples by reverse transcriptase-polymerase chain reaction (RT-PCR) with variable expression in the human endometrium [108]. Close scrutiny of the pictures presented in this manuscript reveal that OCT-4 is not expressed in the epithelial cells, but appears to be seen rarely in some stromal cells or blood vessels. The micrographs of the immuno-staining were not supported by further confirmatory data using a secondary method. The authors simply concluded that OCT-4 staining is present and is mostly expressed in the stromal compartment [108]. In a subsequent study, OCT-4 was found not to be differentially expressed during the menstrual cycle in women and is, therefore, proposed to be uninfluenced by hormones [109]. OCT-4 has also been located in some mouse LRCs in the deeper endometrial stroma, co-localising with c-KIT [82]. Therefore, further work is needed (using a reliable antibody, specific to human OCT4a antigen) to examine the cell-specific expression in the human endometrium, since OCT4a is the particular antigen associated with an undifferentiated phenotype. Therefore, we can conclude that in the present time, there is no robust evidence to suggest OCT-4 expressing cells to be relevant to the endometrial ASC population.

5.2. Musashi-1

Musashi-1 is an RNA-binding protein in neural stem cells and an intra-cellular epithelial progenitor cell marker that regulates self-renewal signalling pathways. The protein is expected to assume an intracellular location, and thus will not be suitable for use in isolation of the cells that express it for functional studies. Musashi-1 was immunolocalised to single epithelial cells, and small clusters of stromal cells in human endometrium [110]. The authors describe the staining as nuclear and cytoplasmic, but the representative figures presented in the manuscript only demonstrated cytoplasmic staining. IF images showed Musashi-1 to be co-localised with its molecular target, Notch1, and telomerase. Musashi-1 positive cells were mainly found in the basalis in the proliferative stage of the menstrual cycle (when compared to the secretory stage), suggesting their possible stem/progenitor cell function. Stromal Musashi-1 positive cells were not found in a perivascular location, although some were in a peri-glandular region, a similar location to some stromal LRC in mouse endometrium [80].

More recently, Musashi-1 expression has been found in the neonatal endometrium from the 12th week of gestation, with the number of positive cells decreasing with increasing gestational age. In the reproductive endometrium, Musashi-1 staining was seen in dispersed single cells and in stromal cell groups adjacent to myometrium [111].

In summary, there is only limited data on cytoplasmic and nuclear IHC staining for Musashi-1 in the endometrium, without any functional studies confirming the stem cell properties of these cells. To date, no further confirmatory work has been undertaken since the preliminary publication in 2008. Therefore, Musashi-1 is yet to be proven as an endometrial ASC marker.

5.3. Notch1/Numb

The family of Notch proteins are ligand-dependent transmembrane receptors that transduce extracellular signals responsible for cell fate and differentiation in a multitude of cellular systems and niches [112–115]. Notch1 is a heterodimeric, 300-kDa type 1 transmembrane receptor which mediates signaling induced by cell-to-cell contact [116]. Numb is an inhibitory regulator of Notch1 signaling that acts by promoting the ubiquitination and degradation of the Notch1 intracellular domain [114].

Positive Notch1 immuno-staining has been found to be concentrated in the cytoplasm of endometrial epithelial cells, whereas very weak staining has been observed in the stromal cells. This immuno-expression was dynamic in the endometrium, with higher Notch1 in the proliferative phase than in the secretory phase in some studies [114,117], although the reverse was reported in others [115], while no significant difference was observed between the proliferative phase in pre-menopausal and postmenopausal samples. Interestingly, the maximal staining intensity was seen in the mid secretory receptive phase of the menstrual cycle [118]. Strong cytoplasmic immunostaining for Numb was limited to the epithelial cells and very weak staining was observed in the stroma. Contrasting results have been reported, suggesting either consistent levels across the menstrual cycle [114] or decreased immuno-expression in the mid secretory phase [118]. These studies only utilized single technique (IHC) without any secondary confirmatory techniques or functional work [114].

The studies that considered Notch1 as a stem cell marker (showing maintenance of cells in an undifferentiated state) used both IHC and qPCR to confirm the presence of Notch1 in endometrial biopsies, and the clones containing Notch1 were able to differentiate into multiple lineages [119]. However, due to their intracellular location, Notch1/Numb are not suitable markers for isolating the potential ASCs for further study.

5.4. MSCA-1

MSCA-1, a bone marrow-derived MSC surface marker, has been identified to be identical to Tissue Non-specific Alkaline Phosphatase (TNAP) [120–125]. When ESC's differentiate, the expression of TNAP decreases [42]. TNAP is expressed on endometrial perivascular cells, the proposed location of endometrial MSC-like cells [42]. The proportion of $W8B2^{+}CD146^{+}$ endometrial stromal cells was compared to the proportion of $CD146^{+}PDGFR\beta^{+}$ MSC-like cells found in the human endometrium, and these were very similar, leading to the conclusion that endometrial MSC-like cells express TNAP. Combined with CD146, this ectoenzyme was proposed to be a suitable marker for the isolation from the $EpCAM^{-}$ endometrial stromal population. Due to TNAP being exclusively expressed in the $CD146^{+}$ subset, but not on other MSC-like/fibroblast-like cells, it would appear that TNAP is developmentally expressed on MSC/pericyte progenitor cells and is down-regulated during further differentiation.

Not only was TNAP expressed on the endometrial perivascular cells, but immuno-staining was detected on endometrial epithelial cells at the apical luminal surface [25]. This has led to TNAP also being proposed as a marker for the isolation of a subset of endometrial glandular epithelial cells. However, the assessment of the ability of TNAP expressing cells to recapitulate endometrial tissue in animal models, and their multilineage potential, would require these cells to be sorted on the basis of their expression of TNAP, as well as EPCAM. Therefore, TNAP may not suitable as a single marker isolation protocol for endometrial MSC. However, the fact that TNAP is expressed in the cells of stromal and epithelial compartments is interesting, and if further studies demonstrate that cells expressing TNAP from both fractions possess stem cell characteristics, it may be a common marker for cells involved in the endometrial regeneration process, and a good therapeutic target. Consequently, further studies are warranted in this area.

5.5. LGR5

Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) is a transmembrane receptor [126] characterised by a large leucine-rich extracellular domain [126], belonging to a family of glycoprotein hormone receptors [127]. Little was known about mammalian LGR5 before 2007 [128] when it was discovered by researchers seeking an intestinal stem cell marker [129]. Subsequently, in the human endometrium, by using RT-PCR, *LGR5* gene was found to be expressed in 26 full thickness mid proliferative to late secretory phase samples [130]. Substantial differences were discovered in expression levels of individual women, but no variation was observed throughout the menstrual cycle, therefore suggesting it was not hormonally regulated. This study was followed by a mouse study demonstrating that: murine *lgr5* gene is dynamically regulated in endometrial epithelium expressed only in immature and ovariectomised mice, and is down-regulated by oestrogen. All of this evidence alludes to a hormonal regulation, and for *LGR5* to be lost with differentiation (such as acquiring hormonal responsiveness, refer to Figure 2) [127]. More recently, a review paper proposed LGR5 to be a potential stem cell marker in the human endometrium. The authors also included original data in this review, using IHC to show a population of stromal and epithelial cells stained by an anti-human LGR5 antibody and LGR5$^+$ cells mainly located in the perivascular regions [131]. In this review, further original data from telomapping (a type of confocal quantitative fluorescence in situ hybridisation that displays the gradient of telomere length in a given adult tissue) to identify cells with the longest telomeres was also included. The authors subsequently claimed that *LGR5* mRNA signal was present in some of the cells containing the longest telomeres, suggesting that LGR5 expressing cells are associated with a long telomere phenotype. Notably, this statement was made without supporting data. No menstrual cycle differences were reported with regards to the expression of LGR5 protein. The authors concluded that perhaps LGR5 could be considered as a universal stem cell marker and possibly a marker of ASCs in the human endometrium [131]. Although this review presented some limited original work on *LGR5* mRNA and protein, without the provision of detailed methodology (e.g., information on the exact antibody used) [132], scientific scrutiny is not possible for the robustness of that data. Since the specificity of the available anti-human LGR5 antibodies are in considerable doubt, the claim that LGR5-expressing cells identified by IHC are ASCs needs further investigation using more suitable methods. The subsequent publication from the same group described xenografting the isolated LGR5$^+$ cells using an anti-human LGR5 antibody into a mouse model [132] to determine their functional relevance. However, the location of the LGR5$^+$ cells in the intact human endometrium using the same anti-human LGR5 antibody was not described. Whether this study used the same antibody that they had used in their previous review is also unclear [131]. Human LGR5$^+$ epithelial and stromal cells from endometrial biopsies (not full thickness endometrium, and thus will contain only the functionalis layer including LE) were sorted, according to their surface expression of LGR5, using an antibody (of unconfirmed specificity) by FACs, and were phenotypically characterised by flow cytometry with haematopoietic and mesenchymal markers. These LGR5-enriched cells were labelled and injected under the kidney capsule of immunocompromised mice. The authors reported LGR5$^+$ cells in the human endometrium to constitute $1.08 \pm 0.73\%$, and $0.82 \pm 0.76\%$ of the total cells in the epithelial and stromal compartments respectively. LGR5-enriched cells showed an abundant expression of CD45 (a mature leucocyte marker) and no expression of more primitive surface markers CD31, CD34, CD133, CD73, and CD90. However, co-expression of LGR5$^+$ with the macrophage marker CD163 was detected. The tissue recapitulation resulted in a weak endometrial reconstitution, and transcriptomic profiling revealed new attributes for LGR5$^+$ cells related to their putative hematopoietic origin. Authors concluded that LGR5 was unlikely to be a universal stem cell marker [132], opposing their previous proposal [131]. They further stated that LGR5$^+$ cells appeared to be recruited from blood to be part of the stem cell niche at the perivascular microenvironment, to activate the endogenous niche [132].

When considering the available evidence, the mouse lgr5 studies may not translate well to humans due to obvious species-specific differences in their endometrial biology. The initial study

examining human *LGR5* mRNA level did not attempt to ascertain the location of the endometrial *LGR5* expression [131]. The specificity of all presently available anti-human LGR5 antibodies to identify the protein are not confirmed, and are of considerable doubt [133].

To overcome the above deficiencies in the literature, earlier this year, the gold standard method of in situ hybridisation (ISH) was utilised alongside qRT-PCR, IHC and in silico analysis of published endometrial microarray datasets to conclusively examine the cellular location of LGR5 expression in full thickness normal human endometrium [85]. *LGR5* expression was limited to the epithelial compartment of the endometrium, with high *LGR5* expressing cells seen in the endometrial LE and in the stratum basalis; the LE expressed significantly higher levels of *LGR5* than all other epithelial compartments. The dynamic spatiotemporal pattern of *LGR5* expression suggested hormonal regulation, with a reduction in *LGR5* expression in the secretory phase (with ISH) in the luminal and functionalis epithelium respectively. Endogenous and exogenous progestogens inhibited *LGR5* expression in the endometrium both in vivo and in vitro in explant culture. When endometrial samples of women taking synthetic progestogen treatment (progesterone only pill, 'POP', or levonorgestrel-releasing intrauterine system, 'LNG-IUS') were compared with the samples of women not on any treatment, a significant reduction of *LGR5* mRNA levels was observed. Data was further confirmed by analysing previously published microarray datasets. The epithelial compartment-specific expression pattern of LGR5 in the full thickness endometrium prompted the novel theory that more than one epithelial stem/progenitor cell pool could exist in the human endometrium; one residing in the basalis (SSEA-1^{++}/$SOX9^{++}$/$LGR5^{+}$) supporting the massive regeneration of the functionalis after menstrual shedding or parturition; while the other ($LGR5^{++}$/SSEA-1^{+}/$SOX9^{+}$) supports the embryo-implantation process, and maintains the LE cells that are likely to be lost on a daily basis [85]. However, this study was an observational study without any functional data. It is therefore important in the future, when a reliable anti-human LGR5 antibody is available, for further work to be carried out to assess the LGR5-enriched cells from the endometrium for stem cell function.

5.6. Telomerase

Telomerase is an RNA-dependent DNA polymerase enzyme responsible for synthesising and maintaining telomeres that exists at the ends of all linear chromosomes (Reviewed in Hapangama et al., 2017 [134]). The expression of telomerase in human cells is essential for maintaining cellular integrity and immortalization. TERT is the catalytic subunit of telomerase holo-enzyme, and has been shown to be expressed in cells with self-renewing potential, including stem cells [135]. $TERT^{+}$ intestinal epithelial cells are considered to be intestinal stem cells which are quiescent and are regenerated in response to tissue injury [136].

In the human endometrium, telomerase activity is limited mainly to the glandular epithelium [137]. Isolated SSEA-1^{+} basalis progenitor epithelial cells grown in culture had a significantly higher telomerase activity, longer mean telomere lengths and the ability to generate endometrial gland-like structures than SSEA-1 depleted epithelial cells [13]. This study suggests that high telomerase activity in SSEA-1^{+} epithelial cells may render this epithelial progenitor population to have an increased replicative lifespan and possibly self-renewal, which are accepted stem cell properties.

Further evidence suggests telomerase activity to mark potential endometrial stem cells, comes from a study where an increased number of epithelial cells co-expressed the ASC marker Mushashi-1 and telomerase reverse transcriptase (TERT) in the proliferative phase endometrial samples when compared with normal secretory endometrium [110]. The reliability of anti-human telomerase antibodies is known to be problematic; therefore, caution should be taken considering this antibody-based telomerase study.

In a transgenic, green fluorescent protein (GFP) reporter mouse model, a small population of $mTERT^{+}$ presumed ASCs were identified in the endometrial luminal and glandular epithelial cells [138]. $mTERT^{+}$ cells decreased in response to ovariectomy of the mouse, suggesting a role of ovarian steroid

hormones in maintaining these cells. In other tissues such as bone marrow [139], *mTERT*-expressing cells are considered to possess ASC qualities. The study, however, did not assess the functional ASC activity of *mTERT*$^{+}$ endometrial cells. Adequate telomerase activity is a prerequisite of proliferating endometrial epithelial cells [140]; therefore, although there is evidence for telomerase activity in the proposed ASC compartment in the human endometrium, further work is needed to clarify if telomerase is a specific phenotypical ASC marker or is merely marking the activation status/proliferation of the ASCs.

6. Involvement of Endometrial Stem Cells in Endometrial Proliferative Disease

The involvement of endometrial ASC in proliferative disease such as endometriosis and endometrial cancer has been postulated [15]. Any recurrent or persistent disease of the premenopausal endometrium, from heavy menstrual bleeding, infertility, or recurrent miscarriage should be originating from abnormalities accumulated in the ASC population that is responsible for the regrowth of a novel functionalis layer each month. Although the theoretical possibility that all endometrial pathologies originate from aberrant endometrial ASCs is widely accepted, the direct evidence available supporting this theory is scarce. Therefore, in this review, we have highlighted two endometrial proliferative conditions, endometriosis and endometrial cancer, to highlight some of the interesting data from the endometrial stem cell marker prospective.

6.1. Endometriosis

Endometriosis is a common, benign proliferative disease of the endometrium defined as having endometrium-like tissue existing outside of the uterine cavity. One in 10 women of reproductive age in the UK suffer from endometriosis, which is responsible for significant morbidity and places a huge economic burden on the women, health services and society in general. Little is known about its aetiology and pathogenesis and this prevents the formulation of novel treatments [141]. The ectopic endometriotic tissue retains hormone responsiveness and may undergo inflammation, proliferation and regeneration, and thus stem cells are thought to be involved in the pathogenesis. Leyendecker et al. proposed that basalis endometrial cells enriched with progenitor potential are shed with menstruation in women with endometriosis and these cells may give rise to ectopic lesions after retrograde menstruation and trans-tubal migration in to the pelvic cavity [142]. The studies demonstrating the expression of epithelial stem cell markers such as SSEA-1, SOX9 and nuclear β-catenin [13] and Musashi-1 [110] in ectopic endometriotic lesions have suggested a possible direct involvement of ASCs in endometriosis lesion formation. High telomerase activity is a feature of endometriosis [143] and is also found in SSEA-1 expressing basalis progenitor epithelial cells [13]. Furthermore, Musashi-1 expressing endometrial epithelial cells have been shown to co-express telomerase catalytic subunit (hTERT) [110]. The involvement of ASC's in the pathogenesis of endometriosis is further supported by increased expression of Numb and Notch1 in eutopic endometrium from patients with endometriosis, when compared with controls, and could be associated with increased severity of the condition. Knock down of Notch1 in human endometrial epithelial and stromal cells resulted in reduced cellular proliferation and migration when injected into the peritoneal cavity of mice, and a reduced size of resulting endometriotic lesion was observed, implicating Notch1 in the pathogenesis of endometriosis [113]. An IHC study also reported increased Notch1 expression in adenomyosis. Studies are awaited to determine the presence or the involvement of cells expressing N-cadherin and other endometrial stromal ASC markers in ectopic endometriotic lesions formation. Interestingly, a recent study which analysed synonymous and missense somatic passenger mutations has suggested that ectopic endometriotic lesions contain clonal populations of epithelial cells originating from, presumably, an ectopically situated epithelial ASC, whereas stromal cells may be continuously regenerated or recruited over the course of disease [144,145]. This data presents a novel concept, in that the primary cell type initiating and regulating the initiation as well as persevering the ectopic lesions could be

the epithelial ASC and they may subsequently recruit stromal ASCs to create the endometrial niche. Further studies are needed to examine this novel hypothesis.

6.2. Endometrial Cancer

Endometrial cancer is the most common gynaecological cancer with an increasing incidence. In 2015 alone, 8984 new cases of endometrial cancer were diagnosed [146] and in 2016 [146], it was the cause of 2360 deaths in the UK. With estimated increases of over 90% in annual costs for endometrial cancer surgery, this places a huge burden on the NHS and society's resources. Furthermore, alternative therapy is urgently needed for the recurrent and metastatic disease that is resistant to both chemo and radiotherapy. Specialised cancer cell sub-populations called cancer stem cells (CSC) are postulated to be responsible for distant metastasis, cancer recurrence and resistance to chemo/radio therapy. High telomerase activity is also implicated in cancer metastasis and CSC. CSC share many features with adult tissue ASC in that they express telomerase, have self-renewal capabilities and higher proliferative potential. SSEA-1 and Musashi-1 expressing potential endometrial epithelial ASCs from healthy endometrium have been shown to have telomerase activity [13,110] and the malignant transformation of these ASC is thought to initiate cancer. High levels of Musashi-1 expression has shown to be associated with poor prognosis in endometrial cancer, suggesting that Musashi-1 expressing CSCs are a possible therapeutic target [110,147,148]. SOX9 is also upregulated in endometrial cancer and upregulation of SOX9 is a feature of the premalignant hyper-proliferative condition, endometrial hyperplasia [98,149], suggesting an involvement of the basalis progenitor ASC in these conditions. Using purely IHC, Xie and colleagues compared N-cadherin expression between patients with endometrioid adenocarcinoma, and normal controls [150]. They showed that N-cadherin was positive when brown/yellow particles were seen in the cytoplasm of a cell (again contradictory to other published studies). For the 50 normal samples that were included in this study, the positive expression rate for N-cadherin protein was 40.0% (8 weakly positive, 9 moderately positive, and 3 strongly positive) and the positive N-cadherin protein expression rate was statistically higher in the endometrioid adenocarcinoma group compared to the normal controls. They also showed that E-cadherin is not commonly expressed in the N-cadherin expressing cells, therefore, a transition may exist between them [150]. An increase in Notch1 expression was reported in endometrial cancer samples [115] in a study using IHC. Stromal Notch1 expression increased in endometrial carcinoma with respect to hyperplasia and polyps. The cell fate determinant Numb, has also been reported to be increased in endometrial cancer, compared to normal endometrium in a study using IHC [151], and the immune-staining gradually increased in correlation with the advancing grade of the endometrial cancer samples. However, functional studies are needed to examine the role of Notch1 and Numb in endometrial carcinogenesis. However, there are no studies to date examining the expression or the involvement of the other proposed normal endometrial ASC markers in endometrial cancer.

7. Conclusions

The human endometrium obviously contains ASCs that are responsible for its frequent, efficient and scar-less regeneration. Recent work suggests that the main endometrial cell lineages, epithelium and stroma, may develop independently; and neither cell types are likely to originate from bone marrow-derived cells. There are many stromal and epithelial ASC markers proposed, with some demonstrating in vitro stem cell properties, yet the in vivo tissue reconstitution ability of these cells has either been poor or not yet fully examined. A summary of postulated endometrial stem cell markers in the epithelia, stroma, and perivascular cell populations can be seen in Table 1. The differentiation potential of the stromal and epithelial cells (isolated to ascertain their therapeutic utility) needs to be fully confirmed in the future. The demonstration that there are epithelial cells expressing the described ASC markers in endometrial proliferative conditions supports their involvement in the pathogenesis of endometriosis and endometrial cancer, yet further studies are needed to ascertain the possibility of targeting them for curative therapy.

Table 1. Summary of postulated endometrial stem cell markers in epithelial, stroma, and perivascular cell populations.

Cell Type	Location	CD146/ PDGFRβ	SUSD2	SP	LRCs	SSEA-1	SOX9	Nuclear β-Catenin	N-Cadherin	OCT4	Musashi-1	Notch/ Numb	MSCA-1	LGR5	Telomerase
Epithelial	Luminal	−	−	?	+	+	+	−	−			+	+	++	
	Functionalis	−	−	?		−	−	−	−		+	+	−	−	+
	Basalis	−	−	?		++	++	+	++		+	+	−	+	+
	Undefined			+											
Stromal	Functionalis				+										
	Basalis				+										
	Undefined			+						+	+				
Peri-vascular	Functionalis	+	+		+						?				
	Basalis	+	+		+								+		
References		[22,42,43]	[15,44]	[1,45, 47–53]	[2,80–82]	[13,85, 87–91]	[13,92– 99]	[13,97,100– 105]	[84,92,106, 107]	[82,108, 109]	[80,110, 111]	[112– 119]	[25,42,112, 120–124]	[85,126– 133]	[13,110, 134–139]

Key: ++ strongly positive, + positive, − negative, ? existing evidence unclear.

Author Contributions: D.K.H. conceived the manuscript, N.T. and D.K.H. prepared the first draft, N.T., A.M. and D.K.H. revised the manuscript critically for important intellectual content and prepared the figures and references. All authors revised and read the manuscript and approved the submitted final version.

Funding: This work was funded by Wellbeing of Women fellowship grant (RTF510 NT and D.K.H.), Wellbeing of Women ELS (ELS706, A.M. and D.K.H.) and Wellbeing of Women project grant (RG1073, D.K.H.).

Acknowledgments: Authors would like to acknowledge help received from John Woodward of Institute of Translational Medicine, University of Liverpool in preparation of the graphical abstract and Druvi Edirisinghe of University of Liverpool in manuscript preparation (language editing).

Conflicts of Interest: The authors declare no conflict of interest.

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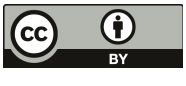

International Journal of
Molecular Sciences

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Review

Vitamin D and Endometrium: A Systematic Review of a Neglected Area of Research

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Received: 6 July 2018; Accepted: 4 August 2018; Published: 8 August 2018

Abstract: Growing evidence supports a role of vitamin D (VD) in reproductive health. Vitamin D receptor (VDR) is expressed in the ovary, endometrium, and myometrium. The biological actions of VD in fertility and reproductive tissues have been investigated but mainly using animal models. Conversely, the molecular data addressing the mechanisms underlying VD action in the physiologic endometrium and in endometrial pathologies are still scant. Levels of VDR expression according to the menstrual cycle are yet to be definitively clarified, possibly being lower in the proliferative compared to the secretory phase and in mid-secretory compared to early secretory phase. Endometrial tissue also expresses the enzymes involved in the metabolism of VD. The potential anti-proliferative and anti-inflammatory effects of VD for the treatment of endometriosis have been investigated in recent years. Treatment of ectopic endometrial cells with $1,25(OH)_2D_3$ could significantly reduce cytokine-mediated inflammatory responses. An alteration of VD metabolism in terms of increased 24-hydroxylase mRNA and protein expression has been demonstrated in endometrial cancer, albeit not consistently. The effect of the active form of the vitamin as an anti-proliferative, pro-apoptotic, anti-inflammatory, and differentiation-inducing agent has been demonstrated in various endometrial cancer cell lines.

Keywords: Vitamin D; endometrium; endometrial cancer

1. Introduction: Vitamin D, Metabolism, and Reproduction

Vitamin D is a well-known steroid hormone whose activated form is the result of the conversion of 7-dehydrocholesterol in the skin, under the influence of ultraviolet B light. To become active, it requires two hydroxylation steps: a 25-hydroxylation occurring mainly in the liver, leading to 25-hydroxyvitamin D_3 ($25\text{-}OHD_3$), and a 1α-hydroxylation occurring in the proximal tubules cells of the kidney, leading to 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). All these hydroxylation steps are catalyzed by cytochrome P450 mixed-function oxidases that are produced by the CYP gene superfamily group. These enzymes are located in the liver either in the endoplasmic reticulum (e.g., CYP2R1/25-hydroxylase) or in the mitochondria (e.g., CYP27A1/25-hydroxylase) or in the kidney mitochondria (e.g., CYP27B1/1α-hydroxylase) [1]. In terms of catabolism, CYP24A1/24-hydroxylase catalyzes the conversion of both $25\text{-}OHD_3$ and $1,25(OH)_2D_3$ into a series of 24- and 23-hydroxylated products targeted for excretion along well-established pathways concluding in the water-soluble biliary metabolite calcitroic acid [2]. However, several extrarenal tissues, such as bone, bone marrow, prostate, and macrophages, also express actively the 1α-hydroxylase enzyme [3].

The biological actions of vitamin D (VD) are mainly mediated by VD receptor (*VDR*) that belongs to the nuclear receptor superfamily. Upon $1,25(OH)_2D_3$ binding, VDR forms a heterodimer complex with the retinoid X receptor (*RXR*) and interacts with regions of the DNA named vitamin D response elements (*VDRE*). These elements recruit coactivators able to regulate the transcription of target genes involved not only in calcium and phosphate homeostasis [4] but also in cell proliferation, differentiation and immune response [5,6]. Moreover, some evidence suggests that non-genomic pathways mediate rapid signaling though cytosolic and membrane VDR [7].

VDR is expressed in different organs and tissues including skeleton, immune system, parathyroid glands and reproductive tissues [8]. Several studies have demonstrated that the ovary could be a target organ for $1,25(OH)_2D_3$ raising the possibility that this active metabolite plays a role in modifying ovarian activity [9]. The role in fertility and reproductive capacity has been investigated in animal models for many years, demonstrating that $25\text{-}OHD_3$-deficient female rats had reduced fertility rates [10]. VDR knockout female mice are unable to reproduce due to defects in uterine development [11]. Furthermore, 1α-hydroxylase-null mice manifest a decrease in ovarian size and uterine hypoplasia [12]. Finally yet importantly, the role of VD in uterine physiology seems to be essential for the normal differentiation of decidual cells [11,13].

Beyond the physiology, there is several evidence on the possible effect of VD in endometrial pathology. For example, the role of VD has been studied in endometriosis and endometrial cancer.

Endometriosis refers to the presence of functionally active endometrial tissue, glands, and stroma in ectopic sites. Its real prevalence in the female population is unknown mainly due to overlooked and delayed diagnosis but it has been reported to affect about 5–10% of women of reproductive age [14]. Several theories have been proposed for its etiopathogenesis with the retrograde menstruation hypothesis being the most accepted [15]. However, this hypothesis has many limitations, which numerous other theories have attempted to circumvent [16,17], including altered Genetic/Epigenetic-based mechanisms [18].

The endometrium of women with endometriosis presents abnormalities on a structural as well as on a functional level affecting proliferation ability, presence of immune components, adhesion molecule expression, steroid and cytokine productions, compared with the endometrium of women without the diseases [19,20]. Moreover, endometriosis possesses features similar to a malignancy and fulfills several criteria of an autoimmune disease. VD is a known anti-proliferative, anti-inflammatory, and even an immunomodulatory agent. Therefore, the association between endometriosis and VD has been the object of some investigation [21]. Unfortunately, data linking VD action and endometriosis on a molecular level is still sparse, both in terms of a potential role in the pathogenesis and therapeutics.

Endometrial cancer is the most common female gynecological malignant pathology in developed countries and its incidence is increasing [22]. A recent systematic review of literature found out a crucial role of VD in cancers as high circulating levels of VD are associated with a reduced risk of developing certain cancer types (breast, colorectal, gastric, hematological, head and neck, kidney, lung, ovarian, pancreatic liver, prostate, and skin cancer) [23]. Nevertheless, the association between VD and endometrial cancer risk is at present controversial [24–26]. Therefore, a better investigation of the potential molecular mechanisms at the basis of the local action of VD in endometrial cancer could be of a value.

Encouraged by the aforementioned information, we aimed to present a systematic review on all available molecular data related to the effect of VD in human endometrium and endometrial diseases, with a focus on endometriosis and endometrial cancer.

2. Methods

The search strategy was agreed upon a priori by the authors. We searched in PubMed Database for articles published in the English language using the following MeSH search terms: ''vitamin D" AND "endometr*" with restriction to the human species. We included articles referring to physiological endometrium, endometriosis, and endometrial cancer with available molecular data.

No time restrictions were applied. Full-length articles were considered eligible for this systematic review if they were written in the English language. Review articles were excluded during the first screening. Studies found to be irrelevant after reviewing the abstracts were likewise excluded. The remaining articles were retrieved in full-length and assessed according to the eligibility criteria. The reference lists of all known primary articles were examined to identify cited articles not captured by electronic searches. Studies referring to animal models, referring to serum levels of VD metabolites and other endometrial diseases were excluded. Papers without available molecular data about VD pathways in endometrial cells were excluded. Titles and abstracts of all identified studies were screened and the full paper of the preselected articles was read by two researchers (A.M.S., G.C.C.). (Figure 1).

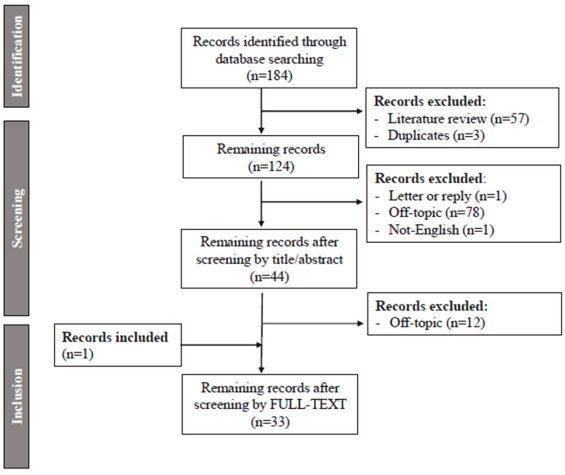

Figure 1. PRISMA flowchart summarizing inclusion of studies in systematic.

3. Vitamin D and Normal Endometrium

During the normal human menstrual cycle, the endometrium undergoes proliferation, differentiation, and finally degeneration. All these events are regulated by changes in steroid hormone levels, mainly estrogen and progesterone. VD, as a steroid hormone, may have a direct role during the modifications that the endometrium undergoes throughout the menstrual cycle [11].

Vienonen and colleagues (2004) were the first to demonstrate the presence of the VDR in human endometrial tissue. They studied the expression pattern of different nuclear transcription factors in normal endometrium including VDR using real-time PCR (RT-qPCR). They evaluated the mRNA expression in uterine samples from three premenopausal women who had undergone hysterectomy (range age 38–50 years). Mainly, they found notable differences in expression levels among individuals, but the levels did not differ between proliferative and secretory phases of the cycle [27]. In 2006, Viganò and colleagues also studied the expression of VDR in the normal endometrium [28]. In that publication, human endometrial samples were collected from women younger than 40 years old, who had not received hormones for at least 3 months and the presence of VDR was demonstrated by RT-qPCR analysis; unfortunately no quantification through the menstrual cycle phases was performed [28]. The group of Linda Giudice conducted a similar study in 2012. They compared the expression of different nuclear receptors in different phases of the endometrial cycle and found that VDR was downregulated in mid secretory phase compared to early secretory phase [29]. Later on, Bergadà and colleagues found different results. In this case, tissue samples in different phases of the endometrial cycle were obtained from 60 women (age ranged from 25 to 55, mean = 43, 20 samples in proliferative and 40 samples in secretory phase), samples were embedded in paraffin blocks and tissue microarrays

were analyzed. A decrease in the total expression of VDR in the proliferative endometrium compared with the secretory phase (fold change 3.17, $p = 0.00002$) as well as an increase in the cytosolic VDR protein expression (fold change 1.81, $p = 0.006$) [30] were observed.

The controversial results found in the literature may be explained by the different models and techniques used. Some studies used the cells isolated from tissue that could be contaminated with immune cells present in the endometrium [30] while others used immunohistochemistry analysis of embedded tissue [31]. The selection of the control patients may be as well a source of variability in the different studies.

The endometrial tissue also expresses the enzymes involved in the metabolism of $1,25(OH)_2D_3$. The mitochondrial enzyme 1α-hydroxylase, encoded by the *CYP27B1* gene, is expressed in the endometrium [28,31]. Moreover, an increase of enzyme expression has been reported during pregnancy, resulting in higher VD serum level necessary to meet the enhanced calcium requirements during this condition [24]. As previously mentioned, the mitochondrial and microsomal 25-hydroxylases (encoded by *CYP27A1* and *CYP2R1*, respectively), typically present in the liver, were found additionally in the human endometrium [30], where their expression is temporal and, specifically, higher in the secretory compared to proliferative phase of the cycle [30].

One of the main functions of the endometrium is to allow for the establishment of pregnancy and changes in human endometrium are essential in this process. Decidualization is the process whereby endometrial stromal cells transform into specialized secretory decidual cells that provide a nutritive and immunoprivileged matrix essential for embryo implantation and placental development [32]. *HOXA10* is a well-known molecule involved in the mechanism of implantation, and a decrease of implantation rates has been observed in women with altered *HOXA10* expression [33]. Indeed, *HOXA10* expression reaches a peak during the window of implantation in response to estrogen and progesterone. In addition, *HOXA10* expression has been found to be regulated by $1,25(OH)_2D_3$ in human endometrial stromal cells. Therefore, the cross talk between sex steroids and VD may converge in the regulation of *HOXA10* [13]. The link between sex steroids and VD during embryo implantation was confirmed by Viganò and colleagues (2006) [28]. They found that the expression of osteopontin, a progesterone-regulated putative adhesion molecule mediating implantation and decidualization [34], was increased in endometrial cells in response to $1,25(OH)_2D_3$ [28]. Beside this evidence, the analysis of the VD system on a molecular level in the normal cycling endometrium as well as in pathophysiological conditions has received very limited consideration.

Special attention is drawn to the endometrium of women undergoing assisted reproduction technology. The intake of VD has been shown to improve the thickness of the endometrium in polycystic ovarian syndrome (PCOS) women but not the probability of pregnancy. Unfortunately, no molecular mechanism explaining this phenomenon was proposed [35]. Interestingly, while different hormonal pathways such as insulin and thyroid hormone signaling pathways have been shown to be dysregulated in the endometrium from assited reproductive technology (ART) patients, the hormonal stimulation treatment does not seem to change the VD pathway at least in terms of *VDR* expression [36]. Additionally VD insufficiency has been in the interest of IVF researchers for many years and it remains unknown which element—the endometrium or the oocyte—is more affected by VD deficiency. One of the first studies addressing this issue was from Rudick and colleagues (2014). In that study, they considered the relationship between circulating VD levels in in vitro fertilization (IVF) recipients with pregnancy outcomes, using only donated oocytes to avoid the embryo-oocyte bias in their assessment. Live birth rate resulted lower in the VD-deficient recipients compared to VD-repleted recipients [37]. In contrast, another study reported no differences in implantation and pregnancy rates in egg donation IVF cycles between recipient women with normal or insufficient VD levels [38]. Similar results in terms of implantation rate were found in the study of Franasiak and collegues (2015), where in the case of euploid blastocyst transfer, no differences in pregnancy outcomes between groups with various levels of VD could be found [39]. At this stage, there is insufficient evidence to confirm that VD levels could influence the receptivity of the endometrium of women undergoing IVF.

4. Vitamin D and Endometriosis

The expression of *VDR* and VD enzymes in endometriotic tissues was demonstrated for the first time by Agic and colleagues in 2007 [40]. Eutopic endometrial tissues were evaluated by immunohistochemistry in patients undergoing laparoscopy during the proliferative or secretory phase. The control tissue was represented by endometrium from women undergoing laparoscopy for other benign gynecological disorders. *VDR* mRNA expression was evaluated by RT-PCR in eutopic endometrium from women with endometriosis ($n = 13$) compared to that of a control group ($n = 14$). A nonsignificant trend towards higher levels of *VDR* mRNA was observed in the endometriosis group ($p = 0.10$). They analyzed separately epithelial and stromal endometrial cells and reported that *VDR* mRNA was significantly higher in epithelial compared to stromal cells isolated from endometrial biopsies from patients with endometriosis ($p < 0.01$) while this difference could not be detected in the endometrium from healthy controls patients. Nonetheless, the expression of epithelial *VDR* mRNA was higher in the endometriosis group compared to the control group and the same applied to the stromal cell expression. The results of transcriptomic analysis were corroborated by western blot analysis. Unfortunately, the authors did not study the *VDR* mRNA expression in the ectopic endometrium of women with endometriosis. The expression levels of the enzymes 24-hydroxylase, 25-hydroxylase, and 1α-hydroxylase were also evaluated and higher levels of 1α-hydroxylase were found in the endometrium of endometriosis patients compared to the control group ($p = 0.03$). It is still pending whether the dysregulated parameters of VD metabolism are constitutively present in patients with endometriosis or are rather the consequence of a secondary response to the local inflammation [40]. In terms of *VDR* expression, similar results were found in the study by Zelenko and colleagues (2007). These authors analyzed proliferative, early secretory, and midsecretory phase eutopic endometrial samples from control women and endometriosis patients by PCR array and did not find any difference in *VDR* expression levels [29].

Whether the metabolism of VD is dysregulated in the eutopic endometrium of women with endometriosis remains to be elucidated.

Genetic evidence suggest that polymorphisms in the *VDR* gene may be associated with an altered susceptibility to diseases such as cancer and osteoarthritis [41]. Therefore, this possibility has been also considered in women with endometriosis. The study conducted by Vilarino and colleagues did not find any difference in the frequency of several *VDR* polymorphisms (SNPs) studied by restriction fragment length polymorphisms among 132 women with endometriosis-related infertility, 62 women with idiopathic infertility, and 133 controls [42]. Similar results were reported by Szczepańska and colleagues (2015), as they did not demonstrate differences in genotype and allele frequencies of several *VDR* SNPs between 154 women with endometriosis-associated infertility and 347 controls [43].

Considering the established inverse correlation between VD levels and cancer development [40,44] and the fact that endometriosis is a disease with similar features of a malignancy, the potential mechanistic anti-proliferative and anti-inflammatory effects of VD for the treatment of endometriosis have been investigated in recent years. The molecular mechanism by which VD could affect the development of the disease has been studied mainly in in vitro models with human endometriotic stromal cells [45,46]. In the study from Miyashita and colleagues, endometriotic tissue samples were obtained from the cyst wall of the ovarian endometrioma. Ectopic stromal cells were treated with $1,25(OH)_2D_3$ and the gene expression profile was analyzed. The authors found a reduction in *IL-1β*, *TNF-α*, metalloproteinase (*MMP*)-2, and *MMP-9* mRNA levels. A reduction of the DNA synthesis was also detected but without affecting the levels of apoptosis [45]. Similar results were reported by Delbandi and colleagues the same year. The ectopic stromal cells isolated from the endometrioma were treated with $1,25(OH)_2D_3$ and this treatment could significantly reduce *IL-1β*- and *TNFα*-induced inflammatory responses, such as prostaglandin activity, *IL-8* and *MMP* mRNA expression. A significantly reduction in terms of cell invasion and proliferation was also reported [46].

Recently, Ingles and colleagues (2017) have further investigated the pathways regulated by VD in endometriosis cells [47]. An endometriosis stromal cell line (ESC22B) derived from peritoneal

endometriosis lesions was treated with the supra-physiologic concentration of 0.1 μM $1,25(OH)_2D_3$ for 24 h. Using Next-Generation Sequencing, 11,627 differentially expressed genes between treated and untreated cells by at least two fold were detected. The most strongly affected pathways were: (a) the axonal guidance pathway involved in neuro-angiogenesis; (b) the RhoDGI signaling pathways involved in actin organization of the cytoskeleton; and (c) the *MMP* inhibition pathway involved in the degradation of the extracellular matrix. The enzyme 24-hydroxylase and *VDR* were both found to be up-regulated while 1α-hydroxylase, responsible for the conversion of 25(OH)D to $1,25(OH)_2D_3$ was down-regulated [40]. Finally, the expression of 24-hydroxylase was also compared between eutopic endometrium of healthy subjects and endometriotic lesions of patients. The enzyme was up-regulated in endometriosis lesions indicating, according to the authors, an intense VD metabolism in endometriosis tissues [47].

Generally, a regression of the endometriotic implants after VD or *VDR* agonist treatment has been described mainly using animal models [48–50] and several mechanisms are postulated to be involved. For this reason, it is quite surprising that the effect of VD has been investigated on very few cellular functions underlying endometriosis development. Very few data refer to the effect on apoptosis, adhesion, and invasion. Moreover, the impact of VD on endometriosis-mediated inflammatory process has been only vaguely considered. There are however some limitations in the in vitro studies described above that need to be taken into consideration. It should be noted that the characterization of the isolated endometriotic cells from endometriomas does not usually receive the necessary attention that would require a first line cell characterization, hence the possibility of contamination by ovarian components or by fibroreactive tissue is high [51]. Therefore, although these studies may be useful to understand whether the VD treatment may influence the development of the disease, information derived from these studies needs to be considered with caution.

Future investigations need to be performed using different models for endometriosis disease, for instance, primate models, to elucidate the real mechanism by which VD and/or *VDR* agonists may exert an "anti-endometriosis" effect. Finally, clinical trials with VD would be helpful to evaluate the possible therapeutic benefit of VD and/or *VDR* agonists in women with endometriosis.

5. Vitamin D and Endometrial Cancer

5.1. VD/VDR Pathway and VD Metabolism in Endometrial Cancer

VDR protein expression and nuclear localization were for the first time established by immunohistochemistry in human endometrial cancer tissue by Yabushita and colleagues (1996). *VDR* proteins were detected in most human endometrial adenocarcinoma tissues studied (14 of 21 samples). Similarly, some immortalized human endometrial cancer cell lines (RL95-2 and Ishikawa lines) but not all of them (AMEC-1 cell line) expressed detectable levels of *VDR* protein [52]. In order to support the presence of *VDR* in endometrial cancer tissues, ten years later, Agic and colleagues showed by RT-PCR that *VDR* mRNA levels were significantly higher in endometrial cancer tissues compared to endometrial tissue from healthy patients (respectively $n = 5$ versus $n = 14$ patients, $p = 0.03$) [40]. On the other hand, a more recent immunostaining analysis of human tissues has found lower levels of nuclear *VDR* protein expression in 137 tumor samples compared with 55 samples from normal endometrium. Nonetheless, cytosolic *VDR* levels remained unvaried [30].

Different components of the VD system have been evaluated in endometrial cancer tissue. As described in healthy endometrium, also in the endometrial cancer tissue the enzyme 1α-hydroxylase has been shown to be expressed in a similar amount between healthy and malignant tissue [31]. On the other hand, a reduced activity of VD has been proposed, resulting from a deficit in the local synthesis due to a reduction of the activity of 1α-hydroxylase and to an increase of VD catabolism. In this context, Agic and colleagues showed significantly increased 24-hydroxylase mRNA levels ($p < 0.05$) in endometrial cancer tissue compared to tissues from healthy control patients [40]. The 24-hydroxylase enzyme is responsible for the catabolism of VD and, potentially, its increase may reduce the cellular

effects of calcitriol. Confirming the mRNA expression analysis, 24-hydroxylase protein expression was higher in endometrial cancer cells compared to healthy endometrium as evaluated by western blot and this increase correlated with tumor progression [53]. These results are consistent with an association between increasing mRNA levels of 24-hydroxylase and poor prognosis in high-grade tumor progression in other kind of tissues [54,55]. In disagreement with these findings, Bergadà and colleagues (2014) detected lower levels of 24-hydroxylase protein in tumoral endometrium compared to normal one by immunohistochemical analysis; no differences emerged among samples characterized by different stages of pathology [30].

Taken together, the presented molecular evidence could suggest an alteration of VD metabolism in this kind of tumor, suggesting VD as a possible target for potential therapeutic treatments. Nevertheless, the inconsistency of results from the published literature revealed that further studies are required.

5.2. Vitamin D Action in Endometrial Cancer

The first study investigating the effects of VD treatment in an in vitro model of endometrial adenocarcinoma cells did not find any alteration in rate of cell proliferation despite of evident VD responsiveness in terms of 24-hydroxylase activity in these cells [56]. Conversely, the subsequent studies reported an anti-proliferative action of VD in different endometrial cancer cell lines. Saunders and colleagues highlighted the inhibitory effect of VD treatment on the growth of endometrial carcinoma cells (RL95-2) for the first time. Unfortunately, these authors did not investigate the presence of *VDR* and the molecular mechanism to account for the observation [57]. Later, Yabushita and colleagues (1996) demonstrated that the growth of RL95-2 cells expressing *VDR* was prevented in a dose-dependent manner by VD treatment (inhibited to 44% following treatment with 50 nM of $1,25(OH)_2D_3$ for 6 days); on the contrary, the growth of AMEC-1 cells not expressing *VDR* was completely uninhibited by the treatment [52].

Similar studies proposed VD as an anti-proliferative drug in endometrial cancer cell lines, mainly reporting a mechanism of growth arrest [30,58] or apoptosis [59,60]. Calcitriol treatment induced cell cycle arrest in endometrial cancer cells suppressing some regulators of the cell cycle progression such as cyclin D1 and D_3 and increasing the expression of p27, a well-known cell cycle inhibitor [59]. In addition, other authors reported variations in expression of proteins involved in several molecular mechanisms such as apoptosis (ex. *hiNT2*), rearrangement of chromatin accessibility (ex. *HIST1H1E*) and differentiation (ex. *EIF2AK2*). Calcitriol was shown to have an additive effect when used in combination with progesterone [59].

Additionally, VD was demonstrated to be able to induce a programmed cell death in endometrial cancer cells activating key actors of the intrinsic apoptotic pathway (such as caspase-3 and caspase-9 proteases) and by disrupting the delicate balance between pro-apoptotic factors and pro-survival defense responses (such as *BAX* versus *BCL-xL* and *Bcl2*) [59,60]. Besides its pro-apoptotic action, Kasiappan and colleagues reported a capacity of VD to suppress the cell survival/proliferation stimuli supported by activation of telomerase typically overexpressed in tumor tissues. In Ishikawa endometrial cancer cells, $1,25(OH)_2D_3$ treatment induced raising levels of mature miR-498 able to promote the degradation of the human telomerase reverse transcriptase (*hTERT*) mRNA and thus preventing the *hTERT*-supported cell survival with a post-transcriptional gene expression regulation mechanism [61].

Vitamin D has been also proposed as a cell-differentiation-inducing agent in endometrial cancer cells. Yabushita and colleagues demonstrated that RL95-2 cells expressing *VDR*, after exposure to $1,25(OH)_2D_3$ for 6 days, expressed high levels of the cytokeratin polypeptide and became columnar with pronounced polarity and formed gland-like structures when cultured in collagen gel [52]. Haselbergerger and colleagues published similar results [62]. The latter demonstrated an up-regulation of genes involved in differentiation pathways such as E-cadherin and lactoferrin. This effect was dependent on *ICB-1*, a gene involved in the differentiation process of endometrial cancer cells [63]. Additionally, the loss of *ICB-1* blocked the inhibitory effect of VD on the process of epithelial to

mesenchymal transition (EMT) [62] that together with migration/invasion and angiogenesis is a fundamental phenomenon in tumorigenesis and cancer progression [64]. In terms of inhibition of angiogenesis, chemotaxis, and endometrial tumor cell growth, a link between semaphorin proteins (*SEMA*) and VD has been as well postulated. Nguyed and collegues reported that *SEMA3B* and *SEMA3F* are strongly induced by $1,25(OH)_2D_3$ in endometrial cancer cells. Lower receptor levels for these proteins were found in endometrial cancer tissue compared to endometrial tissue from control patients. Importantly, the expression of SEMAs further decreased with the tumor progression, suggesting SEMAs as onco-suppressor genes with a key role in molecular mechanisms of transforming processes [58]. More recently, using Ishikawa endometrial cancer cell line, the $1,25(OH)_2D_3$ treatment was shown to affect the reorganization of the cytoskeleton mainly down-regulating the expression and activity of proteins involved in the reorganization of actin structures (such as Actin-Related Protein *ARP2*, Rac Family Small GTPase *RAC-1* and *PAK1* kinase protein) and inducing de-polymerization of actin filaments [65]. Vitamin D may also be a modulator of invasiveness of endometrial tumor cells. Indeed, Bokhari and colleagues found a weak reduction of the invasive potential of endometrial cancer cells (HEC-1B and Ishikawa cells) (15–20%) and a reduction in the levels of tumor invasion molecular markers such as *MMP2* and *MMP9*, upon VD treatment [53]. The inhibitory effect of VD was more effective with a progesterone-induced 24-hydroxylase inhibition in line with the observation that 24-hydroxylase is upregulated in endometrial cancer cells and can control VD cellular responses [53]. This result is consistent with an enhanced anti-proliferative action following a combination of VD and 24-hydroxylase inhibitor treatment in in vitro and in vivo models of prostate and lung tumors [66,67].

Finally, VD is a putative anti-inflammatory agent and inflammation is widely considered as a risk factor for cancer development [68]. In addition, several studies showed a reduction of endometrial cancer risk associated with the use of anti-inflammatory agents [69,70]; therefore, the role of the anti-inflammatory action of VD in endometrial cancer has been closely investigated. VD-induced suppression of NF-κB, the key transcription factor involved in inflammation and innate immunity responses, was associated with a diminished expression of inflammatory cytokines/chemokines involved in metastasis-related processes such as *CXCL1* and *CXCL2* [71].

The results of this review showed the widespread effects of VD of endometrial cancer cells. The complex pleiotropic effect of VD on endometrial cancer cells can be exemplified with the results of the study by Lee and colleagues. These authors revealed that treatment of the HEC-1B cell line with $1,25(OH)_2D_3$ could modify the expression of more than 300 proteins among which oncogenes, tumor suppressor, membrane and structural proteins and actors in a multiplicity of processes such as cell cycle, transcriptional regulation, differentiation, and DNA repair [59].

Although the evidence is suggestive of an inhibitory effect of VD on endometrial cancer, it is important to underline that the majority of the studies concerning VD and endometrial cancer has been done using immortalized endometrial cancer cells. Moreover, the findings on in vivo or ex vivo studies to investigate molecular mechanisms of VD in endometrial cancer are scarce and therefore warrants further investigation.

6. Conclusions

The role of VD and female fertility has been deeply studied. However, the role of VD in physiological endometrium has been less considered. The results of this review demonstrate that the knowledge about the effects of VD in physiological endometrium is poor and that the molecular mechanisms involved are still to be completely defined.

One of the purposes of this review was to elucidate the role of VD in the physiologic endometrium, in endometriosis, and in endometrial cancer. Apparently, *VDR*-mediated signaling pathways seem to be dysregulated in those pathological conditions; nonetheless, the results are contradictory (Table 1). Therefore, more studies are needed to confirm a beneficial role of VD treatment on endometrial cancer and/or endometriosis.

Table 1. Studies addressing the presence/absenceof *VDR* and enzymes that metabolized VD in normal and pathologic endometrium.

Type of Samples	Target	Result	Technique	Reference
Endometrial tissue from control patients (premenopausal)	*VDR*	Presence	PCR-array	Vienonen et al., 2004 [27]
		Presence		Vigano et al., 2006 [28]
		Down-regulated in mid-secretory vs. early secretory	PCR-array	Zelenko et al., 2012 [29]
		Down-regulated in proliferative vs. secretory phase	Tissue array	Bergada et al., 2014 [30]
	1α-hydroxylase	Presence	RT-PCR	Vigano et al., 2006 [28]
		Presence		Becker et al., 2007 [31]
		Down-regulated in proliferative vs. secretory phase	Tissue array	Bergada et al., 2014 [30]
	25-hydroxylase	Down-regulated in proliferative vs. secretory phase	Tissue array	Bergada et al., 2014 [30]
Eutopic endometrium from endometriosis patients	24-hydroxylase	Up-regulated endometriosis vs. control tissue	RT-PCR	Agic et al., 2007 [40]
	25-hydroxylase	No differences between endometriosis vs. control tissue		
	1α-hydroxylase	No differences between endometriosis vs. control tissue		
	VDR	No differences between endometriosis vs. control tissue		
		No differences between endometriosis vs. control tissue	RT-PCR	Zelenko et al., 2012 [29]
Endometrial tissue from endometrial cancer patients	*VDR*	Up-regulated endometrial cancer vs. control tissue	RT-PCR	Agic et al., 2007 [40]
	Nuclear *VDR*	Down-regulated endometrial cancer vs. control tissue	Tissue array	Bergada et al., 2014 [30]
	24-hydroxylase	Up-regulated endometrial cancer vs. control tissue Correlation with tumor progression.	Tissue array	Bokhari et al., 2016 [53]
		Down-regulated endometrial cancer vs. control tissue No differences in tumor progression.		Bergada et al., 2014 [30]
		Up-regulated endometrial cancer vs. control tissue	RT-PCR	Agic et al., 2007 [40]
	1α-hydroxylase	No differences between endometrial cancer vs. control tissue	Immunostaining	Becker et al., 2007 [31]

Author Contributions: G.C.C. and A.M.S. authored the manuscript. The co-authors provided suggestions, comments and participated equally in the critical assessment.

Funding: The research was supported by the Grant of Ministero della Salute-Ricerca Finalizzata Program-Project Code: RF-2013-02358757.

Acknowledgments: We thank Sofia Makieva for carefully reading the manuscript and English editing.

Conflicts of Interest: The authors declare no conflict of interest.

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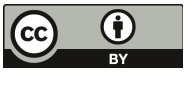

International Journal of
Molecular Sciences

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Review

Non-Coding RNAs in Endometrial Physiopathology

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Received: 22 June 2018; Accepted: 14 July 2018; Published: 20 July 2018

Abstract: The Human Genome Project led to the discovery that about 80% of our DNA is transcribed in RNA molecules. Only 2% of the human genome is translated into proteins, the rest mostly produces molecules called non-coding RNAs, which are a heterogeneous class of RNAs involved in different steps of gene regulation. They have been classified, according to their length, into small non-coding RNAs and long non-coding RNAs, or to their function, into housekeeping non-coding RNAs and regulatory non-coding RNAs. Their involvement has been widely demonstrated in all cellular processes, as well as their dysregulation in human pathologies. In this review, we discuss the function of non-coding RNAs in endometrial physiology, analysing their involvement in embryo implantation. Moreover, we explore their role in endometrial pathologies such as endometrial cancer, endometriosis and chronic endometritis.

Keywords: miRNA; lncRNAs; endometrial cancer; endometriosis; chronic endometritis

1. Introduction

At the beginning of the Human Genome Project (HGP), in the late 1990s, researchers hypothesized that our genome comprised about 100,000 protein-coding genes [1]. Over the years, this number has continuously decreased. In 2001, the International Human Genome Sequencing Consortium (IHGSC) published the initial sequence of the human genome and proposed that the number of protein-coding genes was about 30,000 [2]. The end of the sequencing of the human genome in 2004 revealed that the number of genes encoding for proteins were only 20–25,000 [3]. The latest version of human GENCODE (available online: https://www.gencodegenes.org/) established that the number of genes encoding for protein was 19,901 that is the 34.09% of human genes (Figure 1a). In spite of the low number of gene encoding proteins the ENCODE project, based on High Throughput Sequencing technologies and advances in bioinformatics, has provided a detailed landscape of transcription in human cells. (Figure 1b).

The non-coding RNAs (ncRNAs) could be classified, according on their size, into small ncRNAs (less than 200 nucleotides in length) and in long ncRNAs [(lncRNAs) longer than 200 nucleotides]. Alternatively, a further classification based on their function, split the ncRNAs into housekeeping and regulatory: the housekeeping ncRNAs, including ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA), are expressed in all cell types and carry out essential functions in the cells, while the regulatory ncRNAs, including several classes of small and long molecules, assist in the regulation of gene expression, controlling different points of the central dogma (Figure 2).

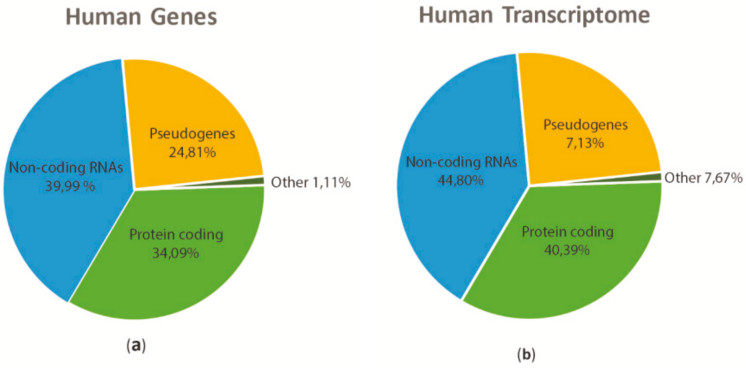

Figure 1. Overview of functional elements of the human genome and of the associated transcriptome. Pie chart showing the percentage of human genes (**a**) and transcripts (**b**) based on the current Release (Release 28, GRCh38.p12) of GENCODE. Classification is referred to Gene/Transcript Biotypes in GENCODE & Ensembl and to VEGA descriptions (available online: http://vega.archive.ensembl.org/info/about/gene_and_transcript_types.html). The non-coding transcriptome includes both small and long non-coding RNAs; pseudogenes incorporate processed, unprocessed, transcribed, translated, polymorphic and unitary sequences; other includes IG/TCR and their pseudogenes, together with non-stop decay and non-sense mediated decay.

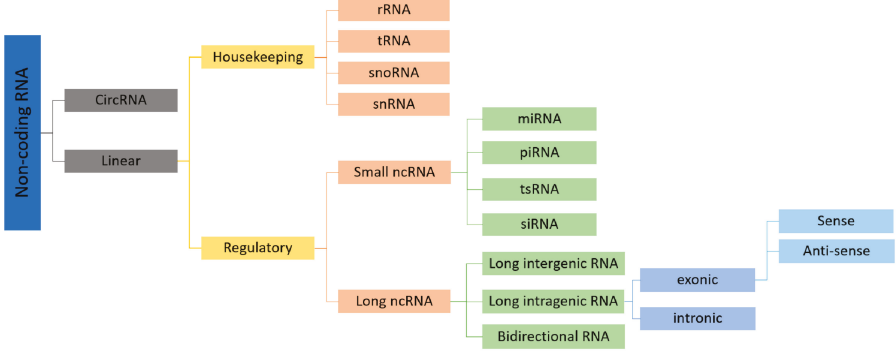

Figure 2. Schematic diagram illustrating the classification of ncRNAs according to their biological role and their length.

The interest of scientific literature in regulatory ncRNAs is generated by the important roles that these molecules perform regulating cell proliferation, differentiation, migration, cell death and angiogenesis. Consequently, their altered expression is involved in different human pathologies. Another interesting characteristic for the potential implications for human health is that ncRNAs are present in all biological fluids associated with protein complexes or enclosed within extracellular vesicles (EVs) such as microvesicles or exosomes. Extracellular ncRNAs also show altered expression in different human pathologies, thus their role has been proposed as non-invasive biomarkers, prognostic factors and also therapeutic targets in cancer or in other complex diseases. Among the regulatory ncRNAs we found: microRNAs (miRNAs), small interfering RNAs (siRNAs), Piwi-associated RNAs (piRNAs), long non-coding RNAs (lncRNAs), circular RNAs (circRNAs) and the tRNA derived small RNAs (tsRNAs) (Figure 2). To date, the most studied molecules are miRNAs, lncRNAs and circRNAs. miRNAs are long 18–25 nucleotide (nt) single-stranded RNAs, evolutionarily conserved, which negatively modulate the expression of their target mRNAs. They bind to the 3′ Untranslated

Region (3′ UTR) of specific mRNA targets, leading to translational repression, or mRNA cleavage [4–6] miRNAs are very important molecules in the regulation of gene expression at the post-transcriptional level, a single miRNA can control the expression of several mRNAs and a single mRNA may be targeted by more than one miRNA, thus creating a complex network of cooperative regulation [7]. lncRNAs are the most heterogeneous class of non-protein-coding RNAs with lengths ranging from 200 to 100,000 nt. They include transcripts that may be classified as: (a) intergenic lncRNAs; (b) intronic lncRNAs; (c) sense or antisense transcripts; (d) pseudogenes; and (e) retrotransposons [8]. lncRNAs regulate gene expression at different levels, including chromatin modification, alternative splicing, protein localization and activity and can protect 3′ UTR of mRNAs from miRNA binding, increasing their stability [8]. Several recent studies have also shown that lncRNAs are critically involved in a wide range of biological processes, such as cell cycle regulation, pluripotency, differentiation and cell death [9–12]. CircRNAs are a recently discovered class of circular single strand RNA molecules, covalently closed, which are resistant by the exonuclease [13]. Their function and the biological process in which they are involved remain mainly unknown but recent evidence suggests that circRNAs may play an important role in RNA–RNA interactions. In some cases, circRNAs exhibit multiple binding sites for the same miRNA and for this reason it has been suggested that they could represent potential molecular sponges for sequestering the most abundant miRNAs [14]. It means that they can act as a negative regulation of miRNAs by competing, using their binding sites, with the miRNA–mRNA target interaction. Their important role in gene regulation is demonstrated by some papers, which show that deregulation of circRNAs is associated in different types of cancer [15].

Scientific community recognized the important role played by regulatory ncRNAs in cell physiology and also the effects caused by their altered expression in all human diseases but many other studies will be necessary. In fact, sometimes, we are able to identify the differential expression of one or more ncRNAs in a particular disease and also to evaluate their prognostic value but we are not able to correlate accurately specific expression profiles to specific phenotype alteration, because of the complexity and the redundancy of the circuits regulating gene expression.

In this review, we will point up the ncRNA, particularly miRNA and lncRNAs, found expressed in endometrium in physiological conditions, focusing on their involvement in embryo implantation. Moreover, we will analyse the alteration of their expression in the pathogenesis of some endometrial diseases, as endometrial cancer, endometriosis and chronic endometritis, trying to understand how their altered expressions can influence cell proliferation, differentiation and apoptosis.

2. ncRNAs and Embryo Implantation

2.1. Embryo Implantation

The success of embryo implantation is related to blastocyst quality as well as to the endometrium receptivity. Both the embryo and the maternal tissues are able to reciprocally exchange signals; the proper secretion of signal molecules and their uptake allow the successful implantation [16,17].

Several mediators such as growth factors, cytokines, chemokines, lipids, matrix-degrading enzymes and integrins, whose expression is regulated by oestrogen (E) and progesterone (P), influence endometrial receptivity [18]. Many genes have been discovered that have important roles in embryo implantation and in 2011 a genomic tool was developed to assess the endometrial receptivity of woman during In Vitro Fertilization cycles (IVF). The Endometrial Receptivity Array (ERA) test, based on microarray technology, analyses the expression profile of 238 genes encoding proteins which are related with the implantation process, during the Window of Implantation (WOI). The ERA test has been shown to be more accurate than histological evaluation to assess endometrial receptivity. This has subsequently led to the new concept of personalized embryo transfer, by using endometrial biomarkers as a therapeutic strategy for patients with recurrent implantation failure [18–22] According to the rising interest in the role of ncRNAs in the regulation of gene expression, different studies have explored the involvement of miRNAs in implantation, focusing on the dialog between embryo and

maternal tissues. Different miRNAs, associated with endometrial receptivity, have been identified in endometrial biopsies as well as in endometrial fluids (Table 1).

2.2. miRNAs in Endometrial Receptivity

Different studies, on animal models and humans, confirmed the important role played by miRNAs in endometrial physiology by regulating the changes in gene expression levels during the different phases of the endometrial cycle. In fact, many of them were found differentially expressed at each stage of the endometrial cycle.

MiR-30b and miR-30d were found to be significantly up-regulated and miR-494 down-regulated in the receptive endometrium (LH+7) compared with the pre-receptive endometrium (LH+2) from healthy fertile women. The bioinformatic prediction of the target genes of these miRNAs showed that they are involved in the cyclic remodelling of the endometrium, including endometrial maturation to the receptive state [23]. Mucin 1 (Muc1) is an integral transmembrane mucin glycoprotein expressed on the apical surface of the endometrium, acting as an inhibitor of embryo attachment. In mice, the expression of Muc1 decreased significantly during the WOI and could be due to a negative regulation mediated by miR-199a, let-7a and let-7b [24,25]. Insulin-like growth factor 1 receptor (IGF1R) is an important receptor, up-regulated in the endometrium during the receptive stage, which is closely related to embryo implantation. In fact, its increase on the surfaces of the endometrium might contribute to adhesive interaction with the embryo. It has been demonstrated that a high expression of miR-145 having as target the mRNA coding for IGF1R, inhibits embryo attachment. Mir-145 up-regulation has been shown in the endometrium of RIF patients [26].

Another very important characteristic for endometrial receptivity is the epithelial-mesenchymal transition (EMT). In EMT, the cells lose their polarity and display a remodelling of cell junctions in order to facilitate the interaction with embryo trophectoderm [27]. As an important suppressor of EMT, miR-429 exhibited a down-regulation during implantation in mice. Enhancement of miR-429 resulted in suppression of the migratory and invasive capacities of cells, probably through targeting protocadherin 8, leading to reduced implantation sites [28]. On the contrary, miR-126-3p was specifically up-regulated in implantation sites, promoting cell migratory and invasive capacity by regulating the expression of integrin α11 [29]. Moreover, we know that sex hormones induce changes in miRNA expression in the endometrium, for example, progesterone induced the expression of miR-125b in human endometrial epithelium cells. The increased expression of miR-125b inhibits cell movement and prevents implantation, by suppressing the expression of Matrix Metallopeptidase 26 (MMP26), a member of the matrix metalloproteinase family, which is involved in degradation of the extracellular matrix [30]. These studies demonstrated that, in addition to the cyclical changes in endometrium morphology and gene expression, there exists a very complex network of miRNAs regulating the expression of many genes encoding proteins related to endometrial receptivity and implantation.

2.3. Extracellular miRNAs in Endometrial Fluid

To assess endometrial receptivity, many researchers are focusing their studies on extracellular ncRNAs present in endometrial fluid. Endometrial fluid is a viscous fluid secreted by the endometrial glands into the uterine cavity and its role consists in nurturing the embryo and represents the important microenvironment in which both embryo and endometrium can interact with each other [31]. The embryo, induced by cytokines and different proteins secreted by the endometrium, modulates, in turn, the secretion of integrins β3, α4 and α1, interleukins, chemokines and leptine. Recently, ncRNAs have been added, as new players, in mediating the dialog between embryo and endometrium. It has been demonstrated that endometrial cells secrete exosomes in endometrial fluids and the secretion reaches a peak during WOI [32]. On the other hand, also embryos secrete extracellular vesicles in vitro that are able to be up taken by cultured endometrial cells [33]. During the last few years, the presence of miRNAs in endometrial fluid has been shown in humans and in animal models [27]. Comparing miRNA profiles between endometrial cells and exosomes present in endometrial fluid,

13 miRNAs out of 227 were exclusively present in exosomes/microvesicles and their mRNA targets were involved in several signalling pathways associated with implantation [34]. In 2015, in a very interesting paper, the authors analysed the RNA transcript present in endometrial fluid by microarray and found that a large number of miRNAs were present in the endometrial fluid. They found 27 miRNAs differentially expressed during the WOI with respect to the four different phases of the menstrual cycle and among them miR-30d was the most up-regulated miRNA [32]. The authors also demonstrated that miR-30d is an exosomal miRNA and it is internalized by trophoblastic cells of murine embryos. Moreover, embryos treated with miR-30d exhibited increased expression of ten genes, including those encoding adhesion molecules such as Integrin Subunit Beta 3 (ITGB3), Integrin Subunit Alpha 7 (ITGA7) and Cadherin 5 (CDH5) [32]. These studies demonstrated the molecular dialog, mediated by miRNAs, between embryo and endometrium, in order to promote embryo implantation.

Table 1. miRNAs involved in embryo receptivity.

miRNAs	Species	Sample	Reference
mmu-let-7a	mouse	endometrium	[23]
mmu-let-7b	mouse	endometrium	[23]
hsa-let-7e*	human	exosomes from endometrial cell lines	[34]
hsa-let-7f-2*	human	exosomes from endometrial cell lines	[34]
hsa-miR-30b	human	endometrium	[24]
hsa-miR-30d	human	endometrium/endometrial fluid	[24,32]
hsa-miR-122	human	exosomes from endometrial cell lines	[34]
hsa-miR-124	human	exosomes from endometrial cell lines	[34]
hsa-miR-125b	human	endometrial cell lines	[30]
mmu-miR-126-3p	mouse	endometrium	[29]
hsa-miR-129*	human	exosomes from endometrial cell lines	[34]
hsa-miR-142-3p	human	exosomes from endometrial cell lines	[34]
hsa-miR-145	human	endometrium	[26]
mmu-miR-199a	mouse	endometrium	[25]
hsa-miR-222*	human	exosomes from endometrial cell lines	[34]
hsa-miR-376c	human	exosomes from endometrial cell lines	[34]
hsa-miR-409-3p	human	exosomes from endometrial cell lines	[34]
mmu-miR-429	mouse	endometrium	[28]
hsa-miR-432	human	exosomes from endometrial cell lines	[34]
hsa-miR-451	human	exosomes from endometrial cell lines	[34]
hsa-miR-494	human	endometrium	[24]
hsa-miR-520h	human	exosomes from endometrial cell lines	[34]
hsa-miR-1248	human	exosomes from endometrial cell lines	[34]

3. ncRNAs and Endometrial Cancer

3.1. Endometrial Cancer

Endometrial cancer (EC) is the most common gynaecological tumour in developed countries, it is the fifth most common cancer and the 14th in terms of mortality [35]. It is becoming clear that EC, as well as many other tumours, includes different subtypes having specific genetic and molecular features. Based on histological characteristics, specific protein expression and grade, EC has been classified into two subtypes [36]. Type 1, also called endometrioid type (EEC), represents 70–80% of new sporadic cases. These cancers are typically well differentiated, associated with increasing oestrogen levels and the patients have a favourable prognosis. On the molecular level, more than 80% type I tumours are associated with a decreased or lacking expression of Phosphatase and tensin homolog (PTEN) and to an overexpressed Oestrogen Receptor (ER), which promote a deregulated cellular proliferation [37–40]. Furthermore, EEC are frequently associated with microsatellite instability (leading to DNA mismatch repair), genetic mutations and epigenetic abnormalities [41]. Type 2, known as non-endometrioid endometrial carcinoma (NEEC), is not related to circulating oestrogen levels

and it is less common than type 1 (10–20% of ECs) [42]. Patients with NEEC, usually have a poor prognosis because the tumour is typically diagnosed when metastases are already present [36]. On the molecular level, NEEC tissue samples show a high expression of Tumour Protein p53 (TP53) and Cyclin Dependent Kinase inhibitor 2A (CDKN2A or P16), resulting in non-functional proteins that accumulate in the cell acting as a double negative inhibitor of the wild-type p53, leading to propagation of aberrant cells and to uncontrolled cell growth [43].

Many papers have investigated the role of ncRNA in EC in an attempt to find new molecular markers able to discriminate the different subtypes, predict prognosis and design new drugs for personalized targeted therapies [44–47]. In this review, we focused our attention on more important biological pathways and processes altered in EC and we discussed the role of ncRNAs in their regulation.

3.2. miRNAs in Endometrial Cancer

The phosphatidylinositol 3-kinase (PI3K) pathway is involved in the control of growth, survival, proliferation and apoptosis. Gain- or loss-of-function mutations of genes encoding proteins involved in the pathway lead to neoplastic transformations and it has been demonstrated that several components are dysregulated also in EC [48]. PTEN is a tumour suppressor that negatively regulates the PI3K-AKT signalling pathway. Loss of its function is implicated in the pathogenesis of a number of different tumours, including endometrial carcinoma [49]. PTEN mRNA is negatively regulated by miR-205 and it has been demonstrated that miR-205 up-regulation leads to increased B-cell lymphoma 2 (BCL2) levels and TP53 down-regulation, inhibiting tumour cell apoptosis and enhancing proliferation [50]. Moreover, by stimulating the AKT pathway and inhibiting glycogen synthase kinase 3β (GSK3B), miR-205 suppresses E-cadherin expression and promotes SNAIL expression [51]. Transfecting endometrial cancer cells with LNA-miR-205-inhibitor (Locked Nucleic Acid-inhibitor of miR-205), Torres et al. recently obtained decreased endometrial cancer cell proliferation in vitro and in vivo [52]. PTEN can also be controlled by miR-21, involved in enhanced malignant transformations and proliferation of type 1 EC cells [53]. The transcription factor Forkhead Box O1 (FOXO1), belonging to the Forkhead Box class O family, is a downstream target of the PI3K pathway and it is able to regulate different biological processes involved in endometrial phases such as menstruation, uterine cell regeneration, tissue remodelling and cell differentiation [54,55]. In EC type 1, FOXO1 is down-regulated and it has been shown that miR-9, miR-27, miR-96, miR-153 and miR-182, able to target FOXO1 mRNA, are over expressed in the same cells [56]. On the other hand, Forkhead Box C1 (FOXC1), another member of the forkhead box transcription factors, is an oncogene controlling tumour cell migration and metastasis and it is inhibited by miR-204 [57]. MiR-204 down-expression has been associated with solid tumour development, such as in the lung and gastric and endometrioid endometrial cancer [57–59]. The hyperactivation of the PI3K pathway leads to an inhibition of apoptosis by the repression of BCL2 associated agonist of cell death (BAD) and the enhancement of BCL2 activity [60]. On the other hand, apoptosis inhibition in EC can occur through BCL2 overexpression and BCL2 associated X (BAX) downregulation promoted by higher oestrogen levels. One of the most important regulator families involved in this mechanism seems to be the let-7 miRNA family, that is, let-7a, let-7b, let-7c, let7d, let-7e, let-7f and let-7g, whose expression grows in response to oestrogen exposure [61]. Nevertheless, high levels of let-7 are related to low malignancy of type I endometrial cancer, suggesting that these molecules could have dual oncogenic and cancer-suppressive effects in endometrial cancer cells. Another important protein, often evaluated to assess an aetiology-specific therapy in type 1 EC patients, is ERα [62]. Its amplification was regarded as a prevalent event in endometrial cancer, however, a decreased ERα level has been detected in high-stage and poorly differentiated cancers [63–65]. ERα seems to be suppressed by miR-206, leading to an anti-proliferation effect and decreased invasion capacity [66]. IGF1R, by binding its IGF1 and IGF2 ligands, is able to activate the PI3K pathway, stimulating cell proliferation and inhibiting apoptosis. Therefore, if it is over-expressed, as in most malignant tissues, it plays an anti-apoptotic role, promoting cancer cell survival and tumour

metastasis [67,68]. Recent studies demonstrated that IGF1R was highly expressed in EC tissues and it was inversely correlated with miR-381 levels [69].

Epigenetic mechanisms seem to be involved in EC: silencing of DNA mismatch repair genes by DNA hypermethylation has been demonstrated [70]. Moreover, the study of methylation profiles in endometrial tumorigenesis showed that, among different tumour suppressor genes, the number of methylated promoters increased in the progression of cancer [71]. For example, miR-129-2 seems to be related to microsatellite instability and mutL homolog 1 (MLH1) methylation, which is frequently observed in EC cells, suggesting an important role in early stages of carcinogenesis [72,73]. Usually, miR-203 suppresses tumour proliferation, invasion and metastasis, through the inactivation of ABL proto-oncogene 1 (ABL1) and BCR-ABL1 oncogenes. In hematopoietic tumours, it has been found silenced by the hypermethylation of its promoter [74]. Huang and collaborators found its hypermethylation in EC cells lines and they observed that it was associated with microsatellite instability and MLH1 methylation in primary endometrioid EC [75]. Mir-152 is a tumour suppressor, which inhibits tumour cell growth both in vitro and in vivo by repressing Cell Division Cycle 25B (CDC25B), an important cell cycle regulator [76]. It is able to target E2F Transcription Factor 3 (E2F3), DNA methyltransferase 1 (DNMT1), met proto-oncogene (MET) and Rapamycin-insensitive companion of mTOR (Rictor) and in EC cells it seems to be down-regulated through CpG hypermethylation, promoting cancer development and progression [77].

Several miRNAs that regulate EMT have been found deregulated in EC. EMT, important in implantation, is also a key process in oncogenesis and tumour metastasis; it has been mainly related to the expression of three markers: epithelial E-cadherin, mesenchymal vimentin and N-cadherin [78]. MiR-93 has been described as a tumour suppressor in ovarian cancer and more recently as an oncomiR in EC cells. MiR-93 up-regulation leads to a downregulated E-cadherin and increased N-cadherin expression [79]. In addition, this miRNA seems to be able to target FOXA1, a negative regulator of EMT, down-regulated in EC cells [79,80]. Another miRNA involved in EMT processes is miR-30c, which targets Metastasis-associated gene-1 (MTA1) inhibiting cell proliferation, metastasis and invasion in EC cells [81]. It is down-regulated both in type 1 and type 2 EC cells and its expression seems to be related to oestrogen concentration [82]. MiR-106b has been related to EMT, too: it has been associated with Twist family bHLH transcription factor 1 (TWIST1) targeting [83]. TWIST1 contributes to the EMT phenotype in EC cells improving cell invasion and it is more expressed in EC cells than in normal ones [84]. These findings are consistent with miR-106b downregulation in EC cells showing EMT phenotypes; profiling its expression levels might be helpful for predicting the risk of metastasis, especially in patients with type II EC [83].

3.3. Circulating miRNA and Endometrial Cancer

To discriminate the different subtypes and predict prognosis by using circulating miRNAs as non-invasive biomarkers of EC, several investigations have investigated their expression profiles in serum or plasma. High serum levels of miR-155 have been found associated with cancer stage and lymph node metastasis [85]. Another study demonstrated that the association of miR-99a and miR-199b, up-regulated in plasma of EEC patients, better discriminate EEC patients [86]. A recent study demonstrated that down-regulated miR-29b expression in plasma correlates with poor EC prognosis and is helpful to evaluate the EC prognosis [87]. A genome-wide study on miRNA expression profiles from plasma of EEC and subsequent validation by quantitative reverse-transcriptase polymerase chain reaction demonstrated the significant up-regulation of 5 miRNAs (miR-15b, miR-27a, miR-223, miR-3145 and miR-4638). Moreover, the authors found that miR-27a and CA125 together have a considerable clinical value in diagnosing EEC [88].

In summary, a lot of miRNAs, cellular and circulating, have been found dysregulated in endometrial cancer and a comprehensive list and the relative references are reported in Table 2 [89–114].

Table 2. miRNAs differentially expressed in endometrial cancer (EC) patients.

miRNAs	Expression	Sample	Reference
Has-let-7 miRNAs	up	EEC	[61]
miR-let-7a	down	EC	[89]
miR-let-7b	down	EC	[90]
miR-9	up	EEC	[56]
miR-10b	up	EC	[91]
miR-15a-5p	down	EC	[92]
miR-15b	up	Plasma in EEC patients	[88]
miR-21	up	EEC	[53]
miR-23a	down	EEC	[93]
miR-24	down	EC	[94]
miR-27a	up	EEC, Plasma in EEC patients	[56,88]
miR-29b	down	EC, Plasma	[87,95]
miR-30c	down	EC	[81,82]
miR-31	up	EC	[96]
miR-34c	down	EC	[97]
miR-96	up	EEC	[56]
miR-99a	up	Plasma in EEC	[86]
miR-106b	down	EEC	[83]
miR-125b	down	EEC	[98]
miR-129	down	EC	[99]
miR-129-2	down	EC	[72]
miR-135a	up	EC	[100]
miR-139-5p	down	EC	[101]
miR-141	up	EEC	[102]
miR-143	down	EC	[103]
miR-145	down	EEC	[104]
miR-148b	down	EC	[105]
miR-152	down	EC	[76]
miR-153	up	EC	[56]
miR-155	Up	Serum	[85]
miR-181c	down	EEC	[106]
miR-182-5p	up	EEC	[56,102]
miR-183-5p	up	EC	[108]
miR-194	down	EC	[109]
miR-199b	up	Plasma in EEC patients	[86]
miR-200a*	up	EC	[102,108]
miR-200b	up	EEC	[102]
miR-200c-3p	up	EC	[110]
miR-203	down	EC	[74]
miR-204	down	EC	[57]
miR-205	up	EEC	[50,51,102]
miR-206	down	EC	[66]
miR-223	up	Plasma in EEC	[88]
miR-361	down	EC	[90]
miR-375	up	EC	[100]
miR-381	down	EC	[69]
miR-424	down	EC	[111]
miR-503	down	EEC	[112]
miR-944	up	EC	[113]
miR-1271	down	EC	[114]
miR-3145	up	Plasma in EEC patients	[88]
miR-4638	up	Plasma in EEC patients	[88]

3.4. lncRNAs in Endometrial Cancer

To date, there are no studies about lncRNA profiles during physiological endometrial changes but their altered expression in EC as well as in endometriosis has been demonstrated [115–117].

In fact, different lncRNAs seem to be differentially expressed in EC and some of them could have a prognostic value. Ovarian adenocarcinoma amplified lncRNA (OVAAL) is overexpressed in many ovarian serum carcinoma, as well as in type 1 EC but less frequently in type 2 EC [46,118]. Its over expression is related to overexpression of p53-regulated genes [118]. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is an 8000 nt lncRNA, upregulated in many different human tumours. It has been positively associated with hyperplasia and negatively with metastasis, thus it can be used as a predictive biomarker. Its expression is regulated by the Wnt/β-catenin signalling pathway, frequently abnormally enhanced in endometrioid type EC. Particularly, MALAT1 has been identified as a functional downstream target of Protocadherin 10 (PCDH10), a tumour suppressor protein, down-regulated in EEC [119]. Growth arrest-specific 5 (*GAS5*) is a tumour suppressor gene, implicated and aberrantly expressed in multiple cancers [120–124]. Recent evidences show that this lncRNA is down-regulated in endometrial cancer cells, being able to induce their apoptosis by inducing PTEN expression through inhibiting miR-103, which usually stimulates cell growth and invasion in endometrial carcinoma [125]. HOX transcript antisense intergenic RNA (HOTAIR) is one of the most studied lncRNAs, because of its involvement in genome modification: in fact, it can repress gene expression through the activation of chromatin modifiers [126]. Its overexpression is associated with increasing oestrogen levels and correlated with poor cancer prognosis. HOTAR has been found upregulated in EC compared to normal endometrium samples. HOTAIR overexpression is also associated with increased metastatic spread and a reduced overall-survival rate in EC [127]. Using a mouse xenograft EC model, treated with HOTAIR siRNA lentivirus, a significant tumorigenesis rate and tumour size reduction occurred both in vitro and in vivo [128]. This shows that HOTAIR may be a prognostic molecular marker for EC, even if further studies are required to demonstrate its involvement in endometrial cancer progression and metastasis [127] H19, a paternally imprinted lncRNA, is located on chromosome 11 and lies within 200 kbp downstream of the *IFG2* gene [129]. H19 is also known as "oncofetal non-coding RNA" because it is primarily expressed during foetal development, so it is absent or poorly expressed in most normal adult tissues. Its re-expression has been detected in various cancers, including ovarian cancer [130–133]. H19 expression levels increase throughout endometrial epithelium tumorigenesis: they are low in normal epithelium, higher in hyperplastic endometrium, very high in EC and even higher in dedifferentiated tumour tissues [115]. Urothelial Cancer-Associated 1 (UCA1) is one of the lncRNAs mostly associated with tumour progression, metastasis and chemo-resistance in several cancer types [134–138]. Lu et al. discovered that UCA1 levels were significantly higher in EC cells than in normal endometrial samples and in metastatic EC they noticed a further level increase if compared to primary tumour, suggesting its involvement in tumour cell migration [139]. The circRNAs, another class of ncRNAs, has been shown to be enriched and stable in exosomes. They act as natural miRNA sponges to decrease miRNA levels, reducing their regulatory effect on mRNAs [140,141]. In a recent study, Xu et al. found that the number of exosomes isolated from serum of EC patients was higher than those of normal samples. In addition, the expression profile of circRNAs in serum of EC patient was dysregulated [142]. Another analysis found several circRNAs differentially expressed in EC cells when compared to normal endometrium [143]. In Table 3, we present a list of significant lncRNAs deregulated in EC and the relative references [144–156].

Table 3. lncRNAs differentially expressed in EC patients.

lncRNAs	Expression	EC Type	Reference
ASLNC04080	up	EC	[144]
BANCR	up	EEC	[145]
CARLo-5	up	EC	[146]
CCAT2	up	EC	[147]
FER1L4	down	EC	[148]
GAS5	down	EC	[125]
H19	up	EC	[115]
HAND2-AS1	down	EEC	[149]
HOTAIR	up	EC	[127]
LA16313D11.11	down	EC	[150]
LINC00672	down	EC	[151]
LINC01016	up	EC	[152]
MALAT1	down	EEC	[119]
MEG3	down	EC	[153]
NEAT1	up	EEC	[154]
OVAL	up	EEC	[46,118]
RP11395G12.3	down	EC	[150]
SNHG8	up	EC	[155]
TUSC7	down	EC	[156]
UCA1	up	EC	[139]

4. ncRNAs and Endometriosis

4.1. Endometriosis

Endometriosis is a disabling disorder characterized by the presence of endometrial tissues outside of the uterine cavity. The most common symptoms are non-menstrual pelvic pain, dyspareunia and infertility [157]. The exact causes are unknown, even if the most shared theory is retrograde menstruation, that is endometrial cells through menstrual blood move in on pelvic cavity. These cells present different characteristics, as the ability of adhesion, aggression, neo-angiogenesis and inhibition of apoptosis, which make endometriosis similar to cancer [158,159].

Although decades of research about the pathogenesis of endometriosis have shown the role of hormonal and non-hormonal mechanisms related to disease development, the therapeutic approach and the methods for early diagnosis are still lacking. Today, the best method for the diagnosis of endometriosis remains laparoscopic surgery but the finding new biomarkers for a minimally invasive diagnosis represent an important scientific challenge [160].

4.2. miRNAs and Endometriosis

Different papers have been published about miRNAs potentially involved in endometriosis but unfortunately, many times the concordance among the results is small. This could be due to the heterogeneity of cell types used in the experiments, as illustrated in a recent review [161]. Another possible explanation is that various technologies (deep-sequencing, microarray, Real-Time PCR) have been used by different researchers.

The first studies on miRNAs and endometriosis demonstrated that different miRNAs were differentially expressed in endometrial tissues with endometriotic lesions with respect to endometrial tissues of the same woman without endometriotic lesions [162,163]. Burney et collaborators were among the first authors to publish the difference in expression profiles of miRNAs between eutopic endometrial tissues of woman with and without endometriosis [164]. They found that four miRNAs (miR-34c-5p, miR-9, miR-9*, miR-34b*) were down-regulated in women with endometriosis compared to controls. In another paper, the authors showed that in patients with ovarian endometriosis, miR-483-5p, targeting IGF2 and miR-629-3p were down-regulated in the eutopic endometrium.

These authors suggest that the dysregulation of these miRNAs and their target genes could contribute to the overgrowth of endometrial tissue outside the uterus [165]. Further studies have been carried out in the last few years on miRNAs and endometriosis. In 2017, Min Kyoung Kim and collaborators demonstrated that the expression of miR-27b-3p, upregulated in human endometrial stromal cells from patients with endometriosis, is reduced after the treatment with Rg3-enhanced red ginseng extract (Rg3E). The authors showed the same effects in vivo in a mouse model [166]. In 2018, by RNA sequencing, it was demonstrated that 107 miRNAs and 6112 mRNAs were differentially expressed in ectopic endometrium. The authors built regulatory networks among Transcription Factors, miRNAs and mRNA targets identifying the most important points of altered regulation in ectopic tissue. Among the miRNAs, they found that some members of miR-449 and miR-34b/c cluster, miR-200 family, miR-106a-363 cluster were dysregulated [167] (Table 4). Members of miR-200 family are also involved in cell migration and EMT which supposedly occurs in the pathogenesis of endometriosis [161]. The downregulation of members of miR-200 family in endometrial tissues with endometriotic lesion results in increased expression of ZEB1/ZEB2 which are transcriptional repressors of E-cadherin. E-cadherin, in the normal endometrial tissue, is required for maintaining the epithelial nature of cells and their adhesion. Its down-regulation leads to epithelial cells to acquire mesenchymal characteristics [161].

Neoangiogenesis regulators such as Vascular Endothelial Growth Factor A (VEGFA) and Thrombospondin-1 (THBS1) have been involved in the pathology of endometriosis [168]. Two different groups of researchers reported that miR-17-5p and miR-20a were down-regulated in the ovarian endometrium compared to eutopic endometrium [165,169] Braza-Boïls and collaborators, in 2013, reported that the miR-17-92 cluster increases tumour neovascularization by decreasing THBS1 expression [170]. MiR-15a-5p, a negative regulator of VEGFA, has been found down-regulated in endometrium samples of woman with endometriosis and VEGFA was found up-regulated in the same endometrial tissues. Moreover, the transfection of endometriosis stromal cells with miR-15a-5p mimics led to reduction in the expression of VEGFA and migration abilities of the endometrial stromal cells, suggesting an important role of this miRNA in the pathogenesis of endometriosis [171].

It has been demonstrated that endometriotic cells in vitro are able to secrete miRNA EV cargo, in culture medium and the role of extracellular miRNAs in the pathogenesis of endometriosis has also been studied. Harp and collaborators observed that miR-21, pro-angiogenic miRNA, is up-regulated in exosomes from endometriosis samples compared with exosomes from controls. The authors suggested that exosomes play autocrine/paracrine roles in the development of endometriosis, potentially by modulating angiogenesis [172].

4.3. Circulating miRNAs as Biomarkers of Endometriosis

Due to the anatomical location of this disease, several closely related biological fluids have been proposed as source for non-invasive biomarkers of endometriosis, for example: urine, plasma/serum and menstrual blood. One potential use of circulating miRNAs as non-invasive biomarkers for endometriosis is an ongoing area of research and its diagnostic and therapeutic implications have been extensively reviewed [164,173–175]. In 2013, Wang and collaborators performed a circulating miRNA array profiling in serum and they found different deregulated miRNAs [176] (Table 4). Moreover, a microarray analysis revealed that miR-17-5p, miR-20a and miR-22 were down-regulated in plasma from patients with endometriosis [177]. It is known that prognostic value increases when two or more biomarkers are evaluated at the same time, in fact, two studies performed in serum and plasma demonstrated that let-7b, let-7d and let-7f altered levels, as well as miR-200a-3p, miR-200b-3p and miR-141-3p down-regulation are able to discriminate patients with endometriosis from controls [160,178] (Table 4).

Table 4. miRNAs differentially expressed in Endometriosis patients.

miRNAs	Expression	Sample	Reference
let-7b	down	serum	[160]
let-7d	down	serum	[160]
let-7f	down	serum	[160]
miR-9	down	endometrium	[164]
miR-9*	down	endometrium\serum	[164,176]
miR-15a-5p	down	endometrium	[171]
miR-17-5p	down	ovarian endometrium\plasma	[165,169,177]
miR-20a	down	ovarian endometrium\plasma	[165,169,177]
miR-20b-5p	down	endometrium	[167]
miR-21	up	endometrial exosomes	[172]
miR-22	down	plasma	[175]
miR-27b-3p	up	endometrium	[166]
miR-30d-5p	down	endometrium	[167]
miR-34b*	down	endometrium	[164]
miR-34c-5p	down	endometrium	[164]
miR-106a-5p	down	endometrium	[167]
miR-122	up	serum	[176]
miR-133b	up	endometrium	[167]
miR-135a	down	serum	[160]
miR-141*	down	serum	[176]
miR-141-3p	down	endometrium\plasma	[167,176]
miR-145*	down	serum	[176]
miR-182-5p	down	endometrium	[167]
miR-183-5p	down	endometrium	[167]
miR-196b-5p	down	endometrium	[167]
miR-199a	up	serum	[176]
miR-200a-3p	down	endometrium\plasma	[161,167,178]
miR-200b-3p	down	endometrium\plasma	[161,167,178]
miR-200c-3p	down	endometrium	[161,167]
miR-363-3p	down	endometrium	[167]
miR-449a	down	endometrium	[167]
miR-449b-5p	down	endometrium	[167]
miR-483-5p	down	endometrium	[165]
miR-542-3p	down	serum	[176]
miR-629-3p	down	endometrium	[165]

4.4. lncRNAs and Endometriosis

In a paper published in 2015, Wang and collaborators compared the expression of several lncRNAs and mRNAs between eutopic and normal endometrium by microarray analysis and validated the obtained data using RT-qPCR. The authors found several lncRNAs and mRNAs differentially expressed between eutopic and normal endometrium. Specifically, among the lncRNAs, 488 were up-regulated and 789 down-regulated and among the mRNAs, 578 were over expressed and 638 down-regulated [117]. The most significantly up-regulated lncRNA is AC068282.3 (fold change 31.3) while RP11-403H13.1 is the most significantly down-regulated one (fold change 44.3). The differentially expressed lncRNAs were involved in cell cycle regulation and immune response [117]. As previously described, H19 is a lncRNA, which is expressed from the imprinted locus that also contains the reciprocally imprinted IGF2 gene. It has been shown that H19, reducing the bioavailability of miRNA let-7, by acting as a molecular sponge, was significantly down-regulated in the eutopic endometrium of women with endometriosis. Its down regulation, increasing let-7 activity, inhibited IGF1R expression. Accordingly, the proliferation of endometrial stromal cells was reduced. This paper represents the first example of lncRNA involvement in the pathogenesis of endometriosis and its association with infertility [179].

Another lncRNA that is important in endometriosis is MALAT1. This is one of the most known lncRNAs, it is evolutionary well-conserved and is overexpressed in many cancers [180–183]. Recently, a paper demonstrated that the expression of MALAT1 is significantly increased during endometriosis. Moreover, the authors showed that miR-200c, which is regulated by MALAT1, was significantly down-regulated in endometrial samples from woman with endometriosis and they were significantly negatively correlated. The functional assay showed that overexpression of miR-200c inhibited the proliferation of Human Endometrial Stromal Cells (HESCs) while the inhibition of miR-200c promoted cellular proliferation. Similar results were also obtained from the migration assay in which MALAT1 knockdown inhibits the proliferation and migration of HESCs [184]. Finally, the presence of deregulated lncRNAs in serum as potential non-invasive biomarkers of endometriosis has been investigated. In 2016, it has been reported that a set of lncRNA in serum can discriminate severe versus mild stages of endometriosis and other associated clinical features. Moreover, the combination of five lncRNAs (NR_038395, NR_038452, ENST00000482343, ENST00000544649, ENST00000393610) is able to discriminate patients with and without endometriosis [116]. In Table 5, we show the list of significant lncRNAs deregulated in endometriosis and the relative references.

Table 5. lncRNAs differentially expressed in Endometriosis patients.

lncRNAs	Expression	Sample	Reference
AC068282.3	up	endometrium	[117]
ENST00000393610	up	serum	[116]
ENST00000465368	down	serum	[116]
ENST00000482343	down	serum	[116]
ENST00000529000	down	serum	[116]
ENST00000544649	down	serum	[116]
H19	down	endometrium	[179]
MALAT1	up	endometrium	[184]
NR_033688	down	serum	[116]
NR_038395	down	serum	[116]
NR_038452	up	serum	[116]
RP11-403H13.1	down	endometrium	[117]

5. ncRNAs and Chronic Endometritis

5.1. Chronic Endometritis

Chronic Endometritis (CE) is a chronic inflammation of the endometrium that is difficult to diagnose, caused by microbial infection sustained by common bacteria such as Escherichia coli, Enterococcus faecalis and Streptococcus agalactiae [185,186]. It has vague symptoms, such as abnormal uterine bleeding, pelvic pain and leukorrhea and, at the moment, hysteroscopy represents the most reliable diagnostic method [187,188]. In spite of the benign prognosis, this pathology can impair endometrial receptivity, in spontaneous and in IVF cycles [189,190]. Moreover, there are evidences showing that CE could be a cause of natural preterm labour, premature birth and recurrent miscarriages [188,191]. To date, the molecular mechanisms with which CE can cause infertility still remain unknown.

In a paper published by our group in 2013, we demonstrated the altered endometrial expression of genes, involved in inflammatory response, proliferation and apoptosis in CE. These findings are in agreement with literature data, showing that the endometrium of CE women results an unusual local microenvironment, due to an altered secretion of paracrine factors [192–194]. Accordingly, endometrial receptivity may be impaired as well as proliferative processes may be increased [195,196].

5.2. miRNAs and Chronic Endometritis

At the moment, very few data are present in the literature about the alteration of molecular pathways associated with CE. In spite of CE is considered a mild pathology, it is related to infertility and it has been suggested the association between CE and endometriosis and between CE and the endometrial micro-polyps [193,195]. Therefore, to investigate the alteration of molecular mechanisms caused by a chronic infection could be interesting.

We showed, for the first time, an up-regulation of miR-27a-3p and miR-124-3p in the endometrium from women affected by CE and demonstrated their ability to discriminate CE women when the 2 miRNAs were analysed together in the serum [197]. Previously, miR-27a has been found highly expressed in cows with subclinical endometritis [198]. Finally, we found that miR-27a up-regulation is significantly related to IGF1 down-regulation in the same endometrial samples [197]. Proper IGF1 levels are required for successful embryonic and placental development; consequently, the down-regulation of protein could be associated with endometrial quality and with infertility observed in women with CE [199]. Interestingly, miR-27a and miR-27b, two isoforms of the miR-27 family, seem to be involved in different endometrial pathologies. Their up-regulation in endometrium and serum has been described in EC, in endometriosis and in CE [56,166,197] (Figure 3). It would be attractive to demonstrate, by functional experiments, its ability to regulate IGF1 and other messengers, in order to understand its role in endometrial physiopathology and evaluate its prognostic potential and the possibility to plan specific therapy based on RNA interference.

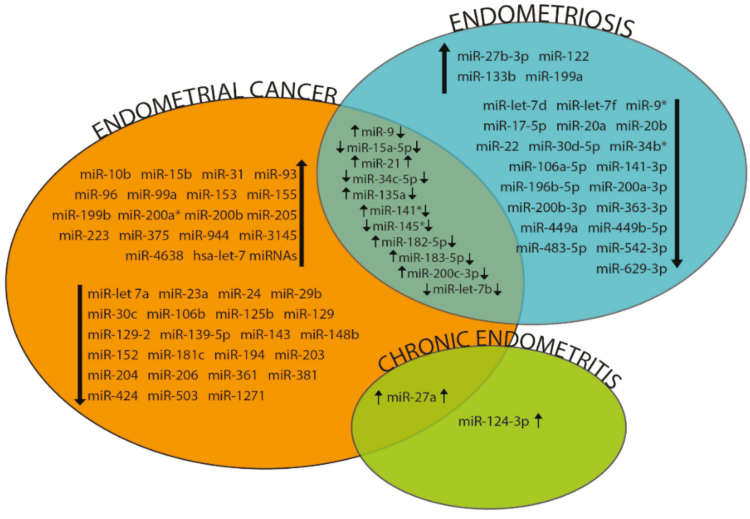

Figure 3. Representative miRNAs involved in endometrial pathologies.

Literature analysis revealed that there are no data about lncRNAs in CE (Figure 4). The overlapping among lncRNAs involved in EC and Endometriosis showed that MALAT1 and H19 are shared across the two pathologies, even if, they show a different trend of expression (Figure 4).

Venn diagram summarizing deregulated miRNAs identified in Endometrial Cancer, Endometriosis and Chronic Endometritis. Intersection areas show molecules differentially expressed in more than one pathology. Up and down-regulated miRNA expression is indicated by black arrows.

Venn diagram summarizing deregulated lncRNAs identified in Endometrial Cancer and Endometriosis. Intersection areas show molecules differentially expressed in more than one pathology. Up and down-regulated lncRNA expression is indicated by black arrows.

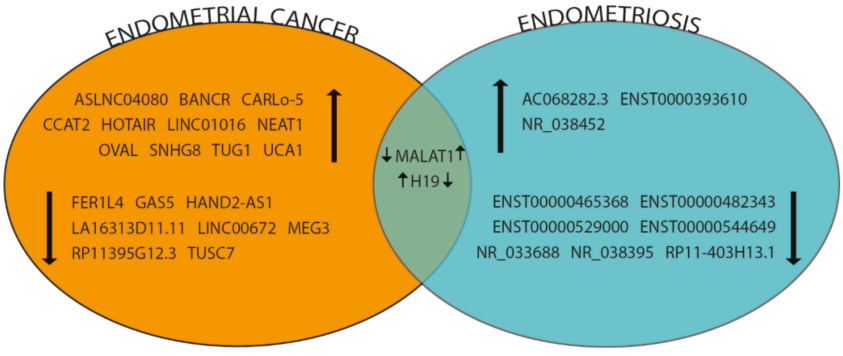

Figure 4. Representative lncRNAs involved in endometrial pathologies.

6. Conclusions

The discovery of ncRNAs in the regulation of gene expression inside the pathway from genotype (DNA) to phenotype (Proteins) has deeply transformed contemporary biomedicine. The countless studies on pathogenesis of tumours and complex phenotypes revealed the involvement of ncRNAs in different human diseases and postulated their role as molecular biomarkers in diagnosis, prognosis and also as possible therapeutic targets. In this review, we reported that several ncRNAs are involved in endometrial physiology and that their altered expression can be related to different endometrial disorders. High-throughput techniques are able to give a general overview, discovering many molecules involved in a specific biological pathway. Nevertheless, the complexity of ncRNA networks makes difficult to understand if deregulation of specific molecules represents the etiological cause of a disease or, alternatively, a secondary effect, depending on the perturbation of physiological pathways. Even if, EC, endometriosis and CE are dissimilar endometrial disorders, an association among them could be supposed. Comparing literature data, we detected ncRNAs found dysregulated in the three pathologies (Figures 3 and 4). For some of them, there is no expression concordance between EC and Endometriosis, probably due to the different regulated targets but interestingly 4 miRNAs show the same expression trends and the up-regulation of miR-27 family seems to be involved also in CE. We believe that in order to understand and resolve particular biomedical problems, it would be appropriate to focus on a limited number of molecules to perform functional analyses, in vitro and in vivo on animals.

Author Contributions: A.L.F., F.A. and R.B. have revised the literature, draft the manuscript and prepared Figures and Tables. S.C., A.C. and M.P. have revised the manuscript. C.D.P. conceived and designed the review, written and revised the manuscript. All authors read and approved the manuscript.

Acknowledgments: The authors would like to thank the Scientific Bureau of the University of Catania for language support.

Conflicts of Interest: The authors declared no conflict of interest.

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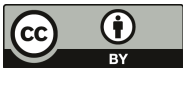

International Journal of
Molecular Sciences

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Review

Chronic Niche Inflammation in Endometriosis-Associated Infertility: Current Understanding and Future Therapeutic Strategies

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Received: 14 July 2018; Accepted: 9 August 2018; Published: 13 August 2018

Abstract: Endometriosis is an estrogen-dependent inflammatory disease that affects up to 10% of women of reproductive age and accounts for up to 50% of female infertility cases. It has been highly associated with poorer outcomes of assisted reproductive technology (ART), including decreased oocyte retrieval, lower implantation, and pregnancy rates. A better understanding of the pathogenesis of endometriosis-associated infertility is crucial for improving infertility treatment outcomes. Current theories regarding how endometriosis reduces fertility include anatomical distortion, ovulatory dysfunction, and niche inflammation-associated peritoneal or implantation defects. This review will survey the latest evidence on the role of inflammatory niche in the peritoneal cavity, ovaries, and uterus of endometriosis patients. Nonhormone treatment strategies that target these inflammation processes are also included. Furthermore, mesenchymal stem cell-based therapies are highlighted for potential endometriosis treatment because of their immunomodulatory effects and tropism toward inflamed lesion foci. Potential applications of stem cell therapy in treatment of endometriosis-associated infertility in particular for safety and efficacy are discussed.

Keywords: endometriosis; infertility; niche; inflammation; immunomodulation; mesenchymal stem cell

1. Introduction

Endometriosis is an estrogen-dependent inflammatory disease characterized by the presence of endometrial glands and stroma outside the uterine cavity. It affects 5–10% of women of reproductive age, up to 80% of women with pelvic pain, and 20–50% of women with infertility [1,2]. Affected women experience impaired quality of life due to chronic pelvic pain and other clinical symptoms

such as dysmenorrhea, menorrhagia, dyspareunia, dysuria, and dyschezia [3]. Endometriosis is also associated with increased risk of certain cancer types and other chronic diseases, including ovarian and endometrial cancer [4,5], cardiovascular diseases [6], autoimmune diseases [7], and allergic disorders [8].

Despite its prevalence and correlation with several diseases, the exact pathogenic mechanism of endometriosis remains unclear. Development of endometriosis may be the endpoint of several combined aberrant biological processes. The most plausible hypothesis is retrograde menstruation, where endometrial fragments regurgitated through the fallopian tubes during menstruation are subsequently implanted in secondary sites [9]. Other possible cellular and molecular mechanisms include coelomic metaplasia, lymphovascular spread, endometrial stem cell implantation, and immune dysregulation [9,10]. All of these theories complementarily explain the complicated and variable nature of endometriosis development and progression.

Current treatment for endometriosis focuses on infertility and pain management. For patients with suspected endometriosis based on presented symptoms and signs, many clinicians begin empirical treatment before making a definitive diagnosis, using medical therapies such as nonsteroidal anti-inflammatory drugs, hormonal contraceptives, progestogens, antiprogestogens, gonadotropin-releasing hormone (GnRH) agonists and antagonists, and aromatase inhibitors [11,12]. These reagents function by inducing hypoestrogenism, amenorrhea, or endometrial atrophy [13]. When empirical therapies fail to alleviate symptoms or long-term medical treatment is warranted, laparoscopic exploration, excision, and adhesiolysis may be performed for definitive diagnosis and curative treatment [14].

Medical management effectively reduces pain in most endometriosis patients. However, for infertility treatment, hormonal medical therapies alone are inadequate. Because these therapies suppress ovarian function and create a contraceptive state along with endometrial atrophy, they do not benefit patients seeking pregnancy. Hughes et al. showed that ovulatory suppressive medications such as oral contraceptive pills, GnRH agonists, and danazol did not improve spontaneous pregnancy and live birth rates for infertile women with endometriosis seeking conception [15]. Currently, conventional medical therapy plays a role only in treating endometriosis-associated infertility in assisted reproductive technology (ART); it was demonstrated that pretreatment with GnRH agonist for 3–6 months before initiation of in vitro fertilization (IVF) or intracytoplasmic sperm injection could improve the pregnancy rate 4-fold [16]. It has been suggested that long-term use of GnRH agonists could improve endometrial receptivity by reducing aromatase and cyclooxygenase (COX)-2 expression in a eutopic endometrium [17]. Using cryopreserved embryo transfer instead of fresh embryos further improves IVF outcomes by circumventing the excessive ovarian suppression caused by long-term GnRH agonist treatment [18,19]. The aromatase inhibitor letrozole may also be used to improve IVF outcomes in patients with low expression of endometrial integrin $\alpha v\beta 3$; this is a common finding in endometriosis cases [20]. Novel nonhormonal medical agents that target other pathways such as inflammation and angiogenesis to treat endometriosis-associated infertility are currently under investigation.

Although the cause of endometriosis-induced infertility remains elusive, several causes have been proposed to explain it, including distorted pelvic anatomy due to adhesions, ovarian dysfunction, defective peritoneal function, and altered endometrial receptivity [21]. Immune dysfunction plays a role in each of these causes. In this review, we first examine the dysregulated niche immune modulation in each anatomical compartment, and then discuss novel treatment strategies that target immune pathways to restore fertility in endometriosis patients.

2. Chronic Niche Inflammation in Endometriosis Development

The tissue niche provides several chronic inflammatory environments for endometriosis development, particularly in the peritoneal cavity, ovaries, and uterus (Figure 1).

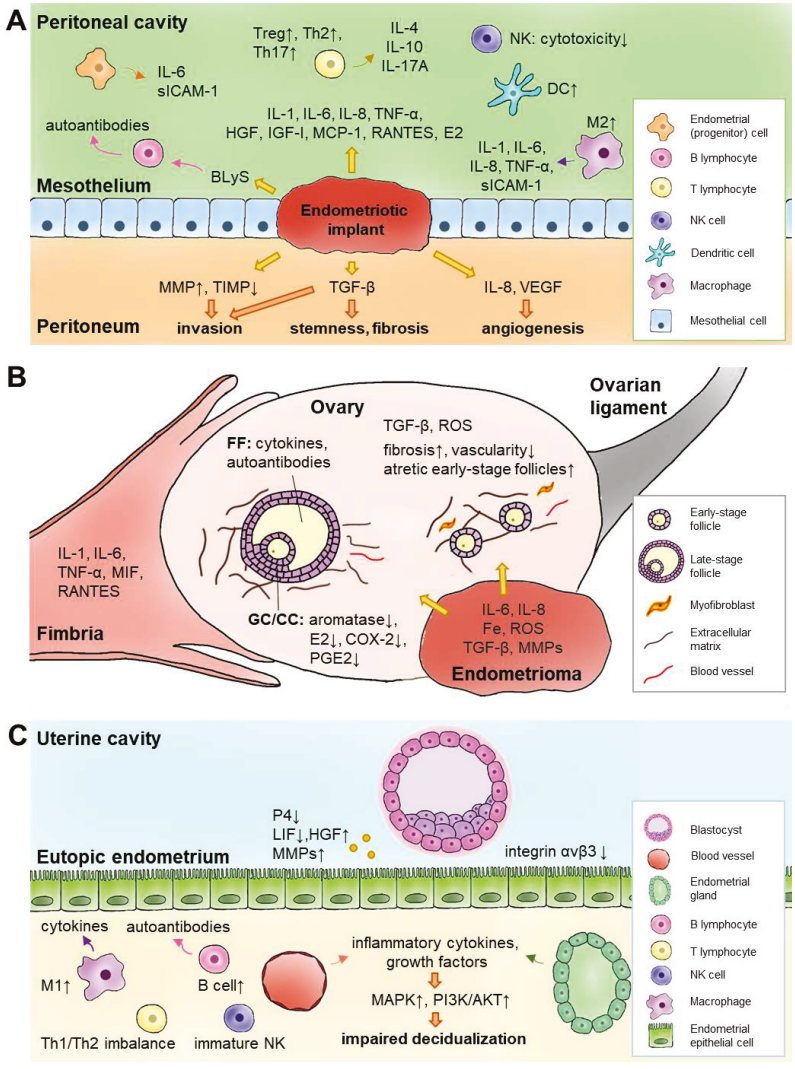

Figure 1. Different inflammatory niche in (**A**) peritoneal cavity, (**B**) ovary, and (**C**) eutopic endometrium in endometriosis. The population of each immune cell type, the level of cytokine/hormone/protein expression, and the activation of cellular pathways are depicted by an up arrow or a down arrow to represent an increase or a decrease, respectively. BLyS, B lymphocyte stimulator; CC, cumulus cell; COX-2, cyclooxygenase 2; DC, dendritic cell; E2, estradiol; FF, follicular fluid; GC, granulosa cell; HGF, hepatocyte growth factor; IGF-I, insulin-like growth factor 1; IL, interleukin; LIF, leukemia inhibitory factor; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; MIF, macrophage migration inhibitory factor; MMP, matrix metalloproteinases; NK, natural killer; P4, progesterone; PGE2, prostaglandin E2; PI3K, phosphoinositide 3-kinase; RANTES (CCL5), regulated on activation, normal T cell expressed and secreted; ROS, reactive oxygen species; sICAM-1, soluble intercellular adhesion molecule-1; TNF-α, tumor necrosis factor alpha; TGF-β, transforming growth factor beta; Th, T helper cell; Treg, regulatory T cell; VEGF, vascular endothelial growth factor.

2.1. Peritoneal Cavity

The peritoneal cavity is immersed in peritoneal fluid, which is mainly ovarian exudate produced by developing follicles and corpus luteum [22]. Its volume and content vary significantly in different phases of the menstrual cycle because vascular permeability increases with estrogen concentration. Peritoneal fluid contains electrolyte, urea, ovarian steroidal hormones such as estrogen and progesterone, and cellular components including endometrial cells, macrophages, lymphocytes, and red blood cells. These cellular components may have their own secretions; for example, endometrial cells secrete glycodelins, and macrophages can produce cytokines and growth or angiogenic factors [22].

Impaired fertility in endometriosis patients mostly results from a chronic inflammatory state caused by an abnormal peritoneal environment. The volume of peritoneal fluid is significantly higher in infertile women with endometriosis than in those without the disease [23]. Numerous aspects of the immune system are altered in endometriosis, including inhibited T-cell-mediated cytotoxicity to endometrial cells, low natural killer (NK) cell activity, and increased numbers of activated macrophages and proinflammatory cytokines [24]. These changes create an oxidative and immunotolerant microenvironment for endometriotic implants to survive [25,26].

The interaction between an endometriotic lesion and its local immune environment was reviewed [1]. Endometriotic implants secrete estradiol, progesterone, monocyte chemoattractant protein (MCP)-1, transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF), and proinflammatory cytokines such as interleukin (IL)-1, IL-6, and IL-8, and tumor necrosis factor alpha (TNF-α), among others [1]. This cocktail of secretions in peritoneal fluid promotes a proliferative and angiogenic environment that enhances endometriosis development and progression. Endometrial cells exposed to the peritoneal fluid of endometriosis patients have been shown to upregulate their VEGF and plasminogen activator gene expression [27,28]. Because fertilization occurs in the ampulla of the fallopian tube with exposure to peritoneal fluid, changes in peritoneal fluid composition directly influence the fertilization process. For example, endometriosis may impair sperm mobilization through macrophage-secreted IL-1, IL-6, and macrophage migration inhibitory factor (MIF) [29,30]. Moreover, TNF-α can damage sperm DNA by inducing apoptosis and oxidative stress [31], and sperm–oocyte binding and fusion are disturbed by TNF-α, IL-1, MIF, and regulated on activation, normal T cell expressed and secreted (RANTES)/C-C motif chemokine ligand 5 (CCL5) [30,32].

Cross-talk between the immune and endocrine systems has been observed in endometriosis because endometriotic lesions are characterized by excessive local estrogen production, abnormal estrogen receptor (ER) expression, and increased cyclooxygenase 2 (COX-2) and prostaglandin expression. Zhao et al. demonstrated that targeting either ER-α or ER-β with isoform-specific ligands significantly reduced inflammation markers such as IL-6, TNF-α, nuclear factor kappa B (NFκB), COX-2, C-C motif chemokine ligand 2 (CCL2), and RANTES and decreased T-cell and macrophage infiltration in endometriotic implants [33]. Burns et al. showed that the development and maintenance of endometriosis could be divided into an early immune-predominant initiation phase mediated by IL-6 signaling and an ensuing hormone-predominant phase mediated by estrogen signaling [34].

Endometriosis is characterized by enhanced humoral immune response with increased B lymphocyte numbers and autoantibody production [35]. Immunohistochemical and gene expression microarray analysis revealed that endometriotic lesions were abundant in plasma cells and activated macrophages, with highly expressed cytokine B lymphocyte stimulator (BLyS) [36]. BLyS is a ligand of the TNF cytokine family that plays a major role in B lymphocyte differentiation, homeostasis, and maturation, and its overexpression is associated with autoimmune diseases [37]. High levels of BLyS result in B lymphocyte production of a plethora of autoantibodies. These autoantibodies include antiendometrial, anti-DNA, antiphospholipid, and antinuclear antibodies [38], and two of them have been identified as antiendometrial autoantibodies against alpha 2-HS glycoprotein and transferrin [39]. Because transferrin functions as an iron transporter in the human body, a high transferrin level in peritoneal fluid could reflect a high iron level in peritoneal fluid in endometriosis patients [39].

The presence of excessive iron in peritoneal fluid could induce oxidative stress and cause tissue injury and subsequent de novo lesion formation; moreover, an autoimmune reaction against transferrin interferes with iron removal from the peritoneal cavity and affects granulosa cell maturation in the ovaries [40]. The presence of autoantibodies also reduces sperm motility [41].

Identifying biomarkers that can assist in noninvasive diagnosis and monitoring of endometriosis remains a challenge because of the heterogeneous nature of the disease process and presentation and the interference of comorbidities, and there has been insufficient evidence to recommend any biomarker for routine clinical practice [42,43]. Numerous studies have analyzed the peritoneal fluid of endometriosis patients to identify cytokine signatures characteristic of the disease. Rakhila et al. demonstrated a distinct cytokine expression profile favoring proliferation and angiogenesis for patients with endometriosis who showed increased levels of EGF, FGF-2, IL-1α, MIP-1β, transforming growth factor (TGF)-α, platelet-derived growth factor (PDGF)-AA, PDGF-BB, monocyte chemotactic protein 3 (MCP-3), soluble CD40 ligand (sCD40L), C-X-C motif chemokine ligand 1/2/3 (Gro Pan), IL-17α, macrophage-derived chemokine (MDC), and RANTES [27]. Several studies also indicated an increased IGF system in the peritoneal environment of endometriosis patients, leading to enhanced endometrial stromal cell proliferation [44–47].

Beste et al. similarly demonstrated that an endometriosis subset had a distinct cytokine profile with IL-1ra, IL-1β, IL-6, IL-8, IL-10, IL-16, hepatocyte growth factor (HGF), MCP-1, MIF, monokine induced by gamma interferon (MIG), granulocyte-colony stimulating factor (G-CSF), growth regulated oncogene (GRO)-α, and RANTES [48]. Network analysis revealed that this cytokine signature was associated with a macrophage-driven process related to NFκB, Jun proto-oncogene (c-Jun), Fos proto-oncogene (c-Fos), activator protein 1 (AP-1), and mitogen-associated kinase signaling [48]. Another study by the same authors aimed to identify endometriosis patients among women assessed for infertility, and concluded that a panel of 11 cytokines (IL-5, IL-9, IL-13, IFN-α2, cutaneous T-cell attracting chemokine (CTACK), HGF, MCP-1, MCP-3, M-CSF, leukemia inhibitory factor (LIF), and SCGF-β) was distinguishable in infertile patients with endometriosis; this profile suggested that dysregulated Th1/Th2 activity could underlie endometriosis-associated infertility [49]. However, whether these aberrant cytokine expressions are the cause or a consequence of the disease remains to be explored, and more studies are warranted to better stratify patients and assess the applicability of these cytokine profiles in clinical use.

2.2. Ovaries

Ovarian endometriomas could alter ovarian function through space-occupying and local effects [50]. The cystic fluid within an endometrioma is a rich source of proinflammatory cytokines (IL-6, IL-8), iron, reactive oxygen species (ROS), growth factors such as TGF-β, and matrix metalloproteases (MMPs) [50]. These cystic fluid contents could affect adjacent ovarian function through diffusion into surrounding tissue. Structural alterations were identified in the neighboring tissue of an ovarian endometrioma, with lower follicular density, greater fibrosis, and loss of cortex-specific stroma [51]. In addition, cortical biopsies of ovaries containing endometriomas revealed atresia of early follicles with increased cleaved caspase-3 immunostaining, which was not seen on contralateral ovaries without endometriomas [52]. Increased oxidative stress due to local inflammatory reactions has been observed in the ovarian cortex surrounding endometriomas [52,53]. Both TGF-β1 and ROS promote fibrosis and adhesion formation through myofibroblast differentiation and profibrotic gene expression of plasminogen activator inhibitor-1 [54]. Structural change including loss of ovarian stroma has a detrimental effect on folliculogenesis due to reduced blood supply to follicles and decreased growth factors secreted by stromal cells [55].

Follicular fluid forms a distinct microenvironment within the ovaries and contains cytokines produced by granulosa and local immune cells. Most studies on the follicular fluid of endometriosis patients have been recruited with patients undergoing IVF following stimulation of gonadotropin. Such reports have suggested high follicular levels of IL-1β, IL-6, IL-8, and IL-18. One study determined that the follicular fluid of naturally matured follicles (without gonadotropin stimulation) in endometriosis patients had higher IL-1β and IL-6. The abnormal presence of intrafollicular IL-1β and IL-6 could affect the follicular biology of endometriosis patients because low intrafollicular IL-6 is associated with a higher pregnancy rate [56]. Proinflammatory cytokines can have detrimental and beneficial effects, and high intrafollicular IL-1β has been found to correlate with higher fertilization and implantation rates [57]. High levels of IL-8, IL-12, and adrenomedullin were detected in one study, correlating negatively with oocyte maturity and embryo quality [58]. Although IL-18 was highly expressed in the peritoneal fluid of endometriosis patients, high amounts were not observed in the follicular fluid of such patients [59]. Inflammatory cytokines could affect folliculogenesis in endometriosis patients by regulating G protein-coupled estrogen receptor (GPER) expression and the fraction of GPER positive macrophages in the ovaries [60]. GPER is a transmembrane receptor that participates in nongenomic estrogen signaling pathway and is upregulated in endometriotic lesion and eutopic endometrium of endometriosis patients [60,61]. GPER signaling plays an important role in follicular maturation, and the lower follicular expression of GPER could explain the lower follicular count in endometrioma patients [60,62]. In moderate/severe endometriosis patients, the follicular environment was also characterized by increased oxidative stress and leukocyte activation marker myeloperoxidase, and this increase was correlated with decreased oocyte quality and fertility [63]. Hormone status in the follicular microenvironment is less certain, with several studies producing different results [59,64].

Both ovarian reserve and response to controlled ovarian stimulation are compromised in endometriosis patients, especially in more severe cases (American Society for Reproductive Medicine, ASRM stage III/IV) [65,66]. In a meta-analysis examining 1039 patients, those with endometriosis had decreased oocyte retrieval, lower metaphase II oocyte numbers, and fewer embryos formed compared with the control group [67]. However, the number of oocytes retrieved from the diseased side of the ovary, the number of metaphase II oocytes, and the embryo count were all similar to those of the healthy contralateral side of the ovary [67]. Significant differences from the healthy contralateral ovary appeared when the endometrioma was large ($\geq$5 cm) [68]. Whether poorer ovarian reserve and response are due to endometriosis or surgical injury to the ovaries during cystectomy are still under debate [69]. Furthermore, coexisting deep-infiltrating endometriosis was shown to further deteriorate ovarian reserve and oocyte retrieval compared with endometrioma alone [70].

2.3. Uterus

Whether endometriosis affects the eutopic endometrial lining to influence implantation has been much debated [71,72]. Several clinical studies have indicated a decreased implantation rate in endometriosis patients undergoing IVF [73–75], and a meta-analysis of 27 studies demonstrated significantly lower implantation and clinical pregnancy rates in patients with severe endometriosis [76]. To exclude factors associated with oocyte and embryo quality, a prospective study using sibling oocytes from the same donor demonstrated that recipients with endometriosis had significantly lower implantation and pregnancy rates than control recipients [77]. However, not all related studies have reached the same conclusion, instead generally attributing poorer IVF outcome in endometriosis patients to compromised oocyte and embryo quality [78–81]. In a large retrospective cohort study involving 22,416 women with or without endometriosis undergoing IVF, no significant differences between groups was found for live birth, clinical pregnancy, and miscarriage rates [81]. Women with endometriosis had significantly lower retrieved oocyte numbers, even though the number of fertilized oocytes did not differ after adjustment for retrieved oocyte numbers [81]. An age-stratified analysis found no difference in reproductive outcomes for women in the under-35 and 35–40 age

groups [81]. Thus, the effect of endometriosis on implantation remains debatable. Possible explanations for discrepancies among studies include different research designs, highly heterogeneous inclusion criteria, and low numbers of enrolled patients.

The effect of endometriosis on endometrial receptivity is based on inflammation. Progesterone exhibits anti-inflammatory activity, and in normal circumstances, an inflammatory response occurs after progesterone withdrawal in the late secretory phase of the menstrual cycle. Decreased progesterone leads to decreased prostaglandin metabolism and increased ROS, which activate an NFκB-mediated inflammatory cascade that induces the processes required for menstruation [82]. The progesterone resistance that characterizes endometriosis mimics this late secretory phase, leading to premature initiation of inflammation [71]. The result is accumulation of proinflammatory cytokines, chemokines, ROS, MMPs, COX-2, and prostaglandins in the endometrium, with all of these inflammatory mediators feeding on one another in a positive feedback loop. Immune transcriptomic profiling showed that compared with a healthy control endometrium, the eutopic endometrium in endometriosis patients was dysregulated for genes related to cytokine and chemokine production, cell adhesion, apoptosis regulation, and wound-healing response [83]. In addition, the eutopic endometrium was also downregulated for genes related to decidualization such as NOTCH1 and NOTCH2 compared with a control endometrium [83]. Dysregulated gene expression related to immune activity has been observed, including increased B-cell signaling and a decreased Treg population [83,84]. To determine the dominant T-cell subtype in infertile endometriosis patients, Koval et al. studied the expression of transcription factors related to differentiation of various subtypes in eutopic endometrial tissues and found increased expression of T-box expressed in T cells (T-bet) and GATA-binding protein 3 (GATA3), the transcription factors involved in Th1 and Th2 differentiation, and decreased expression of forkhead box P3 (Foxp3), which drives Treg differentiation [85]. Abundant immature uterine natural killer cells in the eutopic endometrium also characterized endometriosis patients with infertility [86].

Although the endometrium of a woman with endometriosis is morphologically identical to that of a woman without this disease, it exhibits altered biochemical responses during implantation. Progesterone resistance is generally observed in endometriosis, with altered progesterone receptor (PR) composition for decreased PR-β isoform expression [87]. Abnormally elevated ER-α isoform also occurs in endometriosis patients in the mid-secretory stage, leading to implantation failure [88]. The eutopic endometrium of a woman with endometriosis contains low, but significant, levels of P450 aromatase enzyme activity, which enhances local estrogen activity [89]. Stromal cells derived from the endometrium of a woman with endometriosis have a reduced decidualization capacity [90], possibly because of progesterone resistance and inflammatory cytokines TNF-α and IL-1 [91]. Several preclinical studies have shown that the inflammatory microenvironment maintained by endometriosis compromises implantation ability in animal models. Intraperitoneal injection of peritoneal fluid collected from infertile women with endometriosis decreased the implantation rate in a rodent model, as well as endometrial integrin αvβ3 and LIF expression [92]. During a normal implantation process, endometrial epithelial expression of homeobox A10 (HOXA10) and HOXA11 increases in the luteal phase, but this fails to occur in endometriosis because of dysregulated promoter methylation [93]. Endometrial receptivity is related to integrin expression of the endometrium; however, women with endometriosis have reduced expression of αvβ3, possibly due to decreased HOXA10 [93]. Other implantation-related biomarkers such as glycodelin A, osteopontin, LIF, lysophosphatidic acid receptor 3, and insulin-like growth factor binding protein 1 (IGFBP1) are also affected [94]. Expression of empty spiracles homolog 2 (EMX2) was elevated during the implantation window in endometriosis patients and associated with implantation failure [95]. All of these abnormal gene expressions alter the endometrial receptivity of endometriosis patients. However, none of these individual endometrial markers have been successfully translated to clinical use for receptivity prediction.

Endometrial receptivity array (ERA) has emerged as a new diagnostic tool for determining a woman's implantation window by simultaneously examining the transcriptional expression of a panel of 238 genes related to endometrial receptivity [96]. ERA can guide clinicians to the optimal time frame for performing embryo transfer in recurrent implantation failure patients, and has been demonstrated to outperform endometrial histologic examination [97,98]. Garcia-Velasco et al. conducted a pilot study comparing ERA results between endometriosis patients and healthy women; their results indicated no significant differences in any of the 238 genes in women with endometriosis, irrespective of the disease stage [99]. Therefore, the researchers concluded that endometriosis did not affect endometrial receptivity, at least on a transcriptomic level. Future integrated studies that can capture endometrial status at the proteomic, epigenomic, and hormonal levels may offer greater insight into the effect of endometriosis on the implantation process.

3. Targeting Inflammation for Treatment of Endometriosis-Associated Infertility

Current medical management of endometriosis only treats symptoms rather than cures the disease, and symptoms recur when medication is discontinued. To achieve more effective therapy, novel treatment strategies that target specific pathogenic mechanisms must be investigated. Because endometriosis is considered a chronic inflammatory disease, many studies have explored immunomodulatory agents for restoring balanced immune status. Although this review focuses on systemic pharmacologic treatments, tubal flushing with oil-based contrast media has also been shown to increase short-term pregnancy rate in endometriosis and infertile patients, possibly by increasing the uterine NK cell population and restoring endometrial osteopontin expression [100,101]. Table 1 summarizes drugs targeting inflammatory pathways that have been tested in clinical trials.

Table 1. Potential immunomodulatory pharmaceutical agents for endometriosis treatment in clinical studies.

Treatment	Proposed Mechanism	Indication	Outcome	Comments
Anti-TNF-α Treatment				
Etanercept	Binds and inhibits TNF-α	Infertility	Significantly higher clinical pregnancy rate in patients receiving etanercept	Ref. [102]
Infliximab	Binds and inhibits TNF-α	Pain	No significant effect on pain or lesion size	Phase II trial Ref. [103]
Cytokine Treatment				
Recombinant interleukin-2 (rIL-2)	Enhances cytotoxic activity of macrophages and NK cells	Endometrioma postdrainage recurrence prevention	Significantly longer time to disease recurrence with favorable symptom improvement in rIL-2 and GnRH agonist combination group	Ref. [104]
Interferon-α-2b	Enhances cytotoxic activity of macrophages and NK cells	Postoperative recurrence prevention	No improvement in disease recurrence	Ref. [105]
Angiogenesis Inhibitor				
Simvastatin	Inhibits proliferation and angiogenesis	Postoperative pain	No significant difference from GnRH agonist group	Ref. [106]
Quinagolide	Dopamine receptor agonist for treatment of hyperprolactemia; also has VEGFR2 inhibition effect	Hyperprolactinemic patients with endometriosis	Decreased lesion size with downregulation of angiogenesis markers	Ref. [107]
Cabergoline	Dopamine receptor agonist for treatment of hyperprolactemia; also has VEGFR2 inhibition effect	Pain	N/A	Phase II trial; Recruiting; Clinical Trials.gov: NCT00115661

Table 1. *Cont.*

Treatment	Proposed Mechanism	Indication	Outcome	Comments
Immunomodulatory and Anti-Inflammatory Agents				
Pentoxifylline	Nonselective phosphodiesterase inhibitor; reduces platelet aggregation through platelet phosphodiesterase inhibition; inhibits TNF-α and leukotriene synthesis	Infertility in mild/moderate endometriosis	Nonsignificant increase in cumulative probability of pregnancy in patients receiving pentoxifylline	Phase III trial; Clinical Trials.gov: NCT00632697; Ref. [108]
		Infertility	No significant difference in pregnancy rate or disease recurrence	Ref. [109]
		Postoperative pain	Improved pain score at 2–3 months after surgery	Ref. [110]
Pioglitazone	PPAR-γ agonist; inhibits inflammatory cytokine production and NFκB activity	Infertility	Nonsignificant increase in clinical pregnancy rate; significant increase in embryo implantation rate	Ref. [111]
Rosiglitazone	PPAR-γ agonist; inhibits inflammatory cytokine production and NFκB activity	Pain	Terminated/withdrawn due to adverse cardiovascular events	Clinical Trials.gov: NCT00115661/ NCT00121953
Metformin	Suppresses inflammatory response and aromatase activity; decreases local estrogen production	Pain and infertility	Improved pregnancy rate; improved symptom score	Ref. [112]
Resveratrol	Inhibits hypoxia-mediated ERK1/2, AKT, and MMP2/9 activity	Pain	Nonsignificant decrease in pain score and serum CA-125 level	Phase IV trial; Clinical Trials.gov: NCT02475564
EGCg	Inhibits ROS-induced NFκB activation and MAPK, JNK, and p38 signaling; inhibits angiogenesis	Pain	N/A	Phase II trial; Recruiting; Clinical Trials.gov: NCT02832271
Antioxidants				
Vitamins E and C	Antioxidative activity; decrease peritoneal inflammation	Pain	Improved pain score; decreased peritoneal RANTES, IL-6, and MCP-1	Ref. [113]
Melatonin	Antioxidative activity	Pain	Improved pain score; decreased analgesic use; improved sleep quality	Phase II trial; Ref. [114]

EGCg, Epigallocatechin gallate; GnRH, gonadotropin-releasing hormone; IL-6, interleukin-6; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; MMP, matrix metalloproteinase; NK, natural killer; PPAR-γ, peroxisome proliferator-activated receptor gamma; RANTES (CCL5), regulated on activation, normal T cell expressed and secreted; ROS, reactive oxygen species; TNF-α, tumor necrosis factor alpha; VEGFR2, vascular endothelial growth factor receptor 2.

3.1. Immunomodulators

Pentoxifylline treatment was found to reduce the endometriotic lesion size in an animal model [115]. In addition, periovulatory treatment with pentoxifylline was found to improve the fertilization rate in a rodent model of endometriosis [116]. However, there is still insufficient evidence supporting use of pentoxifylline in management of endometriosis patients with infertility [117]. Luflunomide also appeared to reduce endometriotic implant size in a rodent model [118]. Loxoribine, also an immunomodulator, was found to decrease endometriotic implant size in a rodent model with an increased number of dendritic cells and a decreased number of natural killer cells [119]. Rosiglitazone and pioglitazone are two drugs that belong to the class thiazolidinedione and are clinically used as insulin sensitizer for diabetes treatment [111]. They act as a peroxisome proliferator-activated

receptor gamma (PPAR-γ) agonist that can modulate immune cell activity and cytokine secretion by affecting NFκB activity and have been shown to limit endometriotic lesion development in preclinical studies [120]. Kim et al. showed that pioglitazone could suppress RANTES secretion and improve embryo implantation in stage III and IV endometriosis patients receiving IVF therapy [111]. However, both rosiglitazone and pioglitazone have an adverse effect of developing myocardial infarction, and their use should be carefully assessed in patients predisposed to cardiovascular diseases [121]. Metformin is an antidiabetic drug that potentially can be repurposed for cancer treatment due to its mechanistic target of rapamycin (mTOR) inhibition, cytotoxicity, and immunomodulation effects [122]. Metformin's immunomodulation effect is based on its ability to induce class switching of cytotoxic T cells [122]. Metformin has been found to reduce cytokine effect, aromatase activity, and StAR expression in preclinical endometriosis models, thus regulating local estrogen production [123,124]. In a clinical study involving 69 infertile stage I and II endometriosis patients, metformin was demonstrated to decrease serum cytokine levels and improve pregnancy rate [112].

Natural compounds such as resveratrol have also been studied for endometriosis treatment. Resveratrol is a polyphenolic compound isolated from plants and has been demonstrated to reduce inflammation and oxidative stress in preclinical endometriosis models [125,126]. Epigallocatechin gallate (EGCg) is a green tea extract that exerts immunomodulation activities via increased peritoneal phagocytic activity and NK cell cytolysis [127]. EGCg has been shown to inhibit angiogenesis, fibrosis, and endometriotic cell growth in preclinical models, and a phase II clinical trial is currently ongoing (Clinical Trials.gov ID: NCT02832271) [128].

3.2. Anticytokine Treatment

The peritoneal fluid of endometriosis patients is abundant in proinflammatory cytokines, particularly TGF-β, IL-6, and TNF-α [1]. Thus, targeting proinflammatory cytokines has been proposed as a treatment strategy. An initial study showed that inhibiting TNF-α-mediated pathways could reduce inflammatory cytokine production, metalloprotease expression, and epithelial–mesenchymal transition markers of endometriotic cells [129]. Results from clinical studies have not supported using anti-TNF-α treatment to improve endometriosis-associated pain scores [130]. In a recent retrospective study, endometrioma patients receiving anti-TNF-α treatment etanercept with ART had improved clinical pregnancy rate and nonsignificantly increased liver birth rate [102]. Another study attempted to target IL-6 in a rat endometriosis model and demonstrated that tocilizumab treatment decreased the endometriotic lesion size and VEGF expression in the ectopic and eutopic endometria [131]. Instead of inhibiting proinflammatory cytokines, Quattrone et al. showed that targeted delivery of anti-inflammatory cytokine IL-4 could also reduce ectopic lesion development [132].

Another area where targeting cytokines could be of interest is prevention of postoperative adhesion formation in endometriosis. Pelvic anatomical distortion and adhesion create a physical disturbance between the ovary and fallopian tube, causing tubal blockage and interference with ovum pick-up and transport. Postoperative adhesion is reported in over 60% of patients undergoing gynecological surgery and may present symptoms such as bowel obstruction, abdominal pain, and infertility [133]. Endometriosis may increase the risk of postoperative adhesion [134]. Reduction of postoperative adhesion and inflammation is necessary because adhesion and COX-2 overexpression are significant risk factors for endometriosis recurrence after surgery [135].

Pelvic adhesion formation in endometriosis is largely attributed to local inflammatory reactions and an impaired fibrinolytic system [136]. Levels of proinflammatory cytokines IL-1, IL-6, TNF-α, TGF-β, and VEGF are correlated with adhesion presence and severity of endometriosis [137,138]. TGF-β is a major driver of fibrosis in endometriosis and can promote fibroblast to myofibroblast transdifferentiation via the Smad-dependent and Smad-independent signaling pathways [139]; it also promotes epithelial–mesenchymal transition (EMT) of endometrial cells and increases their migration ability [140,141]. Blockade of TGF-β and its affiliated targets reverses EMT and myofibroblast transdifferentiation in endometriosis and offers a potential therapeutic target [142,143]. Additionally,

an in vivo study using an experimental endometriosis model showed that the presence of IL-1β in the peritoneal fluid of endometriosis patients could contribute to surgery-related adhesion, and inhibiting IL-1β with IL-1 receptor antagonist could significantly reduce postoperative adhesion [144].

3.3. Statins

Statins are a class of drugs that act as inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase. They are commonly used in clinical practice to treat hypercholesterolemia but are increasingly recognized for their anti-inflammatory effects [145]. One study showed that simvastatin had a comparable effectiveness to GnRH agonists in reducing postoperative pain recurrence in endometriosis patients [106]. In preclinical studies, statins decreased immune cell migration and adhesion by reducing chemokine production and surface adhesion molecule expression [146]. Statins may also reduce T-cell activation by decreasing the expression of major histocompatibility complex molecules and altering lipid raft formation [146]. On a molecular level, statins can decrease NFκB nuclear translocation and AP1 activity, thereby reducing inflammation [146]. In vivo animal studies revealed that statins could reduce endometriotic implant size by inhibiting proliferation and promoting apoptosis, reducing VEGF and MMP-9 expression, and improving the pelvic adhesion extent [147]. Studies of its anti-inflammatory effect in endometriosis have shown that atorvastatin can reduce expression of MCP-1, COX-2, RAGE, EN-RAGE, and VEGF, as well as oxidative stress [147–149]. However, the effect of statins on fertility outcomes remains less certain because no clinical study has investigated it in an endometriosis context; nevertheless, preclinical studies have suggested that statins could enhance ovarian granulosa cell apoptosis and inhibit steroidogenesis [150,151]. Furthermore, because statins inhibit COX-2 activity in similar manner to aspirin, whether statins also negatively affect oocyte maturation and embryo quality should be verified [152].

3.4. Tyrosine Kinase Inhibitors

Both the MAPK and phosphoinositide 3-kinase (PI3K)/Akt pathways are enriched in the eutopic and ectopic endometria [153–155], and their relationships with chronic inflammation have been established [156]. These pathways are involved in inflammation, cell cycle progression, cell proliferation and migration, angiogenesis, and fibrosis [156]. Overactivation of these pathways compromises endometrial cells' ability to decidualize, promote progesterone resistance, and increase proliferation and migration of endometrial cells [71]. The MAPK pathway is also involved in IL-1β- and TNF-α-mediated production of IL-6 and IL-8 [156]. Consequently, drugs that inhibit these pathways have potential for application in endometriosis treatment [157,158]. In this regard, tyrosine kinase inhibitors (TKIs) such as sorafenib, sunitinib, pazopanib, and vemurafenib have been tested [159]. These TKIs target Raf kinase and tyrosine kinase receptors such as vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor β (PDGFRβ) and have been used for their antiproliferative and antiangiogenesis effects to treat various tumors [160]. In addition, their immunomodulatory effects have been recognized [161]. Santulli et al. showed that inhibiting B-RAF and MAPK with vemurafenib decreased the endometriotic size in an animal model, with a concomitant decrease in COX-2 level and cell cycle progression [162]. Sunitinib was able to decrease the endometriotic implant volume and adhesion level in an experimental animal model, presumably by inhibiting VEGFR and enhancing apoptosis [159,163]. When various multitargeted TKIs were compared, each seemed to have a different effect on endometriosis extent, VEGF expression, and CD117 expression [164]. Pazopanib and sunitinib significantly reduced endometriotic lesions, whereas sorafenib had no significant effect [164]. Three drugs (pazopanib, sunitinib, and sorafenib) reduced VEGF expression but only pazopanib and sunitinib reduced CD117 expression [164]. Studies published to date have found no negative effects of these drugs on ovarian reserve, but further studies are necessary to assess the immune profile after drug administration and delineate the differential applicability of TKIs for enhanced infertility treatment [165].

3.5. Prostaglandin E2 (PGE2) Inhibitors

In endometriosis patients, there is a significant increase in PGE2 in peritoneal cavity and eutopic endometrium [166,167]. PGE2 is involved in pathogenesis of endometriosis and affects oocyte maturation, ovulation, and fertilization. PGE2 exerts its biological functions via four G protein-coupled receptors EP1, EP2, EP3, and EP4 [166]. Both EP2 and EP4 act through Gs-coupled effects, while EP1 acts via Gq-coupled pathway [166]. EP3 exerts its function via both Gs- and Gq-coupled pathways [166]. The secretion of PGE2 are regulated by passive diffusion, prostaglandin transporter protein (PGT), and multidrug resistant protein 4 (MRP4) [168]. Banu et al. demonstrated that EP2 and EP4 were abundantly expressed, while EP1 and EP3 were lowly expressed in ectopic and eutopic endometrial tissues [169]. Another study found that EP3, EP4, PGT, and MRP4 were overexpressed in ectopic endometrium [168]. Selective inhibition of EP2 and EP4 were demonstrated in preclinical studies to reduce progression of endometriotic lesion and modify the estrogen-dominant and progesterone-resistant microenvironment of eutopic endometrium with decrease in PGE2, E2 biosynthesis, and restoration of PR expression [170]. Inhibition of EP2 and EP4 also decreased inflammatory environment by reducing COX-2, IL-1β, IL-6, and TNF-α expression in both epithelial and stromal compartments of eutopic endometrium [170]. This study suggests the potential use of EP2/EP4 inhibitors to restore endometrial receptivity for improved implantation.

3.6. Antioxidants

Excessive oxidative stress negatively impacts reproductive processes, causing cellular and molecular damages and influencing oocyte maturation, fertilization, and implantation. Oral antioxidant supplements that can modulate inflammatory mediator expression, improve ovulation and endometrial receptivity have therefore attracted growing interests. A recent Cochrane systematic review evaluated 50 randomized controlled studies and concluded that coenzyme Q10 and combined antioxidants (containing multiple vitamins, minerals, and trace elements) may benefit outcomes of infertility treatments [171]. Although the quality of current data is limited, the finding suggested that oral antioxidant supplements can improve live birth and clinical pregnancy rates in subfertile women [171]. Unfortunately, this review did not assess studies targeting endometriosis population.

Women with endometriosis have overproduction of inflammatory mediators, higher oxidative stress induced by ROS and free radicals, and lower total antioxidant potential [172]. One randomized controlled trial showed that combined vitamin E and vitamin C supplementation reduced peritoneal inflammatory markers and improved painful symptoms in endometriosis patients with pelvic pain [113]. Addition of other antioxidative minerals like zinc, copper, and selenium to diet also correlates with positive effects such as improved antioxidant enzyme activity and diminished oxidative stress [173]. A phase II randomized controlled trial demonstrated that oral intake of melatonin was associated with improved sleep quality, reduced pelvic pain levels, and decreased brain-derived neurotrophic factor level in endometriosis women with chronic pelvic pain and dysmenorrhea [114]. Other natural antioxidants include curcumin and resveratrol [174,175]. While only few studies were done, the results showed significant reduction in serum ROS and lipid peroxidation and an increase in total antioxidant capacity [174,175]. Further clinical trials are required to accrue more evidence. Other nondietary antioxidant treatments like cerium oxide nanoparticles, nanoceria, have been studied in an endometriosis animal model [176]. Positive results included decreased systemic oxidative stress, reduced endometriotic lesion angiogenesis, and higher oocyte quality [176].

4. Cell-Based Therapy for Endometriosis Treatment

4.1. Stem Cells in Endometriosis Pathogenesis

The regenerative potential of endometrial tissue during each menstrual cycle has prompted research into stem cells present in the endometrium. Although Sampson's retrograde menstruation theory is the best-known explanation of the pathogenesis of endometriosis, it does not explain

the occurrence of extraperitoneal endometriotic lesions. Studies are increasingly suggesting an association between adult stem cells and endometriosis progression, and such evidence has recently been reviewed [177,178]. Endometrium-derived stem/progenitor cells may be shed through the fallopian tube, thereby establishing endometriotic implants [177]. Endometrial stem or progenitor cells have been identified as clonogenic cells in human endometria and as label-retaining or $CD44^+$ cells in endometria of mice. Several markers for isolation of endometrial mesenchymal stem/stromal cells (emMSCs) have been used, including CD146, PDGFRβ, and SUSD2 [177]. These emMSCs have been isolated from eutopic and ectopic endometria in human biopsies [179–184]. Studies have shown that mesenchymal stem/stromal cells (MSCs) derived from endometriotic implants have higher expression levels of pluripotent octamer-binding transcription factor 4 (OCT4) and responded better to niche cytokines such as TGF-β to increase proliferation, migration, invasion, and angiogenic properties compared with MSCs derived from a eutopic endometrium or healthy control endometrial tissue [140,141,179,182]. Because ectopic emMSCs have greater proliferative, migratory, and angiogenic abilities than eutopic emMSCs with higher expression of HIF-1α and VEGF, preclinical studies have proposed using TKIs such as sorafenib to target ectopic emMSCs [185].

MSCs located within endometriotic ovarian cysts express higher levels of immunosuppressive proteins indoleamine 2,3-dioxygenase 1 (IDO-1), COX-2, and HO-1, and proinflammatory chemokine C-X-C motif chemokine ligand 12 (CXCL-12) compared with eutopic endometria [186]. Moreover, MSCs derived from these endometriotic cysts control monocyte differentiation into immunosuppressive M2 macrophages [186]. Therefore, ectopic endometrial MSCs may contribute to an immunosuppressed environment in the pelvic cavity, thereby enabling immune escape of ectopic lesions and supporting their growth in endometriosis [186].

Adult stem cells of extra-uterine origin contribute to endometriosis pathogenesis. Bone marrow-derived cells (BMDCs) are a collection of hematopoietic stem cells, MSCs, and endothelial progenitor cells; these cells play a crucial role in tissue repair because they can be recruited to distant inflamed sites and either transdifferentiate to replenish injured cell types or modulate the healing process through paracrine effects [187]. In addition, BMDCs participate in normal endometrial regeneration in the menstrual cycle and are incorporated into epithelial, stromal, and endothelial compartments of the endometrium [188]. Whether BMDCs can transdifferentiate to become functional components such endometrial gland is still under debate [189,190].

BMDCs can be attracted to an endometriotic lesion via the signaling axis of CXCL-12 and its receptor, C-X-C motif chemokine receptor 4 (CXCR4), and estradiol enhances chemoattraction by increasing both the endometrial stromal cell secretion of CXCL-12 and the BMDC expression of CXCR-4 [191]. Endometriotic lesions also compete with the eutopic endometrium for BMDCs, and reduced recruitment of BMDCs in the eutopic endometrium possibly impairs its regenerative capability, leading to infertility [192]. Furthermore, estrogen deprivation therapy with GnRH agonist and aromatase inhibitor restores recruitment of BMDCs in the eutopic endometrium, with greater regression of ectopic lesions, whereas progestin does not significantly affect BMDC recruitment, suggesting that targeting stem cells could be a potential treatment strategy [193].

Anglesio et al. demonstrated that 26% of deep-infiltrating endometriotic lesions harbored cancer-associated somatic mutations that were confined within the epithelial compartment and not found in the stromal compartment [194]. Although further research is required for validation, one plausible explanation for this is that ectopic lesions may be derived from two cellular origins, namely initial establishment of the epithelial compartment by endometrial progenitor cells harboring cancer driver mutation, followed by stromal compartment establishment by BMDCs [194].

4.2. Inflammatory Niche–Induced Stemness Re-Expression in Endometriosis Pathogenesis

Evidence suggests that microenvironmental inflammatory cytokines and growth factors may cause significant changes in cellular behavior, promoting stemness, EMT, invasion, and malignant transformation. The interplay between inflammatory niche and stemness has been widely studied in

cancer biology. Factors such as IL-1β, IL-6, IL-17, and TNF-α have been implicated in the maintenance of cancer stemness and promotion of invasive phenotypes in breast cancer [195], ovarian cancer [196], colon cancer [197], hepatocellular carcinoma [198,199], and thyroid cancer [200]. These inflammatory cytokines can be secreted by tumor-associated immune cells, cancer stromal cells, or cancer cells and function via NFκB- or STAT3-mediated pathways [201,202]. Inflammation may also promote stemness properties by stimulation of growth factor production. For example, IL-6 can stimulate IGF-1 production and enhance stemness of HBV-associated hepatocellular carcinoma cells, leading to early recurrence [198].

MSCs themselves are regulated by the inflammatory niche. The cytokine secretion profiles of MSCs change with stimulation by inflammatory signals such as lipopolysaccharides (LPS), and MSCs of different origins respond differently to the same stimulus [203]. For example, BM-MSCs secrete higher levels of VEGF-A, CXCL-12, IL-1RA, IL-6, MCP-1, and MIP-1α after LPS stimulation compared with emMSCs [203]. Proinflammatory cytokines such as IL-6 can maintain BM-MSCs in their proliferative and stemness state by activating the ERK1/2 pathway [204]. In addition, transient treatment of dental progenitor cells with TNF-α increased their stemness phenotype [205]. In an inflammatory microenvironment, MSCs may exhibit a profibrotic phenotype through higher expression of MMPs, IL-1, IL-6, TNF-α, and type-1 collagen [206]. The effects of cytokines on MSCs are not always unidirectional because a high TNF-α concentration may be detrimental to MSC survival [207]. TGF-β secreted by stromal fibroblasts appears to promote differentiation of emMSCs into endometrial stromal fibroblasts [208]. Inhibiting TGF-βR enhances emMSC surface marker expression, promotes cell proliferation, and prevents apoptosis of emMSCs [208]. The inflammatory niche may also contribute to endometriosis, which is characterized by increased stabilization of HIF-1α and HIF-1α-induced expression of VEGF and MMP-9 in eutopic and ectopic endometria [209–212].

4.3. Immunomodulation of MSCs

MSCs are a mixture of various stromal cell types that have pleiotropic properties and are believed to participate in the tissue- and wound-healing process [213]. Because of their self-renewal and differentiation potential, they have been regarded as a potential cell type for regenerative medicine. Moreover, they have been recognized as negative for hematopoietic cell markers with expression of CD90, CD105, CD44, CD73, CD9, and CD80 [214]. Sources of MSCs are diverse; they have been isolated from numerous sources such as bone marrow (BM), adipose tissue, tonsils, umbilical cord, skin, dental pulp, placenta, and endometrium. Pericytes—perivascular cells found in multiple organs and expressing PDGF-Rβ, CD146, NG2, and typical MSC markers—have been suggested as possible progenitors of non-BM-MSCs [215].

MSCs have different effects on different immune cell types, including both innate and adaptive immune systems. They secrete factors such as IDO, PGE2, NO, TGF-β, and HGF to reduce activation and cytotoxicity of NK and T-cells [216]. Moreover, these factors decrease the proliferation and activation of B- and T-cells, leading to reduced antibody and cytokine production. MSCs increase regulatory T-cell numbers, inhibit dendritic cell maturation and the macrophage M1 phenotype, promote polarization of the M2 phenotype, and suppress mast cell degranulation [217]. These anti-inflammatory changes have prompted researchers to investigate MSCs for treatment of inflammatory diseases.

Although numerous studies have focused on the immunosuppressive effects of MSCs and their potential application in treating inflammatory diseases, they also have immunostimulatory properties. MSCs are sensitive to their microenvironment and their interaction with it determines their role in enhancing or suppressing inflammation. For example, the immunosuppressive effect of MSCs on T-cells depends on IFN-γ, IL-1, and TNF-α induction of NO production; however, low concentrations of these cytokines cannot induce sufficient NO production to suppress T-cell proliferation, leading to the activation of T-cell responses [218]. IL-6 expression by MSCs affects their ability to polarize macrophages on cell contact [219]. IL-6 in combination with PGE2 and IDO drives

M1 polarization [219]. In the absence of IL-6, differentiation toward the M2 phenotype because of TNF-α and IFN-γ exposure is favored [219]. Further studies are required to clarify how MSCs might be consistently applied as immunomodulatory agents in clinical use.

Different tissue origins of MSCs are available for research and clinical use. BM represent a common MSC source in clinical practice, with early success in treatment of graft-versus-host disease [217]. However, extraction of BMMSCs requires an invasive and painful BM aspiration procedure, and the number of BMMSCs declines with donor age. Adipose tissue-derived (AT) MSCs have gained significant interest as liposuction has become an increasingly common operation, and the greater stem cell yield from adipose tissue allows faster expansion of ATMSCs for therapeutic use [220]. Neonatal stem cells from biological materials obtained after birth, namely placenta, umbilical cord, and umbilical cord blood, are also attractive cell therapy options for autologous and allogeneic transplantation. Although MSCs of different origins have similar morphology, they can have different biological properties, including epigenetic regulation, gene expression profile, surface receptor expression, proliferation and differentiation capacity [221–223]. However, different studies have reported inconsistent comparison results of different MSC origins, possibly due to differences in cell culturing conditions.

MSCs derived from different sources seemed to have different immunomodulatory effects on different immune cell types. When comparing BMMSCs with ATMSCs, only BMMSCs suppressed NK cell cytotoxic activity, but ATMSCs were more potent at inhibiting dendritic cell differentiation, mononuclear cell proliferation, and B cell immunoglobulin secretion [224–227]. The more potent immunomodulatory capacity of ATMSCs could be attributed to higher level of IDO expression in response to IFN-γ and higher levels of immunosuppressive cytokine IL-10 secretion when co-cultured with monocytes [226]. While both BMMSCs and ATMSCs inhibited B cell activity, umbilical cord MSC seemed to have little effect [228]. Another study showed that, when compared with BMMSC and ATMSC, umbilical cord Wharton's jelly MSCs expressed the lowest level of immune response markers MHC II, JAG1, TLR4, TLR3, NOTCH2, and NOTCH3 and had the most inhibitory effect on T cell proliferation [223]. Although placenta-derived MSCs also have immunomodulatory effects, their relative immunomodulatory capacity compared with other tissue origins is contradictory [229,230]. MSCs isolated from neonatal sources like placenta, umbilical cord, and umbilical cord blood also express high level of HLA-G, a critical surface antigen responsible for inducing immunotolerance and allowing fetal tissues to coexist with maternal immune system during pregnancy [231,232]. The presence of HLA-G on neonatal MSCs may therefore make them favorable cell therapy candidates for allogeneic transplantation.

4.4. Safety and Efficacy of Stem Cell Therapy in Endometriosis-Associated Infertility

Development of MSC-based delivery vectors has generated considerable interest because of their tropism toward inflamed lesion foci such as tumors. Stem cell therapy has been applied to treat infertility, especially Asherman's syndrome [233], which is characterized by endometrium adhesion and fibrosis due to tuberculosis infection or following hysteroscopic procedures such as dilation and curettage; such patients present with absent menstruation and infertility. The objective of MSC transplantation therapy is to reduce the degree of inflammation after adhesiolysis and promote endometrial regeneration [234]. Autologous in utero BM stem cell transplantation facilitated IVF in a case report of a woman with severe endometrial adhesion following dilation and curettage [235]. In addition, CD133+ bone marrow-derived cell transplantation enabled endometrial thickness growth, increased neovascularization, and menses restoration in a cohort study of 11 Asherman's syndrome patients [236]. MSC transplantation also improved ovarian function in premature ovarian insufficiency and aging animal models by increasing folliculogenesis, decreasing granulosa cell apoptosis and the extent of ovarian fibrosis, and modulating cytokine expression [237–239].

Safety always takes priority over efficacy. Application of MSCs for treating endometriosis, and in particular endometriosis-associated infertility, has caused controversy because of stem cell involvement in the disease's pathogenesis [240]. For example, several reports have indicated that bone marrow-derived MSCs can differentiate into carcinoma-associated fibroblasts and promote tumor growth, possibly involving niche TGF-β, CXCL-12, or IL-6 [241–246]. Additionally, MSCs from adipose tissue have been demonstrated to promote cancer proliferation and metastasis through STAT3 activation [247,248]. The effects of different MSC sources on gynecological and breast cancer are summarized in Table 2. Because endometriosis behaves similarly to cancer in many ways and correlates with gynecologic cancer occurrence, considering the risk of disease promotion is vital when determining the optimal cell therapy type. Moreover, carefully designing a cell handling and delivery protocol is essential because different ex vivo expansion methods could affect MSC phenotypes in different manners [249], and systemic infusion provides a better uterine engraft than does local injection [250].

Table 2. Promotive and suppressive effects of MSCs in gynecological and breast cancers.

Cancer type/MSC Source	Surface marker	Effect	Factors/Mechanisms	Ref.
Ovarian cancer				
Omental adipose tissue of healthy donor	$CD73^+$ $CD90^+$ $CD105^+$ $CD34^-$	Increased tumor growth and metastasis	ATMSC increased tumor cell secretion of MMP2 and MMP9	[251]
Umbilical cord Wharton's jelly of healthy donor	$CD44^+$ $CD90^+$ $CD105^+$ $CD34^-$ HLA^-	Decreased tumor growth	WJSC increased tumor cell apoptosis	[252]
Menstrual blood of healthy donor	$CD73^+$ $CD90^+$ $CD34^-$	Decreased tumor growth and angiogenesis	emMSCs induced tumor cell cycle arrest, increased tumor cell apoptosis, decreased AKT phosphorylation, and promoted FoxO3a nuclear translocation of tumor cells	[253]
Endometrial Cancer				
Bone marrow of healthy donor	$CD29^+$ $CD44^+$ $CD73^+$ $CD90^+$ $CD105^+$ $EpCAM^-$ $CD11b^-$ $CD34^-$ $CD45^-$	Increased tumor growth	BMMSC-secreted high level of VEGF, FGF, and SDF1-α; Tumor-secreted IL-8 and CXCL-1 attracted BMMSCs to the tumor site	[254]
Omental adipose tissue of healthy donor	$CD73^+$ $CD90^+$ $CD105^+$ $CD34^-$	Increased tumor growth and metastasis	ATMSC-secreted IL-6 activated STAT3 pathway in tumor cells	[247]
Omental adipose tissue of gynecologic cancer patients, subcutaneous adipose tissue of healthy donor	$CD29^+$ $CD44^+$ $CD73^+$ $CD90^+$ $CD105^+$ $EpCAM^-$ $CD11b^-$ $CD34^-$ $CD45^-$	Increased tumor growth (omental ATMSCs); No significant tumor promotion with subcutaneous ATMSCs	Omental ATMSCs secreted high level of VEGF, FGF, and SDF1-α; Tumor-secreted, IL-8 and CXCL-1 attracted ATMSCs to the tumor site	[254]
Cervical Cancer				
Amniotic fluid in second trimester of gestation	$CD73^+$ $CD90^+$ $CD105^+$ $CD14^-$ $CD34^-$ $CD45^-$ HLA^-	Increased tumor growth	Genetically modified IFN-α-expressing AFMSCs suppressed tumor growth	[255]
Breast Cancer				
Bone marrow of healthy donor	$CD105^+$ $CD31^+$ $CD34^-$ $CD133^-$	Increased tumor growth and metastasis	Tumor increased BMMSC–secreted CCL-5 (RANTES) to increase cell motility, invasion, and metastasis	[256]

Table 2. *Cont.*

Cancer type/MSC Source	Surface marker	Effect	Factors/Mechanisms	Ref.
Bone marrow of healthy donor	Not mentioned	Varying effect in tumor growth and metastasis for different breast cancer cell lines	BMMSC-secreted IL-17 and tumor-expressed IL-17R may stimulate migration of metastatic cancer cells; Tumor-secreted TGF-β1 attracted BM-MSCs	[257]
Subcutaneous abdominal adipose tissue of healthy donor	$CD29^+$ $CD73^+$ $CD90^+$ $CD105^+$ $CD166^+$ $CD11b^-$ $CD31^-$ $CD34^-$ $CD45^-$ $HLA\text{-}DR^-$	Varying effect of tumor growth and angiogenesis for different breast cancer cell lines	ATMSC-secreted CXCL-1 and CXCL-8 promoted tumor angiogenesis	[258]
Umbilical cord of healthy donor	$CD13^+$ $CD29^+$ $CD44^+$ $CD73^+$ $CD90^+$ $CD105^+$ $CD106^+$ $CD166^+$ ABC^+ HLA^- $CD14^-$ $CD31^-$ $CD34^-$ $CD38^-$ $CD45^-$ $HLA\text{-}DR^-$	Increased migration and metastasis (MCF-7)	UCMSC-secreted IL-6 and IL-8 promoted tumor cells to secret IL-6 and IL-8 to increase migration and mammosphere formation	[259]
Umbilical cord of healthy donor	$CD29^+$ $CD44^+$ $CD54^+$ $CD73^+$ $CD90^+$ $CD105^+$ $CD11b^-$ $CD19^-$ $CD31^-$ $CD34^-$ $CD45^-$ $HLA\text{-}DR^-$	Decreased tumor growth and angiogenesis (MDA-MB-231)	Increased apoptosis	[260]
Umbilical cord Wharton's jelly of healthy donor	$CD44^+$ $CD90^+$ $CD105^+$ $CD34^-$ HLA^-	Decreased tumor growth and migration	Increased apoptosis	[252]
Amniotic tissue of healthy donor	Not mentioned	Decreased tumor growth	AMESCs secreted TNF-α, TNF-β, TGF-β, IFN-γ, IL-2, IL-3, IL-4, M-CSF, and IL-8	[261]

AFMSC, amniotic fluid-derived mesenchymal stem cell; AMESC, amniotic membrane-derived epithelial stem cell; ATMSC, adipose tissue-derived mesenchymal stem cell; BMMSC, bone marrow-derived mesenchymal stem cell; CD, cluster of differentiation; emMSC, endometrial mesenchymal stem cell; FGF, fibroblast growth factor; IL, interleukin; M-CSF, macrophage colony-stimulating factor; MMP, matrix metalloproteinase; MSC, mesenchymal stem cell; TGF-β, transforming growth factor beta; TNF, tumor necrosis factor; UCMSC, umbilical cord-derived mesenchymal stem cell; VEGF, vascular endothelial growth factor; WJSC, Wharton's jelly stem cell.

Because MSCs can be derived from multiple organs, the choice of which one to use for clinical application is a challenging problem that requires assessment of safety and efficacy. MSCs of different origin can have different responses to the same niche. Khatun et al. demonstrated that although BM-MSCs and emMSCs exhibit high proliferative and migratory capabilities, BM-MSCs secrete higher levels of cytokines under LPS stimulation, including IL-6, CXCL-12, MCP-1, RANTES, and VEGF-A [203]. Therefore, emMSCs appear to have the greatest potential for use in endometriosis treatment and have been evaluated for their potential use as a cell therapy targeting vector for endometriosis treatment [262]. They did not generate tumors in subcutaneous tumorigenicity in vivo, and were shown to accumulate in an endometriosis implant after systemic intravenous injection [262]. However, no significant differences in endometriotic lesion size, VEGF expression, or microvascular density at sacrifice were observed. Koippallil Gopalakrishnan et al. studied the use of emMSCs as a cell-based targeting vector for endometriosis. These endometrial MSCs were engineered to express sFlt-1—the soluble truncated form of VEGFR—to inhibit angiogenesis. The engineered emMSCs gathered in the endometriotic implant after systemic injection and reduced the implant size and microvessel density [263]. Although few studies have investigated endometrial MSCs, results suggest that they are a safe cell type that does not promote endometriosis progression [262,263].

Other cell origins such as BM and umbilical cord blood have been investigated. BMDCs were the first to be tried in an endometriosis model [264]. Although BMDCs decreased in situ TNF-α and VEGFR levels, they did not affect lesion size [264]. In another study, bone marrow-derived MSCs were injected locally into an endometriotic implant in a rabbit endometriosis model [265]. The MSC group

had significantly more intra-abdominal adhesions and a lower pregnancy rate than did the control group, and no reduction in implant size with MSC treatment was noted [265]. When human umbilical cord mesenchymal stem cells were used to treat cultured endometriotic cells, the cytokine expression of IL-1β and proliferation rate of endometriotic cells increased in vitro [266].

5. Conclusions

Medical therapies for endometriosis commonly focus on relieving its painful symptoms as opposed to curing the disease. Between 5% and 59% of patients undergo ineffective treatment, and 17–34% experience recurring symptoms after treatment cessation [267]. Developing novel therapies would benefit patients with poor response by improving quality of life and infertility outcomes. Therefore, many medical therapy strategies have been devised to target the dysregulated immune system that characterizes endometriosis, but none has proven sufficiently effective to be incorporated into a standard management protocol. MSC-based cell therapy offers an attractive option for addressing the infertility problem of endometriosis patients because of its tropic and immunomodulatory effects. MSC-based cell therapies have been tested in infertility treatment for patients with Asherman's syndrome and premature ovarian failure. However, much remains to be explored where patient safety is concerned. Careful selection and handling of the MSC source to target endometriotic lesions without promoting lesion growth is crucial. Different sources of MSC may respond differently to the inflammatory niche, whereas the same MSCs may alter their behaviors because of their plasticity. Therefore, defining different cytokine–receptor interactions for different MSC origins is necessary. Gene-editing of MSCs to express antiproliferative or antiangiogenic products may also be explored to take full advantage of MSC-based cell therapy (Figure 2).

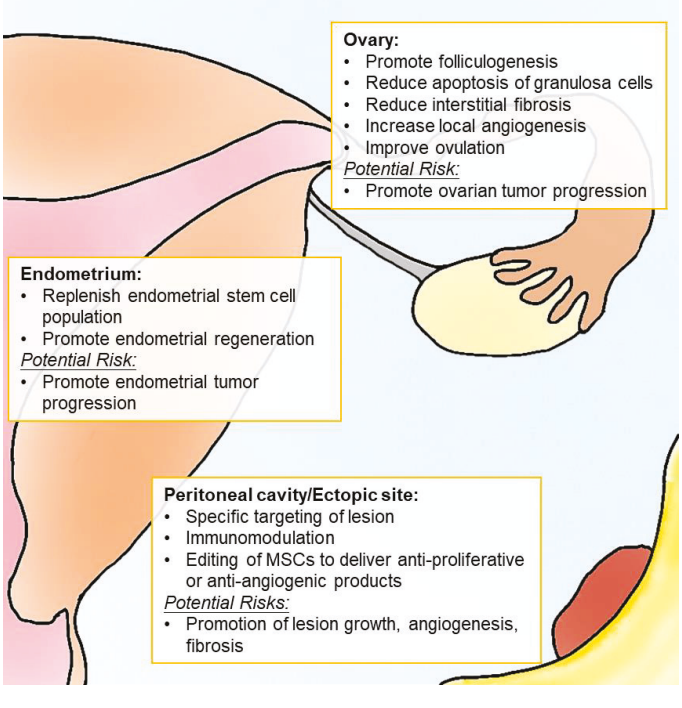

Figure 2. Mesenchymal stem/stromal cell (MSC)-based therapy for treatment of endometriosis-associated infertility: safety and efficacy.

Author Contributions: Y.-H.L., H.-Y.C. and Y.-H.H. formulated the theme and outline of the review. Y.-H.L. and Y.-H.C. reviewed the literature and drafted and revised the manuscript. H.-Y.C., H.-K.A., C.-R.T. and Y.-H.H. reviewed, revised, and finalized the manuscript.

Funding: This work was financially supported by research grants from the National Science Council, Taiwan (NSC102-2628-B-038-008-MY3); the Ministry of Science and Technology, Taiwan (MOST 103-2321-B-038-011, MOST 104-2321-B-038-003, MOST 105-2628-B-038-008-MY3 and MOST 106-3114-B-038-001); the Health and Welfare Surcharge of Tobacco Products (MOHW105-TDU-B-212-134001, MOHW106-TDU-B-212-144001, and MOHW107-TDU-B-212-114014); Taipei Medical University (TMU-T104-06, TMU-T105-06, TMU-T106-03, 105TMU-CIT-01-3, 106TMU-CIT-01-3 and DP2-107-21121-01-T-02); and Taipei Medical University Hospital (103TMU-TMUH-05, 104TMU-TMUH-04, and 105TMU-TMUH-10).

Conflicts of Interest: The authors declare no conflicts of interest.

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International Journal of
Molecular Sciences

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Review

Pathogenomics of Endometriosis Development

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Received: 24 May 2018; Accepted: 21 June 2018; Published: 23 June 2018

Abstract: For over 100 years, endometriosis, as a chronic, estrogen-dependent, inflammatory, heritable disease affecting approximately 5–10% of women in reproductive age has been the focus of clinicians and scientists. In spite of numerous environmental, genetic, epigenetic, endocrine, and immunological studies, our knowledge of endometriosis is still fragmentary, and its precise pathophysiology and pathogenomics remain a mystery. The implementation of new technologies has provided tremendous progress in understanding the many intrinsic molecular mechanisms in the development of endometriosis, with progenitor and stem cells (SCs) of the eutopic endometrium as the starting players and endometriotic lesions as the final pathomorphological trait. Novel data on the molecular, genetic, and epigenetic mechanisms of the disease are briefly outlined. We hypothesize the existence of an endometriosis development genetic program (EMDP) that governs the origin of endometrium stem cells programmed for endometriosis (1), their transition (metaplasia) into mesenchymal SCs (2), and their invasion of the peritoneum and progression to endometriotic lesions (3). The pros and cons of the recent unifying theory of endometriosis are also discussed. Complex genomic and epigenetic interactions at different stages of the endometriosis process result in different forms of the disease, with specific features and clinical manifestations. The significance of the EMDP in elaborating a new strategy for endometriosis prediction, prevention, and treatment is discussed.

Keywords: endometriosis; developmental pathway; pathogenomics; mesenchymal stem cells

1. Introduction

Endometriosis is a common disorder affecting 5–10% of women of reproductive age. By clinical manifestation, it corresponds to chronic, estrogen-dependent inflammation mitigated by the growth of endometrium-like tissue in sites other than the uterine cavity, most commonly in the pelvic cavity. Although studied for a century, many aspects of the pathophysiology and developmental pathogenetics of the disease still remain obscure, and practical achievements in the prediction, prevention or treatment of endometriosis remain rather illusive to date [1,2]. A detailed understanding of the molecular mechanisms underlying endometriosis is also far from complete. Meanwhile, spectacular achievements in molecular diagnostics and system genetics in studies of this common disease have provided a huge bulk of useful information regarding the genetic aspects of endometriosis and the molecular mechanisms of its origin and development [3,4]. Many theories and attractive hypotheses on the pathogenesis of endometriosis are known but they are rather contradictory. Genetic, endocrine, environmental, immune, and epigenetic factors have been studied in numerous articles to explain the mechanistic basis of the origin and development of endometriotic lesions [5,6]. Conspicuous progress in this area has been achieved during the last decade, mainly due to the identification of new candidate genes and numerous SNPs (single nucleotide polymorphism) tightly associated with endometriosis [6], of genetic and epigenetic mechanisms of its regulation [5,7], and of endometrial stem cells [8], and to transcriptome and miRNA analyses of the endometrium and endometriotic

cells [9,10]. The contribution of epigenetic and genetic factors in the pathogenesis of endometriosis has been described in many exhaustive reviews [3,4,10–12].

Studying endometriosis as a problem of developmental genetics is a principal goal of the present paper. The origin of endometriotic cells and the genetic and epigenetic factors contributing to the initiation and growth of endometriotic lesions are briefly reviewed. We hypothesize the existence of a special endometriosis development program (EMDP) which switches on in the progenitor SCs of the endometrium and in SCs descended from the Mullerian duct. EMDP suggests that the cells are prone to giving rise to endometriosis partly through endometrial–mesenchymal transition, their invasion into the peritoneum lining, and differentiation and growth into endometriotic lesions.

Classical embryology and developmental biology postulate that each morphogenetic event has its own critical and sensitive period (SP) which displays a heightened sensitivity to internal and external stimuli [13]. According to further molecular studies, the critical periods precede visible morphogenetic reactions and correspond to massive genome reprogramming [14]. The suggested SPs of EMDP should be considered a suitable timeframe for the prediction and treatment of endometriosis. The epigenetic landscape of endometriosis reflects the complex interactions of genetic and epigenetic factors, which underlies the pathogenomics of endometriosis [15], creates a unique EMDP, substantiates endometriosis clinical manifestations, and provides clues for a personalized treatment of this disease.

2. Key Stages of Endometriosis Development

2.1. Stem Cells in the Pathogenesis of Endometriosis

SCs are defined as undifferentiated cells which possess both self-renewal and differentiation abilities [16]. The possibility for extra-uterine SC to progress into endometriotic lesions may explain endometriosis developing in distant sites such as the lungs. They also support the theory suggesting that SC may travel via lymphovascular spaces [17]. Finding the stemness-related genes, such as *OCT4*, *SOX2*, *SOX15*, *NOTCH1*, *TWIST1*, and others, expressed in endometriotic lesions, may help show that the mechanisms determining the self-renewal rates and SC fates are deregulated in endometriosis, leading to altered SC behavior [18].

According to initial studies, the multi-site origin of endometriotic SCs was repeatedly suspected [3,6,19]. Different types of endometrial SCs were hypothesized, such as endometrial SCs in the peritoneum and pelvic cavity (1), resting embryonic cells descendent from the Mullerian duct (2), SCs in menstrual debris (3), coelomic epithelial cells after metaplasia (4), and mesenchymal bone marrow SC (bmSCs) in inflammation sites in the peritoneum (5). It was postulated that SCs that originated from bone marrow SCs could also be attracted in the human endometrium, but their participation in endometriosis should be proven [3,19]. Several different types of SCs have been suggested in the endometrium itself, including progenitor cells of the endometrium, mesenchymal stem cells, and endothelial stem cells [16,20]. Under appropriate conditions, SCs shed with menstrual blood can differentiate into typical mesenchymal lineages [21]. Thus, although the exact location of endometrial SCs still needs to be explored, some findings suggest that the inner basal layer resting on the myometrium at the endometrium–myometrium interface and known as the "junctional zone", should be treated as a preferential site for the endometrial SC niche [16,22]. Also, bmSCs in the endometrium could contribute to all stem cell kinds in the endometrium [19,23] The existence of own SCs in the endometrium is also postulated, although the specific markers to identify endometrial SCs have not yet been established [19,24].

As might be inferred, little doubt is left with regard to the SC origin of endometriosis. Whether they SCs in the endometrium are endometrial by origin or come from other sources like the bone marrow, peritoneum, or some other tissues, remains unknown. Meanwhile, two major sources of endometriotic SCs should be considered: SCs disseminated throughout the peritoneum lining the pelvic cavity during embryogenesis of the female reproductive tract (endometriosis of extrauterine origin) (1), and SCs from the endometrial layer (endometriosis of intrauterine origin) (2). The hypothesis of

the extrauterine origin of endometriosis from mesenchymal SCs disseminated during embryogenesis that infested the epithelium lining of the pelvic cavity has recently received major support in the novel "unifying theory" of endometriosis pathogenesis [24]. More details of this hypothesis will be given in the Discussion. The second hypothesis is in line with the well-known hypothesis by Sampson (1927), which postulates that the endometriosis originates from the menstrual cells of endometrial tissue disseminated in the pelvic cavity [25].

2.2. Initial Stages of Endometriosis

The most intriguing problem of endometriosis pathogenesis concerns the molecular mechanisms underlying the acquisition of tumor-like properties by otherwise normal SCs. According to the "uterine origin" and the "extrauterine origin" hypotheses, metaplasia of the endometrial (epithelial) cells into mesenchymal cells (so-called epithelial–mesenchymal transition—EMT) may play a key role in the pathogenesis of endometriosis [26].

EMT is a biologic process during which polarized epithelial cells by consecutive changes get a mesenchymal cells phenotype. EMT plays a role in a series of biological settings, such as implantation and embryogenesis and pathogenesis of malignant tumors, and is also associated with wound healing, tissue regeneration, and organ fibrosis [27]. The molecular mechanisms of EMT in epithelial cells involve the functional loss of E-cadherin, desmoplakin, and mucin-1 and increased expression of such mesenchymal markers as N-cadherin, smooth-muscle actin and ohers [28]. Cells of different origin can enter EMT leading to development of endometriosis. These cells can be peritoneum epithelium cells (as according to the metaplastic theory of development of endometriosis), endothelial cells, and also epithelial cells of the endometrium [26]. The molecular mechanisms of EMT have now been studied in detail [18].

Main inducers of EMT are well known [27]. Chronic injury and subsequent inflammation can trigger EMT through the release of some cytokines, such as TGF-β, PDGF, EGF, and FGF-2. A number of authors have reported that that the TGF-β level have increased in peritoneal fluid and serum of women with endometriosis compared to healthy women [29]. Other inducers of EMT are hypoxia and other factors (i.e., the Ras–MAPK (mitogen-activated protein kinase) pathway) leading to hyperexpression of hypoxia-induced factor-1 (HIF-1A) [26].

The principal role in the metaplasia of the endometrial epithelium might be attributed to the *TWIST1* gene (Twist family basic-loop-helix transcription factor 1). It was identified as a key regulator of mesoderm development and later have been implicated in many human diseases. The expression of *TWIST1* is closely related to tumor aggressiveness and metastatic potential [30]. Twist1 has also been shown to function as a key regulator of EMT. Driven by HIF-1, Twist1 realizes its developmental functions by governing cell movement and tissue reorganization [31]. The molecular mechanisms underlying EMT induced by TWIST in epithelial cells involve functional loss of E-cadherin (CDH1) in the eutopic endometrium of endometriosis patients. Reduced level of cadherins accompanied by excessive expression of metalloproteases (MMP) genes provide favorable conditions for cell migration. A mechanosensitive transduction pathway involving β-catenin specifies the early mesodermal conservation, which is required for Twist mechanical identity. Thus, transient hypoxia and mechanical tension switch on EMT through the activation of *TWIST1*. The expression of doublecortin- and Ca^{2+}/calmodulin-dependent protein kinase-like protein-1 (DCAMKL-1), which is known to regulate *TWIST1*, *Myc*, *KRAS*, and other factors, was also recently discovered [18]. Furthermore, it has been pointed out that there might also be some imbalances in micro-RNAs (miRNA) in women with endometriosis, enhancing cell invasiveness due to impaired miR-145 or promoting proangiogenic factors due to the downregulation of miRNA-199a-5p or extracellular matrix regulator miRNA 29a, significant downregulation of mir-200b in the endometrium and in peritoneal lesions, and regulation of *HOX* genes family miRNA196 [10]. Over 600 different miRNAs associated with endometriosis at each stage of development are known so far. The available results in miRNA studies of endometriosis are rather contradictory and need thorough revision [10]. The significant heterogenicity of endometriotic

lesion samples is considered a major problem when analyzing the miRNA signatures of whole endometriotic lesion biopsies [4,9,10].

Thus, during the dormant stage of endometriosis, there are some cells of endometrial origin which might potentially contribute to the growth of endometriotic lesions. The latter is regulated by the activation of specific transcription factors induced by transient hypoxia, chronic inflammation, and mechanical tension switch. The cells lose their polarity and contacts and acquire the migratory and invasive abilities of mesenchymal stem cells. The expression of the *MYC* and *CCND1* (cyclin D1) genes leads to high proliferative activity, while the upregulation of *BCL2* reduces apoptosis and prolongs survival. Thus, as a consequence of EMT, epithelial cells lose their specific features as well as their integrity and acquire mesenchymal traits linked to increased invasion and migration properties [18]. Under appropriate hormonal and immunological stimulation, the SCs shed into the peritoneal cavity during retrograde menstruation gain abilities for invasion, implantation, and growth [19]. It should be reminded that endometriosis might also stem from the stromal cells of the endometrium itself, although their capacity for proliferation, invasion, and endometriotic lesion growth are still not known. There are some data showing that SCs derived from the menstrual blood debris in an endometriosis patient also showed altered SC functions, which favor the establishment of endometriotic implants [16].

2.3. Invasion of Endometriotic SC

The basic signs of endometriosis development include endometriotic SC invasion in the peritoneum, and their proliferation and differentiation into endometriotic lesions. Women with endometriosis are known to have increased macrophage activity, decreased cellular immunity, and reduced natural killer cell counts [8]. Thus, following retrograde menstruation, the immunodeficient condition prevents the clearance of the menstrual debris from the peritoneum, making the ectopic endometrial cells persist [32]. The latter induce inflammation, recruit macrophages and leukocytes, and, thereby, promote the development of endometriosis [33].

The molecular profiling of the eutopic endometrium from endometriosis patients suggests functional alterations in the genes that facilitate proliferation, implantation, and survival of the endometrial tissue in the peritoneal cavity, thus supporting endometriosis pathogenesis from the altered eutopic endometrium. Inflammatory, immune, and angiogenic responses as well as apoptosis reactions are altered in the eutopic endometrium of affected women, thus favoring the survival and the maintenance of ethe ndometriotic tissue [34].

The relocation of SCs from the eutopic endometrium to ectopic sites in the pelvic cavity potentiates the release of several chemokines and cytokines which favor revascularization and thus allow the development of endometriotic lesions [17]. Comparisons between SCs in the eutopic endometrium and ectopic SCs in the peritoneal cavity by analyzing their phenotypes and gene expression of pro-inflammatory cytokines, migration markers, and angiogenic factors proved the increased levels of these molecules, accompanied by the reduced levels of anti-inflammatory cytokines such as TGFβ. The increased levels of pro-inflammatory cytokines such as interleukin-6 (IL-6) and interferon-γ (IFNγ) and the presence of the migration markers matrix metalloproteases (MMP)-2, -3, -9 and of the proangiogenic vascular endothelial growth factor (VEGFA) in ectopic tissue indicate that the abnormal behavior of ectopic mesenchymal SCs may suppress the immune system and enhance angiogenesis [35]. The increased expression of MMPs would also be useful for the ectopic endometrial tissue to activate invasion.

The processes of implantation of endometriotic SC onto the peritoneum and endometriotic lesion growth obviously require angiogenesis. Several studies have reported an increase in VEGFA level in the serum and peritoneal fluid of endometriosis patients in comparison with women without the disease [36]. Endometrial expression of interleukin-8 (IL-8) is responsible for the chemotaxis of neutrophils and partly for angiogenesis. The density of IL-8 receptors is significantly higher in women with endometriosis, as this molecule is involved in endometrial cell proliferation and attachment [17,23].

In a systematic review of different chemokines as markers of endometriosis, IL-8 appeared to be the most significant [9].

The anti-apoptotic *BCL-2* gene, upregulated in the eutopic endometrium of women with endometriosis, enhances cell survival and thus plays a major role in the pathogenesis of endometriosis. Increased proliferation and decreased apoptosis rates in the eutopic endometrium correlate with the expression profile of the *BCL-2* gene in endometriosis patients [37].

The endometriotic lesion cells express high levels of P450 aromatase–a protein which allows estrogen overproduction and decreases the expression of 17β-HSD2 (17β-Hydroxysteroid dehydrogenase), thus inhibiting the response to progesterone ("progesterone resistance") [16]. This is considered a key process through which the maintenance and growth of endometriotic lesions are promoted. It is not known, however, whether these processes are a necessary cause of endometriosis or rather its consequence [32]. These results support the notion that intrinsic abnormalities in the eutopic endometrium cells in women with endometriosis predispose the endometriotic SCs cells to survive in the pelvic cavity, attach, invade, and establish a blood supply in the peritoneum or other areas.

Endometriotic lesions provoke local inflammation of the peritoneum, which attracts bmSCs through the expression of the C-X-C chemokine receptor type 4 (CXCR4) and of the chemokine ligand 12 (CXCL12) which plays a role of chemoattractant in the migration of bmSC towards the endometrial stromal cells. Thus, the deregulation of estrogen combined with local peritoneal injuries may be important in the pathogenesis of endometriosis [23]. Also, bmSCs may migrate from the peripheral circulation and provoke the formation of endometriosis foci in remote sites as well as infiltrate the endometrium of endometriotic lesions [19].

The endometriosis implant can also result from the outgrowths of the dormant SCs disseminated in the pelvic lining during embryogenesis of the female reproductive system [19] (see also Section 1).

Thus, pelvic and extrapelvic endometriosis implants are hypothesized, each with a distinctive epigenetic expression profile. Epigenetics plays a major role in modulating steroid action, and the inflammatory reaction is a key factor for the recruitment of bmSCs [5,38–40]. Whether gene expression profiles in endometriosis cells of the endometrium or bone marrow are similar or different remains unknown. Clarifying this puzzle is important to understand the pathogenetics of endometriosis.

3. Discussion

Genetic and epigenetic data analysis revealed significant differences in various tissues and cell types undergoing the EMDP compared to the normal ones. Complex molecular genetic and epigenetic features constitute the pathogenomic architecture of endometriosis and include gene polymorphisms, peculiarities of their expression, numerous interactions of gene nets, complex combinations of functional protein modules, as well as different metabolic pathways which are altered by sever imbalances in the hormonal and immunologic systems [3,5,32]. Each of these factors is affected at different levels during endometriosis depending on the specific EMDP. On the other hand, common clinical manifestations indicate the existence of some crucial molecular pathways common to all clinical types of endometriosis. Irrespective of the obvious differences in the intermediate events, the EMDP ultimately ends in the typical endometriotic lesions. Thus, the EMDP should be roughly subdivided into three parts: transition of mesodermal embryonic cells into cells of the endometrium within Muller ducts rudiments (1), acquisition of endometrial cells abnormalities and cell transition into endometriotic SCs (2), invasion of the SCs into the peritoneum lining and their differentiation into endometriotic lesions (3).

As it was indicated (see 1), any developmental event should be attributed to a massive genome reprogramming which follows the short critical phases (the epigenetic crises after Waddington) of higher sensitivity to any inducers or noxious triggers [14,41]. Thus, at least three critical phases, corresponding to each of the morphogenetic events described above, should be recognized in the EMDP. The first one corresponds to the initial stages of the development of the reproductive tract in female embryos, while the second and third stages take place in postnatal life (Figure 1).

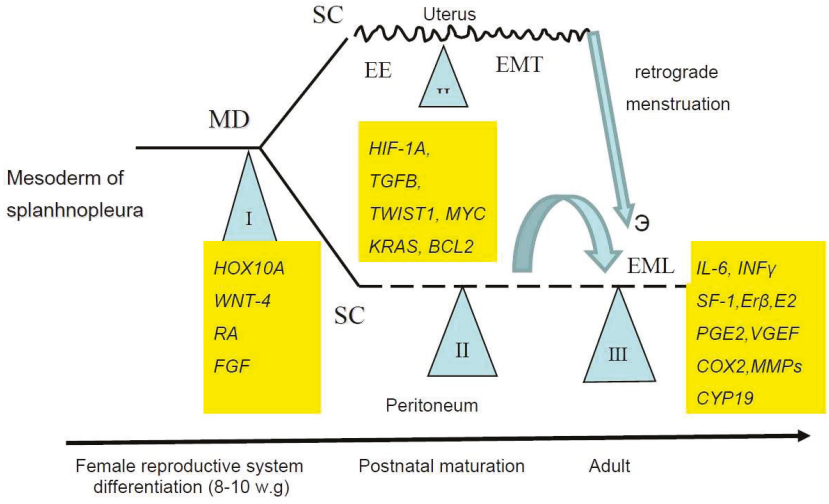

Figure 1. Sensitive periods in the Endometriosis Development Program. SC, stem cells, MD, Mullerian ducts, EE, eutopic endometrium, EMT, epithelial–mesenchymal transition, EML, endometriotic lesions, w.g., weeks of gestation.

The dislocation of the primitive endometrial tissue in female fetuses coincides with human embryonic developmental stages XVII–XX (5–8 weeks of gestation) and lasts into the early postnatal period [42]. Both the coelomic epithelium of the peritoneum and the Mullerian ducts giving rise to all parts of the female reproductive tract generate from the mesoderm layer in the early human embryo. The development of the female urogenital tract is completed only at birth. The genes responsible for female reproductive tract development are well known, and many of them have already been identified [24]. The transcription factors of the *HOX* family, in particular *HOXA10*, are the principal coordinators and regulators of the expression of these genes [3], being responsible for mesoderm segmentation and its axial extension. The next important contributor to the formation of the Mullerian ducts is the *WNT* gene family, with *WNT4* as a key regulator of female sex development. It is located at the 1p36 chromosomal region, wich variants may contribute to endometriosis susceptibility through abnormal differentiation of the female reproductive tract [24]. *WNT4* was shown to be expressed in the normal peritoneum, suggesting that endometriosis can arise through a reversible transformation of the epithelium cells to endometriotic cells (metaplasia) through the developmental pathways associated with the *HOXA9* and *CDKN1A* genes [43]. These data are in line with a recently suggested "unifying hypothesis" of endometriosis [24]. According to this, Müllerian remnants of the endometrium may leak into the peritoneal cavity during embryogenesis of the urogenital system as a result of the deregulation of *WNT* genes and of the Wnt–β-catenin signaling pathway. The latter can lead to aberrations and deregulation within the mesoderm, thus causing the aberrant placement of SCs. Deregulation in the hormonal and immune systems, abnormalities of adhesion, extracellular matrix metalloproteinases, and pro-inflammatory cytokines activate or alter the peritoneal microenvironment, creating the conditions for the differentiation, adhesion, proliferation, and survival of ectopic endometrial cells, thus giving rise to endometriosis in adults. The growth of endometriotic lesions may occur by inclusion and transformation of the mesothelium cells of the peritoneal lining.

Structural variations (polymorphisms) or functional insufficiency of the *HOXA10* and *WNT4* genes and of the genes of their genetic cascade (*MIF*, *VEGFA*, *MMPs*, *VCAM*, *BMP*, etc.) may deregulate highly balanced genetic and epigenetic mechanisms of female reproductive tract embryogenesis, causing disorganization of the endometrium as well as dissemination of mesoderm cells, including

SCs, outside the uterine cavity; this initiates an inborn predisposition to endometriosis in postnatal life. Mullerian embryogenesis-related genes in the uterine endometrium in early life might be associated with endometriosis in the adults.

Direct association of the *HOX* and *WNT* families as well as of 10 other genes with endometriosis was repeatedly confirmed [3,32]. By means of genome-wide association studies (GWAS), 12 single nucleotide polymorphisms at 10 independent genetic loci associated with endometriosis have also been identified [4]. Obviously, mesoderm cells with epigenetic or inborn defects incorporated both in the peritoneal lining and the uterine rudiments are suspected to be associated with the risk of developing endometriosis in adulthood [32].

Thus, endometriosis might be provoked by the failure of the expression of *HOXA10* or *WNTs* genes regulating the initial stages of reproductive tract development in female embryos or also induced by the direct harmful effects of some toxins during embryonic development, which result in the dislocation of the primitive endometrial tissue outside the uterine cavity during early organogenesis [44].

It also might be suspected that endometriotic SCs with inherited disorders of *WNT4* or *HOXA10* genes give rise to clinically forms of endometriosis more severe than those of mostly epigenetic origin [2].

Thus, the first sensitive period (SP) of the EMDP most probably corresponds to the embryonic stages of the female reproductive tract development. An unfavorable combination of endometriosis predisposition genes (predominantly of *WNT* and *HOX* families) and noxious agents (oxidative stress, pesticides, endocrine disruptors) might create conditions for the differentiation, adhesion, proliferation, and survival of eutopic and ectopic endometrial SCs. The direct association of the unfavorable *WNT4* allele with endometriosis has been recently demonstrated [45]. This finding deserves further studies to establish if this allele can be a predictive biomarker of endometriosis.

The second SP of the EMDP concerns the presence of dormant endometriotic cells in the endometrium. The duration of this period is unknown, as progenitors of endometriotic cells may stay dormant for many years until some provocative stimuli trigger their metaplasia into endometriotic SCs. Numerous genetic and epigenetic factors are involved. It was suspected and recently shown that eutopic endometrium cells in endometriosis patients contain aberrantly expressed genes and exhibit deregulated pathways that predispose them to implantation, invasion, and migration outside the uterus [34]. Dysfunctional expression of the genes related to the Mullerian embryogenesis (see SP1) as well as epigenetic immuno-endocrine deregulation of genes in endometrium (*IL11*, *LIF*, *TGF-β*, *FKBP4*, *COX2*, *PGs*, *FOXO1*, and *C/EBPβ*) might appear critical to the development of endometriotic lesions [3,32].

The involvement of external triggers, such as transient hypoxia, chronic inflammation, and mechanic transduction, is also suspected. Transient hypoxia and inflammation induce the *HIF-1A* gene and mechanic transduction upregulate the expression of the *TWIST1* gene. Thus, any measures reducing hypoxia and mechanical stretch of the uterus might be useful in endometriosis prevention. The search for other genes and epigenetic factors in eutopic endometrium cells predisposing to endometriosis should be encouraged.

The third SP of the EMDP includes adhesion, proliferation, invasion, angiogenesis, and growth of endometriotic stem cells into endometriotic lesions. The genes highly expressed at this stage include cell cycle regulators (cyclins and CDKs), angiogenesis factors (*VEGFA*, *ANGPTs*, and *TIEs*), immuno-inflammatory factors (*COX2*), matrix metalloproteinases (*MMP3, MMP9*), and integrins. Their protein products play a critical role in the establishment, maintenance, and development of the endometriotic lesions. Theoretically, interference with the expression of any of this gene might be sufficient for the active prevention and treatment of endometriosis. Clinical practice, however, contradicts these assumptions and favors the view that the EMDP is a well-canalized process, buffered against curative intrusions. At a definite stage of progression, the EMDP becomes irreversible and proceeds to its final stage producing the endometriotic lesions. It should be mentioned that in women receiving a hormonal contraceptive treatment that prevents the implantation, the frequency of

endometriotic lesions on the peritoneum is comparable with that of the controls [46]. In agreement with this, hormonal treatment did not prevent the invasion and implantation of endometriotic SCs. On the other hand, to the best of our knowledge, the implantation of endometriotic SCs per se as well as their invasion into the pelvic lining was never registered, thus giving some credit to the extra uterine origin of endometriosis from the mesenchymal stem cells (meSC) disseminated during embryogenesis of the female reproductive tract (See part 1).

4. Conclusions

As might be inferred from the reviewed studies and suggested hypothesis, each of the three sensitive stages in the EMDP deserves special attention. Intrinsic and external factors interfering with the embryogenesis of the female reproductive tract should be subjected to thorough studies. Of special interest are the inherited forms of endometriosis and their correlation with relevant mutations or polymorphisms of the genes involved in the differentiation of the Mullerian duct and in the development of the urogenital tract, such as *WNT*, *HOXA10*, *HOXA11*, and their signaling pathways, as well as other genes regulating mesoderm differentiation and SC trafficking. The search for teratogenic agents affecting the development of the female reproductive tract should also be encouraged.

More knowledge of SP2 should be drawn from the data on the heterogeneity of eutopic endometrium cells, with special emphasis on the cells prone to induce endometriotic lesions growth. The significance of EMT as a trigger of epigenetic changes amenable to launch the EMDP should be also considered. Both SP1 and SP3 need further global molecular studies of gene expression and its regulation by methylation and microRNA analysis. There are still few reports on these topics, with rather contradictory results for both endometrial transcriptome [9,47] and microRNAs [10]. Large differences between studies can be explained by differences in the study design, subject characteristics, procedures for tissue collection, storage, and processing, assay platforms and data analysis methods. The necessity for the unification of these variables was recently supported by the World Endometriosis Research Foundation initiative that issued the Endometriosis Phenome and Biobanking Harmonization Project, which developed standards for tissue collection, processing, and storage in endometriosis research [48]. It looks very awarding that only –omics analysis of massive endometriosis data stratified according system genetics architecture and collected according to International Conference on Bioinformatics and Biomedicine regulations [7] may pave a reliable way to ultimate solution of endometriosis mystery and maybe give more credit to existence of special developmental program in pathogenomics of endometriosis.

Funding: This research was funded by Russian Science Foundation, Grant Number 14-15-00737.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

EMDP	Endometriosis development program
ESC	Endometrial stem cells
SC	Stem cells
SP	Sensitive period
bmSC	Bone marrow stem cells
meSC	Mesenchymal stem cells
EMT	Epithelial–mesenchymal transition

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International Journal of
Molecular Sciences

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Functional Expression of TRP Ion Channels in Endometrial Stromal Cells of Endometriosis Patients

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Received: 21 June 2018; Accepted: 17 August 2018; Published: 21 August 2018

Abstract: Endometriosis is a common gynecological disease that is characterized by the presence of functional endometrial-like lesions in the abdominal cavity. Aside from epithelial cells, these lesions consist of stromal cells that have the capacity to migrate, adhere, proliferate, and induce neuro- and lymphangiogenesis, which allows them to survive at ectopic locations. However, the exact underlying mechanisms that regulate these changes are yet to be elucidated. The common ground of these processes, however, is the second messenger, calcium. In this regard, members of the superfamily of transient receptor potential (TRP) ion channels, which are known to be calcium-permeable and expressed in the endometrium, have emerged as key regulators. Here, we assessed the molecular and functional expression of TRP channels in stromal cells isolated from the eutopic endometrium of endometriosis patients and controls. Using RT-qPCR, high mRNA levels of TRPV2, TRPV4, TRPM4, TRPM7, TRPC1, TRPC3, TRPC4, and TRPC6 were observed in the whole endometrium throughout the menstrual cycle. Additionally, and in line with previous reports of control patients, TRPV2, TRPV4, TRPC1/4, and TRPC6 were present in human endometrial stromal cells (hESC) from endometriosis patients both at the molecular and functional level. Moreover, proliferation and migration assays illustrated that these parameters were not affected in stromal cells from endometriosis patients. Furthermore, comparison between eutopic and ectopic endometrial samples revealed that the RNA expression pattern of TRP channels did not differ significantly. Collectively, although a functional expression of specific ion channels in hESCs was found, their expression did not correlate with endometriosis.

Keywords: endometriosis; TRP channels; endometrial stromal cells; eutopic and ectopic endometrium

1. Introduction

Endometriosis is a gynecological disease, characterized by the presence of functional, endometrial-like lesions located outside the uterine cavity. This chronic illness burdens 6–10% of

women who are of reproductive-age [1]. Typically, endometriosis is presented with symptoms such as infertility and/or recurrent abdominal pain, which have a crippling effect on patients' lives and an immense impact on their healthcare services [2]. In spite of extensive research, the etiology of the disease remains an enigma. Literature suggests several possible etiological theories for endometriosis; however, no single theory can adequately explain all aspects of this disease. Currently, the most commonly accepted theory is the retrograde menstruation of Sampson, i.e., that lesions occur due to the shedding of the eutopic endometrial lining via the fallopian tubes into the abdominal cavity (Figure 1A) [3]. As this is a natural process which occurs in 90% of reproductive women [4], endometriosis lesions are assumed to acquire additional capacities, such as migration, adhesion, proliferation, and neuroangiogenesis, in order for them to establish and flourish in the abdominal cavity [5].

The endometrium comprises primarily of two different cell types: epithelial and stromal cells. The former can be divided further into luminal and glandular epithelial cells which line the lumen of the uterus and the uterine glands, respectively. Together with the endometrial stem/progenitor cells [6], the stromal cells are the driving force behind the regenerative capacity of the endometrium. They have a mesenchymal background, as stromal cells are vimentin positive [7], bestowing them an inherently migratory and proliferative character. During the follicular phase of the menstrual cycle, the stromal cells are subjected to estrogen, leading to cell proliferation and, subsequently, to the thickening of the endometrium. The exposure to progesterone during the luteal phase will result in the differentiation of the estrogen-primed stromal cells into decidual cells. By undergoing this differentiation process, decidual cells will provide an optimal environment for a possible embryo to be implanted [8]. Several research projects have shown that on several accounts the eutopic endometrium of endometriosis patients is different to that of controls [9]. The most striking difference, is the gain of P450 aromatase expression and activity in the stromal cells of endometriosis patients, which allows for local estrogen production [10–12]. Furthermore, a deficiency of 17β-hydroxysteroid dehydrogenase type II in these cells, which facilitates the inactivation of estrogen into estrone [13], gives the disease an estrogen-dependent character. The endometriotic lesions—presumed to originate from the endometrium—are also comprised of glandular epithelium and stromal cells. Moreover, the ectopic lesions appear to respond in a similar way to cyclic changes of steroid hormones, such as the endometrium [14–16]. However, immunohistochemistry and cDNA microarray studies have shown that the ectopic lesions do not completely resemble their eutopic counterparts [17,18]. They demonstrated an aberrant expression of adhesion molecules [19], anti-apoptotic proteins [20], as well as angiogenic factors, such as the vascular endothelial growth factor [21].

Migration, adhesion, proliferation, and neuroangiogenesis are intricate processes wherein calcium is described as an important regulator [22,23]. Therefore, ion channels are intriguing candidates to regulate these processes, as the activation of ion channels can modulate the intracellular calcium concentrations. The superfamily of transient receptor potential (TRP) channels presents itself as a good candidate to regulate such processes as migration, adhesion, proliferation, and neuroangiogenesis [24,25]. The mammalian TRP-superfamily consists of six subfamilies, based on sequence homology: ankyrin-rich (TRPA1), vanilloid (TRPV1-6), canonical (TRPC1-7), melastatin-like (TRPM1-8), polycystin (TRPP2/3/5), and mucolipin (TRPML1-3) [26]. They can be activated by a variety of stimuli, and are widely distributed throughout the entire body. In endometrial biopsies, TRP channel expression has been shown to fluctuate throughout the menstrual cycle [7]. Furthermore, high mRNA levels for TRPV2, TRPV4, TRPC1/4, TRPC6, TRPM4, and TRPM7, and the functional expression of TRPV2, TRPV4, TRPC6, and TRPM7 was previously illustrated by our group in primary human endometrial stromal cells (hESC) [7]. Interestingly, for some of these stromal TRP channels, their involvement in processes like cell migration (TRPC1/C4 and TRPV2) [27,28], cell adhesion (TRPC4) [29], and cell proliferation (TRPV2, TRPM4, TRPM7) [30,31] has been shown. In addition, Mg^{2+} is involved in essentially every step of cell proliferation, with cancerous cell growth representing the most detrimental effect of deregulated proliferation. Interestingly, TRPM7 represents a major

Mg^{2+}-uptake mechanism in mammalian cells [32,33] and has been implicated as a regulator of cell proliferation [34], inducing cell cycle arrest if blocked. This is based on channel function in Mg^{2+} transport, as cell growth can be restored by Mg^{2+} supplementation [35,36]. Therefore, the characterization of TRP channel expression between (i) the eutopic tissue of endometriosis and controls, and (ii) the eutopic and ectopic endometrium of endometriosis patients would be an interesting feature towards the understanding of the pathogenesis of endometriosis. To this end, mRNA expression studies, and functional Ca^{2+}-microfluorimetry and proliferation/migration assays were performed on human biopsies and the primary cell cultures of both endometriosis patients and controls, to investigate the contribution of TRP channels in stromal cells towards the development of endometriosis.

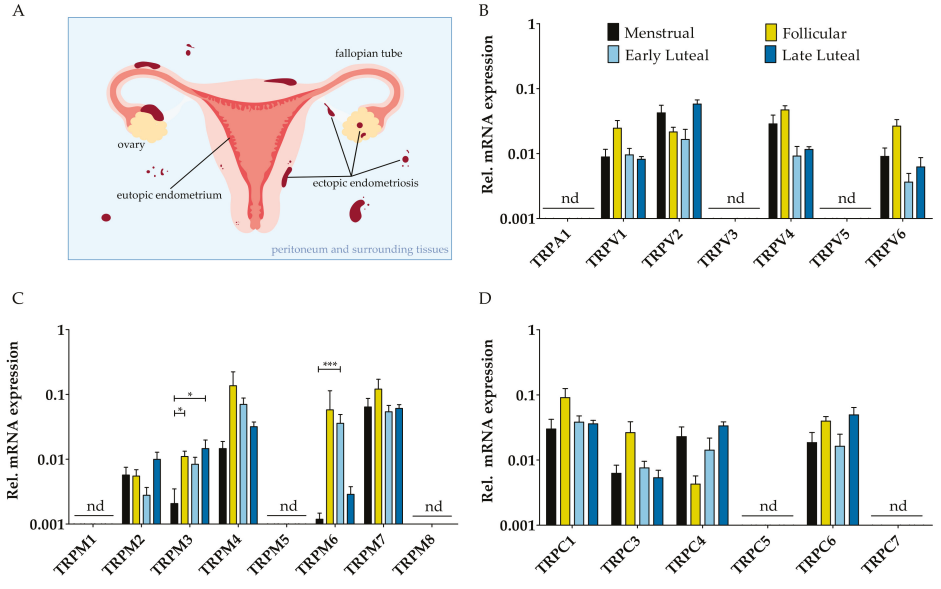

Figure 1. mRNA expression of TRP channels in eutopic endometrium. (**A**) Retrograde menstruation is defined as the regression of eutopic endometrium through the fallopian tubes into the abdominal cavity. In patients with endometriosis, this may ultimately result in ectopic lesions on the peritoneum and surrounding tissues, as indicated in a cartoon; (**B**–**D**) Messenger RNA levels of members of the TRPV, TRPM, and TRPC subfamily, respectively, relatively quantified to the geometric mean of housekeeping genes ACTB, GAPDH, HPRT, PGK-1, and TBP. Biopsies were obtained from endometriosis patients diagnosed with grade II [37] throughout the menstrual cycle, including the menstrual (n = 5), follicular (n = 6), the early luteal (n = 4), and the late luteal phase (n = 3). nd: not detectable. Data are presented as mean + SEM. Statistically significant changes in mRNA expression were assessed using the Two-Way ANOVA test with Bonferroni correction, * $p < 0.05$, *** $p < 0.001$.

2. Results

2.1. mRNA Expression Profile of TRP Channels in Endometrial Biospies and Primary hESC of Endometriosis Patients

Quantitative RT-PCR (RT-qPCR) showed that TRPV1, TRPV2, TRPV4, TRPV6, TRPM4, TRPM6, TRPM7, TRPC1, TRPC3, TRPC4, and TRPC6 were expressed well above the detection limit (Cq < 30) throughout the menstrual cycle in whole endometrial biopsies of endometriosis patients. The mRNA expression level of TRPV3, TRPV5, TRPM2, and TRPM3 was around the detection limit (30 < Cq < 35) of the RT-qPCR analysis, whereas TRPA1, TRPM1, TRPM5, TRPM8, TRPC5, and TRPC7 were not detected

(Cq ≥ 35) (Figure 1B–D). The expression levels of these channels was relatively constant throughout the four different menstrual phases, as no significant differences could be found. The expression of TRPM3 and TRPM6, however, did fluctuate significantly between the menstrual-follicular-late luteal phase and the menstrual-early luteal phase, respectively. When comparing these results with expression data of controls [7], no significant changes in the fold change were observed (Figure S1).

As stromal cells—the most abundant cell type in endometriotic lesions—have adhesive, migratory, and proliferative capacities, human endometrial stromal cells were isolated from both controls and endometriosis patients during the luteal phase. The TRP channel expression pattern was further investigated using RT-qPCR, although only the TRP channels that were present in the whole biopsies were further investigated. mRNA expression of TRPV1, TRPV2, TRPV4, TRPM4, TRPM7, TRPC1, TRPC3, TRPC4, and TRPC6 could be observed in hESC of endometriosis patients (Figure 2). The expression of TRPV6 and TRPM6 was around the detection limit (30 < Cq < 35), while TRPM2 and TRPM3 mRNA levels were below the detection limits (Cq ≥ 35). Furthermore, these levels were not significantly different from the TRP channel expression in hESC from the controls (Figure 2), which is illustrated by a Spearman correlation coefficient of 0.94 (Figure S2). In addition, these results are in line with the expression pattern of the TRP genes in whole endometrial biopsies of endometriosis patients during the luteal phase (Figure 1B–D). Overall, these results showed no difference in the level of TRP channel expression between control and endometriosis patients in endometrial biopsies and in primary cultures of stromal cells of the eutopic endometrium.

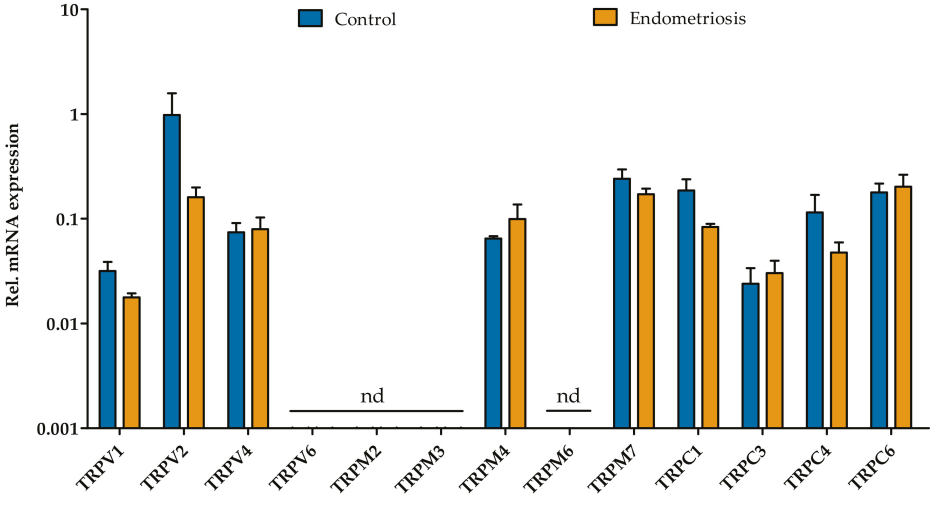

Figure 2. Quantitative RT-PCR of TRP channels in human endometrial stromal cells (hESC) derived from controls and endometriosis patients. Primary hESC were cultured from freshly isolated endometrium obtained during the luteal phase of controls (n = 3) and grade II endometriosis patients [37] (n = 4). mRNA levels were quantified to the geometric mean of housekeeping genes HPRT1 and PGK-1. TRPV6, TRPM2, TRPM3, and TRPM6 were around (30 < Cq < 35) or below the detection limit (Cq > 35). nd: not detectable. Data are presented as mean + SEM. Statistically significant changes in mRNA expression were assessed using the Two-way ANOVA statistical test with Bonferroni correction.

2.2. Functional Expression of TRP Channels in Endometriosis-Derived hESC

In order to assess the functionality of the TRP channels expressed in hESC of endometriosis patients, Ca^{2+} microfluorimetry was performed, using specific pharmacologic agents. The protocol consisted of the stimulation of hESC with a specific TRP channel agonist, followed by a wash-out

period. All primary cells that responded to the application of the positive control stimulus ionomycin (2 μM) at the end of the protocol were used for further analysis.

TRPV2 functionality was investigated by the application of 50 μM THC, which elicited a robust calcium influx in both endometriosis-derived and control hESC, of 314 ± 73 nM in 36 ± 12% of all cells (total of 171 cells) and 244 ± 42 nM in 56 ± 14% of all cells (n = 314 cells) (Figure 3A,E,F and Figure S3A), respectively. GSK (10 nM) was used as a selective TRPV4 agonist, which showed a rapid and reversible calcium influx of 496 ± 366 nM in endometriosis-derived hESC and 484 ± 277 nM in control hESC, in, respectively, 37 ± 18% (total of 305 cells) and 28 + 16% of the cells (total of 241 cells) (Figure 3B,E,F and Figure S3B). The functional expression of TRPC1/4 was tested using 250 nM EA in the abovementioned protocol. Again, the basal calcium increased via a rapid and reversible calcium influx of 228 ± 47 nM in endometriosis-derived hESC and 210 ± 60 nM in control hESC, upon application of EA in, respectively, 14 ± 17% (total of 354 cells) and 20 ± 13% of the cells (total of 102 cells) (Figure 3C,E,F and Figure S3C). Analogously, 100 μM OAG was used to assess TRPC6 functionality, what resulted in an influx in intracellular calcium of 480 ± 198 nM in 32 ± 25% of the endometriosis-derived hESC responders (total of 114 cells). In control hESC, an increase of 557 ± 171 nM was observed in 13 ± 6% of the cells (total of 107 cells) (Figure 3D–F and Figure S3D). Overall, no significant differences were observed between hESC derived from controls or endometriosis patients when the percentage of responders, response amplitude, or basal calcium concentration were compared (Figure 3E–G). In conclusion, these results displayed no significant differences in the functional expression of TRP channels in primary hESC between control and endometriosis groups.

2.3. Proliferative and Migratory Capacity of Endometriosis-Derived hESC

TRP channel activity can elicit physiological reactions in organisms and cells by an influx of extracellular Ca^{2+} or by inducing changes in membrane potential. Moreover, a few of the TRP channels expressed in hESC are involved in the Ca^{2+} and Mg^{2+} homeostasis of the cell and regulate processes like cell migration, adhesion, and proliferation [27–36]. Thus, in light of endometriosis, the proliferative and migratory capacity of hESC was investigated in different Ca^{2+} and Mg^{2+} concentrations. Different media were used in order to further elucidate the contribution of TRP channels in these processes: normal medium, 0 mM Ca^{2+}, 5 mM Ca^{2+}, and 10 mM Mg^{2+}. The mean time to reach 50% confluence was 262 ± 186 h, 269 ± 193 h, 209 ± 90 h, and 208 ± 96 h, respectively, for the control hESC; and 94 ± 5 h, 147 ± 63 h, 118 ± 54 h, and 100 ± 30 h for endometriosis-derived hESC (Figure 4A). No statistical differences were neither observed between the different cell types nor within the different conditioned media. A trend towards faster proliferation of the endometriosis-derived hESC could be observed in the normal culture medium, which should be noted (Two-way ANOVA column factor 0.03). Analogously, the migratory capacity was tested using the scratch-wound assay, resulting in 22 ± 11 h, 21 ± 6 h, 20 ± 11 h, and 24 ± 14 h for the control hESC, respectively; and 15 ± 2 h, 18 ± 3 h, 16 ± 2 h, and 15 ± 1 h for endometriosis-derived hESC to close 50% of the wound. Again, no statistical differences were observed neither between the different cell types nor within the different conditioned media. Overall, these results showed no differences in the proliferative and migratory capacity between control and endometriosis-derived eutopic stromal cells.

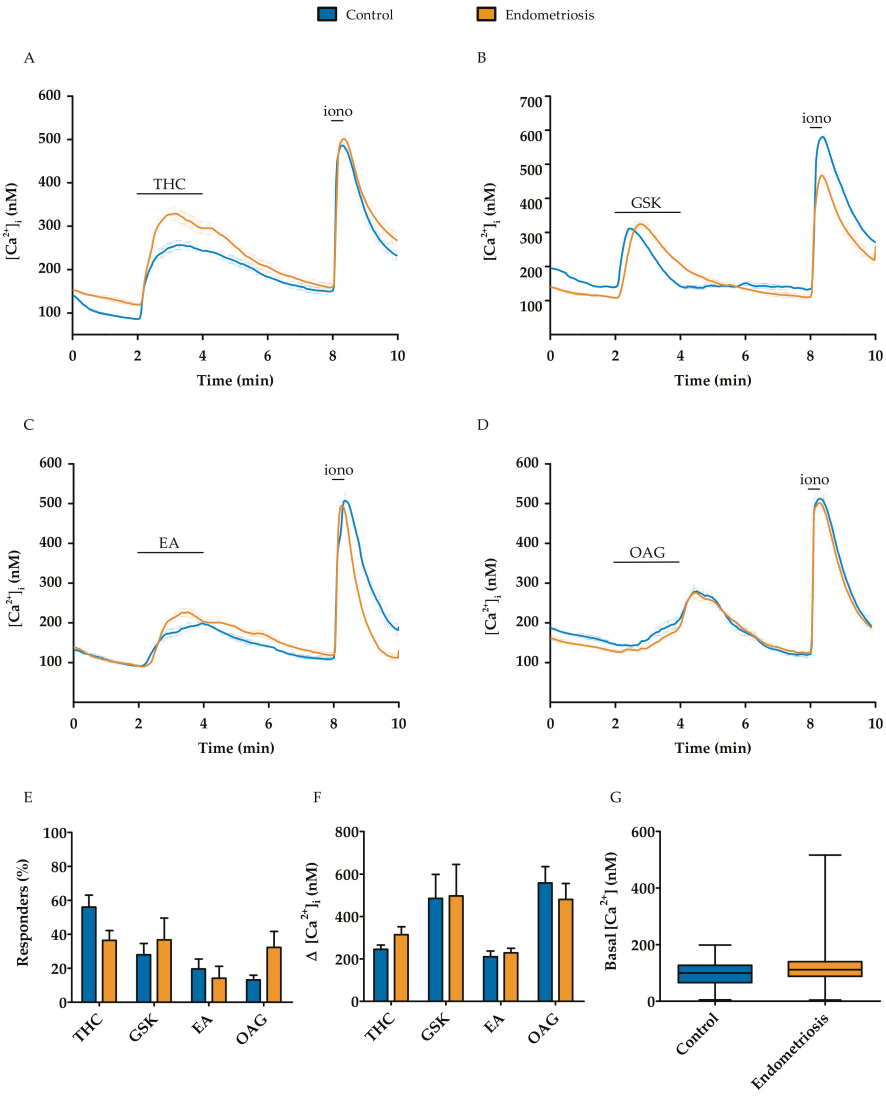

Figure 3. The functional expression of TRPV2, TRPV4, TRPC1/4, and TRPC6 using Ca^{2+} microfluorimetry in hESC of endometriosis patients compared to the controls. (**A**–**D**) Average ± SEM traces of 50 μM THC-, 10 nM GSK-, 250 nM EA-, and 100 μM OAG-induced Ca^{2+} changes ($[Ca^{2+}]_i$), respectively, in control and endometriosis hESC; (**E**) Number of responders in which an increase of at least 100 nM of the intracellular calcium concentration was observed upon application of the agonist; (**F**) Increase in intracellular calcium upon application of TRP agonists; (**G**) Basal intracellular calcium concentration of responders. Data are presented as mean + SEM. Statistically significant changes in responders and calcium influx were assessed using the Two-way ANOVA statistical test with Bonferroni correction, basal calcium levels using a *t*-test.

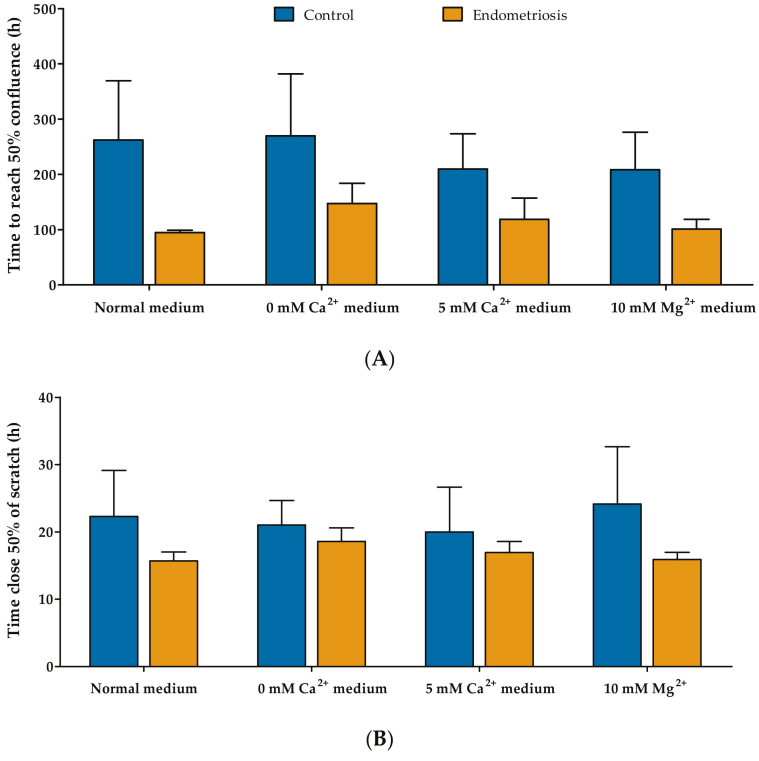

Figure 4. Proliferative and migratory assay of endometriosis-derived hESC. (**A**) The proliferation rate of hESC derived from eutopic endometrium of controls (n = 4) and endometriosis patients (n = 4) was assessed by calculating the time taken to reach 50% confluence in the presence of normal medium, 0 mM Ca^{2+}, 5 mM Ca^{2+}, and 10 mM Mg^{2+}; (**B**) The migratory capacity of hESC derived from endometriosis patients (n = 4) compared to control hESC (n = 4). Using the scratch-wound assay, the time needed for 50% closure was calculated. Data are presented as mean + SEM. Statistically significant changes in proliferative and migratory rate were assessed using the Two-way ANOVA statistical test with Bonferroni correction.

2.4. Eutopic vs. Ectopic

In order to elucidate whether the TRP expression pattern is different in ectopic lesions compared to their eutopic counterparts, RT-qPCR of all TRP channels was performed on paired tissue samples. The expression levels showed no significant difference between whole eutopic and ectopic tissue from endometriosis patients (Figure 5), which is also illustrated by a Spearman correlation coefficient of 0.95 (Figure S4). TRPV1, TRPV2, TRPV6, TRPM2, TRPM3, TRPM6, TRPM7, TRPC1, TRPC3, TRPC4, and TRPC6 were expressed well above the detection limit of the RT-qPCR analysis. Whereas TRPA1, TRPV3, and TRPV5 were observed to be expressed around the detection limit ($30 < Cq < 35$). TRPM1, TRPM5, TRPM8, and TRPC5 expression was not detected ($Cq \geq 35$), which is similar to the findings in the endometrial biopsies.

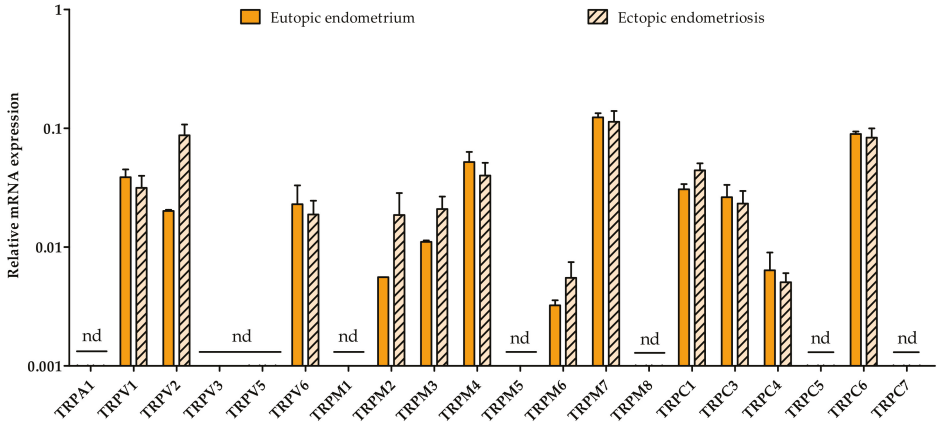

Figure 5. TRP channel expression in eutopic versus ectopic endometrial biopsies. RT-qPCR of TRP channels paired eutopic and ectopic tissue from grade II endometriosis patients during the follicular phase (n = 3) [37]. Messenger RNA levels were quantified to the geometric mean of housekeeping genes ACTB, GAPDH, HPRT, PGK-1, and TBP. TRPM1, TRPM5, TRPM8, TRPC5, and TRPC7 were below the detection limit. nd: not detectable. Data are presented as mean + SEM. Statistically significant changes in mRNA expression were assessed using the Two-way ANOVA statistical test with Bonferroni correction.

3. Discussion

Although endometriosis is a highly prevalent disease, the etiology remains an enigma for researchers and clinicians. Laparoscopic surgery during the menstrual phase has shown that blood can be observed within the abdominal cavity, which makes the case of Sampson's theory of retrograde menstruation a plausible etiological track. As this is a natural process, the endometrial debris that arrives in the abdominal cavity must obtain additional properties in order to thrive in this foreign location, i.e., have an improved ability to migrate, adhere, proliferate, and induce neuroangiogenesis. These processes, which require detailed signaling, use calcium as an indispensable second messenger [22,23]. In this context, members of the TRP superfamily have to be considered as possible contributors due to their role as cellular sensors [38], their functional expression in the endometrium [7], and their involvement in angiogenesis, proliferation, and migration [25,39,40]. In the present study, we assessed the expression pattern of TRP channels at the mRNA and functional levels, together with the proliferative and migratory capacity of the stromal cells in eutopic endometrium from endometriosis patients, in comparison with controls. Furthermore, the similarity of TRP channel expression was assessed between eutopic and ectopic tissue from endometriosis patients.

Using RT-qPCR, the expression profile of TRP channels was determined in whole endometrium biopsies throughout the menstrual cycle in tissue originating from endometriosis patients. TRPV1, TRPV2, TRPV4, TRPV6, TRPM4, TRPM6, TRPM7, TRPC1, TRPC3, TRPC4, and TRPC6 expression levels were observed well above the detection limit. For most TRP channels, fluctuations of the mRNA levels can be observed between the different menstrual phases. Especially for TRPM3 and TRPM6, significant differences between the menstrual-follicular-late luteal phase and the menstrual-early luteal phase, respectively, were measured. Unfortunately, the regulation of TRP channels by steroid sex hormones in the endometrium has only been studied for a limited amount of channels. For example, TRPV6 expression can be positively regulated by estrogen during the follicular phase, both in endometrial biopsies and Ishikawa cells [41]. TRPM2 mRNA expression has been investigated in human endometrium and hESC, revealing an increase in TRPM2 expression upon estrogen

treatment [42]. The co-application of estrogen and progesterone results in an increase of TRPC1 mRNA, whereas TRPC6 expression increases solely by application of estrogen on hESC [43].

In the context of heart and kidney development in mice, in vivo administration of the steroid-derived cortisol to pregnant mice resulted in an increase of TRPM6 and TRPM7 expression in both organs [44]. As glucocorticoid levels are increased in rats when estrogen levels are high [45], the regulation of TRPM6 and TRPM7 in the endometrium might be caused by these steroid hormones. Unfortunately, consensus has not yet been reached regarding the glucocorticoid levels during the menstrual cycle in humans [46]. Regulation of TRPM3 by steroid hormones has only been studied on a functional level, showing TRPM3 inhibition by high progesterone levels in vitro [47]. Moreover, the fluctuation in TRP channel expression levels might not fully be attributed to the menstrual cycle. The local production of estrogen by the stromal cells, giving the disease an estrogen-dependent character, can also influence the regulation of TRP expression. In addition, samples were obtained from different patients with diverse reasons of fertility, which could explain the inter-sample variability.

The overall expression pattern of TRP channels throughout the menstrual cycle of endometriosis patients was synonymous with the earlier findings wherein controls were investigated [7]. In addition, these results are in line with previous reports in which endometrial tissue of control and endometriosis patients was investigated for potential biomarkers [48]. This meta-analysis did not show evidence for TRP channels as a meaningful biomarker to diagnose endometriosis, indicating that there are no significant differences in the RNA expression pattern.

Due to the involvement of TRP channels in processes such as migration, adhesion, and proliferation, the interest arose whether they contributed to the intrinsic characteristics of stromal cells, and hence also to the establishment of endometriosis. To this end, hESC cells were isolated from grade II endometriosis patients [37], which were collected during the luteal phase, as this results in the highest yield. Again, TRP channel expression was determined using RT-qPCR, although only the channels for which expression was present in the endometrial biopsies were taken into account. TRPV1, TRPV2, TRPV4, TRPM4, TRPM7, TRPC1, TRPC3, TRPC4, and TRPC6 mRNA was observed to be present in hESC of both endometriosis patients and controls. No expression of TRPM2 and TRPM3 was observed in both groups, although the former has been described to be expressed in hESC [42]. The ablation of TRPM2 and TRPM3 expression, however, can be explained by the loss of nerve endings when setting up the primary culture of hESC. The absence of TRPV6 and TRPM6 expression confirms the stromal identity of the cells, as these channels are known to be expressed in the epithelial cells [41,49]. Again, no significant differences could be observed between TRP channel expression in hESC derived from controls versus endometriosis patients.

Investigating TRP channel activity can be challenging at times, due to the limited number of selective agonists and antagonists. Therefore, only the functionality of TRPV2, TRPV4, TRPC1/4, and TRPC6 was investigated using Ca^{2+} microfluorimetry in this present study. Moreover, De Clercq et al. has already performed an elaborate screening of TRP channel activity in control hESC [7]. Thus, the present study merely aims to corroborate these findings in hESC derived from endometriosis patients. The functionality of TRPV2 in endometriosis-derived hESC was investigated by using the cannabinoid THC, which revealed a robust increase in intracellular Ca^{2+} concentration. This influx of calcium is similar to that observed in control hESC, and was elicited in a similar percentage of responders. These findings can also be extended to the functionality measurements of TRPV4 and TRPC6. In this study, we showed, for the first time, the functional expression of TRPC1/4, since stimulation by the selective agonist Englerin-A induced robust influxes in the intracellular Ca^{2+} concentrations. However, the EA-induced Ca^{2+} influxes were similar in both the control and endometriosis groups. Additionally, no difference could be observed in the basal Ca^{2+} concentration of the hESC derived from endometriosis patients, indicating no increased basal activity of endogenously expressed Ca^{2+}-permeable TRP channels.

As proliferation and migration are important pathophysiological aspects of endometriosis, two assays were performed to elucidate whether TRP channels are involved in the establishment of the

disease. Literature already showed the importance of TRPM4 in the proliferation of HeLa and PC3 cells, as TRPM4 silencing results in dropping-off proliferation rates of these cell lines, while overexpression caused a reciprocal effect [31,50]. TRPM7 has recently been linked to breast cancer cell proliferation [35]. This ion channel is not only a regulator of the Mg^{2+} homeostasis [36], but intracellular Mg^{2+} has been shown to regulate its activity. TRPC6 was shown to have an impact on the migration, sprouting, and proliferation of endothelial cells [39], while activation of TRPV1 stimulates migration of HepG2 cells [28]. Moreover, TRPC1 has been proposed to be involved in the cell migration process as a mechanosensor [51].

Our results showed that in standard cell culture conditions, hESC derived from endometriosis patients have a trend for improved cell proliferation, compared to control hESC. Similar observations were already made by Wingfield et al., who described increased cell proliferation in the endometrium of endometriosis patients via immunostaining scores [52]. However, since the expression of TRP channels in stromal cells was similar between endometriosis and control samples, this tendency for increased proliferation rates cannot be explained by a difference in TRP channel expression. As differences in TRP channel expression could express itself in the modified ability of hESC to thrive at extreme conditions (i.e., low or high levels of extracellular Ca^{2+} and Mg^{2+}), hESC were subjected to these conditions, and their proliferation rate was measured subsequently. If the proliferation process of endometriosis-derived hESC is influenced by modulation of TRP channel expression, one would expect an altered proliferation rate when these cells are cultured in low Ca^{2+} medium compared to controls. Interestingly, no differences in proliferation rate were measured between control and endometriosis hESC, nor compared to the control situation. Similar reasoning processes could apply to extreme Ca^{2+} and Mg^{2+} concentrations in the extracellular medium—when TRP channel expression is altered, changes in proliferation rate could be expected when these cells are subjected to high calcium and magnesium levels. Overall, these findings might indicate that TRP channels do not play a vital role in the proliferation rate of hESC. Similar results were obtained when the migratory capacity was assessed. The scratch-wound assay indicated that endometriosis-derived hESC migrated at an equal rate, compared to control hESC. Furthermore, the depletion or supplementation of the media with Ca^{2+} and Mg^{2+} did not alter the migratory capacity of the cells. Since the expression pattern of TRP channels was similar between endometriosis and control samples, no differences in calcium and magnesium homeostasis were expected. Nevertheless, these results do not completely brush aside the involvement of TRP channels in proliferation and migration. The epithelial compartment and the endometrial stem/progenitor cells of the endometriotic lesions could still experience an increased proliferative and migratory capacity. For example, the epithelial TRPV6 has been studied extensively in the context of proliferation and cancer, showing increased metastasis and tissue invasion when prostate cancers are TRPV6-positive [53].

To address whether the ectopic environment can affect TRP channel expression in the endometrium, RT-qPCR was performed on paired eutopic biopsies and ectopic lesions from the follicular phase. Between these, no significant differences were found. Furthermore, these results are in line with our results on whole endometrial biopsies.

A first comment, however, is that the expression of TRP channels does not necessarily correlate with the functionality of the channel. The gating of the ion channel can be altered, even when no changes in expression are observed. Furthermore, channels could even lose their characteristic function and serve the cell in other ways, such as protein trapping. Therefore, a lack of alterations in TRP channel expression does not necessarily mean a lack of function for TRP channels, as some channels can employ a non-channel function [54]. Moreover, variations in passage numbers of hESC could also interfere with the observed expression of TRP channels [55]. Secondly, there is a lack of functional data of TRP channels expressed in the hESC of ectopic lesions. Unfortunately, isolation of primary cells from these lesions appears to be challenging and does not allow large yields, making functional measurements rather strenuous. Additionally, due to the scarcity of specific TRP pharmacology, not all expressed TRP channels in hESC could be examined on their functionality. Another remark is

that excised lesions are located in a perimeter of healthy tissue. Although the lesions were cut out of this perimeter before RT-qPCR experiments were performed, not all surrounding tissue can be eliminated. Thus, the lesion comprises a heterogeneous pool of cells, i.e., peritoneal, mesothelial cells from surrounding tissue, nerve endings, endothelial cells, and stromal and epithelial cells. Finally, these results indicate no direct evidence for TRP channels in the development of the disease—however, it is possible that they are of great importance for the subfertility and pain symptoms that endometriosis patients experience in later stages of the disease.

In conclusion, our findings indicated that the endometrium of endometriosis patients and its stromal cells showed an unaltered expression pattern of TRP channels compared to controls. In addition, the proliferative and migratory capacity between the two groups was not significantly different, even in conditions where extracellular Ca^{2+} and Mg^{2+} levels were increased or reduced. Moreover, no differences in expression levels were observed between paired eutopic biopsies and ectopic lesions. Overall, this study suggests a similar TRP channel expression profile in the endometrium of endometriosis patients compared to controls, and provide strong evidence that dysregulation of TRP channel expression in hESC is not a major cause in the development of endometriosis.

4. Materials and Methods

4.1. Patients

Samples were obtained from women of reproductive age undergoing diagnostic laparoscopic surgery for pain and/or infertility at the Leuven University Fertility Centre (LUFC), UZ Leuven, Belgium, and who had received no hormonal therapy in the last 30 days. Women who were diagnosed with an American Fertility Society score grade 0 were considered as controls, whereas those of grade II or more were considered as patients with endometriosis [37]. The use of endometrial and endometriotic tissue was approved by the Institutional Ethical and Review Board of the University Hospital of Gasthuisberg for the protection of human subjects (ML9100-S54776 date 04/10/2012). Written informed consent was obtained from all participating subjects.

4.2. Samples

Methods were based on established protocols from our research group [7].

4.2.1. Whole Endometrium Biopsies from Endometriosis Patients for TRP Expression Studies throughout the Menstrual Cycle

Endometrial biopsies were selected from the biobank of the LUFC. Detailed patient information is provided in Table S1, including the endometriosis grade, AFS score, BMI, age, endometriosis score, and reason for infertility. These samples were obtained using a sterile Novak-curette in the operating room after hysteroscopy and before laparoscopy. The stage of the menstrual cycle was determined from the patient's menstrual history and confirmed by endometrial histology, according to the Noyes criteria [56]. Endometriosis grade II endometrial samples were obtained from the menstrual (days 1–5, $n = 5$), follicular (days 6–14, $n = 6$), the early luteal (days 15–20, $n = 4$), and the late luteal phase (days 21–28, $n = 3$) (Table S1). All samples were collected between 2002 and 2014, snap frozen in liquid nitrogen, and stored in the LUFC biobank at −80 °C until used.

4.2.2. Primary Human Endometrial Stromal Cells

Cultures of primary human endometrial stromal cells (hESC) were started from fresh endometrial biopsies ($n = 16$), obtained from women during the luteal phase, of both endometriosis grade II and III patients and controls (Table S2). After rinsing the biopsy with phosphate-buffered saline (PBS), mucus and blood were removed with scalpels. The tissue was manually minced into pieces smaller than 1 mm^2 and incubated with 0.2% collagenase type IA (Sigma-Aldrich, Bornem, Belgium) in Dulbecco's

modified Eagle's Medium (DMEM) (Gibco, Invitrogen, Ghent, Belgium) for 60 min at 37 °C with constant shaking. The hESC and human endometrial epithelial cells were separated by differential size using gravity sedimentation, as described previously [57]. The hESC were cultured in DMEM/F-12 containing 10% fetal bovine serum (FBS, Gibco), 0.5 µg/mL amphotericin B (Gibco), and 100 µg/mL gentamicin (Gibco), and kept at 37 °C in a humidified, 5% CO_2, 95% air atmosphere [58]. Cells were routinely sub-cultured when they reached 80–90% confluence. The medium was changed every 2–3 days. To assess the expression profile of TRP channels in hESC, cells were frozen, stored at −80 °C and used RT-qPCR. Note that hESC from different patients and different passages (2–9) were used.

4.2.3. Whole Endometrium Biopsies and Endometriosis Lesions from Endometriosis Patients for Paired TRP Expression Study

Endometrial biopsies were obtained using a sterile Novak-curette in the operating room after hysteroscopy and before laparoscopy. The stage of the menstrual cycle was determined from the patient's menstrual history and confirmed by endometrial histology, according to the Noyes criteria [56]. Endometriosis lesions were taken during the laparoscopy using a CO_2-laser. Endometriosis grade II samples were obtained from the follicular phase (days 6–14, n = 3), which were classified as superficial lesions on the peritoneum (Table S3). Both endometrial biopsies and endometriotic lesions were left for 48 h on the RNALater (Qiagen, Venlo, The Netherlands) and stored at −80 °C until further RT-qPCR analysis.

4.3. RT–qPCR Experiments

Methods were based on established protocols from our research group [7].

4.3.1. Whole Endometrial Biopsies and Endometriotic Lesions

RT–qPCR experiments were performed on RNA isolated from frozen whole endometrial biopsies (n = 21) and endometriotic lesions (n = 3). The tissue was homogenized by use of a power homogenizer (Polytron, Montreal, QC, Canada) and total RNA was extracted with TriPure Isolation Reagent (Roche, Mannheim, Germany). RNA concentrations were assessed using the Nanodrop method (Isogen Life Science, Temse, Belgium) and RNA quality was assessed using an Experion RNA StdSens Analysing kit (Bio-Rad, Nazareth Eke, Belgium) (good quality RNA samples included an RNA integrity number 7 for all samples). 1 µg RNA was subsequently used for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies Europe B.V., Ghent, Belgium), and Triplicate cDNA (2.5 × diluted) samples from each independent preparation were used in the StepOne PCR system (Applied Biosystems, Life Technology, Carlsbad, CA, USA) using specific TaqMan gene expression assays for all TRP channels (Table S4). For each gene, 2 µL cDNA was added to 5 µL Mastermix (Life Technology), as well as 2.5 µL diethylpyrocarbonate-treated RNase-free water and 0.5 µL of the specific TaqMan gene primer, resulting in a final volume of 10 µL. ACTB, GAPDH, HPRT1, PGK-1, and TBP were used as endogenous controls. The protocol consisted of a holding stage at 95 °C for 20 min followed by a cycling stage of 40 replication cycles at 60 °C for 20 min (StepOne PCR system, Applied Biosystems, Life Technology). TRP channels with Cq values above 35 cycles were considered as non-detectable. Data were shown as $2^{(-\Delta Cq)}$ (mean + SEM) in which $\Delta Cq = Cq_{\text{TRP channel}} - Cq_{\text{geometric mean of endogenous controls}}$. Statistical tests were performed on the ΔCq values.

4.3.2. Primary Human Endometrial Stromal Cells

hESC were obtained as described above. RT–qPCR experiments were performed on RNA isolated from human endometrial stromal cells derived from endometriosis patients (n = 4) and controls (n = 3) (cell passage 1 or 2). Total RNA was extracted with the RNeasy mini kit (Qiagen, Venlo, The Netherlands). RNA concentrations were assessed using the Nanodrop method (Isogen Life Science, Temse, Belgium) and quality of the RNA was assessed using an Experion RNA StdSens Analysing kit

(Bio-Rad, Nazareth Eke, Belgium) (good quality RNA samples included an RNA integrity number 7 for all samples). 1 μg RNA was subsequently used for cDNA synthesis using the First-Strand cDNA Synthesis Kit (GE Healthcare, Chicago, IL, USA). Triplicate cDNA (2.5 × diluted) samples from each independent preparation were used in the StepOne PCR system (Applied Biosystems, Life Technology) using specific TaqMan gene expression assays for all TRP channels (Table S4). For each gene, 2 μL cDNA was added to 5 μL Mastermix (Life Technology), 2.5 μL diethylpyrocarbonate-treated RNase-free water, and 0.5 μL of the specific TaqMan gene primer, resulting in a final volume of 10 μL. The MS Excel application GeNorm 3.5 indicated PGK-1 and HPRT1 as the most stable endogenous controls for further analysis. The protocol consisted of a holding stage at 95 °C for 20 min, followed by a cycling stage of 40 replication cycles at 60 °C for 20 min (StepOne PCR system, Applied Biosystems, Life Technology). TRP channels with Cq values above 35 cycles were considered as non-detectable. Data were shown as $2^{(-\Delta Cq)}$ (mean + SEM) in which $\Delta Cq = Cq_{TRP\ channel} - Cq_{geometric\ mean\ of\ endogenous\ controls}$. Statistical tests were performed on the ΔCq values.

4.4. Functional Measurements

The protocol and imaging system for standard Ca^{2+}-measurements were performed as described by Vriens et al. [59]. As selective pharmacological agents for TRP channels are limited, not all TRP channels have been tested for their functional expression. The functionality of TRPV2, TRPV4, TRPC1/4, and TRPC6 was assessed by the application of 50 μM Δ9-tetrahydrocannabinol (THC) [60], 10 nM GSK016790A (GSK) [61], 250 nM Englerin A (EA) [62], and 100 μM 1-oleoyl-2-acetyl-glycerol (OAG) [63], respectively. 2 μM ionomycin (iono; Sigma) was applied at the end of every experiment as a positive control.

For intracellular Ca^{2+} measurements, hESC were incubated with 2 μM Fura-2 acetoxymethyl ester (Invitrogen, Eugene, OR, USA) for 30 min at 37 °C. Fluorescent signals were evoked during alternating illuminations at 340 and 380 nm using a Lambda XL illuminator (Sutter instruments, Novato, CA, USA), and recorded using an Orca Flash 4.0 camera (Hamamatsu Photonics Belgium, Mont-Saint-Guibert, Belgium) on a Nikon Eclipse Ti fluorescence microscope (Nikon Benelux, Brussels, Belgium). The imaging data were recorded and analyzed using the NIS-elements software (Nikon). Absolute calcium concentrations were calculated from the ratio of the fluorescence signals at both wavelengths (F340/F380) after correction for the individual background fluorescence signals, using the Grynkiewicz equation [64]. The standard solution contained 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose, and 10 mM HEPES (pH 7.4 with NaOH). Note that the cell passages ranged from 2 to 9.

For all measurements, cells were considered responders if the calcium influx during agonist application exceeded 100 nM and when the highest value of the derivative of the calcium trace during the application of an activator exceeded at least 3 times the standard deviation of the derivative during basal conditions. Calcium amplitudes were calculated as the difference between the maximum Ca^{2+} and basal Ca^{2+} of responding cells during the application of an activator. Only cells that responded to ionomycin at the end of the experiment were considered.

4.5. Proliferation Assay

hESC cell proliferation was evaluated by collecting real-time data of cell confluence using the IncuCyte® ZOOM Live-Cell Analysis System (Essen BiosScience, Ann Arbor, MI, USA). hESC were seeded using a normal growth medium containing 2.9 mM Ca^{2+} and 3.2 mM Mg^{2+} (as described above) into 24-well culture plates (Greiner Bio One, Belgium) at a cell density of 10,000 cells/well. Cells were left to attach for 30–45 min before the medium was replaced to obtain the control, calcium-free, 5 mM calcium, and 10 mM magnesium culture conditions (as described above). hESC were placed into the IncuCyte® ZOOM Live-Cell Analysis System and live images were taken every 2 h over a period of 5 days. Cell proliferation data was obtained via the cell confluence increment in each of the conditions,

after which the time taken to reach 50% confluence was calculated. Note that the cell passages ranged from 2 to 9.

4.6. Migration Assay

The migration potential of hESC derived from endometriosis patients was assessed via the scratch assay [65]. Isolated hESC were seeded into 12-well culture plates and grown to a monolayer in normal growth medium containing 2.9 mM Ca^{2+} and 3.2 mM Mg^{2+} (as described above). When cells reached 100% confluency, the medium was removed and the monolayer was scraped in the middle of the well to create a 'scratch' using a p200 pipet tip. Cells were gently washed with PBS to remove all debris and to smoothen the edges of the scratch, and medium was added onto the wells. Standard growth medium (as described above) was used as the control condition, and was supplemented with 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 4 mM Ca^{2+} or 9.7 mM magnesium, to obtain the calcium-free, 5 mM calcium, or 10 mM magnesium culture conditions, respectively. Markings were made onto the culture wells as reference points to obtain the same field during image acquisition. Images were taken at the time of scratching, and at least 3 different time points thereafter using a Nikon Eclipse Ts2 microscope. In between image acquisition, cells were kept at 37 °C in a humidified 5% CO_2 incubator. At the end of the experiment, images were analyzed using Image J, Fiji Software (National Institute of Health, USA) [66], and the time taken to reach 50% closure of the scratch wound was determined. Note that the cell passages ranged from 2 to 9.

4.7. Data Analysis and Display

For data display, the Origin 9.0 Software package (OriginLab, Northampton, MA, USA) and Graphpad Prism 5.01 (Graphpad software incorporated, La Jolla, CA, USA) were used. The latter was also implemented for statistical analysis.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/19/9/2467/s1.

Author Contributions: Study design: J.V.; Data collection: E.P., A.H., K.D.C., R.V.B., G.V., C.M., C.T.; Data analysis: E.P., A.H., K.D.C.; Data interpretation: E.P., A.H., K.D.C., J.V.; Drafting manuscript: E.P., A.H., K.D.C., J.V.; Revising manuscript content: E.P., A.H., K.D.C., J.V.; Approving final version of manuscript: E.P., A.H., K.D.C., R.V.B., G.V., D.F.O, D.P., A.V., C.M., T.V., C.T., J.V.

Funding: A.H., K.D.C., D.P., D.F.O and A.V.H. acknowledge the support from the Research Foundation–Flanders (FWO). We thank the Research Foundation-Flanders for funding the project for J.V. (FWO, G.0D1417N) and the Research Council of the KU Leuven (OT/13/113).

Acknowledgments: We thank all members of the Laboratory of Endometrium, Endometriosis and Reproductive Medicine (LEERM) and the members of the Laboratory of Ion Channel Research (LICR) Leuven for their experimental help and helpful discussion. We are very grateful to the health care personnel of the Leuven University Fertility Centre for the help in tissue acquisition.

Conflicts of Interest: The authors declare no conflict of interest.

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International Journal of
Molecular Sciences

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Lgr5 Does Not Vary Throughout the Menstrual Cycle in Endometriotic Human Eutopic Endometrium

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Received: 8 November 2018; Accepted: 15 December 2018; Published: 21 December 2018

Abstract: Endometriosis is characterized by the abnormal presence of endometrium outside of the uterus, resulting in pelvic pain and infertility. The leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) has been postulated to be a marker of stem cells in the endometrium. However, $LGR5^+$ cells have a macrophage-like phenotype in this tissue, so it is unclear what role $LGR5^+$ cells actually play in the endometrium. Macrophages serve an important function in the endometrium to maintain fertility, while $LGR5^+$ cells generally have a role in tumor progression and are involved in invasion in some cancers. We sought to determine whether $LGR5^+$ cells vary across the menstrual cycle in women with endometriosis and whether there are implications for LGR5 in the aggressiveness of endometriosis and reproductive outcomes. We performed immunofluorescence, flow cytometry, and primary culture in vitro experiments on eutopic and ectopic endometrium from healthy and endometriosis patients and observed that neither $LGR5^+$ cells nor LGR5 expression varied throughout the cycle. Interestingly, we observed that $LGR5^+$ cell percentage overexpressing CD163 (anti-inflammatory marker) was higher in healthy endometrium, suggesting that in endometriosis, endometrium presents a more pro-inflammatory phenotype that likely leads to poor obstetric outcomes. We also observed higher levels of $LGR5^+$ cells in ectopic lesions compared to eutopic endometrium and specifically in deep infiltrating endometriosis, indicating that LGR5 could be involved in progression and aggressiveness of the disease.

Keywords: LGR5; endometrium; endometriosis; menstrual cycle; macrophages

1. Introduction

Endometriosis is a chronic estrogen-dependent disease characterized by the presence of endometrial tissue outside the uterine cavity. Primary symptoms of the disease, which affects approximately 10% of reproductive age women, are acute pelvic pain and/or infertility/subfertility [1]. Endometrium, which is composed of stromal and epithelial compartments, contains many immune cells and is a very dynamic tissue that is tightly regulated by ovarian hormones.

The leucine-rich repeat containing G protein-coupled receptor 5 (LGR5) is a seven transmembrane receptor described as a stem cell marker in a variety of tissues, including the small intestine and hair follicles [2–4]. LGR5 has been identified in endometrium [5,6], but its role in endometrial function

is unclear. Recently, it was discovered that LGR5-positive cells (LGR5$^+$) from healthy endometrium have a hematopoietic origin [7]. Approximately half of the population of LGR5$^+$ cells found in the endometrium co-express CD45, a leukocyte marker, and CD163, a monocyte and macrophage specific marker, suggesting a myeloid nature to the cells. This expression pattern was also observed by our group in LGR5$^+$ cells from eutopic endometrium from women with endometriosis [8]. Interestingly, LGR5$^+$ cells seem to remain constant throughout the menstrual cycle in normal human eutopic endometrium [7,9], although it was recently described that LGR5 expression decreases during the secretory phase [6].

We previously described a special subset of LGR5$^+$ cells that express unique genes in the eutopic endometrium of women with deep infiltrating endometriosis (DIE) compared to other types of endometriosis. These genes are related to immune system responses, hematological system development, and infertility [8]. Thus, we believe that LGR5$^+$ cells could be implicated in aggressiveness and reproductive outcomes of the disease based on the function of these co-expressed genes.

LGR5$^+$ cells have a macrophage-like phenotype, and macrophages have been shown to increase in the secretory and menstrual phases of the menstrual cycle in normal eutopic endometrium [10]. However, this increase has not been observed in eutopic endometrium of women with endometriosis [11]. Macrophages have a role in embryo implantation failure and are involved in poor reproductive outcomes [12]. Moreover, this immune cell population is increased in the peritoneal fluid and endometriotic lesions of women with endometriosis [13]. However, variation of these types of cells throughout the menstrual cycle in women with endometriosis has not been investigated. We assessed whether LGR5$^+$ cells vary throughout the menstrual cycle, along with the percentage of LGR5$^+$ cells in ectopic versus eutopic endometrium, as these cells may have implications for reproductive outcomes in endometriosis.

2. Results

2.1. LGR5 Does Not Vary Throughout the Menstrual Cycle in Women with Endometriosis

To determine whether LGR5 varies throughout the menstrual cycle, we used three experimental approaches: immunofluorescence, in vitro assay, and flow cytometry. We used endometrial biopsies and ectopic lesions in order to answer this question. The characteristics of the patients are shown in Table 1.

Table 1. Patients used in the study.

Patient ID	Type of Patient	Type of Endometriosis	Eutopic Endometrium	Mached Ectopic Endometrium	Technique Used
1	Control	-	yes	-	FC
2	Control	-	yes	-	FC
3	Control	-	yes	-	FC
4	Control	-	yes	-	FC
5	Control	-	yes	-	FC
6	Control	-	yes	-	FC
7	Control	-	yes	-	FC
8	Control	-	yes	-	FC
9	Control	-	yes	-	FC/CC
10	Control	-	yes	-	FC/CC
11	Control	-	yes	-	FC/CC
12	Control	-	yes	-	FC/CC
13	Control	-	yes	-	IF
14	Control	-	yes	-	IF
15	Control	-	yes	-	IF
16	Control	-	yes	-	IF

Table 1. *Cont.*

Patient ID	Type of Patient	Type of Endometriosis	Eutopic Endometrium	Mached Ectopic Endometrium	Technique Used
17	Control	-	yes	-	IF
18	Control	-	yes	-	IF
19	Control	-	yes	-	IF
20	Control	-	yes	-	IF
21	Control	-	yes	-	IF
22	Control	-	yes	-	IF
23	Control	-	yes	-	IF
24	Control	-	yes	-	IF
25	Control	-	yes	-	IF
26	Control	-	yes	-	IF
27	Control	-	yes	-	IF
28	Control	-	yes	-	IF
29	Control	-	yes	-	IF
30	Control	-	yes	-	IF
31	Control	-	yes	-	IF
32	Control	-	yes	-	IF
33	Control	-	yes	-	IF
34	Control	-	yes	-	IF
35	Control	-	yes	-	IF
36	Control	-	yes	-	IF
37	Endometriosis	Ovarian	yes	-	FC
38	Endometriosis	Ovarian	yes	-	FC
39	Endometriosis	Ovarian	yes	-	FC
40	Endometriosis	Ovarian	yes	-	FC
41	Endometriosis	Ovarian	yes	yes	FC
42	Endometriosis	Ovarian	yes	yes	FC
43	Endometriosis	Ovarian	yes	yes	FC
44	Endometriosis	Ovarian	yes	yes	FC
45	Endometriosis	DIE	yes	yes	FC
46	Endometriosis	DIE	yes	yes	FC
47	Endometriosis	DIE	yes	yes	FC
48	Endometriosis	DIE	yes	yes	FC
49	Endometriosis	DIE	yes	yes	FC
50	Endometriosis	DIE	yes	yes	FC
51	Endometriosis	DIE	yes	-	FC
52	Endometriosis	DIE	yes	-	FC
53	Endometriosis	DIE	yes	-	FC
54	Endometriosis	DIE	yes	-	FC
55	Endometriosis	Pelvic	yes	yes	FC
56	Endometriosis	Pelvic	yes	-	FC
57	Endometriosis	Pelvic	yes	-	FC
58	Endometriosis	Adenomyosis	yes	-	FC
59	Endometriosis	Adenomyosis	yes	yes	FC
60	Endometriosis	Adenomyosis	yes	-	FC
61	Endometriosis	Adenomyosis	yes	yes	FC
62	Endometriosis	DIE	yes	-	CC
63	Endometriosis	DIE	yes	-	CC
64	Endometriosis	DIE	yes	-	CC
65	Endometriosis	ND	yes	-	IF
66	Endometriosis	ND	yes	-	IF
67	Endometriosis	ND	yes	-	IF
68	Endometriosis	ND	yes	-	IF
69	Endometriosis	ND	yes	-	IF
70	Endometriosis	ND	yes	-	IF
71	Endometriosis	ND	yes	-	IF
72	Endometriosis	ND	yes	-	IF
73	Endometriosis	ND	yes	-	IF
74	Endometriosis	ND	yes	-	IF

Table 1. *Cont.*

Patient ID	Type of Patient	Type of Endometriosis	Eutopic Endometrium	Maches Ectopic Endometrium	Technique Used
75	Endometriosis	ND	yes	-	IF
76	Endometriosis	ND	yes	-	IF
77	Endometriosis	ND	yes	-	IF
78	Endometriosis	ND	yes	-	IF
79	Endometriosis	ND	yes	-	IF
80	Endometriosis	ND	yes	-	IF
81	Endometriosis	ND	yes	-	IF
82	Endometriosis	ND	yes	-	IF
83	Endometriosis	ND	yes	-	IF
84	Endometriosis	ND	yes	-	IF
85	Endometriosis	ND	yes	-	IF
86	Endometriosis	ND	yes	-	IF
87	Endometriosis	ND	yes	-	IF
88	Endometriosis	ND	yes	-	IF

FC: flow cytometry; CC: cell culture; IF: immunofluorescence; ND: non-determined.

2.1.1. Immunofluorescence Analysis

Calculations of fluorescence intensity mean (FIM) for LGR5 throughout the menstrual cycle showed no significant differences between epithelial and stromal compartments in control endometrium and no significant differences in the case of endometriosis (Figure 1A). Additionally, there were no significant differences between phases of the menstrual cycle in either group (Figure 1B). However, when comparing LGR5 expression in the eutopic endometrium from healthy patients and endometriosis patients, we observed a statistically significant increase in LGR5 expression in proliferative, secretory, and menstrual phases in healthy women (Figure 1C). An example of the immunofluorescence in eutopic endometrium is shown in Figure 1D.

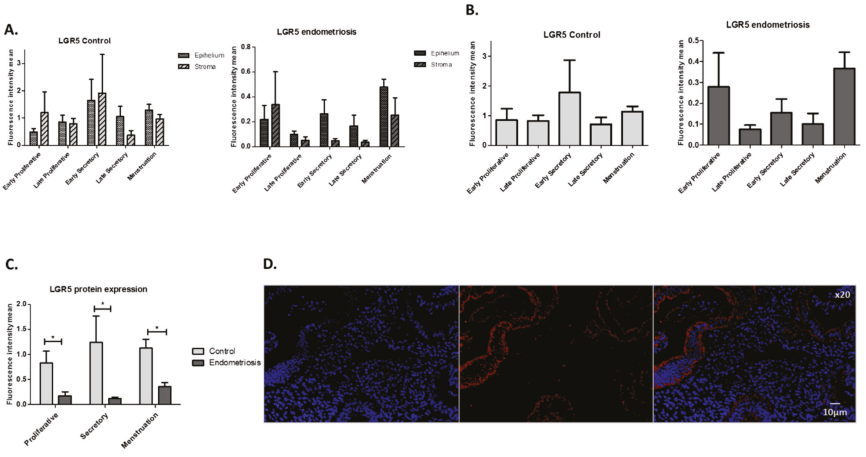

Figure 1. Immunofluorescence measurement of LGR5 expression throughout the menstrual cycle in eutopic endometrium. (**A**) Epithelial and stromal expression of LGR5 in control and endometriosis tissue in five phases of the menstrual cycle. (**B**) Total tissue expression across five phases of the menstrual cycle in control and endometriosis tissues. (**C**) Differences in LGR5 expression throughout the menstrual cycle between control and endometriosis tissue groups (*t*-test for each phase; proliferative: $p = 0.0242$; secretory: $p = 0.0424$; menstruation: $p = 0.0121$). (Control: $n = 24$; endometriosis: $n = 24$). (**D**) Example of immunofluorescence of LGR5 in eutopic endometrium (early secretory phase). In blue: DAPI; in red: LGR5. * $p < 0.05$.

2.1.2. In vitro Studies

In vitro experiments using endometrial stromal fibroblasts (eSF) cells treated with estradiol (E_2) for six days, and with E_2 and progesterone (P_4) for six more days, showed that primary culture cells mimicked proliferative and secretory phases of the menstrual cycle. Although non-significant, a tendency in increase in expression of CYR61, which increases during the proliferative phase, was observed and a tendency in increase of DKK1, which increases during the secretory phase, was also observed at days 6 and 12, respectively, in both groups when compared to the untreated control (Figure 2A). No differences in cell morphology were observed. In addition, no significant variation in LGR5 throughout the menstrual cycle was observed in control or endometriosis samples. Interestingly, a non-significant decrease in LGR5 was observed in the proliferative phase of the treatment group in control samples, while we observed a non-significant increase in the secretory phase of endometriosis samples (Figure 2B).

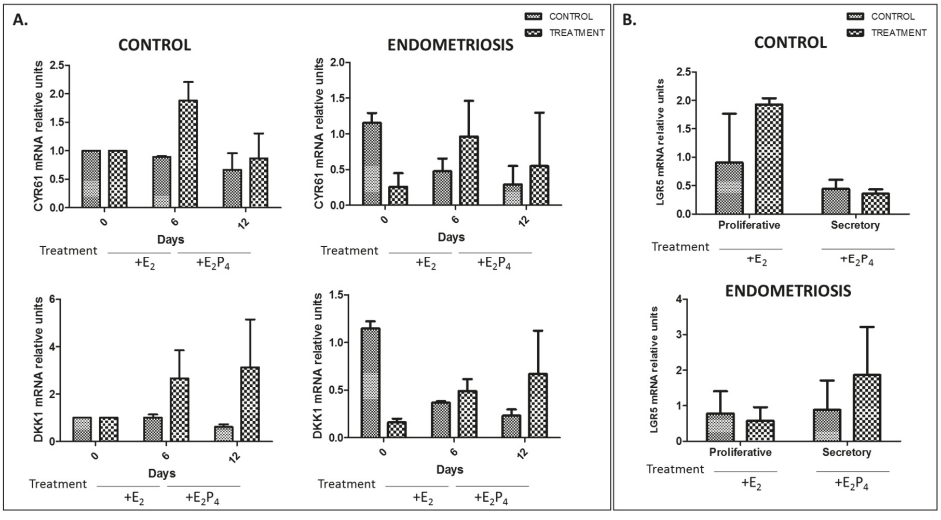

Figure 2. In vitro analysis of LGR5 expression throughout the menstrual cycle in endometrial stromal fibroblast primary culture after treatment (E_2 and E_2P_4 for six and six more days, respectively). (**A**) Left panels show expression of CYR61 (marker of the proliferative phase) and DKK1 (marker of the secretory phase) in the control group (n = 4); right panels show expression of CYR61 and DKK1 in the endometriosis group (n = 3). (**B**) Upper panel shows expression of LGR5 in proliferative (E_2) and secretory (E_2P_4) phases of the control group; lower panel shows LGR5 expression in both phases of the endometriosis group (E_2: estradiol; P_4: progesterone).

2.1.3. Flow Cytometry Analysis

In order to determine the variation throughout the cycle, we next performed flow cytometry analysis to determine the percentage of $LGR5^+$ cells in eutopic and ectopic endometrium. We measured a mean of 5.9% $LGR5^+$ cells (range: 2–7%) from each sample. In the preliminary study, no variation in percentage of $LGR5^+$ cells was observed throughout the menstrual cycle in nine eutopic endometriosis samples. Interestingly, no variation in $LGR5^+$ cells across the menstrual cycle was observed (Figure 3A). Moreover, no significant differences were found between $LGR5^+$ cells in eutopic endometrium of women with endometriosis (differentiated by different types of disease) (Figure 3B) and control women (Figure 3C). However, comparison between ectopic lesions and eutopic endometrium in patients with endometriosis (ovarian and DIE) revealed a significant increase of $LGR5^+$ cells in ectopic lesions (Figure 3D). These comparisons were only performed for ovarian endometriosis and DIE due to the

lack of ectopic samples from pelvic endometriosis ($n = 1$) and adenomyosis ($n = 2$). A *t*-test showed that DIE lesions contained a significantly higher percentage of LGR5$^+$ cells than ovarian lesions (Figure 3E).

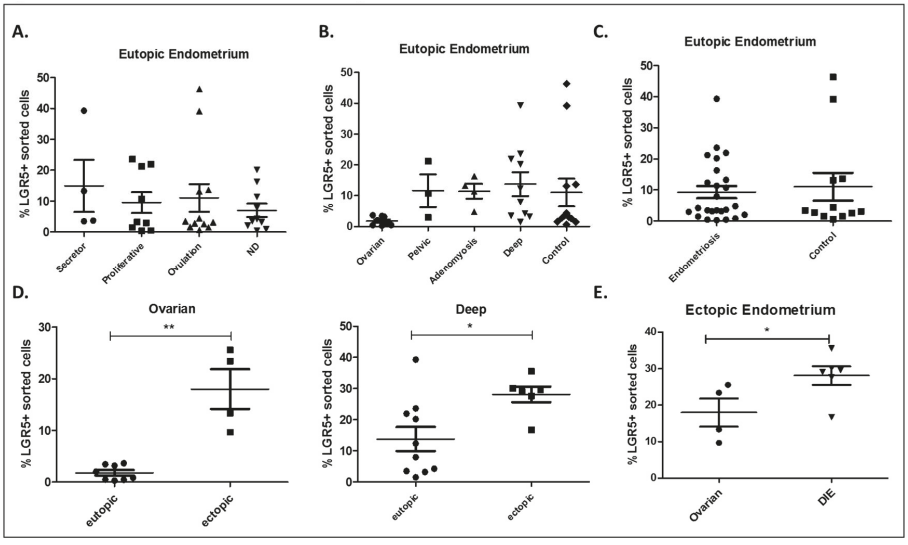

Figure 3. Percentage of FACS-sorted LGR5$^+$ cells. (**A**) Percentages of LGR5$^+$ cells in different phases of the cycle (proliferative: $n = 9$; secretory: $n = 4$; ovulatory: $n = 12$; ND: $n = 9$). (**B**) Percentage of LGR5$^+$ cells in eutopic endometrium of different types of endometriosis and controls (ovarian: $n = 8$; pelvic: $n = 3$; adenomyosis: $n = 4$; DIE: $n = 10$; control: $n = 12$). (**C**) Percentage of LGR5$^+$ cells identified in eutopic endometrium of women with and without endometriosis (control: $n = 12$; endometriosis: $n = 25$). (**D**) Differences in LGR5$^+$ cells between eutopic and ectopic endometrium. Left panel: ovarian endometriosis (eutopic: $n = 8$; ectopic: $n = 4$; $p = 0.0286$). Right panel: DIE (eutopic: $n = 10$; ectopic: $n = 6$; $p = 0.0411$). (**E**) Difference between ovarian and DIE ectopic endometrium (ovarian: $n = 4$; DIE: $n = 6$; $p = 0.0381$). ND: non-determined; DIE: deep infiltrating endometriosis. * $p < 0.05$, ** $p < 0.03$.

3. Discussion

The endometrium is a highly dynamic tissue that changes throughout the menstrual cycle. LGR5 is an interesting cellular marker in endometrial cells that has been shown not to vary throughout the cycle at RNA and protein levels in healthy endometrium [5,9]. However, a recent study reported that LGR5 is regulated by progesterone and showed that it does decrease in the secretory phase of the menstrual cycle and that there are progesterone binding sites in the promoter of LGR5 [6]. Interestingly, stromal fibroblasts show progesterone resistance in women with endometriosis [14]. Despite these controversial findings, the behavior of LGR5$^+$ cells in eutopic and ectopic endometrium throughout the menstrual cycle had yet to be described in women with endometriosis.

Only four studies on LGR5 have been performed in healthy human eutopic endometrium and, to our knowledge, this is only the second work to explore LGR5 in the eutopic endometrium of women with endometriosis—the first study was published by our group [8]. Our previous work demonstrates an abnormal epithelial phenotype in the stromal compartment of eutopic endometrium of women with endometriosis [8], similar to what has been observed in mice with induced endometriosis [15]. LGR5 is aberrantly co-expressed with cytokeratin (CK) or E-cadherin (ECAD) in endometriotic eutopic endometrium, but this co-localization is not found in healthy women, suggesting that eutopic LCR5$^+$ cells could have different behavior in endometriosis compared to healthy tissue [8]. Interestingly, we observed this process in both follicular and secretory phases of the menstrual cycle.

Cervelló et al. showed that LGR5$^+$ cells in healthy eutopic endometrium present a monocyte-macrophage-like phenotype and, surprisingly, do not express any typical stem cell markers, indicating that these cells do not act as traditional stem cells in this tissue [7]. However, these cells do seem to be involved in stem cell niche modulation [7]. These results, together with those from our group, are comparable to our findings in patients with endometriosis, in which we also observed a predominant myeloid phenotype of these cells [8], indicating they are likely monocytes and macrophages.

In the endometrium, macrophages make up approximately 10% of the total immune cell population [10,16–18], making them the second most abundant endometrial leukocyte population after T cells [12]. Macrophages comprise 1–2% of endometrial cells in the proliferative phase, 3–5% in the secretory phase, and 6–15% in the menstrual phase [10]. Depending on the activation state and surface markers, they are classified as either classically activated macrophages (Mϕ1) or alternatively activated macrophages (Mϕ2) [19]. Mϕ1 secrete pro-inflammatory factors, whereas Mϕ2 are involved in angiogenesis, anti-inflammatory processes, and coordination of tissue repair [19,20]. This plasticity in phenotype is due to environmental cues [21]. In normal endometrium, the majority of macrophages are CD163$^+$CD14Low, which correspond to Mϕ2 [20,22]. Interestingly, LGR5$^+$ cells overexpress CD163 in normal endometrium [7], suggesting an anti-inflammatory phenotype in this tissue.

In accordance with other groups [5], we did not observe significant differences in LGR5$^+$ cell percentages between epithelial and stromal compartments as measured by flow cytometry and gene expression after RNA-high-sequencing analysis [8]. In the present work, we also assessed differences between percentages of LGR5$^+$ cells in eutopic endometrium in healthy women and women with endometriosis in both menstrual phases. We did not observe significant differences in LGR5$^+$ cells throughout the cycle in control or endometriosis groups, as similarly observed by other authors in normal, healthy endometrium [5]. However, we did observe a significant increase in LGR5 in healthy endometrium as measured by immunofluorescence in all phases of the menstrual cycle compared to women with endometriosis. This suggests that healthy eutopic endometrium has a higher anti-inflammatory phenotype than endometriotic endometrium, where the percentage of LGR5$^+$ cells is lower. We hypothesize that a lower percentage of LGR5$^+$ cells would lead to a pro-inflammatory phenotype throughout the menstrual cycle in eutopic endometrium of diseased women, which may have a negative impact on reproductive outcomes.

We did not find differences in LGR5$^+$ cells between healthy and endometriotic endometrium by flow cytometry, although immunofluorescence did show a significant increase in LGR5 in healthy endometria in all phases of the menstrual cycle compared to endometriosis. This discrepancy may be due to: (1) different forms of sample processing (fresh tissue or FFPE endometrium); (2) different sample sizes, since in the flow cytometry study the control group represented only one third of total endometriosis samples; (3) use of two different antibodies for each technique; and/or (4) the FACS control group being comprised of egg donors. Egg donor women were stimulated with follicle stimulating hormone (FSH), which could produce differences in LGR5 expression. Immunofluorescence is likely more reliable in this case because there is no bias of sample processing and control group members were healthy women without stimulated endometrium. Moreover, the sample size is slightly larger in the immunofluorescence analysis.

To avoid possible effects from FSH in the stimulated endometrium, when we mimicked the menstrual cycle in vitro, we grew cells in DMEM without phenol red and we treated cells with charcoal-stripped serum, which inhibits androgen receptor expression. CYR61 and DKK1 expression was measured to find out if the treatment was having any effect in terms of gene expression in endometrial stromal fibroblasts. In a previous in silico study performed by our group [8], we determined that these two markers were varying across the menstrual cycle in healthy human eutopic endometrium. CYR61 was overexpressed in proliferative phase and DKK1 in secretory phase. Although it is not significant, after the treatment with E_2 (mimicking proliferative phase) and E_2P_4 (mimicking secretory phase) we observed an increase of CYR61 at day 6 and an increase of DKK1 at day 12 in both groups when compared to the control stromal fibroblasts, indicating that the treatment

of E_2 and P_4 was having the desired effect on the primary cells. Interestingly, we observed a slight decrease (not statistically significant) in LGR5 expression in the secretory phase when E_2P_4 was added to the medium in the control patients in in vitro experiments (Figure 2B), in accordance with Tempest et al. [6], that states that LGR5 could be regulated by progesterone. In women with endometriosis, we also observed no significant variation throughout the menstrual cycle in the in vitro experiments, although a trend for increased marker in the secretory phase suggests that LGR5 could be regulated by progesterone, because it has progesterone binding sites in its promoter [6] and there is resistance to progesterone in women with endometriosis [23].

Although we are aware that the number of ectopic lesions obtained is low, we found our results interesting because the average percentage of $LGR5^+$ cells was increased in endometriotic lesions compared to their matched eutopic endometrium. Additionally, DIE ectopic lesions displayed a higher average of $LGR5^+$ cells than ovarian endometriosis. Our previous findings show that $LGR5^+$ cells from eutopic endometrium of women with DIE present a special subset of $LGR5^+$ cells [8] that could not only play a role in endometriosis in the eutopic endometrium, but also in the development of ectopic lesions and their aggressiveness.

Other works have supported this idea since $LGR5^+$ seems to be related to progression of different cancers, such as colon [24], papillary thyroid [25], breast [26], and ovarian cancers [27] by promoting epithelial ovarian cancer proliferation, metastasis, and epithelial–mesenchymal transition (EMT). Furthermore, LGR5 is overexpressed in ovarian cancer tissue compared to normal tissue [28]. Moreover, it is well known that patients with endometriosis have a higher risk of developing ovarian cancer [29]. These findings, together with our results, suggest that LGR5 could also be involved in the pathophysiology of endometriosis and its eventual progression to ovarian cancer. However, the role of LGR5 in ectopic lesions and its relation to the promotion of endometriosis and/or ovarian cancer is less understood. LGR5 should be further considered as a marker of ovarian endometriosis lesions, and its role in the development of ovarian cancer should be further studied.

In conclusion, this work shows that LGR5 does not vary across the menstrual cycle in healthy and endometriotic eutopic endometria at either RNA or protein levels using three different approaches: immunofluorescence, RT-qPCR, and flow cytometry. It seems that healthy eutopic endometria have more $LGR5^+$ cells, suggesting that endometriotic eutopic endometria could have a pro-inflammatory phenotype that would lead to poorer obstetrical outcomes. Our results open a new window to study $LGR5^+$ cells in ectopic lesions and discover their role in the pathophysiology and aggressiveness of endometriosis.

4. Materials and Methods

4.1. Sample Collection

A total of 101 samples were obtained from 24 eutopic endometrium embedded in paraffin (formalin-fixed paraffin tissue, FFPE) from women with endometriosis and 24 from healthy women that were provided by the Department of Anatomical Pathology of the University Hospital Vall d'Hebron, Barcelona. From those samples, we obtained five slides from phases of the menstrual cycle (early proliferative, late proliferative, early secretory, late secretory) and four slides from menstrual phases to perform immunofluorescence assays. We also obtained 12 fresh eutopic endometrium from healthy egg donors, provided by IVIRMA-Barcelona (Barcelona, Spain), for flow cytometry assays. Four of these samples were also used for in vitro assays. We also collected 28 eutopic and 13 ectopic endometrium from women with endometriosis who underwent laparoscopy from the University Hospital of Vall d'Hebron, Barcelona. Ectopic tissue samples were categorized as follows: four samples were ovarian endometriosis, six were DIE, two were adenomyosis, and one was pelvic endometriosis. These samples were mostly used for flow cytometry assays, except for three samples that were used for in vitro assays. Different types of endometriosis were collected, and all patients and their designations are listed in Table 1. All patients signed a consent form, and use of the uterine specimens

after surgery was approved by the Ethics Committee of Vall d'Hebron Research Institute, Barcelona, Spain (PR(AMI)410/2016) and by the ethics committee of IVI Barcelona S.L. (1611-BCN-080-XS) on 7 July 2017.

4.2. Immunofluorescence

Three cuts of 3 μm each were made from all paraffin blocks. A xylene/ethanol circuit and 5 min in H_2O was used to deparaffinize and rehydrate tissues. Slides were then blocked with 5% bovine serum albumin (BSA) and 5% normal goat serum (NGS) in 1× phosphate buffered saline (PBS) for 30 min. We stained tissue with anti-LGR5 antibodies at 1:30 dilution (anti-LGR5/Gpr49 (loop 2) rabbit, Abgent, San Diego, CA, USA) overnight (ON) at 4 °C. After three washes with 1× PBS, anti-rabbit Alexa647 (Invitrogen, Carlsbad, CA, USA) secondary antibody was added at 1:500 dilution and incubated for 45 min at room temperature. All antibody dilutions were made using 3% BSA solution with 3% NGS in 1× PBS. We used ProLong Gold antifade reagent with 6-diamino-2-phenylindole (DAPI; Invitrogen) to visualize nuclear DNA and mount the slides. As negative controls, we stained the tissue only with secondary antibody and used unstained tissue. Two independent observers using an OlympusBX61 (Tokyo, Japan) microscope evaluated the slides. We took photos from five different fields for each sample. Next, images were analyzed with ImageJ software K1.45 (GPL). We took photos for each sample and split color channels in red for LGR5 and blue for DAPI. We selected epithelial and stromal regions and calculated intensity (fluorescence intensity mean (FIM)) levels of LGR5 in relation to DAPI to compare epithelial versus stromal LGR5 expression. Data were analyzed with GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA) using two-way ANOVA and Bonferroni tests. A p-value of <0.05 was considered statistically significant. Different red signal intensity in relation to blue was used to determine the expression level for each cell type.

4.3. Tissue Digestion and Primary Culture

Endometrial tissue (four samples from controls and three from DIE) was minced mechanically and digested with DMEM high glucose and collagenase (1 mg/mL). After digestion at 4 °C ON, samples were separated by gravity sedimentation, and single cells were filtered in a 40-μm mesh and washed with 1× PBS. Stromal cells were collected as they were filtered through the mesh. Epithelial cells remained in the mesh, so the mesh was washed upside down with 1× PBS to recover epithelial cells. Afterwards, epithelial cells were incubated with accutase to obtain singe cells. Pellets were washed with 1× PBS and resuspended with serum containing medium (SCM).

Using endometrial stromal fibroblast (eSF) primary cell cultures, an assay reproducing menstrual phases was performed to determine LGR5 expression throughout the menstrual cycle. After tissue digestion, samples were centrifuged for 5 min at 1200 rpm, and single stromal cells were resuspended with SCM and placed in p100 plates. The next day, media was changed, and cells were grown to confluence. Primary cultures were passaged no more than twice to preserve integrity of the eSF, and 2×10^5 cells were cultured in p6 well plates with SCM containing 2% FBS. Cells were treated with 10^{-8} M estrogen (E2) β-estradiol (Sigma, Saint Louis, MO, USA) for six days and with 10^{-8} M E2 and 10^{-6} M progesterone (P4) (Sigma) from days 6–12; a second group was treated only with ethanol as a vehicle, as both hormones were diluted in ethanol. Every two days, media was changed and hormones were added. Samples were obtained at days 0 (control), 6 (proliferative phase), and 12 (secretory phase). The experiment was performed in duplicate with 84 wells total. After treatment, cells were harvested using trypsin, and 106 cells were collected in 350 μL of lysis buffer with 1% β-mercaptoethanol and stored at −80 °C for subsequent RNA extraction.

4.4. RNA Extraction

RNA from 84 samples from the in vitro study described above was isolated using an RNeasy micro kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions, and concentration of RNA was determined using a Nanodrop® photometer (Thermo-Fisher Scientific, Waltham, MA, USA).

4.5. Real Time Quantitative PCR (RT-qPCR)

RT-qPCR of specific markers for the proliferative phase (CYR61) and secretory phase (DKK1) was performed. LGR5 expression was also studied to determine its variation throughout the menstrual cycle. Primer pairs are shown in Table 2. A total of 84 samples were reverse transcribed into complementary DNA (cDNA) using the SuperScript (Invitrogen) synthesis system, and RT-qPCR was performed using SYBR-green (Roche Life Sciences, Basel, Switzerland). Samples were analyzed in triplicate. GAPDH was used as a reference gene, and all data were normalized to its levels. Experimental data were compared to untreated cells as controls and were analyzed using one-way or two-way ANOVA followed by a Bonferroni comparison test, with $p \leq 0.05$ indicating statistical significance.

Table 2. Primers used for RT-qPCR.

Gene	Primer Name	Sequence 5′–3′	Tm
CYR61	hCYR61-For-25	CTCGCCTTAGTCGTCACCC	57.6
	hCYR61-Rev-226	CGCCGAAGTTGCATTCCAG	57.1
DKK1	hDKK1-For507	ATAGCACCTTGGATGGGTATTCC	56.6
	hDKK1-Rev-560	CTGATGACCGGAGACAAACAG	55.5
LGR5	hLGR5-For-71	CACCTCCTACCTAGACCTCAGT	57
	hLGR5-Rev-274	CGCAAGACGTAACTCCTCCAG	57.5
GAPDH	hGAPDH-For	CGT CTT CAC CAC CAT GGA GA	61.1
	hGAPDH-Rev	CGG CCA TCA CGC CAC AGT TT	56.7

Forward (For) and reverse (Rev) primers used for RT-qPCR.

4.6. Immunocytochemistry and Fluorescence Activated Cell Sorting (FACS)

Eutopic endometria from 12 healthy women and 25 women with endometriosis were stained and cells were sorted using a BD FACS-ARIA I instrument (BD Bioscience, San Jose, CA, USA). We also stained 13 ectopic endometria from women with endometriosis. To elucidate whether LGR5 varied across the menstrual cycle, we performed a trial with five samples in the proliferative phase and four samples in the secretory phase. After tissue digestion, samples were treated with erythrocyte lysis buffer and blocked with 5% BSA for 1 hour at room temperature. Samples stained with monoclonal rabbit anti-LGR5 primary antibody (1 µL per million cells was used; BioNova Scientific, Fremont, CA, USA) and goat Alexa647 anti-rabbit secondary antibody (Invitrogen) at a 1:500 dilution. To discard dead cells, samples were stained with DAPI (5 mg/mL). $LGR5^{+/-}$ cells were collected separately in TRIzol (Invitrogen) and stored at −80 °C. To confirm that $LGR5^{+}$ cells were specifically sorted, we performed a cytospin of 5000 cells on a slide and stained them with the same antibody and concentration used for immunofluorescence (anti-LGR5/Gpr49 (loop 2) rabbit, Abgent) (Figure S1). Percentage of $LGR5^{+}$ cells was analyzed by FCS Express5.0 software (De Novo Software, Glendale, CA, USA), and data analysis was performed using Prism software (GraphPad, San Diego, CA, USA). Two-way ANOVA and a Bonferroni post-test ($p \leq 0.05$ indicated statistical significance) was used to find significant differences between phases of the menstrual cycle and between different types of endometriosis. A *t*-test ($p \leq 0.05$) was used to determine differences between eutopic endometrium in control and endometriosis samples and between eutopic and matched ectopic endometrium in women with endometriosis.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/20/1/22/s1.

Author Contributions: Conceptualization, J.V.-J. and X.S.; Methodology, J.V.-J. and X.S.; Software, J.V.-J., C.B., X.S.; Validation, J.V.-J. and X.S.; Formal Analysis, J.V.-J., C.B., X.S.; Investigation, J.V.-J., C.B., J.C., X.S.; Resources, E.S.-S., J.C., A.B.; Data Curation, J.V.-J., A.G.-M., X.S.; Writing-Original Draft Preparation, J.V.-J. and X.S.; Writing-Review & Editing, J.V.-J., X.S.; Supervision, J.V.-J., X.S.; Project Administration, X.S.; Funding Acquisition, J.V.-J., A.B., A.G.-M. and X.S.

Funding: This research was funded by Centro de Desarrollo Tecnológico Industrial (CDTI) grant (IDI-2013039), Spain. The Industrial Doctorate AGAUR (Agency for Administration of University and Research Grants, Catalonia, Spain) also supported this work (DI2015_074).

Acknowledgments: We also aknoledge the collaboration in of Miquel Verdaguer and José Amich who have partially and temporaly contributed in the lab work.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

DIE	Deep infiltrating endometriosis
DMEM	Dulbecco's modified Eagle medium
ECAD	E-cadherin
FIM	Fluorescence intensity mean
ND	Non-determined
FSH	Follicular stimulating hormone
DAPI	4′,6-diamidino-2-phenylindole
CK	Cytokeratin
Mϕ	Macrophages
eSF	Endometrial stromal fibroblasts
SCM	Serum containing media
ON	Overnight
NGS	Normal goat serum
RT-qPCR	Real time-quantitative PCR
E_2	Estradiol
P_4	Progesterone
LGR5	Leucine-rich repeat containing G protein-coupled receptor 5
IF	Immunofluorescence
PBS	Phosphate buffered saline
FBS	Fetal bovine serum
BSA	Bovine serum albumin
FC	Flow cytometry

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Differentially-Expressed miRNAs in Ectopic Stromal Cells Contribute to Endometriosis Development: The Plausible Role of miR-139-5p and miR-375

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Received: 19 October 2018; Accepted: 24 November 2018; Published: 28 November 2018

Abstract: microRNA (miRNA) expression level alterations between endometrial tissue and endometriotic lesions indicate their involvement in endometriosis pathogenesis. However, as both endometrium and endometriotic lesions consist of different cell types in various proportions, it is not clear which cells contribute to variability in miRNA levels and the overall knowledge about cell-type specific miRNA expression in ectopic cells is scarce. Therefore, we utilized fluorescence-activated cell sorting to isolate endometrial stromal cells from paired endometrial and endometrioma biopsies and combined it with high-throughput sequencing to determine miRNA alterations in endometriotic stroma. The analysis revealed 149 abnormally expressed miRNAs in endometriotic lesions, including extensive upregulation of miR-139-5p and downregulation of miR-375 compared to eutopic cells. miRNA transfection experiments in the endometrial stromal cell line ST-T1b showed that the overexpression of miR-139-5p resulted in the downregulation of homeobox A9 (*HOXA9*) and *HOXA10* expression, whereas the endothelin 1 (*EDN1*) gene was regulated by miR-375. The results of this study provide further insights into the complex molecular mechanisms involved in endometriosis pathogenesis and demonstrate the necessity for cell-type-specific analysis of ectopic tissues to understand the interactions between different cell populations in disease onset and progression.

Keywords: endometriosis; ectopic stroma; microRNA; small RNA sequencing; *EDN1*; *HOXA10*; miR-139-5p; miR-375

1. Introduction

Endometriosis is a fibrotic condition defined by the presence and growth of endometrial-like tissue outside the uterine cavity [1]. Current endometriosis treatment strategies are rather general and mainly alleviate pain symptoms, but there are no specific approaches to cure the disease [2]. Therefore, the elucidation of aberrant molecular processes in endometriotic tissues is necessary to find new molecules enabling targeted endometriosis therapies. Various molecular aberrations

between endometriotic lesions and eutopic endometrium have already been detected, which can partly explain the disease pathogenesis (reviewed in [3,4]); nevertheless, the complete molecular etiology of endometriosis is still unclear.

microRNAs (miRNAs) are non-coding RNA molecules of ~22 nucleotides in length that have a regulatory function in gene expression. One miRNA can regulate more than 100 genes [5] and, in turn, a single gene can be regulated by multiple miRNAs [6]. miRNAs regulate the translation of target mRNAs negatively; however, through a combinatorial action of miRNAs and transcription factors (TFs), more complex regulatory networks are often involved in various biological events [7]. Accumulating evidence also indicates the involvement of miRNAs in the development and persistence of endometriosis [8–12]. Nevertheless, there is a lack of consistency among reported lists of aberrantly-expressed miRNAs, with the most likely underlying cause being the variation in the cellular composition of the studied tissue [13]. To overcome the issue of study material heterogeneity, Logan et al. investigated mRNA and miRNA expression in pure fractions of uncultured eutopic endometrial epithelial and stromal cells of endometriosis patients compared to control women and found that differentially-expressed miRNAs in stromal cells were distinct from miRNAs in epithelial cells [14]. Recently, we demonstrated that the investigation of uncultured eutopic and ectopic stromal cells of endometriosis patients unveils transcriptomic differences that may remain unnoticed in whole-tissue examination, stressing the importance of cell-type-specific analysis [15]. However, there are no high-throughput studies revealing miRNA expression patterns from distinct cell types in ectopic tissues.

In the current study, we applied small-RNA sequencing to uncultured paired eutopic and ectopic endometrial stromal cells to reveal disease-specific alterations. To understand the regulatory networks between genes and miRNAs, we implemented an integrated analysis of mRNA data from our previous study [15] and differentially-expressed miRNAs from this study.

2. Results

2.1. miRNA Profile of Eutopic and Ectopic Endometrium

miRNA expression profiles from uncultured endometrial stromal cells from paired samples of eutopic endometrium ($n = 4$) and endometriomas ($n = 4$) were determined by small RNA sequencing. In total, 719 miRNAs were detected in eutopic and 637 miRNAs in ectopic stroma (present in at least 50% of samples, Table S1). Most abundant miRNAs were highly similar in both groups (Table 1), where let-7a-5p was the most highly-expressed miRNA in stromal cells of eutopic and ectopic origin.

Table 1. Most abundantly expressed miRNAs in endometrial eutopic and ectopic stromal cells.

miRNAs in Eutopic Stroma	Average Raw Read Count	miRNAs in Ectopic Stroma	Average Raw Read Count
let-7a-5p	262,062	let-7a-5p	336,278
miR-148a-3p	251,377	miR-10b-5p	185,323
let-7f-5p	171,896	miR-21-5p	149,525
miR-10b-5p	119,737	let-7f-5p	97,019
miR-21-5p	116,936	miR-148a-3p	89,056
miR-26a-5p	102,057	miR-99a-5p	88,792
miR-143-3p	98,860	miR-26a-5p	88,348
let-7i-5p	89,804	miR-143-3p	67,660
miR-99a-5p	89,793	let-7b-5p	51,762
miR-199a-3p	76,412	miR-126-3p	49,937

High-throughput sequencing revealed 149 differentially-expressed miRNAs (recognized by at least 2/3 analysis methods, adjusted p-value < 0.05, $\log_2$ fold change ($|\log_2 FC| \geq 1$)), where 71 miRNAs were downregulated and 78 miRNAs upregulated in ectopic stromal cells (Table S2, Figure S1). Twenty-one miRNAs were recognized as differentially-expressed between eutopic and

ectopic endometria with all three methods used (Table 2). miR-139-5p was statistically most significantly upregulated [$\log_2$FC = 5.0, false discovery rate (FDR) = 1.4×10^{-24}] and miR-375 most significantly downregulated ($\log_2$FC = −4.9, FDR = 1.4×10^{-14} in ectopic stroma.

Table 2. Differentially-expressed miRNAs between ectopic and eutopic stromal cells identified by edgeR, DESeq2 and BaySeq programs.

miRNA ID	$\log_2$FC	FDR (edgeR)	padj (DESeq2)	FDR.DE (BaySeq)	Average CPM Eutopic Stroma	Average CPM Ectopic Stroma
Upregulated miRNAs in ectopic stroma						
hsa-miR-139-5p	5.0	1.4×10^{-24}	7.2×10^{-39}	1.5×10^{-2}	57	1292
hsa-miR-139-3p	6.1	4.9×10^{-24}	8.5×10^{-29}	1.6×10^{-2}	6	242
hsa-miR-202-5p	9.3	2.8×10^{-19}	5.8×10^{-11}	7.2×10^{-3}	0	51
hsa-miR-506-3p	5.8	1.4×10^{-17}	9.9×10^{-17}	1.8×10^{-2}	4	204
hsa-miR-150-5p	4.3	2.5×10^{-14}	7.7×10^{-17}	9.5×10^{-3}	14	203
hsa-miR-202-3p	9.1	3.1×10^{-14}	3.5×10^{-9}	3.9×10^{-2}	0	41
hsa-miR-150-3p	7.3	6.5×10^{-12}	5.2×10^{-6}	4.7×10^{-3}	0	15
hsa-miR-513c-5p	5.6	1.1×10^{-9}	2.2×10^{-6}	1.9×10^{-2}	1	19
hsa-miR-193a-5p	2.7	1.2×10^{-9}	3.5×10^{-14}	3.8×10^{-2}	44	194
hsa-miR-584-5p	3.1	9.1×10^{-7}	6.5×10^{-5}	3.4×10^{-2}	3	23
hsa-miR-371a-5p	4.5	1.1×10^{-6}	7.2×10^{-4}	2.9×10^{-2}	1	11
hsa-miR-216b-5p	4.3	7.5×10^{-5}	1.8×10^{-3}	4.5×10^{-2}	1	9
Downregulated miRNAs in ectopic stroma						
hsa-miR-375	−4.9	1.4×10^{-14}	3.7×10^{-11}	5.9×10^{-3}	162	3
hsa-miR-105-5p	−4.7	1.6×10^{-13}	4.4×10^{-9}	2.1×10^{-2}	104	3
hsa-miR-1298-5p	−5.8	2.5×10^{-9}	5.6×10^{-5}	1.3×10^{-2}	18	0
hsa-miR-6507-5p	−4.8	5.2×10^{-8}	3.6×10^{-4}	3.6×10^{-2}	27	1
hsa-miR-767-5p	−4.7	8.5×10^{-8}	5.5×10^{-4}	2.3×10^{-2}	25	1
hsa-miR-675-3p	−3.3	1.9×10^{-6}	7.7×10^{-4}	3.1×10^{-2}	29	2
hsa-miR-429	−4.4	1.9×10^{-6}	2.3×10^{-3}	4.1×10^{-2}	23	1
hsa-miR-141-3p	−3.8	3.7×10^{-5}	1.0×10^{-2}	8.4×10^{-3}	12	1
hsa-miR-873-5p	−3.5	3.9×10^{-4}	4.6×10^{-2}	4.3×10^{-2}	9	1

miRNAs in **bold** were chosen for validation by qRT-PCR. FC—fold change, CPM—count per million, FDR—false discovery rate, DE—differentially expressed.

Eight potential novel miRNAs were determined from small RNA sequencing data of eutopic and ectopic stromal cells (Table 3). One candidate miRNA (provisional ID: 3_18752) was detected only in eutopic stromal cells. All other sequences were detected from both eutopic and ectopic stroma; however, none of the novel miRNAs were differentially expressed. Three potential novel miRNAs showed similarities with other human miRNAs according to the miRBase database.

Table 3. Novel miRNAs detected from eutopic and ectopic stroma.

Provisional ID	Average Read Count in Eutopic Stroma	Average Read Count in Ectopic Stroma	Similarities with Other Human miRNAs	Consensus Mature Sequence	Precursor Coordinate, Forward (+) or Reverse (−) Strand
10_4598	1	2	-	gucauagacuagugcuuccga	10:106043903..106043987:−
12_4331	630	67	-	guucugggcuguagugagcuaugc	12:24706803..24706886:+
11_4914	9	11	-	aacugcucuucucuaauuuaa	11:101346555..101346607:−
19_4246	8	9	hsa-miR-25-3p	gugugugcaccugugucugucugu	19:18284682..18284741:+
3_22611	17	14	-	cucugggcugcagugcgcuaugc	3:49863521..49863597:−
3_18752	4	0	-	ugugguggcugcugcuggugc	3:53763045..53763105:+
6_24262	4	17	hsa-let-7b-5p	ugagguaguagguguguugc	6:158493843..158493925:−
8_30909	8	2	hsa-miR-9903	ccagccuacuggaggauaagagg	8:98393666..98393724:−

2.2. miRNA Validation by Quantitative Real-Time PCR (qRT-PCR)

Small RNA-sequencing data validation from six pairs of fluorescence-activated cell sorting (FACS)-isolated eutopic and ectopic stromal cells by qRT-PCR confirmed the upregulation of miR-139-5p (FC = 19, p = 0.03) and downregulation of miR-375 (FC = −42, p = 0.03) in ectopic stroma (Figure 1A). To verify whether the detected miRNA alterations could also be identified in cultured cells, miR-139-5p and miR-375 levels were determined in six paired cultured eutopic and ectopic stromal cells. A slight upregulation of miR-139-5p (FC = 3.2, p = 0.03) in cultured ectopic cells was determined, but no differential expression was detected for miR-375 (> 0.05, Figure 1B), indicating the effect of cell culturing on miRNA expression levels.

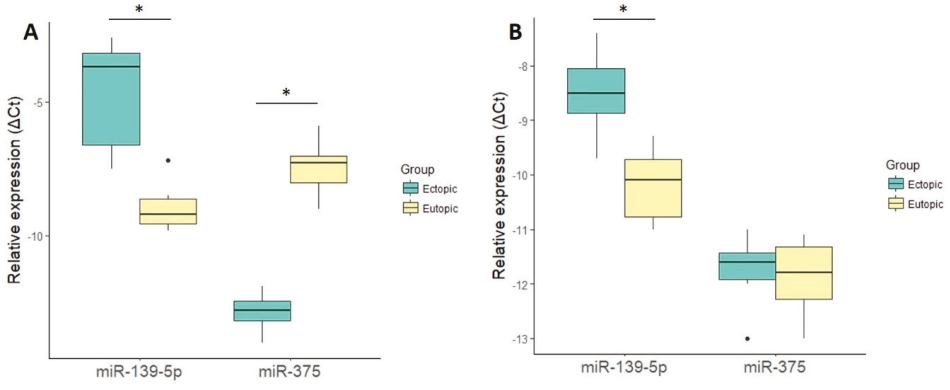

Figure 1. Relative miRNA expression levels (log2 scale) in (**A**) paired uncultured eutopic (n = 6) and ectopic (n = 6) stromal cells and (**B**) paired cultured eutopic (n = 6) and ectopic (n = 6) stromal cells. The ΔCt values were calculated as follows: miRNA Ct value − average Ct value of reference genes (RNU44 and RNU48). * p-value < 0.05. Outliers (defined as datapoints outside 1.5 times the interquartile range above the upper quartile and below the lower quartile) are pointed out with black dots. For illustrative purposes, relative expression levels (ΔCt) were multiplied by −1.

2.3. Integrated miRNA–mRNA Analysis for Target Identification

Target gene prediction was performed using MAGIA^2 (http://gencomp.bio.unipd.it/magia2), which identifies two types of regulatory circuits: (a) TFs that regulate both miRNAs and their targets; and (b) miRNAs that regulate both TFs and their targets. In the current study, MAGIA^2 identified 5914 significant links (q-value < 0.05) between TFs and their targets (miRNAs or mRNAs) and 1183 significant links between miRNAs and their targets (miRNA/mRNA or miRNA/TF, Table S3). The top 200 interactions are visualized in Figure S2. Among others, several interesting TFs including estrogen receptor 1 (*ESR1*), signal transducer and activator of transcription (STAT) genes (*STAT2*, *STAT3*, *STAT5A*, *STAT5B*), v-rel avian reticuloendotheliosis viral oncogene homolog A (*RELA*) and nuclear factor kappa B subunit 1 (*NFKB1*) were predicted to regulate miRNAs that were determined to be differentially-expressed in our study. In addition, various mRNAs such as mitogen-activated protein (MAP) kinases and insulin like growth factor 1 (*IGF1*) were determined as targets for differentially-expressed miRNAs (Table S3).

As no targets for miR-139-5p and miR-375 were predicted by the MAGIA^2 program, an additional target prediction analysis for these miRNAs was conducted using the DIANA microT (https://bio.tools/DIANA-microT), TargetScan (http://www.targetscan.org), miRanda (https://omictools.com/miranda-tool) and miRDB (http://www.mirdb.org) programs. Target genes predicted by at least two programs and that showed differentially-expressed levels (downregulated for miR-139-5p and upregulated for miR-375) in our previous study [15] were considered as potential targets for miR-139-5p

and miR-375. For miR-139-5p, 16 potential targets were found, and for miR-375, 19 potential targets were found (Table S4).

2.4. miRNA Target Validation

To examine the impact of miR-139-5p and miR-375 on target gene expression in stromal cells, the cell line ST-T1b was transfected with selected miRNA precursors or negative control precursors and the expression of seven and 10 potential target genes for miR-139-5p and miR-375, respectively, were selected for validation by qRT-PCR (Table S4). The overexpression of miR-139-5p resulted in 2.1-fold and 1.8-fold downregulation of *HOXA9* ($p = 0.0005$) and *HOXA10* ($p = 0.001$) expression, respectively. The overexpression of miR-375 resulted in a 1.9-fold downregulation of *EDN1* gene expression ($p = 0.01$). The expression of the other tested target genes (*CDH20*, *ESRRG*, *FBN2*, *LRFN5*, *GNAO1* for miR-139-5p and *ZFPM2*, *GATA6*, *FZD4*, *AHR*, *CD200*, *CTGF*, *DUSP6*, *IL1RAP*, *NCAM1* for miR-375) did not differ between the transfected cells (all $p > 0.05$).

3. Discussion

To the best of our knowledge, this is the first study utilizing an uncultured cell-type specific approach and high-throughput small-RNA sequencing for miRNA analysis of endometriotic lesions. We demonstrated distinct alterations in miRNA expression patterns between uncultured stromal cells from the endometrium and endometriomas and their potential involvement in miRNA-mediated pathological processes occurring in endometrial cells in ectopic locations.

According to our analysis, the statistically most significantly upregulated miRNA in ectopic stromal cells was miR-139-5p. The involvement of this miRNA in endometriosis had previously not been determined; however, decreased expression of miR-139-5p occurred in endometrial cancer tissues [16] where its levels are inversely correlated with *HOXA10* expression [17]. The suppressive impact of miR-139-5p on *HOXA10* gene expression was confirmed in our study, and in addition we observed a downregulation of another homeobox gene, *HOXA9*. A lower level of *HOXA10* expression in ovarian endometriomas and peritoneal endometriotic lesions in contrast to eutopic endometrium has been previously detected and it has been speculated that aberrant *HOXA10* expression might contribute to endometriosis pathogenesis through progesterone resistance [18] or by the induction of autophagy [19]. Both HOX genes are also highly expressed in the endometrium and play important roles in endometrial receptivity [20]. *HOXA10* expression is lower in the mid-secretory endometrium during the implantation window in endometriosis patients [21], but the levels are restored after surgical resection of endometriotic tissue [22]. Therefore, aberrant HOX gene levels likely contribute to the etiology of infertility in patients with endometriosis.

Furthermore, an integrated analysis of miRNA–mRNA expression data by MAGIA^2 predicted that miR-139-5p is regulated via estrogen receptor alpha (encoded by *ESR1* gene; Table S3). Although there are contradicting results regarding *ESR1* expression in ectopic endometrium, in particular ovarian endometriosis [23–25], it is widely accepted that the *ESR1* gene has a pivotal role in endometriosis pathogenesis. *HOXA10* expression is also regulated by estrogen receptor alpha [26], suggesting sophisticated molecular interactions between miRNAs, their targets and transcription factors.

The most significantly downregulated miRNA in our dataset was miR-375. In contrast to miR-139-5p, the downregulation of miR-375 was only observed in FACS-isolated cells, but got lost upon in vitro culture, emphasizing the importance of performing investigations in uncultured cells. The downregulation of miR-375 has been consistently reported in previous endometriosis studies involving whole eutopic and ectopic tissues [8–10,12]; however, the possible function of miR-375 in endometriosis has not been elucidated. We found that one of the miR-375 predicted targets was the *EDN1* gene, which is expressed more highly in stromal cells from ectopic origin compared to eutopic endometrium [15], and confirmed the potential regulatory link between the miR-375 and *EDN1* gene by transfection experiments. However, a comprehensive review has revealed that besides tissue-specific miRNA-mediated regulation, *EDN1* gene transcription may be modulated by DNA methylation and

histone modification patterns, as well as being influenced by different transcription factors responding to a wide variety of stimuli [27]. Therefore, the impact of miR-375 overexpression, detected in in vitro conditions, is probably less straightforward in in vivo situations. In order to determine whether the downregulation of miR-375 results in higher levels of *EDN1* in ectopic stroma, experiments with anti-miR-375 should be performed. However, as the baseline level of miR-375 in ST-T1b endometrial stromal cell line is low, it is unlikely that the further repression of miR-375 with antagomiR could show a considerable effect on *EDN1* gene levels.

Nevertheless, endothelin-1 (ET-1), which is encoded by the *EDN1* gene, has been associated with endometriosis pathogenesis, as the cystic fluid of endometriomas contains a higher amount of ET-1 compared to ovarian cysts other than endometriomas, and in vivo experiments in mice demonstrated that blocking ET-1 activity was effective in decreasing endometriosis-related pain [28]. Also, ET-1 supports the survival, angiogenesis and migration of mesenchymal stem cells [29], which are also proposed to be involved in endometriosis development [30,31]. Thus, the suppression of *EDN1* transcription by the overexpression of miR-375 could potentially be used as a therapy for endometriosis-related pain or as a strategy to prevent the dissemination of endometrial mesenchymal stem cells outside the uterus.

Besides miR-375, we detected several other dysregulated miRNAs that have been previously reported as being differentially-expressed in endometriosis studies investigating whole eutopic and ectopic tissues. Although the overlap between previously published miRNA studies has remained minimal, constant downregulation of miR-200-family members (miR-200a, miR-200b, miR-200c and miR-141), miR-196b-5p, miR-183-3p, miR-34c-5p, and upregulation of miR-202 in ectopic compared to eutopic tissue has been reported [13]. As our study confirmed the differential expression of these miRNAs in endometriotic stromal cells, we suggest that the aforementioned miRNAs most likely contribute to endometriosis pathogenesis and/or the persistence of the disease.

In conclusion, our cell-type-specific analysis revealed remarkable differences in miRNA expression patterns between stromal cells isolated from the endometrium and endometriomas. Based on our findings, we propose that two molecular mechanisms are involved in endometriosis pathogenesis, where, firstly, *HOXA9* and *HOXA10* genes are regulated by miR-139-5p among other factors and are potentially involved in endometriosis-associated infertility. Secondly, the aberrant expression of miR-375 in ectopic stromal cells may contribute to higher levels of *EDN1* in lesions, which can be associated with pain mechanisms or be involved in the regulation of invasive growth and cell proliferation in endometriosis development. Further functional studies are still needed to prove the connections between these miRNAs and endometriosis development. Nevertheless, the current results provide evidence that further studies are needed to learn about the interactions within and between all cell populations of endometriotic lesions and to uncover the exact molecular mechanisms behind the disease pathogenesis.

4. Materials and Methods

4.1. Patients and Sample Collection

The study was approved by the Research Ethics Committee of the University of Tartu, Estonia (approval no. 278/M-18; approval date: 19 February 2018). Patients undergoing laparoscopic surgery at Tartu University Hospital Women's Clinic with symptoms of endometriosis were recruited and signed informed consent was obtained from all women who entered the study. In total, 12 patients aged 32.0 $\pm$ 6.6 years (mean $\pm$ standard deviation) and with a body mass index of 22.4 $\pm$ 2.4 kg/m^2 were enrolled. According to the revised American Society for Reproductive Medicine classification system [32], the severity of the disease was classified as moderate–severe (stage III–IV) in all cases. None of the participants had received hormonal treatments for at least three months prior to the time of sample collection.

Biopsies from endometriomas and eutopic endometria were obtained at the proliferative menstrual cycle phase and were processed and preserved as described previously [15]. Briefly, the collected tissue samples were subdivided, immediately immersed into formalin for histopathological assessment or into the cryopreservation medium, cooled down at −80 °C freezer overnight and subsequently kept in liquid nitrogen until further use. Histopathological evaluation was performed on endometrioma samples and the diagnosis of endometriosis was confirmed in all cases.

4.2. Stromal Cell Isolation from Eutopic and Ectopic Endometria

Stromal cells were isolated from paired endometrial (n = 6) and endometrioma (n = 6) biopsies using fluorescence-activated cell sorting (FACS) as described previously [15]. Cells were stained with phycoerythrin-conjugated mouse anti-human CD10 antibody (1:20 dilution, clone HI10a, BD Pharmingen, San Diego, CA, USA) and were sorted into 1× PBS (without Ca^{2+} and Mg^{2+}). Total RNA was isolated immediately using miRNeasy Micro kit (Qiagen, Hilden, Germany). The quality and quantity of isolated RNA was assessed with 2200 TapeStation RNA ScreenTape (Agilent Technologies, Palo Alto, CA, USA).

4.3. Small RNA Sequencing

Endometrial stromal cells from paired samples of eutopic endometrium (n = 4) and endometriomas (n = 4) isolated by FACS were subjected to high-throughput small RNA sequencing. Library construction and sequencing were performed by an external service provider (Admera Health LLC, South Plainfield, NJ, USA). Small RNA libraries were prepared using NEBNext Small RNA Library Prep kit (New England Biolabs, Ipswich, MA, USA) and sequencing was performed with 1 × 76 bp NextSeq High Output kit on NextSeq 500 platform (Illumina, Inc, San Diego, CA, USA).

4.4. Sequencing Data Analysis

Small RNA sequencing data were deposited into the Gene Expression Omnibus (GEO accession number GSE121406). The quality of the input reads before and after the read trimming was assessed with FastQC v0.9.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Read trimming and filtering was performed with Cutadapt v1.8.1 (https://cutadapt.readthedocs.io/en/stable/) [33]. All reads shorter than 15 and longer than 35 base-pairs, adapter sequence and sequence read ends with quality value less than 15 were filtered out. The read-mapping tool STAR aligner v2.4.0j (http://code.google.com/p/rna-star/) [34] was used to align quality-controlled sequences to the human reference genome (GRCh38). miRNA alignment filtered out reads with (a) fewer than 17 bp matched to reference (–outFilterMatchNmin), (b) alignments matched to more than five locations (–outFilterMultimapNmax) and (c) if more than 5% of the total read length was mismatched (–outFilterMismatchNoverLmax). No separate restrictions were set on the number of matched bases relative to the read length or alignment score. Raw counts of the miRNA regions were quantified using featureCounts v1.5.2 (bioinf.wehi.edu.au/featureCounts/) that allowed strand-specific and multiply-aligned reads. Successfully-aligned reads were then quantified against mature *Homo sapiens* miRNA coordinates retrieved from miRBase version 21 (http://www.mirbase.org/) [35]. Novel miRNA sequences were predicted with miRDeep2 (https://www.mdc-berlin.de/n-rajewsky#t-data, software&resources) [36] using default settings. Reads predicted as potential candidate miRNAs by miRDeep2 were subjected to BLAST to discriminate the sequences corresponding to other human coding or non-coding RNAs. Sequences were considered as candidate novel miRNAs if detected in at least two out of eight sequenced samples.

Differentially-expressed miRNAs between stromal cells from eutopic and ectopic endometria were identified using edgeR v3.16.5 [37], DESeq2 v1.14.1 [38] and baySeq v2.8.0 [39] packages. Final p-values were reported as corrected for multiple testing with FDR for edgeR and baySeq and the Benjamini–Hochberg method for DESeq2. miRNAs were considered as differentially-expressed if

recognized by at least two out of three methods (adjusted p-values ≤ 0.05). A heatmap was generated with ClustVis (https://biit.cs.ut.ee/clustvis/) [40].

4.5. miRNA Target Prediction

An integrated analysis of differentially-expressed miRNAs combined with mRNAs from paired eutopic and ectopic endometrial stromal cells from our previous study [15] was used to predict miRNA targets. The software MAGIA^2 [41] was implemented to construct post-transcriptional regulatory networks including circuit components of TF regulating both miRNA and its target mRNA, and miRNA regulating both TF and its target. Raw counts for both miRNA and mRNA samples were normalized using weighted trimmed mean of M-values (TMM) normalization implemented in edgeR. A combined meta-analysis was applied as the samples used for mRNA and miRNA analysis originated from different women. Three different target prediction methods within MAGIA^2 were used with mean stringency: RNA22 (threshold: −27.4) [42], DIANA microT (threshold: 2.7) [43] and TargetScan (threshold: 0.7) [44].

4.6. miRNA Transfection

Transfection experiments were performed for the validation of the predicted miRNA targets. The immortalized endometrial stromal cell line ST-T1b [45] was cultured in medium containing 70% Dulbecco's Modified Eagle's medium (PAA Laboratories, Pasching, Austria), 18% MCDB-105, 10% fetal bovine serum, 1% glutamine, 1% penicillin/streptomycin and 5 µg/ml insulin. For miRNA transfection, cells were plated in six-well plates one day before transfection to reach 70% confluency. Cells were then transfected with the miRNA precursors miR-139-5p, miR-375 or pre-miR precursor negative control #2 (Thermo Fisher Scientific, Waltham, MA, USA), via lipotransfection with DharmaFECT reagent (Thermo Fisher Scientific, Waltham, MA, USA) in OPTI-MEM media (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. Twenty-four hours later, the medium was replaced by respective normal culture medium. Expression analyses of predicted miRNA targets for miR-139-5p and miR-375 (Table S4) were performed 48 to 72 h after transfection. To confirm the efficiency of miRNA transfection, miR-139-5p and miR-375 levels were determined by qRT-PCR from ST-T1b stromal cells transfected with miR-139-5p, miR-375 or precursor negative control. The experiments were conducted as three separate transfections resulting in a total of eight replicate samples for both miRNAs and eight control samples. RNA was isolated from the cells using innuPREP RNA Mini Kit (Analytik Jena AG, Jena, Germany) following the manufacturer's instructions.

4.7. Primary Cultures of Human Endometrial Stromal Cells

Primary cultures of human endometrial stromal cells were prepared from paired endometrial ($n = 6$) and endometrioma ($n = 6$) biopsies. Single cell suspensions of endometrial stromal cells for primary culture were isolated and cultured as described previously [46]. Confluent cells were collected and stored in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) at −80 °C until further analysis.

4.8. qRT-PCR

miR-139-5p (Applied Biosystems, Assay ID 005364) and miR-375 (ID 000564) expression levels from uncultured and cultured stromal cells were validated by qRT-PCR. cDNA synthesis was conducted with TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) and qRT-PCR was performed with TaqMan Universal PCR Master Mix, No AmpErase UNG (Thermo Fisher Scientific, Waltham, MA, USA). RNU44 (ID 001094) and RNU48 (ID 001006) were used as references for normalization. Real time PCR experiments were performed in duplicate using 7500 Fast or ABI PRISM 7300 Real Time PCR Systems (Applied Biosystems, Foster City, CA, USA).

For the quantitative analysis of the predicted target genes of miR-139-5p and miR-375, the expression levels were analyzed from cells transfected with respective miRNA precursors or negative control. RNA was converted into cDNA using the High-Capacity cDNA Reverse Transcription

Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR analysis was performed with a 2× SYBR Select Master Mix (Applied Biosystems, Foster City, CA, USA) using ABI PRISM 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). *ACTB* was used as a reference gene. The primer sequences used in the study are listed in Table S4.

Relative miRNA and mRNA expression levels were compared between the studied groups by Wilcoxon test (eutopic vs. ectopic) or the Mann–Whitney U test (cells transfected with miRNA precursor vs. negative precursor control) and a p-value ≤ 0.05 was considered significant. The FC was calculated according to the $2^{-\Delta\Delta Ct}$ method [47].

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/19/12/3789/s1.

Author Contributions: K.R. participated in designing the study, performed experiments and data analysis, and wrote the manuscript; T.T. performed data analysis; M.S. participated in designing the study; K.S. performed experiments; Ü.K. critically revised the manuscript; H.K. critically revised the manuscript; M.G. participated in designing the study, and critically revised the manuscript; A.S. participated in designing the study, and critically revised the manuscript; M.P. participated in designing the study, performed experiments and data analysis, and wrote the manuscript. All authors contributed to drafting the manuscript and have approved the final version of the article.

Funding: This study was funded by the Estonian Ministry of Education and Research (grant IUT34-16); Enterprise Estonia (grant EU48695); the European Commission Horizon 2020 research and innovation programme under grant agreement 692065 (project WIDENLIFE); MSCA-RISE-2015 project MOMENDO (grant no 691058), and University of Tartu base funding.

Acknowledgments: The authors are very grateful to the staff of Tartu University Hospital's Women's Clinic for collecting the samples and to the women who participated in this study.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

CPM	count per million
FACS	fluorescence-activated cell sorting
FC	fold change
FDR	false discovery rate
miRNA	microRNA
qRT-PCR	quantitative real-time PCR
TF	transcription factor

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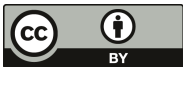

International Journal of
Molecular Sciences

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Review

Mitochondrial Dysfunctions in Type I Endometrial Carcinoma: Exploring Their Role in Oncogenesis and Tumor Progression

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Received: 28 June 2018; Accepted: 14 July 2018; Published: 17 July 2018

Abstract: Type I endometrial cancer (EC) is the most common form of EC, displaying less aggressive behavior than type II. The development of type I endometrial cancer is considered a multistep process, with slow progression from normal endometrium to hyperplasia, the premalignant form, and endometrial cancer as a result of an unopposed estrogenic stimulation. The role of mitochondria in type I EC tumor progression and prognosis is currently emerging. This review aims to explore mitochondrial alterations in this cancer and in endometrial hyperplasia focusing on mitochondrial DNA mutations, respiratory complex I deficiency, and the activation of mitochondrial quality control systems. A deeper understanding of altered mitochondrial pathways in type I EC could provide novel opportunities to discover new diagnostic and prognostic markers as well as potential therapeutic targets.

Keywords: endometrial cancer; mtDNA mutations; deficit of complex I; antioxidant response; mitochondrial biogenesis; mitochondrial dynamics; mitophagy

1. Introduction

Mitochondria are double membrane organelles that supply ATP for key cellular processes in all eukaryotic cells, through the oxidative phosphorylation system (OXPHOS), thus functioning as the fulcrum of cellular homeostasis. In addition, mitochondria are primary sources of reactive oxygen species (ROS), and regulate intracellular calcium, apoptosis, signal transduction and redox balance [1–3].

Mitochondria contain mitochondrial DNA (mtDNA), a circular, double-stranded DNA of approximately 16569 bp [4]. mtDNA is inherited exclusively from the mother [5,6]. The number of mtDNA copies per cell varies (about 2–4000); this feature is called polyploidy [5]. Moreover, the mtDNA molecules may be all the same type (homoplasmy), wild-type or mutant, or may be of different types (heteroplasmy) in cells or tissues.

MtDNA codes for 24 RNAs (12S and 16S ribosomal RNA, 22 tRNAs), and 13 protein subunits of the respiratory chain complexes. Therefore, the subunits of the respiratory complexes are encoded both by nuclear and mitochondrial DNA with the exception of complex II encoded only by the nuclear genome. Other mitochondrial proteins (about 1500) are coded by nuclear DNA and delivered to mitochondria by a localization signal in the amino-terminus of the polypeptides. The most variable part of the DNA molecule is the noncoding region (D-loop region), which is 1.1 kbp long and comprises regulatory regions implicated in mtDNA replication and transcription [7]. MtDNA is located near the mitochondrial respiratory chain—the major source of ROS in the cell—therefore it is more affected by mutations (point mutations and deletions) induced by ROS than nuclear DNA [8]. Some mutations are deleterious to cells because they result in mitochondrial dysfunction, others have no important functional consequences and are considered neutral polymorphisms. However, it is important to know the percentage of deleterious mtDNA mutations (threshold) that can lead to a dysfunction of the mitochondrial respiratory apparatus because it is reported that a very high mutation load may have phenotypic effect [9–11].

In reference to neutral polymorphisms, their de novo sequential accumulation in a single mtDNA molecule generates a mtDNA haplotype. A group of related haplotypes gives rise to haplogroups, which can be specific for ethnic groups or geographic areas [12]. Haplogroups may be related to the individual predisposition to diseases [13].

To ensure maximal mitochondrial function, the mitochondrial quality control systems protect mitochondria from ROS damage at the protein, DNA, and organelle level. At the protein level, mitochondria are protected by antioxidant systems, DNA repair, protein folding and degradation. At organelle level, damage activates mitochondrial biogenesis, mitochondrial dynamics (fusion and fission) and mitochondrial autophagy, also known as mitophagy [14].

The best-characterized metabolic phenotype of tumor cells is aerobic glycolysis (the so-called Warburg effect) where cancer cells, even in presence of oxygen, metabolize glucose and produce an excess of lactate. Warburg explains this phenomenon by hypothesizing the presence of defects in the mitochondrial respiratory chain compensated by the increase in glycolytic rate [15]. Aerobic glycolysis may have a key role in supporting the biosynthetic programs of the fast-growing tumor cells. However, the Warburg effect is not a consistent feature in all cancer types and in different cell populations, in fact, cancer cells may be glycolytic, partially mitochondrial OXPHOS-dependent or completely OXPHOS-dependent. In recent years, reprogramming of metabolism has emerged as a new hallmark of tumor development [16].

In cancer, several alterations of mtDNA (deletions, point mutations and copy number variation) cause mitochondrial dysfunction [17]. In addition to mutations that directly affect mtDNA, mutations in nuclear genes coding for mitochondrial proteins, such as tricarboxylic acid cycle genes (succinate dehydrogenase, fumarate hydratase, and isocitrate dehydrogenase1 and 2), have been described in cancer [18–21]. The mutated proteins contribute to tumorigenesis via stabilization of hypoxia inducible factor 1alpha (HIF1α) [22], thereby probably driving the glycolytic shift that depends strictly on this transcription factor.

Endometrial carcinoma (EC) is one of the most frequently occurring gynecological malignancies in the Western world whose incidence has increased significantly during the last few decades. Endometrioid carcinoma (type I, estrogen-dependent) is the most common form; it displays a less aggressive behavior than type II. The development of type I EC is correlated with unopposed endogenous estrogen exposure; risk factors are chronic anovulation, tamoxifen treatment, nulliparity, early age of menarche, and late age of menopause, age, high body mass index, hypertension, type II diabetes [23]. The unopposed estrogenic stimulation is considered at the basis of a slow progression from normal endometrium to hyperplasia and endometrial cancer [24–26]. It is known that estrogens exert direct and indirect effects on mitochondrial function by differential expression and localization of estrogen receptors [27].

In this review, we will provide an overview and update from our previous review [26] on the mitochondrial alterations in type I EC and in endometrial hyperplasia. Moreover, we will highlight the involvement of mitochondrial DNA mutations and respiratory complex I deficiency in activating mitochondrial quality control systems and the role of these mitochondrial alterations in oncogenesis and tumor progression.

2. MtDNA Mutations in EC Development and Progression

MtDNA mutations have been described in almost all types of cancer and could play different roles in tumor development and progression.

MtDNA mutation could arise either in the female germ line (germline mutations), and may predispose to cancer, or in the affected tissues, representing tumor-specific somatic mutations. Tumor-specific somatic mtDNA mutations may contribute to tumor development and progression as drivers or as complementary gene mutations according to the multiple-hit model [28]. In particular, they can be tumorigenic or adaptive mutations. Pathogenic mtDNA mutations in genes coding subunits of the mitochondrial respiratory complexes may be tumorigenic, since they may lead to dysfunction in the mitochondrial respiratory chain and may stimulate ROS production. ROS in turn may induce mutations in genes that regulate cell replication in proto-oncogenes and in tumor-suppressor genes, leading to cancer development. Adaptive mutations may be mild mtDNA mutations that may participate in metabolic remodeling and may influence tumor progression conferring to cancer the ability to metastasize [29]. However, some mtDNA mutations may be casually present in cancer, thus having no role in cancer development and progression.

Initial studies describing mtDNA mutations in EC did not distinguish between type I and type II. However, most of the analyzed EC samples were of type I. These mutations included deletions, insertions and point mutations and were located in the D-loop region, or in regions coding for rRNAs, tRNAs, or subunits of mitochondrial respiratory complexes.

Germline mtDNA mutations were investigated in EC to evaluate whether they have the potential to represent markers for predicting the risk of developing EC. MtDNA mutations that predispose or protect from EC are reported in Table 1. Base change (m.16189T>C) located in the D-loop region was associated with susceptibility to EC [30]. A mitochondrial polymorphism associated to haplogroup D (m.5178A>C) in the *ND1* gene was reported to predispose to EC in a southwest Chinese population [31]. Three polymorphisms (m.16223C>A, m.207G>A and m.16126T>C) located in the D-loop region of mtDNA, were associated with an increased risk of EC in the Polish population, whereas the polymorphism m.7028C>T located in the *COI* gene seemed to be a EC protective factor [32].

Table 1. Germline mitochondrial DNA mutations that may predispose or protect from endometrial cancer.

Mutation	Gene	Mutation Type	Population	Effect	References
m.16189T>C	*D-loop*	Point mutation	Chinese	Predispose	[30]
m.16223C>A	*D-loop*	Point mutation	Polish	Predispose	[32]
m.207G>A	*D-loop*	Point mutation	Polish	Predispose	[32]
m.16126T>C	*D-loop*	Point mutation	Polish	Predispose	[32]
m.5178A>C	*ND2*	Point mutation	Chinese	Predispose	[31]
m.7028C>T	*COI*	Point mutation	Polish	Protect	[32]

Abbreviations: m., mitochondrial.

However, these studies lack functional proof that these common polymorphisms are really able to predispose to EC. Therefore, to exclude a mere association between the above reported mtDNA variants and EC, it would be very interesting to monitor the daughters of these patients harboring these mtDNA polymorphisms to verify whether this risk is increased in the generations to whom the mutation is passed.

Studies on somatic mtDNA mutations in EC have attempted to demonstrate their role as possible molecular markers for cancer detection. In Table 2, somatic mtDNA mutations found in EC are reported.

Table 2. Somatic mitochondrial DNA mutations in endometrial cancer.

Mutation	Gene	Mutation Type	AA	References
m.152T>C	*D-loop*	Point mutation	-	[33]
m.251G>A	*D-loop*	Point mutation	-	[33]
m.294T>C	*D-loop*	Point mutation	-	[33]
m.289-346del	*D-loop*	50bp deletion	-	[33]
m.305C>A	*D-loop*	Point mutation	-	[34]
m.306C>G	*D-loop*	Point mutation	-	[34]
m.303-309	*D-loop*	mtMSI	-	[33–37]
m.309C>A	*D-loop*	Point mutation	-	[34]
m.514-523	*D-loop*	mtMSI	-	[33–35,37]
m.16153G>A	*D-loop*	Point mutation	-	[32]
m.16182A>C	*D-loop*	Point mutation	-	[34]
m.16183A>C	*D-loop*	Point mutation	-	[34]
m.16184-16193	*D-loop*	mtMSI	-	[33–35,37]
m.16188A>C	*D-loop*	Point mutation	-	[32]
m.16189T>C	*D-loop*	Point mutation	-	[34]
m.650T>C	*12S rRNA*	Point mutation	-	[33]
m.817G>A	*12S rRNA*	Point mutation	-	[33]
m.879T>C	*12S rRNA*	Point mutation	-	[33]
m.956-965	*12S rRNA*	mtMSI	-	[33–35,37]
m.961T>C	*12S rRNA*	Point mutation	-	[34]
m.1474G>A	*12S rRNA*	Point mutation	-	[38]
m.3163G>A	*16S rRNA*	Point mutation	-	[33]
m.3470T>Y	*ND1*	Point mutation	L55P	[39]
m.3730T>Y	*ND1*	Point mutation	Y142H	[39]
m.3670G>A	*ND1*	Point mutation	A122T	[39]
m.3425T>Y	*ND1*	Point mutation	V40A	[39]
m.4722A>G	*ND2*	Point mutation	Y85A	[38]
m.5212T>C	*ND2*	Point mutation	L248P	[39]
m.5567T>C	*TW*	Point mutation	-	[38]
m.6129G>R	*COI*	Point mutation	G76stop codon	[39]
m.6562T>C	*COI*	Point mutation	F220S	[39]
m.6822T>A	*COI*	Point mutation	S307T	[39]
m.6991T>Y	*COI*	Point mutation	L363P	[39]
m.7962T>Y	*COII*	Point mutation	L126S	[39]
m.8545G>A	*ATP6*	Point mutation	A7T	[39]
m.10290G>A	*ND3*	Point mutation	A78T	[39]
m.11863insC	*ND4*	Point mutation	-	[38]
m.11873insC	*ND4*	Point mutation	-	[38]
m.12425insA	*ND5*	Point mutation	-	[38]
m.12439T>C	*ND5*	Point mutation	Y35H	[39]
m.13718G>A	*ND5*	Point mutation	S461N	[39]
m.13994T>C	*ND5*	Point mutation	L553P	[38]
m.14279G>A	*ND6*	Point mutation	S132L	[39]
m.14510delA	*ND6*	Point mutation	-	[38]
m.15172G>A	*CYB*	Point mutation	S	[38]
m.15247C>T	*CYB*	Point mutation	S	[38]
m.15573T>C	*CYB*	Point mutation	F276S	[38]
m.15831T>C	*CYB*	Point mutation	I362T	[39]

Abbreviations: mtMSI, mitochondrial microsatellite instability; AA, aminoacidic change; -, no change; S, synonymous mutation.

Some somatic mtDNA mutations are changes in length of short base-repetitive sequences of mtDNA (mitochondrial microsatellite instability, mtMSI) located in the D-loop region and in the 12S rRNA gene [33,35–37]. Interestingly, the occurrence of these mtMSI was significantly higher in EC (48.4%) than in breast (29.4%), ovarian (21.9%), and cervical (25.4%) cancer [35]. However, the sequencing of mtDNA of cells isolated from different areas of EC and from adjacent normal tissue by laser-capture microdissection, demonstrated that mtDNA mutations occurred randomly and independently in single cells [34]. Therefore, the authors suggested that it is very unlikely that mtDNA mutations may be involved in EC development, but they may be a secondary event during tumor progression.

Moreover, in endometrial hyperplastic and cancer tissues part of the D-loop region, 16S rRNA, tRNAs and the *ND4L* gene were analyzed by single-strand conformation polymorphism (SSCP) technique to study the incidence of mtDNA mutations [40]. Somatic mtDNA mutations were found in 10% of analyzed patients, however, they were not detected in hyperplastic endometrial tissues. When the relationship between somatic mtDNA mutations and clinical and pathological variables (age, clinical stage, histological grade and type or depth of myometrial invasion) of women affected by EC were studied, no correlation was found.

A more informative picture of the role of mtDNA mutations in cancer development and progression was achieved through the sequencing of the entire mtDNA molecule in type I EC samples and in matched typical hyperplastic samples as control [39]. Tumor-specific mtDNA mutations, identified only in endometrial cancer tissue and not in matched endometrial control tissue, were found in 69% of the analyzed EC patients. Many of these mutations were located in complex I genes, predicted to be pathogenic by *in silico* analysis and had not been previously reported in the literature. Interestingly, pathogenic mutations were absent in hyperplastic tissues and all mtDNA variants detected in hyperplasia were haplogroup determinants. No correlation between the occurrence of tumor-specific mtDNA mutations and clinical data was found, even if low-grade (G1–G2) tumors harbor more pathogenic mtDNA mutations than high-grade (G3) tumors [39].

We have suggested that estrogen may favor the appearance of mtDNA mutations in EC by two mechanisms [26]: (1) estrogen increases mitochondrial ROS [41] that may directly damage mtDNA; (2) estrogen stimulates mitochondrial biogenesis [27] that may cause excessive mtDNA replication and consequently mutations, since mitochondrial DNA polymerase is prone to insert incorrect bases during replication. Accordingly, the mtDNA mutational pattern seems to be related more to mtDNA replication errors than to mutagenic agents in human tumors [42].

The mechanism through which mtDNA mutations are selected and accumulated in cancer cells is still debated; it is likely they expand under the selective pressure of the tumor microenvironment, suggesting they may confer a selective advantage to cancer cells, or they may be subjected to a relaxed selection [43]. In type I EC tissue, but not in hyperplasia, mtDNA mutations may reach detectable values, probably due to these mechanisms, and therefore have the potential to become useful biomarkers for the distinction of tumor versus hyperplastic tissues.

Tumor-specific mtDNA mutation could be an additional diagnostic tool to reveal synchronous nature of simultaneously detected endometrial and ovarian cancer [38,44]. A comparison of tumor-specific mtDNA mutations present in endometrial and ovarian cancer tissues of the same patient would allow us to understand the origin of the two cancers. Since it is improbable the same somatic mutation may occur synchronously and independently in EC and ovarian cancer, the presence of the same tumor-specific mtDNA mutations in both tissues suggests these mutations have a common clonal origin and that one of these cancers is the metastasis of the other.

Nuclear genes commonly involved in progression from hyperplasia to tumor (*KRAS*, *PTEN*, *TP53* and *CTNNB1*) were screened for point mutations in the hyperplastic and tumor samples of the same patients in order to place mtDNA mutations in the EC tumor progression model [39]. About 39% of tumor samples harbored point mutations in the *PTEN* gene. In two cases, the mutation was also detected in the matching hyperplastic tissue, suggesting an early inactivation. Mutations in *KRAS*, *TP53*, and *CTNNB1* genes were found only in tumor samples and not in hyperplastic tissues. Since

mtDNA mutations were identified in 69% of cases, while mutational events in nuclear analyzed genes occurred in 56% of the cases, the authors suggested that mtDNA mutations may precede the genetic instability of these genes. The ROS increase, due to mtDNA mutations, may be responsible for nuclear DNA damage and may induce genetic instability and tumor development. However, the authors pointed out that even if a high percentage of EC patients harbor tumor-specific mtDNA mutations, several tumor-specific mtDNA mutations were not potentially pathogenic and finally that not all mutations were homoplasmic or had a high mutation load that imply a mitochondrial dysfunction [39].

Therefore, the role of tumor-specific mtDNA mutation in EC is still a matter of controversy: although it seems likely that they contribute to cancer, inducing nuclear DNA damage, they may be merely a side effect of tumorigenesis. To address this topic more research is needed.

3. Deficit of Respiratory Complex I in Type I EC

The main site of energy production in cells, the mitochondrial oxidative phosphorylation system (OXPHOS), is localized in the inner membrane of mitochondria. OXPHOS machinery is composed of four complexes (complex I, II, III, and IV) responsible for electron transport and proton translocation and for the adenosine triphosphate (ATP) synthase complex (complex V). From Warburg's observations at the beginning of the last century to the most recent research, the role of the OXPHOS system and, in particular, of respiratory complex I (CI) emerges as central in cancer development and progression [22,43]. CI is the largest complex, being composed of 44 subunits, seven of which (ND1-6 and ND4L) are encoded by mtDNA.

A disassembly of CI has been demonstrated in oncocytomas, tumors characterized by mitochondria hyperproliferation (oncocytic-like foci) and by high load of pathogenic tumor-specific mtDNA mutations (nonsense and frameshift) in CI [45–47]. These results suggested the altered mitochondrial function due to mtDNA mutations can be compensated by mitochondrial hyperproliferation. Also in type I EC, most EC samples, characterized by the presence of pathogenic tumor-specific mtDNA mutations, showed oncocytic-like foci and a partial or total loss of immunohistochemical staining for the ND6 subunit of complex I in some of them, suggesting a deficit of CI [39]. Recently, CI has been investigated in two type I EC patients [48] by nondenaturing Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) and enzymatic colorimetric reactions, confirming a deficit of CI activity in cancer samples compared to matched controls. Western blotting analysis on respiratory complexes separated by BN-PAGE with antibodies against subunits of respiratory complexes I, IV and II showed a decrease in CI amount. These results confirmed an association in type I EC between pathogenic mtDNA mutation, loss of CI, and oncocytic-like transformation as already reported in oncocytomas. The mtDNA mutations in complex I genes associated to deficit of CI are reported in Table 3.

Table 3. Mitochondrial DNA mutations in complex I genes associated to deficit of complex I.

Mutation	Gene	Mutation Type	AA	References
m.3730T>Y	*ND1*	Point mutation	Y142H	[39,48]
m.3425T>Y	*ND1*	Point mutation	V40A	[39,48]
m.5212T>C	*ND2*	Point mutation	L248P	[39]
m.10844A>C	*ND4*	Point mutation	T29P	[39]
m.14510delA	*ND6*	Point mutation	-	[39]

Abbreviations: AA, aminoacidic change; -, no change.

It has been suggested CI can be considered an "*oncojanus*" [43]. Mild CI dysfunction may contribute to tumor metabolism and to tumorigenic properties of cancer cells enhancing oxidative stress and activating the oncogenic Akt/mTORC1 pathway. Conversely, since cancer cells are characterized by a high-energy demand for proliferation, the severe CI defects in oncocytomas may induce a metabolic short-circuit preventing tumor progression, thus leading to an almost benign phenotype.

Therefore, it can be envisioned that also in EC a combined action of estrogens and complex I dysfunction may contribute to maintain the tumor in a less aggressive state and can explain how type I EC prognosis is generally more favorable. However, the functional role of CI dysfunction in EC deserves in-depth investigation.

4. Mitochondrial Biogenesis Increase in Hyperplasia and Type I EC

Changes in mitochondrial number, mtDNA content and mRNA expression for OXPHOS genes have been reported in solid tumors [49].

The master regulator of mitochondrial biogenesis is the nuclear transcriptional coactivator belonging to PPARγ coactivators (PGC) family, namely PPARγ-coactivator-1 alpha (*PGC-1α*) [50]. PCG1-α is a coactivator of nuclear respiratory factors 1 and 2 (*NRF-1* and *NRF-2*) and by means of these factors enhances the expression of many nuclear genes, in particular, that of the mitochondrial transcription factor A (*TFAM*), which is a key factor in regulating mtDNA transcription and replication. [50].

An increase in TFAM, NRF-1 and PGC-1α protein content was found in a pooled group of type I EC endometrial tissues compared with a pooled group of endometrial proliferative control tissue suggesting, in type I EC tissue, an upregulation of the PGC-1α signaling pathway and an increase in mitochondrial biogenesis [51]. The increase in mitochondrial biogenesis is generally measured by an increase in the mtDNA/nuclear DNA ratio (mtDNA cellular content) and in citrate synthase (CS) activity (marker of mitochondrial mass). In fact, the mtDNA cellular content was measured in EC cells collected by laser-capture microdissection revealing a twofold increase in EC compared with normal endometrial cells [52]. Moreover, a twofold increase in mtDNA content and in CS activity was found in a pooled group of type I EC endometrial tissues compared to a pooled group of endometrial proliferative control tissue [51]. An increase in mtDNA content in structural mitochondrial proteins TFAM and voltage-dependent anion channel 1 (VDAC1). In some nuclear DNA-encoded respiratory subunits NADH:ubiquinone oxidoreductase subunit A9 (NDFUA9), succinate dehydrogenase complex flavoprotein subunit A and B (SDHA, SDHB), Core II was also found in EC samples compared to matched control tissues, especially, in the EC samples harboring pathogenic tumor-specific mtDNA mutations. In 72% of these analyzed patients, oncocytic-like foci were also found confirming the association between mtDNA mutations and the increase in mitochondrial biogenesis in type I EC [39]. Different results were found by Reznick et al. [49] reporting a decrease of OXPHOS mitochondrial genes expression and no increase of mtDNA in EC compared to adjacent-normal tissue.

The mtDNA content and CS activity were also measured in control, hyperplastic (with or without atypia) and cancer endometrial tissues to verify if they could be considered possible markers for progression from benign to premalignant lesions [53]. This analysis revealed an increase in mtDNA content in hyperplasia and, in particular, in ECs compared with controls. The same trend was found for CS activity. These data also revealed that an mtDNA content increase preceded the increase in CS activity, since a statistically significant increase was observed for mtDNA content already in typical hyperplasia, while an analogous increase for CS activity was found only in atypical hyperplasia. No statistically significant correlation was found between the mtDNA content or CS activity and prognostic factors (grade, depth of myometrial invasion, stage). However, in high-grade tumors, mtDNA content was slightly decreased, probably due to the high rate of cell division and the consequent lower number of mitochondria per cell and to the lower estrogen exposure.

Estrogen has an important role in mitochondrial homeostasis. The genomic activity of estrogen is mediated by estrogen receptors (ERα and ERβ). They have been identified in different cell compartments and also in cell-type-dependent manner colocalize within mitochondria. They stimulate mitochondrial biogenesis by activating *NRF-1* transcription and, by directly interacting with D-loop, increasing mtDNA transcription [27,54–56]. In particular, in breast and lung adenocarcinoma estradiol, by stimulating directly *NRF-1* that increase *TFAM* genes expression, enhanced mitochondrial biogenesis

and oxygen consumption [57]. Moreover, in breast cancer cells, estradiol increased mitochondrial ROS, which stimulated NRF-1 activity [58].

Estradiol regulates also ion homeostasis increasing intracellular Calcium uptake, expression of the antiapoptotic factor *Bcl-2*, which augments the maximal mitochondrial calcium uptake capacity [59]. Increased intracellular calcium, through activation of Calcium dependent protein kinases and phosphatase, may regulate corepressors and coactivators as PGC-1α modulating gene transcription [27]. Calcium can cause also changes in cellular function and may contribute to cancer progression and metastasis [60].

Therefore, it can be envisioned that the moderate increase in mitochondrial biogenesis reported in endometrial hyperplasia could be a direct result of estrogen stimulation. In type I EC cancer tissues, the effect of estrogen stimulation on the increase in mitochondrial biogenesis may be reinforced by the occurrence of pathogenic tumor-specific mtDNA mutations. These mutations, in fact, may lead to respiratory dysfunction and ROS increase, thus triggering a retrograde signaling to the nucleus, through the upregulation of the PGC-1α signaling pathway [26].

Since mtDNA content and CS activity increased in cancer and also in atypical hyperplasia it could be envisioned that they represent possible molecular markers to establish the risk of malignant transformation in endometrial hyperplasia and may have a clinical value in patient management. However, due to the high interindividual variability of these markers, further analysis in a wider panel of patients and prospective longitudinal studies is necessary to address this topic.

5. Activation of Antioxidant Response in Type I EC

In cancer, oxidative stress condition induces an increase in antioxidant enzymes as a compensatory defensive mechanism to counteract ROS increase and to maintain mitochondrial function [61].

It is plausible to hypothesize that oxidative stress conditions may occur in EC, since estrogens and mitochondrial respiratory dysfunction may increase ROS production in mitochondria, and may activate the mitochondrial redox defense system. This system consists of antioxidant proteins namely peroxiredoxin 3 (Prx3), peroxiredoxin 5 (Prx5), manganese superoxide dismutase, and thioredoxin 2. These proteins eliminate ROS that have been generated in the oxidative phosphorylation system. Indeed, an increase in Prx3, manganese superoxide dismutase and catalase was reported in type I EC, especially in patients harboring pathogenic tumor-specific mtDNA mutations [39]. Moreover, an increase in Prx3 and Prx5 was also reported in endometrial cancer [62] and Prx3 was found upregulated in EC cells and in endometrial cancer stem cells (CSCs) [63]. The knockdown of the Prx3 gene in these endometrial CSCs resulted in the death of cells by causing mitochondrial dysfunction. This result indicated that Prx3 eliminated ROS and it was required for the maintenance of mitochondrial function and the survival of CSCs.

Furthermore, an increase in the expression level of augmenter of liver regeneration (ALR) protein was recently reported in type I EC [48]. It was reported that, in other tissues, ALR has an antioxidant activity, stimulates mitochondrial biogenesis [64] and, by inducing the antiapoptotic protein Bcl-2, acts as antiapoptotic factor [65,66]. Moreover, in glioma cells, it was demonstrated that ALR had antioxidative activity by reducing ROS and protecting cells from ROS-induced oxidative damage, since it stimulated the expression of clusterin, a reducing agent [67]. Concordantly with these findings, the increase in clusterin mRNA [68] and in Bcl-2 protein [47] was also reported in endometrial cancer suggesting that the increase in ALR might be a protective response to the ROS increase.

Therefore, it can be envisioned that Prx3 and ALR might represent valuable therapeutic targets and could provide new insights into the development of new therapeutic strategies for patients with endometrial cancer.

6. Activation of The Mitochondrial Quality Control Systems in Type I EC

It has been suggested that mitochondrial dysfunction in cancer cells may activate mitochondrial quality control systems, such as mitochondrial biogenesis, mitochondrial dynamics (fusion and fission), mitophagy and protein turnover, as a compensatory response [14,69,70].

A marked increase in mitochondrial fission protein Dynamin related protein 1 (Drp1) and a decrease in fusion protein mitofusin protein 2 (Mfn2) were found in type I EC patients, characterized by the deficit of respiratory complex I and oncocytic-like foci, compared with matched controls, suggesting an increase in mitochondrial fission [48]. This analysis was extended to a pooled group of type I EC, of endometrial hyperplasia and of nonmalignant tissues revealing an increase in the mitochondrial fission proteins Drp1 and Fission protein 1 (Fis1) in cancer compared with control and hyperplastic tissues. Mfn2 was also found to be significantly decreased in cancer compared to control and hyperplastic tissues. Moreover, an increase in the expression level of Bcl-2 and adenovirus E1B 19 kDa-interacting protein (BNIP3), the molecular mediator implicated in promoting mitophagy, and in the caseinolytic mitochondrial matrix peptidase proteolytic subunit (CLPP) was also observed. These results suggested that, not only the increase in mitochondrial biogenesis, but also fission, mitophagy and proteolysis may be activated in type I EC to ensure a sufficient number of functional mitochondria to survive mitochondrial dysfunction better [48].

A key question is how mitochondrial dysfunction might regulate mitochondrial dynamics to facilitate a fragmented mitochondrial network. It has been suggested that oncogenic K-Ras, via Extracellular signal-regulated kinase 1 and2 (ERK1/2)-mediated phosphorylation of Drp1, promotes mitochondrial fragmentation and forces cellular metabolism towards glycolysis [71]. Supporting this hypothesis, an increase in phosphorylated Drp1 on serine 616 [48] and heterozygous mutations in the critical amino acids of K-Ras [39] were detected in type I EC, suggesting an increase in mitochondrial fragmentation via the K-Ras pathway.

The clinical utility of mitochondrial dynamics, biogenesis and mitophagy as biomarkers for cancer progression is only at the beginning and requires substantial future efforts.

7. Conclusions

Estrogens may have a role in type I EC development through direct and indirect effects on mitochondrial function.

We propose (Figure 1) that hyperestrogenism may stimulate ROS production and may increase mitochondrial biogenesis because of a direct interaction of estrogens with NRF-1 and of ROS activation of NRF-1. Estrogen-related ROS increase and excessive mtDNA replication, due to increased mitochondrial biogenesis, may lead to an increase in tumor-specific mtDNA mutations. These mutations may reach a threshold value and affect respiratory complexes, in particular complex I, and may lead to respiratory dysfunction and ROS increase. Mitochondrial dysfunction and ROS increase, in a vicious cycle, may in turn reinforce the occurrence of mtDNA mutations and trigger a retrograde signaling to the nucleus that stimulates further mitochondrial proliferation and activates antioxidant response as a compensatory mechanism. Moreover, as an adaptation process to mitochondrial dysfunction, mitochondrial fission may be stimulated in order to segregate damaged mitochondria components that can be discharged by proteolysis and mitophagy.

We have also highlighted the fact that pathogenic mtDNA mutations are hallmarks of EC and are potentially useful tools for tumor diagnosis and prognosis. They could be useful biomarkers for the distinction of tumor versus hyperplastic tissues, since they are present in high percentages only in type I EC, they can be markers of low-grade tumors. Moreover, mtDNA mutations may provide an additional diagnostic tool to reveal synchronous cancers.

A key unresolved question is whether tumor-specific mtDNA mutations may play a role in oncogenesis and tumor progression processes, through ROS increase and genetic instability, or whether they are merely a side effect of tumorigenesis. Certainly, they could provide an explanation for altered mitochondrial phenotype and for the activation of mitochondrial quality control systems in EC.

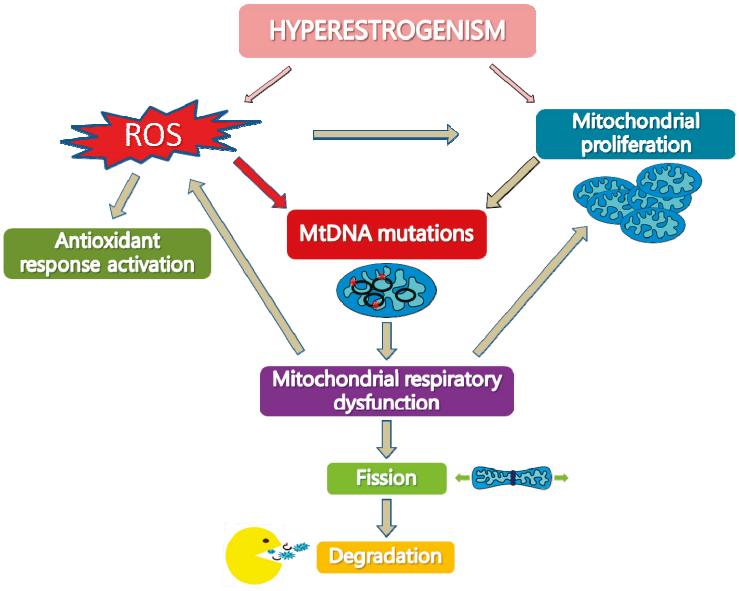

Figure 1. Hyperestrogenism and mitochondrial dysfunction in type I endometrial cancer. Hyperestrogenism may stimulate reactive oxygen species (ROS) production and increase mitochondrial biogenesis. ROS increase and excessive mitochondrial DNA (mtDNA) replication due to increased mitochondrial biogenesis may lead to mtDNA mutations. These mutations may affect respiratory complexes, in particular complex I, and may induce mitochondrial dysfunction that reinforces ROS production and stimulates mitochondrial proliferation in a vicious cycle. ROS increase activates the antioxidant response. Mitochondrial dysfunction may also increase mitochondrial fission in order to segregate damaged mitochondria components, which can then be degraded by proteolysis and mitophagy.

There are grounds to state that within the field of studies on EC, future studies on mtDNA mutations and on the expression level of proteins involved in altered mitochondrial pathways should be implemented, since they could open new horizons in the diagnosis and in the prognosis of EC and, most importantly, could represent potential therapeutic targets.

Author Contributions: Concept, definition of intellectual content and manuscript review: A.C.; literature review: C.M.; manuscript preparation: C.M., G.C., V.P., V.L., E.C., L.R., G.R. and A.C.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflicts of interest.

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International Journal of
Molecular Sciences

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Review

Liquid Biopsy in Endometrial Cancer: New Opportunities for Personalized Oncology

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Received: 15 June 2018; Accepted: 2 August 2018; Published: 7 August 2018

Abstract: The identification of new molecular targets and biomarkers associated with high risk of recurrence and response to therapy represents one of the main clinical challenges in the management of advanced disease in endometrial cancer. In this sense, the field of liquid biopsy has emerged as a great revolution in oncology and is considered "the way" to reach personalised medicine. In this review, we discuss the promising but already relatively limited advances of liquid biopsy in endometrial cancer compared to other types of tumours like breast, colorectal or prostate cancer. We present recent data analysing circulating tumour material in minimally-invasive blood samples, but also in alternative forms of liquid biopsy like uterine aspirates. Proteomic and genomic studies focused on liquid-based uterine samples are resulting not only in optimal diagnostic tools but also in reliable approaches to address tumour heterogeneity. Likewise, circulating tumour cells (CTCs) and circulating tumour DNA (ctDNA) represent an opportunity for the correct stratification of patients, for the assessment of early recurrent disease or for the real-time monitoring of therapy responses. Appropriately designed studies and implementation in clinical trials will determine the value of liquid biopsy for precision oncology in endometrial cancer.

Keywords: liquid biopsy; uterine aspirate; circulating tumour cells (CTCs); circulating tumour DNA (ctDNA); exosomes

1. Challenges in Endometrial Cancer

Endometrial cancer (EC) is the fourth leading cancer in women from developed countries. In Europe, the number of new cases was about 100,000 in 2012, with an incidence of 13.6 per 100,000 women [1]. This tumour originates in the inner layer of the uterus when epithelial cells lining the myometrium start to proliferate abnormally. Although most ECs are diagnosed early, mainly due to symptomatic postmenopausal metrorrhagia, up to 20% of the lesions progress to a high-stage carcinoma. Unfortunately, the five-year survival in this group of women drops to 15%, compared to 90% in women diagnosed with confined disease. Myometrial infiltration and the appearance of disseminated aggressive tumour cells are crucial events for prognosis and death in EC [2]. Surgery represents the primary treatment. In addition, patients with high risk of recurrence also receive adjuvant radiotherapy, together with chemotherapy that is restricted to metastatic/recurrent disease and high-grade ECs. However, traditional chemotherapy regimens are less effective in comparison with other cancers. This overview situates the clinical challenge on the identification of new molecular

targets and biomarkers associated with a high risk of recurrence and/or with response to therapy as valuable tools to improve our management of advanced disease in endometrial cancer.

EC is classified into two distinct groups, type I and type II, which differ in molecular, clinical and histopathological characteristics. Type I tumours are low-grade and estrogen-related endometrioid carcinomas (EEC), while type II are non-endometrioid (NEEC), mainly serous and clear cell carcinomas. To date, this classification has been demonstrated to be an important predictor of survival, but also a determinant for the extent of the initial surgical procedure and subsequent use of adjuvant therapy. However, the molecular heterogeneity associated with the histological diversity of this type of cancer makes the current treatment options insufficiently personalised. To this regard, the integrated genomic, transcriptomic and proteomic characterisation of EC performed by The Cancer Genome Atlas Research Network (TCGA) revealed four groups of tumours [3]. The first group (EEC1) includes EEC with somatic inactivating mutations in POLE exonuclease and very high mutation rates (hypermutated) (7%); it is associated with a good prognosis. The second group (EEC2) includes EEC with microsatellite instability, frequently with MLH-1 promoter hypermethylation and high mutation rates (28%). The third group (EEC3) is composed of EEC with low copy number alterations (39%). Importantly, both the second and third groups show similar progression-free survival rates. Finally, the fourth group (serous-like or copy-number high) (26%) shows low mutation rate but frequent *TP53* mutations. This group has worse prognosis, being predominantly composed of serous carcinomas with some sporadic cases of ECC (mainly EEC3 and some EEC1–2). The incorporation of TCGA surrogate classification into clinical practice should carry important advantages in the management of EC patients [4]. The prognostic value of TCGA in EC has been corroborated in large cohorts included in studies developed by the Vancouver and PORTEC (Post Operative Radiation Therapy in Endometrial Carcinoma) groups [5,6]. This can be especially relevant in adjuvant treatment choices for high to intermediate-risk EC patients that are likely to be impacted by the integrated molecular classification [7], while recurrent disease may continue to represent an additional challenge. Despite these stratification conditionings, and as demonstrated in the majority of solid tumours, EC shows intratumour heterogeneity with different neoplastic cell components within the same tumour. These cells have different morphologic and molecular features that may present a relevant clinical impact, especially for the assessment of prognosis and clinical management of EC patients [8]. In this sense, the use of liquid biopsies to diagnose and characterise EC can facilitate the integration of tumour heterogeneity into the therapy selection and monitoring.

2. Liquid Biopsy

Nowadays, research efforts are focused on the discovery of new non-invasive methods for the diagnosis and comprehension of the tumour molecular architecture in real time. In comparison with traditional biopsies, the study of the tumour material present in bodily fluids can provide valuable information for the diagnosis of tumours with low accessibility, or for a more complete overview of tumours in advanced stages where there are different tumour locations to be interrogated. Liquid biopsies also offer advantages to monitor the tumour evolution and the response to therapy with more accuracy than current clinical imaging techniques. In this sense, the field of liquid biopsy has emerged as a great revolution in oncology and is considered "the way" to reach precision medicine. In addition to blood, several other bodily fluids such as saliva, urine, cerebrospinal fluid (CSF), uterine aspirates, pleural effusions or even stool have been shown high interest as a non-invasive source of tumour-derived material [9]. This tumour circulating material is mainly composed of circulating tumour cells (CTCs), circulating tumour DNA (ctDNA), circulating tumour miRNA, proteins and exosomes [10].

The analysis of these different types of liquid biopsy has been successfully applied in oncology research during the last two decades, closely linked to the development of ultrasensitive methods for their detection. In fact, the main limitation to working with liquid biopsy is the low quantity of tumour material present in circulation. For example, in metastatic patients, the mean CTC level is 1 CTC/10^{6-8}

mononuclear cells, while ctDNA is normally less than 0.01%. Fortunately, nowadays we have highly sensitive techniques to tackle liquid biopsy analyses with enough guarantees [9].

CTC research is considered the start-point of the liquid biopsy field. Early in the formation and growth of a primary tumour, cells are released into the bloodstream. Several groups are studying the clinical benefit of CTC monitoring. CTCs have been validated as a prognostic marker in metastatic breast cancer and other solid tumours such as prostate, colorectal, and lung cancer, showing even more accuracy than conventional imaging methods for response evaluation. However, there are still technological challenges to use CTC monitoring to detect minimal residual disease in patients at early stages. On the other hand, the molecular characterisation of CTCs is of great value to guide the selection of targeted therapies since it allows clinicians to have a dynamic view of different molecular targets such as ERBB2, EGFR, AR or PD-L1, among others [11,12].

Despite all the studies demonstrating the relevance of CTCs for cancer management, the results from clinical trials have failed to demonstrate a clear clinical benefit. In comparison, the younger brother of CTCs, the ctDNA, has already been implemented in routine clinical practice after EMA (European Medicines Agency) approval of the EGFR mutation test (Therascreen EGFR Plasma, Qiagen) in plasma of patients with non-small cell lung cancer (NSCLC) [13]. Highly sensitive and specific methods have been developed to detect ctDNA, including beads, emulsion, amplification and magnetics based digital PCR (BEAMing) [14], safe sequencing (Safe-Seq) [15], tagged amplicon deep sequencing (TAm-Seq) [16], and digital PCR [17] to detect point mutations or whole-genome sequencing [18]. Using these technologies, several studies have demonstrated that ctDNA may be a useful tool for drug development and for the study of intratumour heterogeneity and clonal evolution in tumours such as breast, colon, melanoma and NSCLC [13]. In gynaecological tumours, and especially for ovarian cancer, TAm-Seq has demonstrated high sensitivity to detect point mutations [16].

On the other hand, the interest in characterising circulating exosomes and miRNAs is continuously increasing. These tumour entities contribute to cancer development and metastasis, and their detection in a variety of biological fluids represents a promising strategy to identify specific biomarkers with diagnostic and prognostic relevance. An additional advantage of circulating exosomes versus CTCs or ctDNA is that these extracellular vesicles can provide higher amounts of tumour material for genetic analyses. Many kits have been commercialised for improved and simplified isolation, such as ExoQuick (System Bioscience). Furthermore, tumour exosome biomarkers such as HSP60 and GPC1 have been described as valuable candidates for colorectal, pancreatic and breast cancer detection. However, these studies on exosomes and miRNA in blood are still quite exploratory and further validation in clinical studies with standardised protocols is mandatory before the routine use of these biomarkers in the clinic [19,20].

3. Liquid Biopsy in Endometrial Cancer

Liquid biopsies will play a key role in managing EC patients in the coming years, in addition to multiple other tumour types. This includes different clinical scenarios such as early diagnosis, tumour phenotyping, therapy selection and disease monitoring in real time. Below, we provide an overview of the different forms of liquid biopsy that can be exploited in patients with endometrial cancer towards personalised medicine (Table 1, Figure 1).

3.1. Uterine Aspirates

In addition to peripheral blood as the prototypical form of liquid biopsy and main source of tumour material with clinical utility, the uterine aspirate represents an alternative form of liquid biopsy with high relevance in gynaecological malignancies. This is of special importance in EC diagnosis to address an increasing incidence in developed countries [1]. Risk factors for EC include age $\geq$40 years, obesity, diabetes, hypertension, estrogen using, tamoxifen treatment, and family history of malignant tumours. Many of these factors are tightly linked to current lifestyles in developed countries. An effective screening strategy for women with high-risk factors may contribute to the early detection and

management of EC, and a screening policy using liquid-based cytology could be considered in selected high-risk groups of patients in developed countries (Table 1). The diagnostic procedure consists of pelvic examination and transvaginal ultrasonography, followed by the histopathologic observation of an endometrial biopsy, which is preferably obtained by a minimally invasive aspiration from the uterine cavity using a Cornier pipelle (i.e., uterine aspirate or pipelle biopsy). Diagnosis is achieved by the observation of abnormal cells in the uterine aspirate, which presents high sensitivity to detect EC [21]. However, high failure rates with an average of 22% of histologically inadequate specimens have been reported, and a more invasive test such as dilatation and curettage (D&C) or hysteroscopy must be performed, with the added risks of anesthesia, infection and perforation, and higher health care costs. There is a high concordance in molecular subtype assignment between hysterectomy specimens and diagnostic endometrial specimens as obtained by office biopsy (e.g., pipelle) or dilatation and curettage [22]. This makes liquid-based endometrial cytology, which prepares samples for cytology examination by depositing the collected sample into a preservative liquid, a useful method for detecting endometrial pathologies as a first-line approach compared to the more invasive, painful and expensive endometrial biopsy, D&C and hysteroscopy. Cytology sampling performed by brushing the uterus cavity followed by a liquid-based smear has demonstrated an optimal diagnostic accuracy [23].

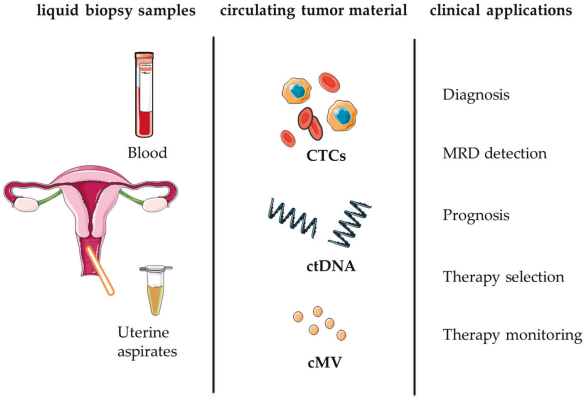

Figure 1. Liquid biopsy for personalised medicine in endometrial cancer. CTCs (circulating tumor cells), ctDNA (circulating tumor DNA), cMV (circulating microvesicles), MRD (minimal residual disease).

The endometrial fluid is a non-invasive sample which contains numerous secreted proteins representative of endometrial function and reflects the state of the endometrium. This type of liquid biopsy can result in a comprehensive catalogue of proteins of the endometrial fluid during the secretory phase of the menstrual cycle [24], but also as a promising biological fluid in which to identify potential endometrial cancer biomarkers for its early diagnosis, such as costars family protein ABRACL and phosphoglycerate mutase 2 [25]. The fluid fraction of uterine aspirates are minimally invasive samples with an important value for the screening of EC protein biomarkers, leading to uterine aspirate-based signatures to diagnose EC and classify tumours in the most prevalent histologic subtypes [26]. This will improve diagnosis and assist in the prediction of the optimal surgical treatment. In addition to proteomics in uterine aspirates as an alternative form of liquid biopsy, the potential of targeted genetic sequencing of uterine aspirates has been assessed as a pre-operative tool to obtain reliable information regarding the mutational profile of a given tumour, even in samples that are not histologically classifiable [8]. Notably, the genetic analysis of uterine aspirates captures the high intratumour genetic heterogeneity associated with endometrial cancer, solving the potential problem of incomplete genetic characterisation when a single tumour biopsy is analysed. PapSEEK, a recently developed test that interrogates for mutations in 18 genes as well as for aneuploidy after Tao and

Pap brush, showed 81% (95% CI, 76–84%) of EC patients with detectable mutations, including 78% of patients with early-stage disease and 89% of the patients with late-stage disease. In addition, comparing intrauterine sampling with a Tao brush and endocervical sampling with a Pap brush, the former showed an improved detection rate of 93% of 123 (95% CI, 87–97%) patients with endometrial cancer. The most commonly mutated genes in both Tao and Pap brush samples were: *PTEN* (63%), *TP53* (42%), *PIK3CA* (36%), *PIK3R1* (20%), *KRAS* (17%), *CTNNB1* (15%), *FGFR2* (15%), *RNF43* (11%), *PPP2R1A* (7%), *POLE* (7%), and *FBXW7* (6%), clearly representing the endometrial cancer mutational landscape [27].

Table 1. Clinical research approaches to liquid biopsy in endometrial cancer.

Liquid Biopsy	Technology	Clinical Setting	Biomarkers	References
Uterine Aspirate	Targeted Proteomics	Diagnosis	ABRACL and PGAM2; KPYM, MMP9, to identify the disease; CTNB1, XPO2, and CAPG to discriminate between endometrioid endometrial carcinomas (EEC) and serous endometrial carcinoma (SEC)	[25,26]
	Targeted Sequencing	Diagnosis	*PTEN*, *PIK3CA*, *CTNNB1*, *TP53*, *FGFR2*, *KRAS*, *CDKN2A* (most common mutated genes in endometrial cancer (EC))	[8,27]
Circulating Tumour Cells (CTC)	EpCAM-Based Immunoisolation (CellSearch®) and IF	Prognosis	CK-8, CK-18, CK-19, *ETV5*, *NOTCH1*, *SNAI1*, *TGFB1*, *ZEB1* and *ZEB2*	[28–31]
	Density-based Enrichment (Oncoquick) and RTqPCR	Prognosis	*CCNE2*, *DKFZp762E1312*, *EMP2*, *MAL2*, *PPIC*, and *SLC6A8*	[32]
	RTqPCR and flow cytometry	Prognosis	TTF-1 and the mRNA expression of: survivin, β-catenin, miR-15a, and *PTEN*	[33]
	Size-Based Enrichment (Metacell®) and Immunodetection	Prognosis	CTCs were defined based on: (i) cell size $\geq$ 15 µm; (ii) nuclear size $\geq$ 10 µm); (iii) irregularity of the nuclear contour; (iv) visible cytoplasm; (v) prominent nucleoli; (vi) high nuclear-cytoplasmic ratio; (vii) cluster presence; (viii) mitosis presence.	[34]
Endothelial Progenitor Cells (EPC)	Flow Cytometry	Diagnosis	VEGFR2/KDR and CD34	[35]
Cell Free DNA (cfDNA)	PCR-RFLP RTqPCR Alu-RTqPCR NGS	Prognosis Prognosis Prognosis Diagnosis	*KRAS* Alu sequences cfDNA content and integrity index Copy number variations (CNVs)	[36] [37] [38] [39]
Circulating Tumour DNA (ctDNA)	Droplet Digital PCR	Response to Treatment	Various tumour-specific fusions and mutations in ctDNA	[40]
Circulating miRNA	RTqPCR	Diagnosis/Prognosis	miR-99a/miR-199b, miR-9/miR-1228 and miR-9/miR-92a, miR-222, miR-223, miR-186, miR-204 and miR-21	[38–41]

3.2. Circulating Tumour Cells (CTCs)

The presence of circulating tumour cells (CTCs) has been evaluated with the FDA approved technology CellSearch. The findings consistently point to a small number of high-risk EC patients presenting with EpCAM positive CTCs in circulation at the time of diagnosis, although limited cohort studies have been conducted: a study with 7% (n = 28) CTC-positive grade 3 EC patients with an association between positive CTCs and both deep myometrial infiltration and positive lymph nodes has been described [28]. Similarly, 15% (n = 40) CTC-positive high-risk EC patients have been observed, associated in this case with cervical involvement [29]; in addition, no significant correlation was found between CTCs and serum CA125/HE4 and no CTCs were detected after the first cycle of standard chemotherapy. The ENITEC (European Network for Individualized Treatment in EC) Consortium described a study with 22% (n = 32) CTC-positive high-risk EC patients [30]. Finally, another study described 60% (n = 30) CTC-positive advanced EC patients with detectable circulating tumour cells, generally associated with non-endometrioid versus endometrioid histology, tumour size $\geq$5 versus <5 cm, higher-stage disease and worse survival [31]. Although the detection of CTCs in the blood might be of help to determine the potential risk of recurrence in EC patients and to assess

the prognosis and possibly guide postoperative treatment, no conclusive information is available, thus limiting their utility in the clinical setting (Table 1).

The combination of isolated CTCs and RTqPCR of a panel of genes has also been explored in EC. Obermayr et al., performed a multimarker analysis using a panel of six genes (*CCNE2*, *DKFZp762E1312*, *EMP2*, *MAL2*, *PPIC*, and *SLC6A8*) for the detection of CTCs which positively identified 64% of a cohort of 25 EC patients [32]. Importantly, gene-expression profiling characterised a strong CTC-plasticity phenotype with stemness and epithelial-to-mesenchymal transition (EMT) features that may provide an advantage in the promotion of metastasis for CTC dissemination and homing. The in vitro recapitulation of this phenotype indicated an improved metastasis efficiency. Moreover, the CTC expression of *CTNNB1*, *STS*, *GDF15*, *RELA*, *RUNX1*, *BRAF* and *PIK3CA* suggested potential therapeutic targets [30]. Regarding biomarkers associated with circulating tumour cells (CTCs), the expression of thyroid transcription factor-1 (TTF-1) correlated with TNM staging, vascular infiltration, and lymphatic metastasis. Progression-free survival (PFS) and the median survival time decreased in the TTF-1-positive group compared with the TTF-1-negative group. Additionally, the recurrence rate increased in the TTF-1-positive group [33].

Finally, using size-based enrichment (MetaCell system), Kolostova et al., demonstrated the feasibility of isolating CTCs from ovarian, endometrial and cervical cancers, and culturing them in vitro for a short time [34]. More recently, and using the same enrichment strategy, peripheral blood samples from 92 patients who underwent a surgical procedure were evaluated for the presence of CTCs, showing an improved detection rate compared to previous studies. In addition, the authors claimed that endometrial CTCs were successfully cultured for further downstream functional and molecular characterisation [42].

In addition to CTCs, other circulating cells have been described as potential EC biomarkers (Table 1). Besides their role in cardiovascular diseases, circulating endothelial cells (CEC) are considered a biomarker for different neoplasms as they play a relevant role in tumour angiogenesis, which is essential for invasive tumour growth and metastasis [43]. Endothelial progenitor cell numbers (CD34, VEGFR2/KDR) in the peripheral blood of women with early endometrial carcinoma were significantly augmented compared with those of healthy control women, while circulating endothelial cell numbers (CD31, CD45) were similar in both groups [35]. By contrast, no prognostic significance or association with clinicopathological features have been demonstrated for the presence of disseminated tumour cells (DTC) in the bone marrow of endometrial carcinoma patients [44,45].

3.3. Cell free DNA (cfDNA)

Changes in circulating cell-free DNA (cfDNA) levels have been associated with cancer development and progression. However, very few studies have been developed for evaluating the cfDNA content in EC patients (Table 1). Dobrzycka et al., demonstrated the feasibility of cfDNA detection using the PCR-RFLP and enriched by the PCR-RFPL method in a cohort of 109 patients with EC (87 patients with type I and 22 patients with type II) [36]. In this cohort, *TP53* mutations were also identified in plasma, frequently in early serous carcinomas, and a high frequency of KRAS mutations in grade 2 endometrioid tumours. This was one of the first studies focused on EC that suggested the value of cfDNA monitoring as a marker for predicting the prognosis and selecting individualised treatment regimens [36]. Tanaka et al. also evaluated the cfDNA in 15 healthy individuals, nine with benign gynaecologic diseases, and 53 with EC. They analysed Alu sequences in free DNA fragments by RTqPCR as surrogate markers and found that cfDNA levels in EC tended to be higher than in healthy and benign conditions, although there was no significant difference in cfDNA among stage or histological grade of EC, and no significant changes before and after surgery [37]. A recent report detected augmented levels of total cfDNA and mitochondrial cell-free DNA (cfmtDNA) in serum of patients with EC compared to benign lesions using a SYBR Gold assay and qPCR, respectively. Importantly, they observed that this increase was significantly larger in high grade EC [46]. The same group also explored the cfDNA

integrity index as a rapid and noninvasive biomarker that might provide complementary information for diagnosis, prognosis, and treatment stratification in cancer patients [38].

Recently, Zou et al., developed an algorithm called eTumorType to identify different cancer types, including 149 cervical squamous cell carcinoma and endocervical adenocarcinoma and 401 uterine corpus endometrial carcinoma [39]. This test is based on copy number variations (CNVs) of the cancer founding clone, modelling cancer hallmark-associated genes, and integrates cancer hallmark concepts and a few computational techniques. Relatively high accuracies from 0.63 to 0.92 were obtained for these gynaecologic tumours using eTumorType, indicating its value in non-invasive diagnosis [39]. More interestingly, the use of personalised ctDNA biomarkers in gynaecologic cancers including EC could demonstrate the presence of residual tumour; in addition, ctDNA predicted response to treatment in a more dynamic manner relative to currently used serum and imaging studies [40]. Patient/tumour-specific mutations were identified using whole-exome and targeted gene sequencing, and ctDNA levels were quantified using droplet digital PCR. Of particular interest, ctDNA was an independent predictor of survival. Early detection of disease persistence and/or recurrence and the ability to stratify patients in better and worse outcome groups by ctDNA surveillance may improve survival and quality of life in patients with endometrial cancer [40].

Epigenetic markers have also demonstrated great potential for the identification of different tumours. In fact, the methylation status of a number of genes has been described as an accurate tool for cancer detection. For example, SEPT9 showed value for colorectal cancer identification, while MGMT methylation detected brain tumours [47,48]. Margolin et al., described a specific hypermethylation at the ZNF154 CpG island in EC tissues compared to normal controls. These results in tissue were also validated in silico for blood testing [49]. Although these are promising results and methylation markers present advantages in comparison with point mutations, there is still a need for a methodological standardisation to implement these markers in clinical routine.

3.4. Circulating Exosomes/miRNAs

Extracellular vesicles containing proteins, lipids, and DNA/RNAs involved in intercellular communication are also considered as alternative forms of liquid biopsy [50]. Among them, exosomes (approximately 100 nm nanovesicles) released from endometrial epithelial cells are an important component of these interactions, as not only are they restricted to tumour cells, but endometrial cancer cells can transmit small regulatory RNAs to endometrial fibroblasts via exosomes [51]. Isolated exosome-like vesicles could become an attractive source of biomarkers, including RNA, by the analysis of the specific inner cargo by RTqPCR from uterine aspirates [52].

For diagnostic purposes, circulating miRNAs (particularly in plasma/serum) have appeared as an important source of clinical material [53]. Torres et al., studied microRNA expression in plasma samples of patients with EC for the first time [54]. They found high expression levels of miR-99a, miR-100 and miR-199b in plasma samples from patients in comparison with healthy controls. The combined analysis for plasma miR-99a/miR-199b resulted in 88% sensitivity and 93% specificity discriminating patients vs. controls, indicating a good diagnostic potential. A more recent study analysed 16 miRNAs in plasma of 34 EC patients and 14 controls, finding miR-9/miR-1228 and miR-9/miR-92a signatures as a good diagnostic tool (Area Under Curve, AUC values ~0.9) [55]. After a genome wide serum miRNA expression analysis, Jia et al., identified miR-222, miR-223, miR-186 and miR-204 up-regulation as a powerful signature for EC detection (AUC of 0.927) [56]. More recently, in a meta-analysis including EC patients, Gao et al., also demonstrated that serum miR-21 could be serve as a novel biomarker for EC. They found higher serum miR-21 levels in patients with benign lesions ($p = 0.003$) and EC ($p < 0.001$) than healthy controls, showing that EC patients also have higher expression levels ($p < 0.001$) than benign lesions [41]. Interestingly, in addition to serum and plasma samples, urinary miRNAs were also explored in patients with EC, finding a specific down-regulation of miR-106b in comparison with healthy donors [57]. All these results evidence a great potential of miRNA signatures in liquid biopsies

as valuable information in EC, although until now there is no consistent and clinically validated signature of miRNAs for a reliable clinical management of EC patients.

4. Conclusions

Although liquid biopsy can be considered a reality in the clinical setting of some types of cancers, such as breast, colorectal or prostate, it remains as a promising field in gynaecological oncology. The value of liquid biopsy-related technologies in endometrial cancer, such as diagnostic/screening tools based on tumour material in uterine aspirates and as prognostic/monitoring tools based on tumour material in circulation like CTCs or ctDNA (Table 1), needs to be analytically and clinically validated in large clinical trials. Moreover, the potential combination of different and complementary types of liquid biopsy for surgery stratification and during follow-up in intermediate/high-risk and advanced EC patients might result in a comprehensive strategy targeting the identification of mutations for the evaluation of residual disease, the early detection of recurrence, the selection of personalised therapies and disease relapse associated with therapy resistance (Figure 1). These type of strategies appropriately designed and validated in clinical trials, including accurate technical and cost-effective evaluations, represent an opportunity for precision medicine in gynaecological oncology.

Funding: This research was funded by Instituto de Salud Carlos III, grants PI14/01942 and PI17/01919, co-financed by the European Regional Development Fund (FEDER).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Abbreviations

BEAMing	Beads, Emulsion, Amplification and Magnetics based digital PCR
CEC	Circulating Endothelial Cells
cMV	Circulating Microvesicles
CNVs	Copy Number Variation
CTCs	Circulating Tumour Cells
CK	Cytokeratin
ctDNA	Circulating Tumour DNA
DTC	Disseminated Tumour Cells
D&C	Dilatation and Curettage
EC	Endometrial Carcinoma
EEC	Endometrioid Endometrial Carcinoma
EMA	European Medicines Agency
EMT	Epithelial to Mesenchymal Transition
EPC	Endothelial Progenitor Cells
NEEC	Non-Endometrioid Endometrial Carcinoma
NGS	Next Generation Sequencing
NSCLC	Non-Small Cell Lung Carcinoma
PFS	Progression-free Survival
TCGA	The Cancer Genome Atlas
TAm-Seq	Tagged-amplicon deep sequencing

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Review

Targeted Therapies in Type II Endometrial Cancers: Too Little, but Not Too Late

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Received: 29 June 2018; Accepted: 8 August 2018; Published: 13 August 2018

Abstract: Type II endometrial carcinomas (ECs) are responsible for most endometrial cancer-related deaths due to their aggressive nature, late stage detection and high tolerance for standard therapies. However, there are no targeted therapies for type II ECs, and they are still treated the same way as the clinically indolent and easily treatable type I ECs. Therefore, type II ECs are in need of new treatment options. More recently, molecular analysis of endometrial cancer revealed phosphorylation-dependent oncogenic signalling in the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways to be most frequently altered in type II ECs. Consequently, clinical trials tested pharmacologic kinase inhibitors targeting these pathways, although mostly with rather disappointing results. In this review, we highlight the most common genetic alterations in type II ECs. Additionally, we reason why most clinical trials for ECs using targeted kinase inhibitors had unsatisfying results and what should be changed in future clinical trial setups. Furthermore, we argue that, besides kinases, phosphatases should no longer be ignored in clinical trials, particularly in type II ECs, where the tumour suppressive phosphatase protein phosphatase type 2A (PP2A) is frequently mutated. Lastly, we discuss the therapeutic potential of targeting PP2A for (re)activation, possibly in combination with pharmacologic kinase inhibitors.

Keywords: endometrial cancer; type II endometrial carcinoma; targeted therapy; kinase inhibitor; molecular marker; protein kinase; protein phosphatase; PP2A; *PPP2R1A*; SMAP

1. Introduction

Cancer is responsible for 1 in 6 deaths worldwide, and is thereby the second leading cause of mortality [1]. In 2012, there were on estimation 6.6 million new cancer cases and 3.5 million cancer deaths, specifically for women. Endometrial cancer accounted for 2.1% of these cancer deaths, and 5% of these cancer cases, making it the fourth most common cancer in women. Moreover, global incidence rates for endometrial cancer are still rising each year. In the USA for example, incidence rates have risen about 24% over a period of 23 years and are expected to rise another 35% by 2030 [2]. Additionally, even though technological advancements have improved standard therapies, mortality rates for endometrial cancer are still increasing. Over the last 15 years, there has been an increase in endometrial cancer related deaths of about 15% [1–5], supporting the notion that therapies for endometrial cancer need to be urgently revised and improved. Recent advances in the understanding of the molecular mechanisms of gynaecological cancers and the discovery of potential molecular markers through large-scale genomics studies have paved the way to implementing targeted therapeutics, an approach already successfully used in other cancers.

In this review, we discuss the therapeutic potential of targeting phosphorylation-dependent oncogenic signalling, particularly in type II endometrial cancers. The initial focus is on the use of pharmacologic kinase inhibitors as targeted therapeutics. However, the biochemical antagonists of

protein kinases, the protein phosphatases, have more recently also come into the limelight as potential targets in cancer, particularly in type II endometrial carcinomas, where the tumour suppressive phosphatase protein phosphatase type 2A (PP2A) is frequently mutated. Moreover, PP2A (re)activation therapies may be of significant relevance to improve kinase inhibitor treatments. Therefore, we argue that targeted therapies, that take the tumour type and molecular profile of endometrial cancers into account, should be implemented on a more rational basis in clinical trials.

2. Endometrial Carcinomas: Histologic Classification and Diagnosis

Most endometrial cancers (97%) are epithelial lesions arising from the lining of the uterus, known as endometrial carcinomas (ECs) [2]. In 1983, Bokhman classified ECs into two types, based on the histopathology of the tumour [6]. Type I EC is composed of oestrogen-dependent, mostly low-grade endometrioid tumours and represents up to 80% of all ECs [6,7]. Endometrioid EC generally has a favourable prognosis with a five-year-survival rate of more than 80% due to its indolent clinical course and early stage detection [8]. On the other hand, type II ECs are high-grade by definition, with a poor prognosis, and mainly include three distinct histologies: Serous adenocarcinomas (10–20%), clear cell adenocarcinomas (<5%) and carcinosarcomas (<5%) [9–16]. Carcinosarcomas consist of a sarcoma as well as a carcinoma component. This carcinomatous component can display serous or endometrioid histology. However, most carcinosarcomas develop from a serous precursor and consequently have more similarities with high-grade serous tumours [17]. Hence, carcinosarcomas are treated like type II ECs [18]. Here, we will mainly focus on the serous ECs, which are most common within the type II ECs. Generally, type II ECs are oestrogen-independent and have an aggressive clinical course with late stage detection and a high tendency for early metastasis and extra-uterine spread. For example, several studies reported extra-uterine disease at the time of diagnosis in over 65% of patients with serous EC compared to only 4% of patients with type I endometrioid EC [9,19–21]. Serous EC was found to spread most often to the pelvic and paraaortic lymph nodes (in more than 40% of cases) but also to the cervix, ovaries and lungs [16,21–23]. Owing to these aggressive characteristics, serous EC has a high recurrence rate and a very poor five-year survival rate of less than 30% [9,12,19,24]. Consequently, serous ECs are responsible for almost half of the EC-related deaths, even though they represent only 10–20% of all ECs [9]. This indicates more effort has to be made in terms of treatment for patients with type II EC.

To date, standard treatment for all endometrial cancers is surgery, followed by adjuvant therapy based on grade and stage of the tumour [23]. Therefore, it is important to diagnose patients with the correct type of EC, in order to avoid over- or undertreatment. Several tools are used for diagnosing patients with endometrial cancer. Ideally, an accurate diagnosis is made without the need for surgery. In these pre-operative analyses, tools like the Papanicolaou (Pap) test can be used. However, the sensitivity of Pap tests in the detection of suspicious glandular cells is very low [25]. Additionally, transvaginal ultrasound (TVU), magnetic resonance imaging (MRI) and computed tomography (CT) scans can help with pre-operatively diagnosing tumour stage. However, the results from these tests are not always conclusive, resulting in incorrect clinical estimations in over 20% of cases [26–28]. Therefore, the golden standard technique for diagnosing endometrial cancer consists of two procedures. First, an endometrial biopsy tissue is taken, and histological analysis of this tissue is used to decide on tumour grade and subtype based on Bokhman's classification. Second, surgical staging is performed to accurately determine the extent of the disease according to the staging system developed by the International Federation of Obstetrics and Gynaecology (FIGO) [23,29]. Determining tumour subtype solely based on histological information is, however, rather subjective and prone to error, resulting in poor reproducibility. This was demonstrated in a study by Gilks et al., in which 3 independent reviewers disagreed about the grade of the tumour (low-grade versus high-grade) in 36% of the cases [30]. Moreover, also serous versus endometrioid histology was found to be a frequent point of disagreement. This means that patients with high-grade serous EC could be diagnosed as low-grade endometrioid EC and consequently receive inadequate surgery and treatment. Hence, there is a clear

need for a more objective classification of the tumours, which recently became feasible through analysis of the genomic profiles of endometrial cancers. For example, Ratner et al. proposed the use of miRNA signatures to differentiate between EC subtypes [31]. However, mutational analyses of cancers made an even more elaborate classification possible, based on the molecular gene profiles of the tumours.

3. Genomic Classification of Endometrial Carcinomas

Since the human genome project in 1990 and the emergence of next-generation sequencing, the focus of cancer research has shifted towards the molecular level [32]. This led to the identification and characterisation of many molecular alterations associated with different cancers. In turn, these molecular alterations helped us to understand the underlying mechanisms of tumorigenesis.

One of the pioneering initiatives investigating the molecular profile of several cancers was The Cancer Genome Atlas (TCGA). Initially, they analysed 3281 tumours from twelve cancer types, among which endometrial carcinoma [33]. TCGA objectively classified endometrial tumours based on their molecular profiles, revealing four major genomic groups: Polymerase ε (POLE) ultra-mutated, microsatellite instability (MSI), copy number low and copy number high [34]. The first three groups display endometrioid histology while the last group mostly involves serous histology. Based on this classification, Kommoss et al. designed the molecular classification tool ProMisE (Proactive Molecular Risk Classifier for Endometrial Cancer) [35]. This tool can be applied to endometrial biopsies and consistently identifies four prognostic genomic groups. ProMisE was successfully validated in a retrospective cohort and is now being clinically evaluated. Correct genomic classification of the tumour can help to form a more objective and accurate diagnosis of the tumour type, consequently leading to the right therapy. Nevertheless, high-grade tumours like serous ECs are always treated with adjuvant therapy (e.g., chemotherapy and/or radiation therapy), even though they show low response rates [36–41]. This emphasises the need for new predictive biomarkers and improved targeted therapies within this fourth EC subgroup, in particular for patients with resistant and recurrent type II ECs.

4. Molecular Markers in Endometrial Carcinomas

Molecular analysis of endometrial tumours allowed for the identification of mutations in oncogenes and tumour suppressor genes, thereby exposing the oncogenic signalling pathways for these cancers. ECs revealed to be most frequently mutated in *TP53*, *PPP2R1A*, *FBXW7*, *PIK3CA*, *PTEN*, *ARID1A*, *CTNNB1* and *KRAS*. Additionally, type II ECs also frequently have *HER2* gene amplifications. An overview of the frequency of these mutations in type I and II ECs can be found in Table 1.

Table 1. Most common genetic alterations in type I and type II endometrial carcinomas (EC). Percentages in the header refer to all EC cases; percentages in the table refer to each EC subtype.

Common Genetic Alterations	Type II Serous Carcinoma (10–20%)	Type II Clear Cell Carcinoma (<5%)	Type II Carcinosarcoma (<5%)	Type I Endometrioid Carcinoma (±80%)
TP53	57.7–92% [34,42–46]	29–46% [47–50]	64.3–91% [17,42]	10.1–14% [34,42,51]
PPP2R1A	15.4–43.2% [34,42–46,52–54]	15.9–36% [47–50,55]	0–28.1% [17,42,54,56,57]	2.5–6.9% [34,42,52–54]
FBXW7	17.3–29% [34,43–45]	7.9–25% [43,48–50,55]	39% [17]	10–12% [42,51]
PTEN	2.7–22.5% [34,42,45,46]	11–21% [50,55,58]	19–33.3% [17,42]	67–84% [34,42]
ARID1A	0–10.8% [34,42]	15–21% [48–50,55]	12–23.8% [17,42]	40–46.7% [42,59]
PIK3CA	10–47% [34,42–46]	23.8–36% [48–50,55]	17–35% [17,42,60]	38–55% [42,51]
CTNNB1	2.7% [42]	0% [47,48]	4.8% [42]	23.8–52% [34,42,48,51]
KRAS	2–8% [42,51]	12–16.7% [17,42]	14% [17]	16.6–26% [42,51]
HER2	17–44% [34,45,61–63]	12–50% [61–64]	0–20% [61,65]	1.4–30% [62,66]

TP53 encodes the transcription factor and tumour suppressor p53, and is the most commonly mutated gene in human cancers [67]. However, *TP53* mutations occur at a much lower frequency in type I ECs (<15%) (Table 1). Remarkably, high-grade endometrioid ECs have more frequent mutations

in *TP53* (up to 30%) [34]. This indicates *TP53* mutations are associated with a poor prognosis in endometrial cancer, which is also demonstrated by cBioportal survival data [56,57]. These survival data report a five-year overall survival rate of 60% for patients with *TP53* mutations compared to up to 90% for patients without *TP53* mutations. So far, therapeutic targeting of p53 has mostly been limited to pre-clinical studies testing small molecules, but toxicity towards healthy cells was a frequent problem [68].

The second most mutated gene in type II ECs turned out to be *PPP2R1A*, encoding the Aα subunit of the Ser/Thr-specific PP2A phosphatase, a known tumour suppressor [69]. PP2A phosphatases, for instance, regulate growth factor-induced Raf/Extracellular signal-Regulated Kinase (ERK) signalling, phosphatidylinositol-4,5-bisphosphate 3-Kinase (PI3K)/Akt signalling, mammalian target of rapamycin (mTOR) signalling, and WNT signalling [70–72]. Remarkably, somatic mutations in *PPP2R1A* occur at high frequencies in type II ECs (up to 40%), while only a low percentage is found in type I endometrioid ECs (<7%) (Table 1). Additionally, the few *PPP2R1A* mutations found in endometrioid ECs are mostly correlated with high-grade endometrioid EC, suggesting *PPP2R1A* mutations are associated with aggressiveness of the tumour and poor patient outcome [73]. Moreover, cBioportal survival data indicate a five-year survival rate of 50% for patients with serous EC harbouring *PPP2R1A* mutations compared to 80% for patients without *PPP2R1A* mutations [56,57]. However, these data only include 12 patients. Therefore, a larger group of patients with type II ECs will need to be investigated in order to get more conclusive results about the prognostic marker potential of *PPP2R1A*. Interestingly, *PPP2R1A* mutations occur early during progression in the precursor lesions and are able to distinguish serous EC from the clinicopathological similar ovarian high-grade serous carcinomas, which rarely harbour *PPP2R1A* mutations [44,52].

FBXW7 encodes the tumour suppressive FBOX protein, a component of the Skp, Cullin, F-box (SCF)-ubiquitin ligase complex [74]. This complex targets phosphoprotein substrates for ubiquitination and subsequent proteasomal degradation. *FBXW7* mutations are most frequently reported in type II ECs (Table 1) and mainly affect the substrate binding WD repeats of the FBOX protein resulting in loss of function of the SCF-complex and hence (onco)protein accumulation. Interestingly, mTOR is one of the substrates of this SCF-complex. Consequently, inactivating mutations in *FBXW7* can result in PI3K pathway activation through mTOR stabilisation [75].

The PI3K pathway in type II ECs is also often affected by recurrent mutations in *PIK3CA* and *PTEN* (Table 1). *PIK3CA* encodes the p110α catalytic subunit of the class IA PI3Ks, which catalyse phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP_2) resulting in phosphatidylinositol 3,4,5-trisphosphate (PIP_3). Thus, *PIK3CA* mutations lead to the constitutive activation of PI3K signalling [76]. *PTEN* encodes the phosphatase and tensin homolog (PTEN), a lipid as well as a protein phosphatase. As a lipid phosphatase, PTEN is the functional antagonist of PI3K, and specifically dephosphorylates PIP_3. Hence, inactivating mutations in *PTEN* mostly result in overactivation of PI3K signalling. *PTEN* is mutated at low frequencies in type II ECs while mutated at very high frequencies (up to 84%) in type I endometrioid ECs (Table 1). The higher frequency of *PTEN* mutations reported in type II carcinosarcomas compared to the other type II ECs could be explained by its biphasic nature, containing carcinoma and sarcoma elements. Specifically, *PTEN* mutations were reported in the carcinoma component resembling endometrioid histology and not in the component resembling serous histology [77]. However, here we made no distinction between the mutational profiles of the serous-like and endometrioid-like carcinomatous component within the carcinosarcomas. Nevertheless, most carcinosarcomas resemble type II serous tumours. This is also indicated by their general mutational profile, which is more closely related to type II serous ECs than to type I endometrioid ECs (Table 1) [17,78].

ARID1A encodes the BAF250A tumour suppressor and is functionally involved in the SWI/SNF chromatin-remodelling complex [79]. *ARID1A* mutations are less common in type II ECs than in type I ECs (Table 1). Interestingly, *ARID1A* mutations can result in PI3K pathway activation via downregulation of PI3K interacting protein 1 (PIK3IP1). Furthermore, inhibition of the EZ2H

methylesterase in *ARID1A* mutated ovarian cancer cells results in synthetic lethality, suggesting EZ2H as a potential new therapeutic target for *ARID1A* mutated tumours [80–83].

CTNNB1 encodes β-catenin and is mutated at lower frequencies in type II ECs compared to type I ECs (Table 1). β-catenin is an important component of the canonical WNT signalling pathway and stimulates transcription of genes associated with proliferation and cell survival [84]. β-catenin is negatively regulated by glycogen synthase kinase 3β (GSK-3β) which phosphorylates β-catenin leading to its degradation. Most *CTNNB1* mutations result in evasion of GSK-3β-mediated phosphorylation resulting in nuclear accumulation and consequently, increased cell proliferation [85].

Furthermore, *KRAS* mutations have been reported in ECs, although at low frequencies for both EC types (Table 1). *KRAS* encodes the small GTPase K-Ras, which is an oncoprotein involved in the activation of several signalling pathways, including the PI3K and mitogen-activated protein kinase (MAPK) pathways, and in the activation of other small GTPases, such as RalA [86]. *KRAS* mutations result in Ras proteins with constitutively bound GTP, consequently activating these downstream oncogenic pathways.

Lastly, also *HER2* gene amplification was identified as a common genetic alteration in ECs, with a higher frequency in type II ECs compared to type I ECs (Table 1). *HER2* encodes the human epidermal growth factor receptor 2 (HER2), which belongs to the Epidermal Growth Factor Receptor (EGFR) family of Receptor Tyrosine Kinases (RTKs) and stimulates signal transduction via the PI3K and MAPK pathways [87]. Consequently, *HER2* amplification/overexpression can lead to oncogenic overactivation of the PI3K and MAPK pathways. Furthermore, *HER2* amplification/overexpression was found to be correlated with a worse prognosis for patients with EC [88].

In summary, the identification of these nine most altered genes and related signalling pathways involved in tumorigenesis of type II ECs, opens opportunities for the rational development of targeted therapies in the clinical management of these tumours. Remarkably, seven out of nine (*PPP2R1A*, *FBXW7*, *PIK3CA*, *PTEN*, *ARID1A*, *KRAS*, *HER2*) of the type II EC-associated genes encode proteins involved in regulation of the PI3K pathway. Furthermore, some of these genetic alterations (*PPP2R1A*, *KRAS* and *HER2*) can also lead to activation of the MAPK pathway. Thus, the logical next step would be to test therapeutics targeting the affected PI3K and/or MAPK pathways in patients with type II ECs. Specifically, based on the specific nature of the most recurrent molecular alterations found in type II ECs, the therapeutic targeting of phosphorylation-dependent oncogenic signalling seems a particularly promising strategy to improve current treatments—especially, since a large variety of pharmacologic kinase inhibitors, targeting activated PI3K or MAPK signalling, have already been developed. In addition, targeting of the counteracting protein phosphatases has recently moved into the limelight as novel promising cancer therapeutics, and may also be applied to specific molecular subsets of type II ECs.

5. Therapeutic Potential of Targeting Kinases and Phosphatases in Endometrial Carcinomas

5.1. Successes of Kinase Inhibitors as Targeted Cancer Therapies

During the last three decades, protein kinases have gained enormous interest as potential therapeutic targets in many common cancer types. A well-known example is the use of the US Food and Drug Administration (FDA)-approved Tyrosine Kinase Inhibitor (TKI) imatinib in treatment of patients with chronic myeloid leukaemia (CML) [89]. More than 90% of these patients are characterised by the presence of the Philadelphia chromosome and its oncogenic product, the constitutively active BCR-ABL tyrosine kinase. Imatinib inhibits this kinase and significantly improves outcome of patients with Philadelphia chromosome-positive CML. Another example can be found in treatment of non-small cell lung cancer (NSCLC), where two main targeted therapies affecting protein kinases are currently clinically applied. The first ones are the FDA approved EGFR tyrosine kinase inhibitors (e.g., erlotinib and gefitinib), which show high response rates in patients with NSCLC harbouring *EGFR* mutations [90,91]. The second ones are the FDA approved anaplastic lymphoma kinase (ALK)

inhibitors (e.g., crizotinib and ceritinib), which show positive effects in tumours harbouring *ALK* gene rearrangements [92,93]. Lastly, also breast cancer therapy has successfully implemented targeted therapies in a molecularly stratified group. For example, patients with breast cancer tumours overexpressing the HER2 receptor (in up to 30% of cases) showed favourable response to the FDA-approved anti-HER2 monoclonal antibody Trastuzumab [94–96]. A list of other FDA-approved drugs targeting cancers with specific mutations can be found on the online tool OncoKB [97]. Therefore, from these examples, the overall image has emerged that by stratifying patients based on the affected gene/pathway, targeted therapies can prove to be much more effective. However, despite these positive results, this strategy is still not applied for treatment of endometrial cancers.

5.2. Targeted Therapies in Endometrial Carcinomas: Mainly Geared towards PI3K Signalling

Therapies for endometrial cancer still tremendously lag behind. So far, the only FDA-approved targeted therapies for ECs are hormonal intervention (for hormone-dependent endometrioid ECs) and the immune checkpoint inhibitor pembrolizumab [98]. However, for type II ECs, there are no approved targeted therapies, even though these patients need it the most. Molecular analysis revealed the PI3K pathway to be most frequently involved in tumorigenesis of ECs (Table 1) [99]. This led to the clinical evaluation of targeted therapies against this pathway, which can be affected at different levels via multiple kinase inhibitors (Figure 1) [100]. Initially, clinical trials mainly tested derivatives from rapamycin (e.g., everolimus, temsirolimus), single mTOR kinase inhibitors primarily targeting the mTORC1 complex, in unstratified groups. However, the outcome with these single agent inhibitors turned out to be rather disappointing as reviewed by several research groups [101–104].

Two main biochemical mechanisms inherent to the PI3K pathway could explain for these disappointing results. The first mechanism is the presence, as in many signalling pathways, of negative feedback loops preventing overactivation of the PI3K pathway under normal conditions (Figure 1). Briefly, stimulation of the PI3K pathway leads to mTORC1 activation and, consequently, to phosphorylation and activation of its downstream substrate p70S6 kinase. In turn, p70S6 kinase inhibits PI3K signalling via two feedback loops [105]. The first one acts via phosphorylation and inhibition of the insulin receptor substrate (IRS)-1, a docking protein for PI3K. The second one acts via phosphorylation of mTORC2, leading to its inhibition. mTORC2 is located upstream of mTORC1 and is involved in Akt phosphorylation and activation. Pharmacologic mTORC1 inhibitors disrupt these negative feedback loops, resulting in constitutive activation of the PI3K pathway, thereby abolishing the effect of mTOR inhibition [106]. Furthermore, mTOR independent targets of Akt (e.g., Forkhead box (FOXO), GSK-3β) can also contribute to increased cell proliferation and survival [107].

Therefore, additional blocking of the PI3K pathway via dual mTORC1/2 inhibitors or PI3K/mTOR inhibitors was thought to be able to circumvent these problems associated with single mTORC1 inhibition. Indeed, pre-clinical studies testing mTORC1/2 and PI3K/mTOR inhibitors demonstrated improved anti-tumour activity compared to single agent mTOR inhibitors, although Shoji et al. reported no persistent effect of the PI3K/mTOR inhibitor in vitro and in vivo [108,109]. Furthermore, a phase II clinical trial where a dual PI3K/mTOR inhibitor was used had limited success due to its poor tolerability [110]. Nevertheless, patients with confirmed response all had mutations in the PI3K pathway. Additionally, Weigelt et al. showed endometrioid EC cell lines with mutations in the PI3K pathway to be more sensitive to PI3K and mTOR inhibitors [111], while *KRAS* mutant ECs did not react to mTORC1 treatment in a phase II clinical trial [112]. This all suggests that PI3K/mTOR pathway inhibitors are more efficient in tumours with activated PI3K signalling.

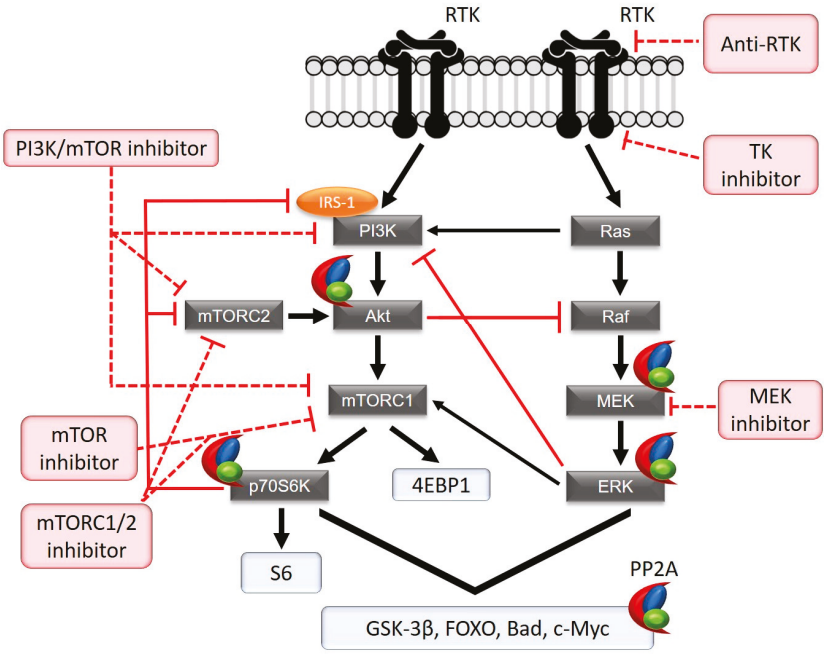

Figure 1. Schematic overview of the PI3K and MAPK pathways and important substrates. Black arrows represent activation. Red lines represent endogenous inhibition through feedback/cross-talk. Dotted red lines represent inhibition with pharmacologic kinase inhibitors. The heterotrimeric PP2A complex is represented in red (A subunit), blue (B subunit) and green (C subunit). Both pathways can be targeted at several levels using pharmacologic kinase inhibitors. However, single agent inhibitors targeting mTORC1 (e.g., everolimus) will deactivate the negative feedback loops from p70S6K to mTORC2 and PI3K. The use of a dual PI3K/mTOR inhibitor could circumvent this problem. There is also cross-talk between the PI3K and MAPK pathway, which could be evaded by using the combination of a PI3K and MAPK (e.g., MEK inhibitor) pathway inhibitor. Anti-RTKs (e.g., Trastuzumab) target the extracellular domain of the RTKs. TK inhibitors (e.g., Lapatinib) target the intracellular tyrosine kinase activity of the RTKs. Furthermore, PP2A acts as a tumour suppressor on many components of both pathways and should therefore be considered when targeting kinases. Additionally, it is an attractive target for activation, and hence PI3K and MAPK pathway downregulation, potentially in combination with kinase inhibitors. The PI3K and MAPK pathways have several substrates in common (GSK-3β, FOXO, Bad and c-Myc), which are involved in cell proliferation and cell survival. Some substrates, like FOXO and GSK-3β, are activated by Akt, independently of mTORC1. 4EBP1: eukaryotic translation initiation factor 4E-binding protein 1, Bad: Bcl-2-associated death promoter, FOXO: forkhead box protein, GSK-3β: glycogen synthase kinase 3β, IRS-1: insulin receptor substrate 1, mTOR: mammalian target of rapamycin, PI3K: phosphatidyl-4,5-bisphosphate 3-kinase, PP2A: protein phosphatase 2A, RTK: receptor tyrosine kinase, S6: ribosomal protein S6, TK: tyrosine kinase, MAPK: mitogen-activated protein kinase; ERK: extracellular signal-regulated kinase; MEK: mitogen-activated protein kinase kinase.

This view was further sustained by several pre-clinical trials in which serous EC cell lines with and without *HER2* amplifications were used, and three PI3K pathway inhibitors were tested (mTORC1/2, PI3K and a dual PI3K/mTOR inhibitor) [113–115]. In all of these studies, the *HER2* amplified cells (with activated PI3K pathway) were more sensitive towards the PI3K pathway inhibitors than the wildtype *HER2* cell lines. Furthermore, some of these cell lines had additional mutations in

PIK3CA, which rendered them even more sensitive in case of dual PI3K-mTORC1/2 inhibition [113]. This certainly advocates for the implementation of genotype-dependent patient stratification in clinical trials, so that kinase inhibitors could be tested on a more rational basis. Nevertheless, in two phase II studies of mTORC1 inhibitors, *PIK3CA* and *PTEN* mutations in EC tumours could not predict patient outcome [112,116], but this could be due to the use of an agent targeting just a single kinase (as explained above). Until now, however, most clinical trials for endometrial cancers had no biomarker restrictions [101], likely also explaining the poor results.

The second mechanism, likely to be responsible for the poor response rates to mTOR inhibitors involves the phenomenon of "cross-talk", in which there is (altered) signalling to other pathways—here, most often, the MAPK pathway. Cross-talk between the PI3K and MAPK pathways is well-established and elaborately reviewed by Mendoza et al. [107]. Briefly, both PI3K and MAPK pathways can regulate each other positively as well as negatively (Figure 1). For example, Akt (PI3K pathway) phosphorylates and inhibits Raf (MAPK pathway), thereby blocking MAPK signalling. On the other hand, ERK (MAPK pathway) negatively regulates PI3K and stimulates mTORC1. Therefore, in case of PI3K pathway inhibition, cross-inhibition from Akt to Raf is relieved, resulting in activation of the MAPK pathway and hence, treatment resistance. Furthermore, since the PI3K and MAPK pathways often target the same substrates (e.g., FOXO, c-Myc, Bad, GSK-3β), cross-activation will ensure these substrates remain activated even after PI3K pathway inhibition. Such cross-activation due to PI3K pathway inhibition is also reported in other cancers. For example, inhibition of the PI3K pathway resulted in MAPK pathway activation in NSCLC cell lines [117]. Additionally, also breast cancer studies reported this inhibitor-induced activation of the MAPK pathway after administration of a single mTOR inhibitor [118]. Furthermore, the authors of this study observed that a Mitogen-activated protein kinase kinase (MEK) inhibitor was able to reduce MAPK activation and reduce cell growth. Remarkably, the combination of the mTOR and MEK inhibitor had an additive effect, resulting in stronger cell growth reduction. Furthermore, in EC cell lines, PI3K/mTOR inhibition resulted in MAPK pathway activation [109], and the combination of a MEK inhibitor and PI3K/mTOR inhibitor improved anti-proliferative effects.

Furthermore, pharmacologic targeting of HER2 has also gained interest as a potential therapeutic strategy for patients with type II ECs, especially since positive results were obtained for patients with *HER2* positive breast cancer as mentioned in Section 5.1. Therefore, anti-HER2 compounds like Trastuzumab (anti-HER2 antibody) and Lapatinib (small molecule TKI) were also tested in ECs. However, single agent targeting of HER2 with Trastuzumab or Lapatinib had no effect in vitro (serous EC cell lines) and in vivo (xenografts) [119]. In contrast, dual inhibition with Trastuzumab and Lapatinib in serous EC xenografts showed significant anti-tumour activity and resulted in decreased phosphorylation of downstream PI3K and MAPK signalling proteins. Remarkably, this effect was only observed in the *HER2*-amplified serous EC cell lines, indicating the importance of patient stratification based on *HER2* status of the tumour. This important paradigm was further underscored in several publications of Santin et al., in which the responses of serous EC cell lines with or without *HER2* amplification to HER2 inhibitors were assessed [120–124]. They consistently observed that the *HER2* amplified cell lines showed a higher sensitivity for these targeted inhibitors than the cell lines without *HER2* amplification. Furthermore, *HER2* amplified cells with additional *PIK3CA* mutations showed resistance to the anti-HER2 monoclonal antibody Trastuzumab, while *HER2* amplified cells with wildtype *PIK3CA* were sensitive towards Trastuzumab [120]. This clearly indicates the benefit of molecular stratification and advocates the implementation of such stratification in clinical trials. Not surprisingly, clinical trials testing the efficacy of Trastuzumab and Lapatinib in unstratified patient populations had poor response rates [125,126]. Furthermore, these trials tested single agent inhibitors of the PI3K pathway, probably activating several resistance mechanisms (as mentioned above).

Ongoing or recently completed clinical trials for type II serous ECs registered in ClinicalTrials.gov were searched using the keyword "serous endometrial adenocarcinoma". Strikingly, out of 36 matching clinical trials, only 16 focussed on targeted therapies with or without combination with adjuvant

therapy. The other 20 trials solely used adjuvant therapies. Remarkably, only three out of 16 trials using targeted therapies (NCT02491099, NCT00506779, NCT01367002) specifically recruited type II serous ECs and had biomarker restrictions. Currently, two of these clinical trials (NCT02491099 and NCT01367002) are ongoing and promising results in favour of molecular stratification have already been obtained in NCT01367002 [127]. The other 13 trials testing targeted therapies, unfortunately, included both type I and type II ECs in their study and did not select patients based on their mutational profiles. Once more, this clearly illustrates that, even up to date, with molecular data of type II ECs at hand, and despite the proven clinical successes of targeted therapies in molecularly well-defined tumours, a change in mind-set among clinical oncologists is definitely still needed to improve the setup, and thereby (hopefully) the outcome, of novel clinical trials in (type II) EC.

Summarised, several (pre-)clinical results have indicated that dual pathway inhibition could evade the problem of cross-talk and improve treatment efficacy in PI3K or MAPK pathway driven endometrial cancers. Furthermore, these studies have illustrated that molecular stratification of ECs is critical in efficient testing of kinase inhibitor therapies. On the other hand, it is also known that the clinical success of kinase inhibitors in cancer therapy is often of limited duration, as over time, many patients develop therapy resistance, for example by acquiring mutations in the drug target [128]. However, based on the biochemical logic that not just a kinase, but both kinases and phosphatases regulate the phosphorylation of any phosphoprotein under normal conditions, there is an emerging view that inhibition of phosphorylation-dependent oncogenic signalling may only be efficiently achieved by targeting both kinases and phosphatases [129,130]. Specifically, one cannot expect to efficiently revert hyperphosphorylation of an oncoprotein just by inhibiting the oncogenic kinase involved, if the counteracting tumour suppressive phosphatase is no longer active. In terms of avoiding acquired kinase inhibitor resistance, a combination therapy of a kinase inhibitor and a phosphatase activator may indeed be much more effective [130]. Particularly for type II ECs, where *PPP2R1A* is mutated at high frequencies, opportunities may be ahead to exploit the phosphatase PP2A for therapeutic purposes.

5.3. Targeting the Phosphatase PP2A in Type II Endometrial Carcinomas

The tumour suppressive nature of PP2A was first established through the observation that the tumour promotor okadaic acid (OA), which specifically inhibits PP2A, resulted in cellular transformation in mouse skin [69]. Later on, functional studies in several human epithelial cells have underscored that inhibition of PP2A (typically achieved by expression of SV40 small T antigen) is an absolute requirement to fully transform an immortalised human cell, despite the overt activation of oncogenic kinases (e.g., typically downstream of an established oncogene, such as *RAS*) [131]. PP2A phosphatases are heterotrimers composed of a catalytic C subunit, a scaffolding A subunit, and a regulatory B subunit (Figure 2) [132]. SV40 small T antigen was shown to inhibit PP2A through displacement of the PP2A B subunits [69]. The A and C subunit each have two isoforms, α and β, of which the α isoform is the most common. The A subunit forms a scaffold for the catalytic C and regulatory B subunits and is composed of 15 HEAT repeats, each consisting of two anti-parallel α-helices connected by an intra-repeat loop (Figure 2) [132]. These intra-repeat loops allow protein-protein interactions with the catalytic C subunit and regulatory B subunits. More precisely, the B subunits are known to bind to HEAT repeats 1–10 while the C subunit binds to HEAT repeats 11–15. [133]. The regulatory B subunits are divided into four families (B/PR55/B55, B′/PR61/B56, B′′/PR72, B′′′/Striatins), each containing several isoforms, and thereby resulting in a vast array of PP2A holoenzymes [132]. The B subunits are responsible for subcellular localisation and determine substrate specificity of the PP2A complexes. This way, PP2A complexes negatively regulate a variety of signalling pathways involved in carcinogenesis (Figure 1) [71,72,134]. Consequently, inactivation of PP2A has been associated with several human cancers [135–137], and increased rates of spontaneous or carcinogen-induced oncogenesis in mice [138–141]. Therefore, therapeutic targeting of PP2A has gained interest and has been focussing on the direct or indirect (re)activation of PP2A (reviewed

in [142]). How exactly this should be achieved, might in part be determined by the mechanism(s), if any, by which PP2A is inactivated in the tumour.

Strikingly, most type II EC-associated heterozygous missense mutations in *PPP2R1A*, encoding the Aα subunit isoform of PP2A, cluster in HEAT repeats 5 and 7, encoded by exon 5 and 6, respectively. These *PPP2R1A* mutations frequently recurred at the same positions (P179, R183 and S256) across different cancer types, as indicated by several comprehensive studies [143–145]. In type II ECs, these so-called *PPP2R1A* hotspot mutations were recurrently found in codons: P179, R182, R183 (HEAT repeat 5) and S256, W257 (HEAT repeat 7) (Figure 2) [17,44,45,48,52,54,144,146,147]. Remarkably, based on structural studies, the same residues or neighbouring residues were predicted to be important for interaction with the B subunits [133,148]. However, experimental evidence for this hypothesis revealed a much more sophisticated image, in that mutations at these positions indeed resulted in binding deficiencies of Aα mutants with several regulatory B subunit types, but specifically preserved binding to others, most notably to B56δ and B56γ [149,150]. Some mutations, e.g., p.(P179R), also diminished binding to the catalytic C subunit [149]. Moreover, Haesen et al. suggested a dominant negative mechanism for these mutants, as the PP2A trimers that could still be formed proved catalytically impaired through the increased recruitment of a cellular PP2A inhibitor, TIPRL1 [149]. Accordingly, ectopic expression of the Aα mutants increased anchorage-independent cell growth in vitro, xenografted tumour growth in vivo, and resulted in hyperactivation of, again, the PI3K/Akt/mTOR pathway. Interestingly, downregulation of the MAPK pathway was seen in these conditions, suggesting cross-activation might not be an issue. Thus, type II ECs with *PPP2R1A* driver mutations might be sensitive towards single pathway (PI3K/mTOR) inhibitors. Hence, these results open a window of opportunity for the use of kinase inhibitors targeting the PI3K/mTOR pathway in type II ECs, harbouring an oncogenic mutation in *PPP2R1A*, potentially in combination with pharmacologic PP2A activators.

5.3.1. Direct Targeting of PP2A

Recently, a lot of interest has gone into the discovery of Small Molecule Activators of PP2A (so-called SMAPs) that appear to be able to allosterically activate PP2A. In 2014, the FDA-approved tricyclic neuroleptic drug perphenazine was found to have anti-proliferative effects in T-cell acute lymphoblastic leukaemia (T-ALL) through binding and activation of PP2A, and subsequent downregulation of both the PI3K and MAPK pathways [151]. At first, the extrapyramidal side effects and high concentrations necessary for PP2A activation made the perphenazines not appealing as anticancer drugs. However, Kastrinsky et al. reengineered these drugs to abrogate the CNS activity and enhance the anti-proliferative effects, resulting in the new molecular class of "small molecule activators of PP2A" (SMAPs) [152]. SMAPs were able to increase PP2A activity in *KRAS* mutant lung cancer cells, resulting in significantly decreased cell survival [153]. This was further established in lung cancer cell xenografts in mice, where SMAP treatment significantly inhibited tumour growth. Moreover, single agent SMAP treatment was as efficient as the combination treatment of an Akt (PI3K pathway) and MEK (MAPK pathway) inhibitor. This indicates SMAPs are able to inhibit both PI3K and MAPK pathway simultaneously, hence eliminating the problem of cross-activation of oncogenic pathways when using a single kinase (pathway) inhibitor, as well as avoiding potential tolerance problems of combinations of more than one kinase inhibitor. Furthermore, castration-resistant prostate cancer cells showed sensitivity towards SMAP treatment in vitro, as well as in xenografts [154]. The exact mechanism by which SMAPs are able to activate PP2A is not known yet, although Sangodkar et al. convincingly reported direct binding of SMAPs to the Aα subunit of PP2A (Figure 2). More precisely, residues K194 and L198 within HEAT-repeat 5 were necessary for the interaction with these compounds [153].

These pre-clinical data indicate it might be interesting to test SMAPs as treatment for ECs, which are mainly MAPK and PI3K pathway driven. Moreover, SMAPs might be able to re-activate PP2A in type II ECs which have frequent *PPP2R1A* mutations. However, SMAPs bind in close proximity (K194,

L198) to the residues of the Aα subunit that are most frequently mutated (P179, R182, R183) in type II ECs [153]. Therefore, these mutations might influence SMAP binding to the Aα subunit and result in resistance to SMAPs. Future research into this potential issue should eventually allow to clarify whether *PPP2R1A* could be used as a predictive marker for SMAP treatment in type II ECs.

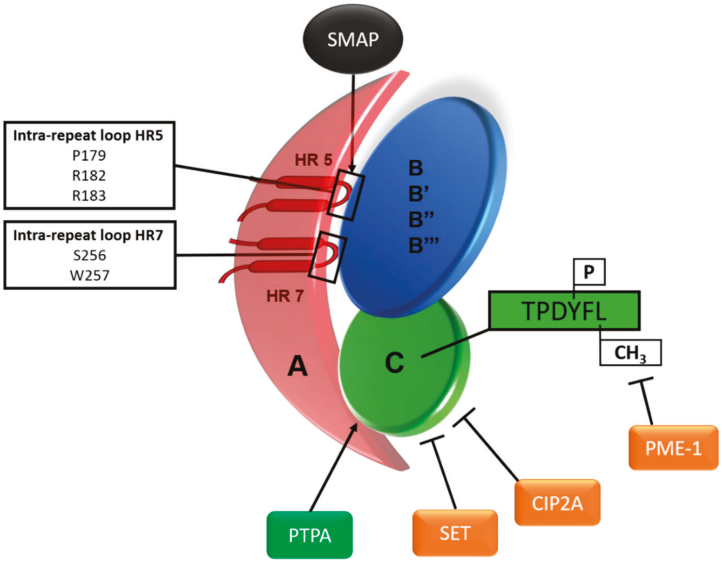

Figure 2. The heterotrimeric PP2A complex with activating and inactivating mechanisms. Heat repeat (HR) 5 and 7 are represented with their intra-repeat loops in subunit A. These intra-repeat loops harbour the most recurrent *PPP2R1A* hotspot mutations identified in type II ECs. Endogenous inhibition of PP2A can occur via SET, CIP2A and PME-1, which act on the C subunit. TPDYFL is the conserved motif in the C-terminal tail of the catalytic C subunit. Phosphorylation of the tyrosine (Y) is thought to cause inactivation, while methylation of the carboxyterminal leucine (L) of the C subunit promotes binding of specific B subunits, and thereby assembly of active trimers. PME-1 demethylates this leucine, and stabilises inactive PP2A complexes. PTPA is necessary for endogenous activation of inactive PP2A complexes. SMAP is a small molecule activator of PP2A, which binds at heat repeat 5 in close proximity to the hotspot mutations in *PPP2R1A*. The vast array of regulatory B subunits allows for the targeting of PP2A to many different substrates. A: Scaffolding A subunit; B, B′, B″, B‴: The four families of regulatory B subunits, each containing several isoforms; C: catalytic C subunit; CH_3: methyl group; CIP2A: cancerous inhibitor of PP2A; HR: heat repeat; P: phosphate group (PO_4^{3-}); PME-1: PP2A methylesterase 1; PTPA: phosphatase 2A phosphatase activator; SET: Suvar/Enhancer of zeste/Trithorax; SMAP: small molecule activator of PP2A.

5.3.2. Indirect Targeting of PP2A

Besides *PPP2R1A* mutations, other mechanisms of PP2A dysfunction in endometrial cancers have been described (Figure 2). Very commonly, PP2A inactivation in cancer occurs through overexpression of the endogenous PP2A inhibitors SET (Suvar/Enhancer of zeste/Trithorax) and CIP2A (cancerous inhibitor of PP2A), for example in chronic myeloid leukaemia and in many solid tumour types [136]. Therefore, many therapeutic strategies to target PP2A focus on the inhibition of these inhibitors, thereby indirectly re-activating PP2A. For example, FTY720 is a compound that is able to inhibit SET, resulting in increased PP2A activity [142]. In endometrial cancer, CIP2A overexpression is observed in endometrioid ECs, and siRNA mediated knockdown resulted in a decreased oncogenic phenotype [155]. However, to our knowledge no studies have investigated

CIP2A or SET overexpression in type II ECs yet. cBioportal data reveal *CIP2A* gene amplification in 4.65% of serous ECs, albeit in a total of just 43 cases [56]. Therefore, involvement of CIP2A or SET in carcinogenesis of type II ECs needs to be further explored, potentially leading to more therapeutic options for this disease.

PP2A inactivation can also occur through aberrant post-translational modifications of the C-terminal tail of the catalytic C subunit (Figure 2). These modifications occur on the conserved C-terminal motif (TPDYFL), where phosphorylation of the tyrosine (Y) results in PP2A inactivation, and methylation of the carboxyterminal leucine (L) is required for binding select B-type subunits and assembly of active PP2A trimers [156]. Consequently, components inhibiting phosphorylation or promoting methylation generally lead to activation of PP2A.

PP2A demethylation is catalysed by the PP2A methylesterase PME-1, and increased PME-1 expression has been associated with tumour progression in human malignant gliomas [157]. Notably, PME-1 overexpression was also observed in 83% (24/29) of tumours from patients with type I endometrioid ECs [158], and PME-1 overexpression in endometrioid EC cell lines resulted in increased cell proliferation and anchorage-independent growth. Xenograft experiments confirmed these in vitro data, showing increased tumour growth in case of PME-1 overexpression. PME-1 overexpression was associated with a strong reduction in PP2A activity, consequently leading to increased ERK and Akt phosphorylation. As expected, PME-1 knockdown increased PP2A activity leading to decreased phosphorylation and hence, decreased activation of ERK and Akt. This indicates that targeting PME-1 could be feasible for re-activation of PP2A. Furthermore, addition of an Akt inhibitor further decreased Akt phosphorylation, suggesting combination therapy of a PME-1 and an Akt inhibitor could enhance anti-tumour activity even more [158]. Recently, another study confirmed the potential of PME-1 inhibition as an anti-cancer treatment in EC. They reported decreased tumour growth in xenografts after PME-1 depletion using shRNA [159]. Furthermore, they tested two pharmacologic inhibitors of PME-1, ABL-127 and AMZ-30, in type I EC cell lines. ABL-127 was the most potent inhibitor, increasing PP2A activity by 45%. However, pilot studies testing ABL-127 did not reduce tumour growth. Altogether, these date indicate the potential of PME-1 inhibition as treatment for type I ECs, while not tested for type II ECs. cBioportal reports *PPME1* (encoding PME-1) amplification in 2.33% of type II serous ECs on a total of 43 cases [56]. Despite apparent low frequencies, it is definitely worthwhile to further explore PME-1 inhibition in type II ECs, which could lead to new therapies for this disease.

Another important recurrent mechanism for genomic PP2A inactivation found in several human cancers, involves haploinsufficiency of *PPP2R4*, caused by heterozygous loss or mono-allelic loss-of-function mutations. Heterozygous loss of *PPP2R4* was found in about 20% of all endometrial cancers with up to 70% for specifically the type II endometrial carcinosarcomas [56,57,139]. *PPP2R4* encodes the phosphatase 2A phosphatase activator (PTPA), an essential cellular PP2A activator, necessary for the generation of active PP2A complexes. Sents et al. demonstrated loss-of function in five cancer-associated PTPA mutants [139]. These mutants had decreased PP2A-C binding and a reduced ability to reactivate PP2A in vitro. Furthermore, ectopic expression of these PTPA mutants in PTPA depleted HEK-TER cells could not rescue the oncogenic phenotype in vitro (anchorage-independent growth) and in vivo (xenograft growth), while ectopic expression of WT PTPA could. Moreover, PTPA-deficient mice had significantly impaired PP2A activity, decreased methylation, and spontaneously developed tumours [139]. These data further indicate PP2A could be a new therapeutic target, in this case especially for type II endometrial carcinosarcomas, where heterozygous loss of *PPP2R4* occurs in up to 70% of cases. In particular, it would be of interest to investigate whether SMAPs might be able to activate the inactive PP2A complexes thereby compensating for the lack of functional PTPA.

6. Conclusions and Future Perspectives

Molecular alterations in type II ECs hold major potential for the development of targeted therapies which, according to the most recurrent alterations, should be focused on the inhibition of phosphorylation-dependent oncogenic signalling in the PI3K and MAPK pathways. To date, there are still too few clinical trials specifically for type II ECs. Additionally, most of these trials mainly focus on kinase inhibitors without stratifying patients based on the affected pathway, even though this is successfully done in other cancers. Consequently, clinical trial results for ECs have been rather disappointing. Furthermore, there is emerging evidence that the enzymes counteracting the protein kinases, the protein phosphatases, also need to be considered in order to efficiently inhibit these oncogenic pathways. This seems particularly important for type II ECs, which harbour frequent mutations in the tumour suppressive phosphatase PP2A.

In order to improve clinical trials, we propose the implementation of a dual stratification system based on the molecular profile of the tumours. Firstly, patients need to be stratified based on tumour type, in which tools like ProMisE could help to make a more objective stratification possible. To date, most clinical trials do not distinguish between endometrial cancer types. This is particularly disadvantageous for type II ECs, since they account for only 10–20% of ECs. Accordingly, results are biased towards the more common type I ECs and no conclusive results can be obtained for patients with type II EC even though they need targeted therapies the most. Since type II ECs are less common, the number of available patients may be a problem. Therefore, the implementation of multi-institutional clinical trials should be encouraged. Secondly, patients need to be stratified based on their molecular alterations and, hence, affected pathways. Accordingly, kinase inhibitors can be tested on a more rational basis, targeting the pathway that indeed shows oncogenic alterations. Furthermore, it will also be important to take the status of the tumour suppressive phosphatase PP2A into account, whose inactivation in fact also results in upregulation of the PI3K/Akt/mTOR pathway. Additionally, direct or indirect targeting of PP2A for (re)activation could be a potential new therapy for type II ECs. In particular, the new molecular class of PP2A activators, SMAPs, seem promising to achieve direct activation of PP2A, and consequently, suppression of oncogenic signalling. The combination of both a kinase inhibitor and a phosphatase activator is emerging as the most promising novel therapeutic approach, which could help to circumvent problems like pathway cross-talk and acquired kinase resistance, thereby improving treatment efficacy.

Therefore, therapeutic approaches for type II ECs, so far, have benefited too little from the available molecular data, but it is definitely not too late to improve future clinical trials in order to obtain effective targeted therapies for type II ECs.

Author Contributions: Writing, M.R.; Review & Editing, V.J.

Funding: M.R. is a recipient of an F.W.O.-SB fellowship of the Research Foundation-Flanders. V.J. is supported by grants from the Belgian Foundation against Cancer (FAC-F/2016/822), the Research Foundation-Flanders (F.W.O. G.0B01.16N) and the KU Leuven Research Fund (C24/17/073).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study, or in the writing of the manuscript.

Abbreviations

4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
ALK	Anaplastic lymphoma kinase
Bad	Bcl-2-associated death promotor
CIP2A	Cancerous inhibitor of PP2A
CML	Chronic myeloid leukaemia
CT	Computed tomography
EC	Endometrial carcinoma
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase

FDA	US Food and Drug Administration
FIGO	International Federation of Obstetrics and Gynaecology
FOXO	Forkhead box
GSK-3β	Glycogen synthase kinase 3β
HER-2	Human epidermal growth factor receptor 2
IRS-1	Insulin receptor substrate 1
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
mTOR	Mammalian target of rapamycin
NSCLC	Non-small cell lung cancer
OA	Okadaic acid
Pap	Papanicolaou
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIK3IP1	PI3K interacting protein 1
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-trisphosphate
PME-1	PP2A methylesterase 1
POLE	Polymerase ε
PP2A	Protein phosphatase 2A
ProMisE	Proactive Molecular Risk Classifier for Endometrial Cancer
PTEN	Phosphatase and tensin homolog
PTPA	Phosphatase 2A phosphatase activator
RTK	Receptor tyrosine kinase
S6	Ribosomal protein S6
SCF	Skp, Cullin, F-box
SET	Suvar/Enhancer of zeste/Trithorax
SMAP	Small molecular activator of PP2A
SV40	Simian virus 40
T-ALL	T-cell acute lymphoblastic leukaemia
TCGA	The Cancer Genome Atlas
TVU	Transvaginal ultrasound

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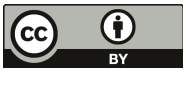

International Journal of
Molecular Sciences

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Review

Class I Phosphoinositide 3-Kinase *PIK3CA*/p110α and *PIK3CB*/p110β Isoforms in Endometrial Cancer

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Received: 30 October 2018; Accepted: 3 December 2018; Published: 7 December 2018

Abstract: The phosphoinositide 3-kinase (PI3K) signalling pathway is highly dysregulated in cancer, leading to elevated PI3K signalling and altered cellular processes that contribute to tumour development. The pathway is normally orchestrated by class I PI3K enzymes and negatively regulated by the phosphatase and tensin homologue, PTEN. Endometrial carcinomas harbour frequent alterations in components of the pathway, including changes in gene copy number and mutations, in particular in the oncogene *PIK3CA*, the gene encoding the PI3K catalytic subunit p110α, and the tumour suppressor *PTEN*. *PIK3CB*, encoding the other ubiquitously expressed class I isoform p110β, is less frequently altered but the few mutations identified to date are oncogenic. This isoform has received more research interest in recent years, particularly since PTEN-deficient tumours were found to be reliant on p110β activity to sustain transformation. In this review, we describe the current understanding of the common and distinct biochemical properties of the p110α and p110β isoforms, summarise their mutations and highlight how they are targeted in clinical trials in endometrial cancer.

Keywords: phosphoinositide 3-kinase; *PIK3CA*; *PIK3CB*; p110α; p110β; endometrial cancer

1. The Phosphoinositide 3-Kinase Pathway

The phosphoinositide 3-kinase (PI3K) signalling pathway is essential for a myriad of cellular processes and is among the most altered pathways in human cancers, including endometrial cancer [1,2]. Consequently, many cellular processes, coined as hallmarks of cancer [3], are dysregulated due to increased PI3K signalling and contribute to tumour development and progression [4–6].

In this review, we aim to give an overview of the mechanism of action of PI3K enzymes in the context of endometrial cancer, in which the PI3K pathway is highly dysregulated. We first summarise current understanding on the two ubiquitously expressed isoforms of class I PI3Ks, p110α and p110β, at the biochemical level and review mutational and other events affecting these two isoforms in endometrial cancer. Finally, clinical trials employing selective inhibitors for p110α and p110β in advanced endometrial carcinomas are summarised and outcomes are reviewed when reported.

1.1. Class I PI3K Enzymes

PI3Ks are lipid kinases which phosphorylate the 3′ hydroxyl group on the inositol ring of the glycerophospholipid phosphatidylinositol (PtdIns), or its derivatives, polyphosphoinositides (Figure 1A). This family is divided into three main classes: class I, II and class III depending on their structure and substrate preference [7,8]. Class I PI3Ks, which are further divided into two sub groups,

IA and IB, are heterodimers consisting of a catalytic and a regulatory subunit. Class IA consists of a catalytic subunit, p110 (α, β or δ, each encoded by separate genes *PIK3CA*, *PIK3CB* and *PIK3CD*) interacting with one of the regulatory subunits (p85α and splice variants p55α and p50α: encoded by *PIK3R1*, p85β: encoded by *PIK3R2*, p55γ: encoded by *PIK3R3*). The class IB catalytic subunit p110γ (encoded by *PIK3CG*) associates with p84/p87 or p101. In vivo, class I PI3K phosphorylates PtdIns(4,5)P_2 to generate PtdIns(3,4,5)P_3 [9,10] (Figure 1B). Both class II and III generate PtdIns3*P*, whereas class II can also produce PtdIns(3,4)P_2 [11–13]. We refer the reader to other reviews on the two latter PI3K classes [14,15] as the focus of this review is on class I PI3Ks.

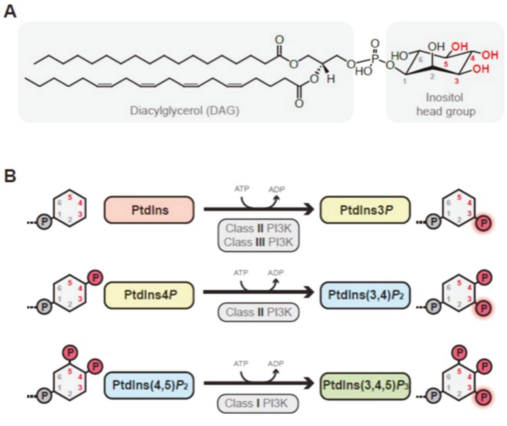

Figure 1. Chemical structure of phosphatidylinositol and phosphoinositide 3-kinase (PI3K) enzyme reactions. (**A**) Phosphatidylinositol chemical structure PI(18:0/20:4(5Z,8Z,11Z,14Z)) downloaded from the LIPID MAPS Structure Database (LM ID: LMGP06010010) [16,17]. Hydroxyl groups located at positions 3, 4 and 5 of the myo-inositol head group that are targeted by phosphorylation by polyphosphophoinositide kinases are highlighted in red. (**B**) Main enzymatic reactions carried out by the different phosphoinositide 3-kinase (PI3K) classes. Only the inositol head groups are shown with sites of phosphorylation marked in red. Phosphate groups are indicated with a circled P.

1.2. Activation of Class I PI3Ks and the PI3K Pathway

The activation of class I PI3Ks occurs downstream of the activation of receptor tyrosine kinases (RTK) (for p110α, p110β and p110δ) or G protein coupled receptors (GPCR) (for p110β and p110γ) [7,18] (Figure 2). Upon stimulation of tyrosine kinase receptors, phosphorylation of several tyrosine residues in the intracellular domains leads to the recruitment of the p85/p110 dimer, which releases the inhibition of the p110 catalytic subunit by p85. The mode of activation by GPCR involves the interaction between the Gβ/γ heterodimer and the linker present between the C2 and helical domains in the catalytic unit and their recruitment to the membrane [19,20]. When active and on the membrane, the p110 catalytic subunit can phosphorylate PtdIns(4,5)P_2 to generate PtdIns(3,4,5)P_3 [21]. Target proteins, such as Akt (alias Protein Kinase B), phosphoinositide-dependent protein kinase 1 (PDK1), as well as Sin1 (component of the mammalian target of rapamycin complex 2 (mTORC2), can bind to PtdIns(3,4,5)P_3 through their plextrin homology (PH) domain, thus recruiting them to the plasma membrane [22–26]. Akt is then activated by PDK1 and mTORC2 by phosphorylation at Thr308 and Ser473, respectively [27,28]. Activated Akt, in turn, acts as a downstream signalling molecule, which triggers the activation of multiple other downstream pathways that participate in many cellular processes.

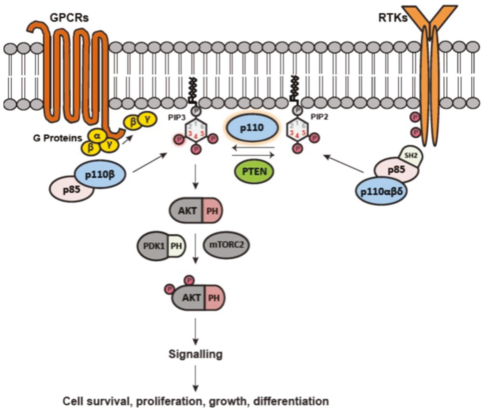

Figure 2. Class IA PI3K activation. Class IA PI3Ks consist of a catalytic subunit (p110) and a regulatory subunit (p85). Upon stimulation of receptor tyrosine kinase (RTK) or G-protein-coupled receptor (GPCR), the p85-p110 complex is targeted to the membrane via different mechanisms (due to tyrosine phosphorylation of RTKs or interaction with G-proteins β/γ). The p85 subunit loses its inhibitory effect on the catalytic activity of p110, and thereafter the p110 subunit phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP2) to generate phosphatidylinositol (3,4,5)-triphosphate (PIP3). PIP3 then targets proteins containing the pleckstrin homology (PH) domain—such as AKT, phosphoinositide-dependent kinase 1 (PDK1), as well as Sin1 (not shown), part of the mammalian target of rapamycin complex 2 (mTORC2) and locates them to the plasma membrane. AKT is then phosphorylated on Thr308 and Ser473 by PDK1 and mTORC2 respectively. Once these proteins are activated at the membrane, they trigger a signalling cascade that leads to multiple cellular functions. Phosphate groups are indicated with a circled P.

To precisely control the pathway, lipid phosphatases regulate the levels of PtdIns(3,4,5)P_3. The tumour suppressor phosphatase and tensin homolog (PTEN) dephosphorylates PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2, thereby negatively regulating the pathway [29]. Other phosphatases that dephosphorylate PtdIns(3,4,5)P_3 are the 5′ phosphatases, SH2 (Src homology 2)-domain-containing inositol phosphatase (SHIP) 1 and 2, that produce PtdIns(3,4)P_2 [30,31]. The action of these phosphatases allows PtdIns(3,4,5)P_3 to be kept at low levels in resting cells [32].

1.3. Properties of PI3K p110α and p110β and Mode of Activation in Cancer

Both p110α and β are ubiquitously expressed, unlike p110δ and p110γ, for which the expression tends to be restricted to the immune system [33]. Due to the context of this review, the focus is on p110α and β. Similarities and differences are summarised for these two isoforms (Table 1 and Figures 3 and A1) and we refer to two reviews for more detailed information [34,35]. Both p110α and β are embryonically lethal in homozygous mice knockouts, suggesting non-redundant functions [36,37]. Following these early studies, homozygous knockin mice with inactivating mutations in the ATP binding site in p110α (D933A) or p110β (D931A) both demonstrated embryonic lethality in an activity-dependent manner, albeit with different penetrance [38–40]. This strategy had the advantage of not disrupting the expression and stoichiometry of the catalytic/regulatory PI3K complex. In cell studies, p110α was shown to have a role in cell survival and p110β in DNA synthesis or cell proliferation [41–44]. Their distinct cellular localisation can be a feature explaining their different cellular roles. p110α is predominantly found in the cytoplasm, but can be detected at very low levels in the nucleus in some cells [45]. p110β is distributed in both the cytoplasm and nucleus including the nucleolus [43,45,46]. p110β has a nuclear localisation signal in the C2 domain, which is absent

in p110α ([45], shown in Figures 3 and A1) and plays multiple nuclear roles such as in cell cycle progression, DNA replication, and repair of DNA double-strand breaks [42,43,45,47].

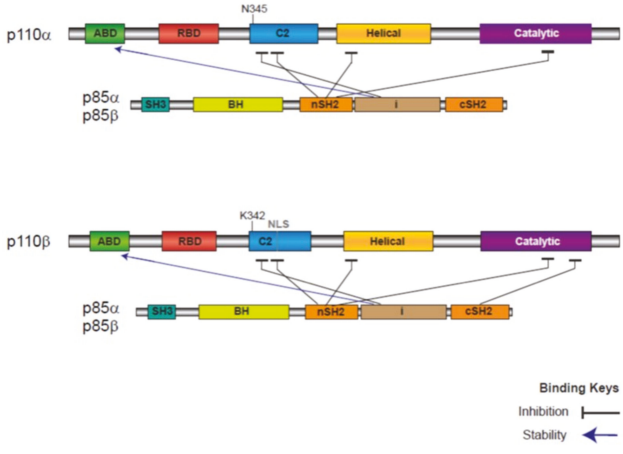

Figure 3. Domain structure of class IA PI3K catalytic and regulatory subunits. Domain structure and interaction of the catalytic subunits p110α and p110β and regulatory subunits p85α and p85β. The regulatory p85 subunit binds to p110 to inhibit and stabilise the lipid kinase. The interlinker domain of p85 binds to the ABD domain of the p110 subunit to stabilise the kinase (shown with an arrow). On the other hand, the nSH2 and interlinker domains in p85 have an inhibitory effect on both p110α and β. In addition, the p110β isoform can be inhibited by the cSH2 domain of p85. The figure is adapted from Reference [18]. Abbreviations: ABD, adaptor binding domain; RBD, Ras-binding domain, C2; protein-kinase-C-homology-2 domain, Helical; helical domain, Catalytic; kinase domain; NLS, nuclear localisation sequence; SH, Src homology; BH, breakpoint-cluster region homology; nSH2, N-terminal SH2; cSH2, C-terminal SH2, i, interlinker SH2 coiled-coiled domain.

Both enzymes share many structural and biochemical properties, since they use the same substrate to generate the same product, and thus activate the same effector proteins. They are multidomain proteins which have the same domain organisation and share the same mode of activation ([48], Figure 3, see also alignment of p110α and p110β in Figure A1). In particular, class IA p110 catalytic enzymes harbour an adaptor-binding domain (ABD) which interacts with the inter-SH2 linker (iSH2) of p85 and promotes stability. In addition, interaction of the N-terminal SH2 domain and iSH2 in p85 with the C2 and helical domain of p110 blocks basal catalytic activity [48]. Phosphorylated tyrosine residues on activated RTKs bind to the SH2 domains of p85s and release the inhibitory interaction of these domains with the p110 catalytic subunit. In p110β, an additional inhibitory contact between the C-terminal catalytic domain of p110 and the C-terminal SH2 domain of p85, entails a different mechanism to release the inhibitory interaction by phosphorylated RTKs [48,49].

In cancer, alterations in both catalytic isoforms have been reported, albeit at different frequencies [6]. Since the observation of high mutational frequency in human cancers suggests *PIK3CA* as a driver, much research effort has focused on this gene [50]. The most common activating mutations in *PIK3CA* are found in the helical domain (E542 and E545) and the kinase domain (H1047) and activate p110α through different mechanisms, such as reducing the inhibitory effect of p85 or facilitating the interaction with the lipid membrane [51–53]. In addition, mutations in p85 can lead to the stimulation of the p110 subunit, as shown not only for p110α but also for p110β [54,55]. A recent study by Thorpe et al. demonstrated that a decrease in p85α resulted in elevated p85-p110 complex signalling in vitro, correlating to increased tumour development in breast cancer mouse models [56]. As for *PIK3CB*, mutations are in general less frequent compared to *PIK3CA*, and *PIK3CB* can be

amplified or overexpressed in some solid tumours [6]. Importantly, p110β can promote oncogenic transformation when overexpressed in its wild type (WT) form, in contrast to p110α, which requires the presence of activating mutations [57].

A few key biochemical differences help explain the distinct mode of contribution in tumourigenesis for both isoforms. Firstly, a critical difference was identified in the C2 domain and this may explain their differential activation mode. Indeed, N345 in p110α is involved in hydrogen bond interactions with the iSH2 domain of p85. This residue aligns with K342 in p110β, which corresponds to oncogenic mutations found in p110α in some cancers, from N345 to K345 ([58], shown in Figures 3 and A1). This one residue difference decreases the inhibitory interaction of p110β with p85 [58] and may explain the transforming capacity, at least partially, of WT p110β compared to p110α, which requires mutation for transformation [57]. Secondly, in contrast to p110α, p110β can be activated by GPCRs through its direct association with the G protein subunits β/γ [19,59–62] and through the RAC (ras-related C3 botulinum toxin substrate) small GTPAse [63]. Importantly, activation of p110β by GPCR was required for cell invasiveness in breast cancer cells [62]. Cell proliferation was also dependent upon GPCR-mediated activation of p110β in PTEN-negative prostate and endometrial cancer cells, but not PTEN-positive cells [19].

A few mutations have recently been validated as oncogenic for *PIK3CB*. These include the helical domain mutation (E633K), identified in a Her2-positive breast tumour, which leads to increased p110β association with the membrane and increased basal activity [64]. A mutation in the *PIK3CB* kinase domain (D1067V), was shown to occur in several tumour types at low rates [65]. A gain-of-function mutation in the kinase domain of *PIK3CB* (E1051) was found to be an oncogenic driver and to promote cell growth and migration [66]. While studying the selective p110β inhibitor (GSK2636771) on solid tumours, a patient with castrate-resistant prostate cancer was found to have a mutation (L1049R) in the *PIK3CB* gene [67]. Functional characterisation of variants identified from cancer genome sequencing showed malignant transformation due to a rare mutation (A1048V) in the *PIK3CB* gene [68]. p110β also plays a role in the resistance of tumours to inhibitors of p110α [69,70]. A mutation (D1067Y) in *PIK3CB* has also been detected in cells resistant to pan-PI3K inhibition, which induced the activation of the PI3K signalling pathway [71]. Furthermore, p110β activity contributes to tumour progression and its expression correlates with poor prognosis and metastasis in breast cancer [59,72].

In 2008, a study which aimed to find the lipid kinase required to sustain and drive PI3K signalling in PTEN-deficient cancers showed that p110β played an essential role in these cancers and that its lipid kinase activity was required [73]. Later studies showed that, indeed, p110β activity is required in PTEN-deficient breast, prostate and haematological tumour growth [61,74,75], but not in other tissues which reported reliance on p110α or both isoforms [76,77]. A recent study showed that the PI3K pathway in PTEN-null tumours that rely on p110β are regulated by the Crk-like protein (CRKL) adaptor protein [78]. The study showed that CRKL has a preference to associate with p110β over p110α, and through its interaction with p110β/p85, it regulates the PI3K signalling pathway.

Table 1. Differences and similarities between p110α and p110β.

Property Description	p110α	p110β
Gene name	*PIK3CA*	*PIK3CB*
Regulatory subunit [1]	p85α, p55α, p50α p85β p55γ	p85α, p55α, p50α p85β p55γ
Cellular localisation	cytoplasm	cytoplasm, nucleoplasm, nucleolus
Receptor activation	RTKs	GPCRs (dominant) and RTKs
Mutations in carcinomas	frequent	rare

[1] p110α and p110β can, in theory, form heterodimers with any of the regulatory subunits, depending on the tissue of interest [33,79,80]. p85α and p85β are ubiquitously expressed, whereas the expression of the shorter isoforms p55α, p50α or p55γ is restricted to certain tissues [33,81]. Abbreviations: RTK: receptor tyrosine kinase. GPCR: G protein-coupled receptor.

2. Alteration of the PI3K Pathway in Endometrial Cancer

2.1. Endometrial Cancer

Endometrial cancer arises from lesions in the lining of the uterus (also known as the uterine corpus) which are, in up to 95% of cases, carcinomas with the remaining being sarcomas [82,83]. This gynaecological cancer is highly prevalent in developed countries, is highly associated with obesity and its incidence is rising [84,85]. Endometrial carcinomas have different histologies and were traditionally divided into two subtypes, type I and type II, according to the Bokhman classification [86]. Type I accounts for the majority of endometrial cancers and consists of low-grade tumours of endometrioid histology which are positive for hormone receptors and have a good prognosis [86,87]. These tumours are often referred to as endometrioid endometrial cancers (EEC). In contrast, type II tumours, also known as non-endometrioid (NEEC), are less common, of high grade, and hormone receptor negative with a poor prognosis [86,87]. Type II can display the following histologies: serous adenocarcinomas, clear cell adenocarcinomas and carcinosarcomas [86,87]. More recently, large-scale sequencing studies of primary endometrioid tumours (UCEC), initiated by The Cancer Genome Atlas (TCGA) genome network, suggested a classification of endometrial cancers into four major groups depending on different molecular signatures: (1) Polymerase ε (POLE), ultramutated with the highest survival outcome; (2) microsatellite instability (MSI), hypermutated with an intermediate outcome; (3) low copy number (endometrioid) with an intermediate outcome; and (4), high copy number (serous-like) with the poorest outcome [88]. This molecular classification has been further compared to and integrated with previous classification properties such as grade, genetic alterations and histology [87]. Type I tumours are hence considered endometrioid tumours with the following molecular characterisation: ultramutated POLE, microsatellite instability (MSI), hypermutated and a low copy number. Type II, including serous or clear cells, consists of the high copy number group. Specific molecular alterations representing each group can now be selected to evaluate endometrial tumours for diagnosis.

2.2. Alteration of PI3K PIK3CA and PIK3CB in Endometrial Cancer

The PI3K pathway is the most frequently altered in endometrial cancer [87–89]. The most frequent mutations are found in the *PTEN*, *PIK3CA* and *PIK3R1* genes, particularly in type I endometrioid tumours [88,90–93]. The distribution of mutations of these genes obtained from the endometrial cancer TCGA study is shown in Figure 4A [88,94,95]. PTEN loss is the most frequent alteration in endometrioid tumours with a frequency of up to 80% and mutations are often seen in hyperplasias, considered precursor lesions to endometrial cancer [96–100]. *PIK3CA* is the second most mutated gene, with overall frequencies of 25% of tumours according to the Catalog of Somatic Mutations In Cancer (COSMIC, v86 [101]) as well as of 51% and 53% in TCGA-UCEC (release 13) and the TCGA endometrial cancer genomic data, respectively ([88,95] Figure 4A,B and Figure A1). Mutations occur in both type I endometrioid and serous carcinomas ([88,92,98,102–105], Figure 4A). The effect of *PIK3CA* mutations on clinical variables is conflicting, as positive and negative associations have been shown in relation to survival [106]. However, in a recent study of *PIK3CA* mutations in exon 9, corresponding to part of the helical domain, mutations were associated with poor survival [106]. *PIK3CA* mutations occur early in endometrial cancer progression and are of high clonality from primary lesions to metastasis [99,100,106]. Other types of alterations in *PIK3CA* have also been reported. *PIK3CA* mRNA levels were higher in non-endometrioid tumours and increased from primary tumours to their corresponding metastasis, and high mRNA levels correlated with lower survival [44,106]. In addition, *PIK3CA* amplification, which is associated with high level PI3K signalling, correlates with NEEC/type II aggressive endometrial cancer phenotype [88,104,107,108] (Figure 4A).

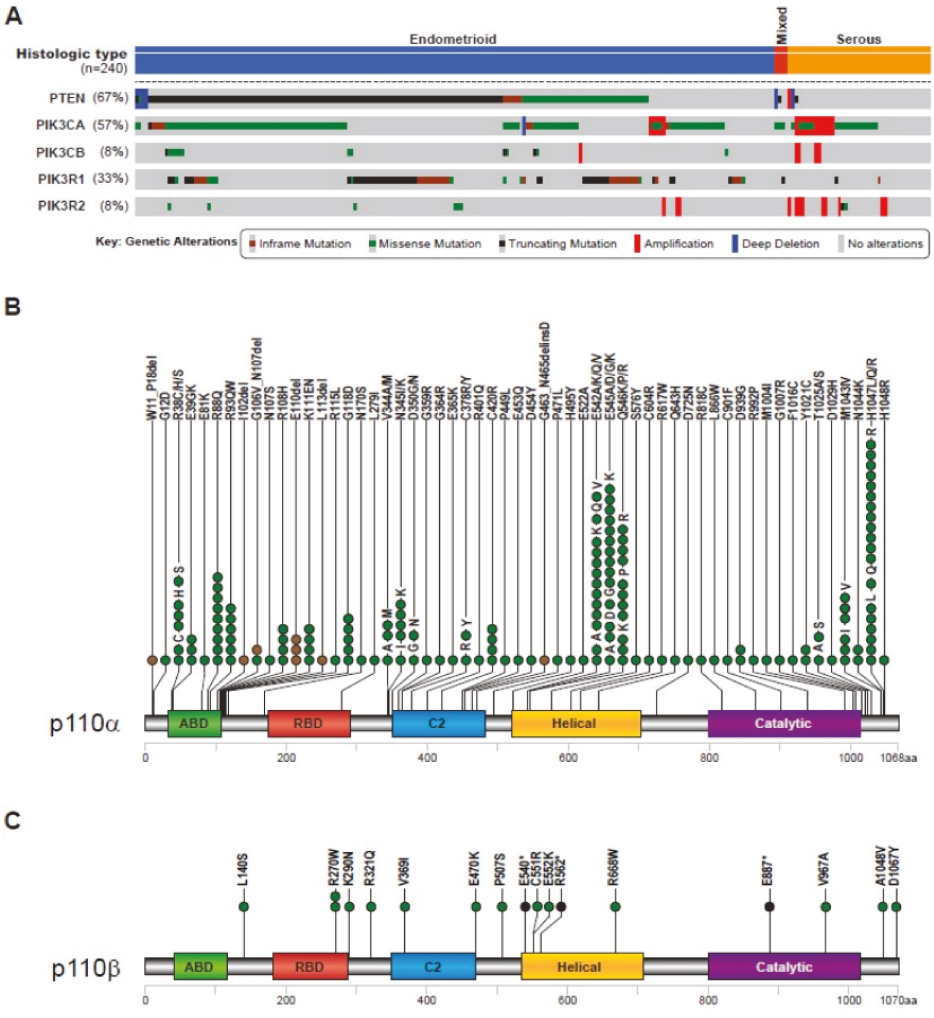

Figure 4. Overview of genomic alterations in PI3K pathway genes in endometrial cancer. (**A**) Overview of alterations in selected PI3K pathway genes in endometrial cancer, showing mutations and copy-number alterations, as well as the frequency of alterations. (**B**) Overview of mutations in *PIK3CA* displayed on the p110α protein structure. Note the hotspot mutational sites in p.E524, p.E545, p.Q546 and p.H1047, i.e., those with the highest number of lesions and the corresponding mutation. (**C**) Overview of mutations in *PIK3CB* displayed on the p110β protein structure by amino acid position. Panels were generated using the cBioPortal [94,95] with modifications with data from The Cancer Genome Atlas (TCGA) from a total of 240 patients with endometrial cancer [88]. Each dot corresponds to the occurrence of a specific mutation in a lesion. Mutations in (**B**) and (**C**) are colour-coded according to the key shown in (**A**) (green for missense mutations and brown for other in-frame mutations such as insertion and deletion). Abbreviations: ABD, adaptor binding domain; RBD, Ras-binding domain, C2; protein-kinase-C-homology-2 domain, Helical; helical domain, Catalytic; kinase domain.

The frequency of *PIK3CB* mutation is lower compared to the *PIK3CA* gene, with reported frequencies of 2% (COSMIC), 10% (TCGA-UCEC, release 13) and 8% (TCGA endometrial cancer genomic data [88,95], Figure 4B). So far, only two studies have reported the occurrence of *PIK3CB*

mutations in endometrial cancer, D1067V and A1048V within the kinase domain, and shown that they are oncogenic [65,68]. Other mutations were detected in the TCGA genomic study, highlighting other potential oncogenic mutations, which are to date uncharacterised for their oncogenic properties (Figure 4C). A more common alteration is an increase of its mRNA levels reported in two studies [44,109] and by COSMIC (v86) with a frequency of 6.8%. In particular, the mRNA levels of *PIK3CB* were shown to be higher in grade 1 endometrioid endometrial lesions when compared to complex hyperplasias and remained high in higher grades as well as in NEEC tumours [44]. Importantly, high levels of *PIK3CB* mRNA correlated with lower survival [44]. In cell line studies, the protein levels of p110β, but not of p110α, were elevated in endometrial cancer cells compared to non-transformed cells [44]. In addition, gene amplification was also detected, particularly in serous tumours (Figure 4A). Considering the transforming ability of p110β in its WT state, overexpression of this isoform may account for tumour development in endometrial cancer. Consistently, knock down of p110β in endometrial cancer cell lines induced cell death [109].

3. Targeting p110α and p110β in Endometrial Cancer

About 20% of endometrial tumours recur and respond poorly to currently available systemic therapy. Because the PI3K pathway is frequently altered in endometrial cancer, it is an attractive target for therapy, as recently validated by large-scale genomic sequencing reports [88,89,100,110,111]. PI3K chemical inhibitors have hence been tested in pre-clinical studies and used to target tumours with aberrant activation of the pathway.

3.1. PI3K Inhibitor Studies in Endometrial Cancer Cell Lines

A number of pre-clinical studies have been performed using endometrial cancer cell lines to test the efficacy of pan-PI3K inhibitors as well as selective p110α and p110β inhibitors [44,112,113]. *PIK3CA* mutant cancer cells were more prone to respond to pan-PI3K or selective p110α inhibition compared to WT cells [44,113].

Considering the importance of *PIK3CB*/p110β in PTEN-deficient tumours and the high frequency of PTEN mutations in endometrial cancer, the effect of p110β selective inhibition was evaluated in endometrial cancer cell lines, with or without PTEN loss [44,113]. Selective inhibition of p110α and p110β led to different effects on cell signalling and cell outcomes. p110α activity was correlated with cell survival and its inhibition led to decreased cell survival in *PIK3CA* mutant cells but not in WT cells [44,113]. In contrast, p110β inhibition had no effect on cell survival but rather decreased cell proliferation in PTEN-deficient cells [44,113]. Considering that endometrial tumours can harbour alterations in both *PIK3CA* and *PTEN*, combination treatment with both p110α and p110β may increase response efficacy compared to monotherapy [113]. The determination of the genetic status of *PIK3CA* and *PTEN* is of great importance for the most appropriate personalised treatment. In addition, the presence of genetic alteration in *PIK3CB* as well as increased levels of p110β may also influence treatment outcome in a few cases.

3.2. Clinical Trials in Endometrial Cancer

The pan-PI3K inhibitor Pictilisib (BKM120) was administered to patients with advanced or metastatic endometrial cancer but adverse side effects were observed, and the clinical trial was stopped ([114] NCT01397877). In contrast, Pilaralisib (SAR245408; XL147), another pan-PI3K inhibitor, used in a clinical trial of advanced or recurrent endometrial carcinomas, did not show any severe adverse events on patients but had only minimal anti-tumour activity ([115] NCT01013324). Finally, a trial planning to use the pan-PI3K inhibitor Copanlisib (BAY 80-6946) in patients with persistent or recurrent endometrial cancer with *PIK3CA* hotspot mutations was suspended (NCT02728258). Selective inhibitors for p110α and p110β are being used in clinical trials but the results in patients with endometrial cancer are scarce as they are part of large studies including different types of cancer. Patients with *PIK3CA*-altered advanced cancer were treated with the p110α selective inhibitor Alpelisib

(BYL719) and one patient with endometrial cancer showed a complete response and another showed a partial response to treatment ([116], NCT01219699). However, the study does not clearly state the total number of patients with this type of cancer and the respective mutational status.

A phase I trial with the p110β selective inhibitor GSK2636771 in PTEN-deficient solid advanced cancers, including three patients with endometrial cancer, was completed and reported (Reference [67] and Table 2). One patient out of three with endometrial cancer benefitted from progression-free disease for 33 weeks. Intriguingly, this patient did not harbour any mutation or gene copy variation in *PTEN*, *PIK3CA*, *PIK3CB* or *AKT2*. As we are writing this review, other clinical trials are currently recruiting patients with advanced solid tumours harbouring PTEN loss or with *PIK3CB* mutation and/or amplification and may include patients with endometrial cancer (Table 2).

Table 2. Completed and planned clinical trials using p110β inhibitors.

Drug Name	Molecular Condition for Trial	Types of Cancer	Phase	ID Number
GSK2636771	PTEN deficiency	advanced solid tumours	I	NCT01458067 (completed and results published [67])
GSK2636771	PTEN loss, mutation or deletion	Advanced-stage refractory solid cancers	II	NCT02465060 (recruiting patients)
AZD8186	PTEN loss, mutation or deletion	Advanced-stage refractory solid cancers	II	NCT02465060 (recruiting patients)
AZD8186 (with docetaxel)	PTEN loss or mutation, or *PIK3CB* Mutation	Advanced-stage solid cancers metastatic or unresectable	I	NCT03218826 (recruiting patients)

Information on clinical trials was retrieved from www.clinicaltrials.gov.

4. Conclusions

Alteration in the PI3K pathway is undoubtedly a key event in endometrial carcinomas with differences in molecular genetic features throughout histologies, stages and grades. Much of the research and clinical efforts have focused on *PIK3CA*/p110α but are starting to include *PIK3CB*/p110β in light of its association with loss of PTEN, the most frequent genetic alteration in endometrial carcinomas. To date, clinical trials include few patients with endometrial cancer, making it challenging to draw any reliable conclusions on the correlation between the genetic alteration status of the PI3K pathway and outcomes. Still, a positive outcome was reported for one patient treated with a selective p110β inhibitor who responded well to the treatment [67]. Several clinical trials with different p110β inhibitors are recruiting patients with advanced solid cancers with PTEN loss but also harbouring *PIK3CB* mutations or amplification. However, it is not yet known whether these will include endometrial carcinomas.

Author Contributions: F.M.G. and A.E.L. drafted the manuscript. All authors provided suggestions and comments on the manuscript and generated figures. All authors agreed to the final version.

Funding: Research in our laboratories is funded by the University of Bergen (AEL and CK), the Meltzer foundation (AEL, FMG and RGJ), the Norwegian Cancer Society (AEL and CK), the Helse Vest (EAH) and the Norwegian Research Council (CK). This work was also partly supported by the Centre of Cancer Biomarkers, a Centre of Excellence funded by the Research Council of Norways funding scheme, project number 223250.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

GPCR	G protein coupled receptor
PI3K	Phosphoinositide 3-kinase
PtdIns	Phosphatidylinositol
PtdIns(4,5)P_2	Phosphatidylinositol (4,5) bisphosphate
PtdIns(3,4,5)P_3	Phosphatidylinositol (3,4,5) triphosphate
PTEN	Phosphatase and tensin homologue
RTK	Receptor tyrosine kinase
SH2	Src homology 2

Appendix A

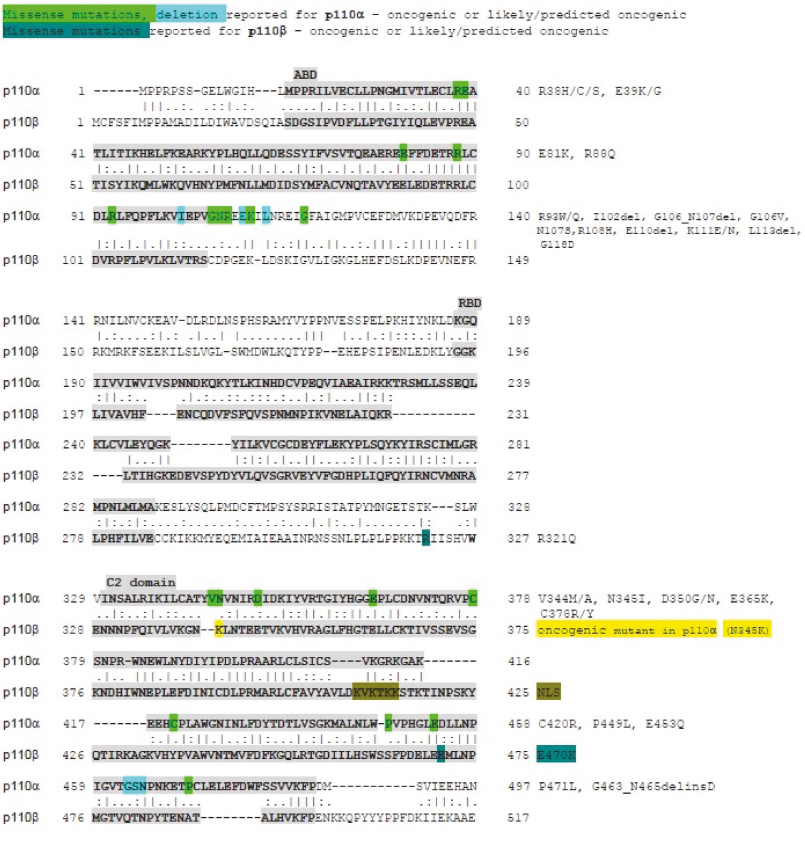

Figure A1. *Cont.*

Figure A1. Alignment of human p110α (UNIPROT P42336) and p110β (UNIPROT P42338) protein sequences using the pairwise sequence alignment web tool EMBOSS Needle. Mutations identified in the TCGA genome-wide sequencing in endometrial cancer are highlighted here for both isoforms with different colour code.

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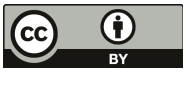

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Review

Patient-Derived Xenograft Models for Endometrial Cancer Research

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Received: 30 June 2018; Accepted: 13 August 2018; Published: 17 August 2018

Abstract: Endometrial cancer (EC) is the most common malignancy of the genital tract among women in developed countries. Recently, a molecular classification of EC has been performed providing a system that, in conjunction with histological observations, reliably improves EC classification and enhances patient management. Patient-derived xenograft models (PDX) represent nowadays a promising tool for translational research, since they closely resemble patient tumour features and retain molecular and histological features. In EC, PDX models have already been used, mainly as an individualized approach to evaluate the efficacy of novel therapies and to identify treatment-response biomarkers; however, their uses in more global or holistic approaches are still missing. As a collaborative effort within the ENITEC network, here we describe one of the most extensive EC PDX cohorts developed from primary tumour and metastasis covering all EC subtypes. Our models are histologically and molecularly characterized and represent an excellent reservoir of EC tumour samples for translational research. This review compiles the information on current methods of EC PDX generation and their utility and provides new perspectives for the exploitation of these valuable tools in order to increase the success ratio for translating results to clinical practice.

Keywords: orthoxenograft; uterine cancer; avatar; murine models; personalized medicine; targeted therapy; preclinical studies; translational research

1. Introduction

1.1. Cancer Models

Establishing suitable models is one of the cornerstones for cancer research. Among the most important and challenging are mouse models, since they have to mimic different steps of the disease and are used as tools for biomarker identification as well as preclinical models for therapy drug screening [1,2]. In the last 60 years, murine cancer models have evolved from cell-line-derived to genetically engineered mice (GEM) and tissue allo- or xeno-graft models [3,4]. The main differences among these models are the genetic similarity between tumours and the host (isogenic, allogenic, or xenogenic); the site of tumour injection, implantation, growth, and development (heterotopic versus orthotopic); and the immunological status or immunocompetence of the host. Although cell-line-derived and GEM models have led to significant advances in cancer biology and are still crucial for cancer research [5], they often fail to recapitulate key aspects of human malignancies and thus do not adequately predict drug effects in the clinic. In fact, the high failure rate of preclinical compounds in clinical trials clearly demonstrates the limitations of existing preclinical models [6,7]. Thus, there is an urgent need to develop more realistic and clinically relevant mice models that reliably represent the patient's tumour according to its genetic and molecular profile, its histopathology, the disease course and metastatic progression profile, and the therapy response [8]. The satisfaction of all these criteria will result in models with a close resemblance to human disease, enabling their use in preclinical trials with a high predictive value and significance for transferring results into the clinic. In this context, patient-derived xenograft (PDX) models have emerged as an excellent alternative to overcome these shortcomings [9,10]. PDX development is based on transplanting fresh cancer patient tissue samples directly into immunocompromised mice. In short, tumour tissue obtained directly from the operating room or from a biopsy is sliced into small fragments or disaggregated into cell suspension and surgically implanted or inoculated into immunocompromised mice. The most common mice strains used are SCID, NOD/SCID, NSG, and athymic nude mice [11]. The implantation or injection of the tumour fragment could be performed heterotopically or orthotopically. Tumours typically engraft over the course of weeks to months, depending mainly on tumour features (stage, grade, and aggressiveness). Upon engraftment and during exponential growth, the tumour is harvested and prepared for transplantation into one or various animals to develop a mice cohort of PDX that could be used for molecular characterization, biobanking, or as preclinical models. Serial expansions can take place for several passages maintaining tumour genetic fidelity [10,12].

As we have mentioned before, some research prefers to use tumour tissue fragments for PDX development, while other research uses a tumour cell suspension as the starting material. Each method has its advantages and disadvantages; tumour fragments retain cell–cell interactions as well as conserve tissue architecture, therefore mimicking better the tumour microenvironment. Alternatively, a single-cell suspension is a more heterogeneous sample that would represent unbiasedly the whole tumour; however, to obtain this type of sample, it is necessary to chemically or mechanically process the tissue, which affects cell viability and has the risk of decreasing engraftment success [13]. Also, the tumour implantation site is a crucial issue for PDX development. Heterotopic implantation occurs when the tumour fragment is implanted into an area of the mouse unrelated to the original tumour site, generally subcutaneously, in the interescapular region, the mammary fat pad, or the sub-renal capsular site. In contrast, orthotopical transplantation refers to when the patient's tumour tissue is put into the corresponding anatomical organ as the original primary tumour. Subcutaneous PDX rarely develop metastases in mice and it is difficult to use them to simulate the initial tumour microenvironment.

In contrast, orthotopic models can mimic the natural environment of a primary tumour, turning it into an ideal model to study the metastatic process. Nevertheless, these models can be difficult to generate depending on the organ of implantation [14].

In summary, the main features and advantages of PDX models are: (1) preservation of the genetic profile of the primary tumour and stability along passages into several animals; (2) retention of the histological and phenotypic features of the tumour, such as its tissue architecture, and the maintenance of stromal and stem cell components, cell-to-cell interactions, and spatial distribution; (3) amplification of tumour tissue biomass to facilitate tumour biology analysis, such as tumour characterization and biomarker identification; and (4) generation of a mice cohort with the same tumour that can be used as a preclinical model to test and predict anticancer drug response [15].

1.2. Endometrial Cancer

Endometrial cancer (EC) is the most common gynaecological cancer in developed countries and the sixth in mortality among all cancer types in women [16]. The majority of ECs are diagnosed at early stages when the tumour is still confined to the uterus and are frequently associated with vaginal bleeding as an initial symptom [17–19]. EC early detection is crucial to increase patient survival: the 5-year overall survival rate is around 80–95% in early stage tumours and falls to 20–60% in more advanced tumours [20,21].

Classically, EC has been classified into a dualistic model according to its biological, molecular, and clinical features. Type I or endometrioid endometrial carcinomas, comprising 80% of cases, are mainly represented by low-grade and hormone-receptor-positive tumours, while type II or non-endometrioid endometrial carcinomas are represented by papillary serous carcinoma, clear cell carcinoma, and carcinosarcoma, among other minor histologies, and are characterized by high-grade tumours and loss of hormone receptors [22–26]. Although serous, clear cell, and carcinosarcoma histologies represent only 10–15% of all EC cases, these minority subtypes account for up to 40% of all EC-related recurrences and subsequent deaths. This lower survival rate in comparison to endometrioid EC tumours is due, despite the fact that a growing list of evidence indicates the distinct nature of these subtypes at the molecular and clinical level, to non-endometrioid EC subtypes still being managed with the same adjuvant treatment as endometrioid EC tumours [27]. Similarly, the International Federation of Gynaecology and Obstetrics (FIGO) established a stratification system of EC according to its risk of recurrence based on postoperative pathologic information, such as histologic type, tumour grade, stage, and myometrial and lymphovascular invasion [26]. Nonetheless, 8% to 10% of early stage endometrial carcinoma develops recurrence and distant metastasis. Current classification systems have a limited potential to predict recurrence of EC patients, hence the need for more reliable systems to categorize and classify EC tumours to better tailor the clinical management of each individual patient.

Recently, the Cancer Genome Atlas Network (TCGA) performed an integrated genomic, transcriptomic, and proteomic analysis focusing on endometrioid EC and serous histologies, demonstrating that EC is a heterogenic disease. The TCGA identified and classified EC into four distinct molecular subgroups: POLE ultramutated (DNA polymerase epsilon), microsatellite instability hyper mutated, copy-number-low microsatellite stable, and copy-number-high serous-like [28]. Interestingly, TCGA molecular characterization data demonstrated that a quarter of the tumours classified by the dualistic model, as high-grade endometrioid EC, have a molecular phenotype similar to Type II serous non-endometrioid EC, including frequent TP53 mutations and extensive somatic copy number alterations (SCNA), thus suggesting that among these subtypes of patients similar clinical management should be considered beyond the histological classification. The molecular classification of EC has shown great promise, proving to be reproducible, demonstrating a higher association with clinical outcomes, and providing more valuable prognostic and predictive information in comparison to the dualistic classification system.

Considering that, researchers and clinicians are now enthusiastic and believe that a combination of both classification systems would be promising to precisely classify EC, thus improving the

management of EC patients. However, the road to an ideal scenario should still be paved with a deeper understanding of the ability to predict treatment-response and with an increased variety of effective therapeutic options for the different types of EC. Moreover, inter and intra-tumour genetic heterogeneity represents a challenge that should be faced in the current classification systems as this might have implications for the response to standardized or personalized treatments [29]. In this sense, the development of clinical relevant models for translational research is of great importance, since they represent the disease and could translate the beneficial results of preclinical trial and drug screening assays into clinics to improve EC treatment therapy.

2. Endometrial Cancer PDX Models

2.1. Strategies for EC PDX Model Development

Even though PDX development is widely described [10,30], several steps may differ across different research groups regarding EC PDX development. In this review, which is co-authored by European Network of Individual Treatment in Endometrial Cancer (ENITEC) members, we analyze strategies for PDX model development and provide an overall perspective on the value of PDX models for EC research.

Cabrera et al. was the first to describe the development of orthotopic PDX models using human EC tumour tissue [31]. To develop this model, tumour tissue resected from an EC tumour was grown subcutaneously in nude mice previously to orthotopic implantation. Once the tumour engrafted subcutaneously, tumour was removed, mechanically crumbled, and injected transvaginally or transmyometrially into nude mice, generating the orthotopic PDX models. Between the two different methods for orthotopic PDX model generation, the transmyometrial implantation of the tumour had a higher engraftment rate compared to the tranvaginal injection. They showed that orthotopically implanted tumours produced myometrial infiltration, lymph-vascular invasion, and dissemination in the pelvic cavity. In addition, tumours retained the molecular and histological characteristics of the original samples, reproducing glandular patterns and expressing hormone receptors.

Similarly, Haldorsen et al. [32] reported the development of an orthotopic PDX by mechanical dissociation of a primary tumour biopsy into a cell suspension, which was injected into the left uterine horn of NSG mice. Unlike Cabrera et al., in which PDX models have to be euthanized after 63 days as a consequence of tumour invasion, presence of pelvic mass, and ascites development, Haldorsen et al. showed that their PDX developed from a cellular suspension took longer to reach this phenotype: almost 10 months. However, they showed that their orthotopically grown tumours could be excised, disaggregated into a cell suspension, and reinjected to develop a next-generation cohort of orthotopic PDX mouse models.

Later on, Depreeuw et al. [33] developed and fully characterized a panel of 24 subcutaneous EC PDX models that includes more frequent histologic and genetic subtypes of EC. The authors demonstrated that EC PDX models can be successfully established from both primary, metastatic, and recurrent endometrioid EC and non-endometrioid EC tumours, with an overall engraftment rate of 60%. They also showed that these models closely resemble the tissue architecture and genomic features of the original human tumours. By whole-exome sequencing focused on cancer consensus genes, they found that most of the mutations were common between the primary human tumour and its paired PDX model in four cases. In addition, they evaluated genomic copy number alteration in both samples and found that, on average, 90% of the genome had the same copy number alterations between the primary tumour and the xenograft. Similarly, by immunohistochemistry staining of PDX and a primary sample with a human vimentin antibody, they showed that human EC stroma is replaced by murine stroma after engraftment.

On the other hand, Unno et al. [34] established a xenograft model by transplanting fragments of four different EC histologies, from patients undergoing surgery, into the renal capsule of NSG mice. Following this approach, the authors reported that tumour xenografts retain characteristics of the

original tumour and display features that are unique to endometrioid EC or non-endometrioid EC. In addition, they showed that each model has a different invasive and metastatic capacity according to its histology as well as a distinct dependence on β-estradiol [34]. Despite the technical difficulties presented by this type of approach, a renal capsule xenograft model is suitable for studying aggressive EC since tumour cells are in an environment susceptible to invasion and metastasis.

Recently, Pauli et al. [35] described the development of PDX from patient-derived tumour organoids (PDTOs). The authors collected metastatic and primary tumours from 18 different tumour types (two EC) and established culture organoids characterized by cytology and histology. Once established, PDTOs were subcutaneously injected into the flanks of nude mice. The xenograft take rate varied from 2 weeks to 16 weeks based on tumour type, and they reported an 86.4% engraftment rate of the PDTOs. They found that PDTOs and PDXs had similar histopathology to the parental tumours from which they were derived. The whole-exome sequencing genetic profile and single nucleotide variants analysis of PDTOs and PDXs showed excellent concordance with the patient's tumour.

2.2. Strategies for EC PDX Model Monitoring

The ability to monitor disease development is one of the major challenges when using mouse models. Tumour xenografts growing subcutaneously could be followed simply by visual inspection or palpation; however, the monitoring of orthotopic tumour xenografts requires the utilization of imaging techniques to follow-up tumour progression. In this section, we will summarize the most commonly used in vivo imaging techniques that are currently available to monitor tumour growth in PDX models. A full description of these techniques is reviewed by Dall'Ara et al. [36].

- Micro-computed tomography is a high image resolution technique with great potential for in vivo use since it could be coupled with other imaging modalities providing three-dimensional (3D) reconstruction of bone and soft tissue. It has been used to study bone metastases [37] in mice and also has been applied to monitor the progression of lung and liver tumours [38–40].
- Magnetic resonance imaging (MRI) also offers in-vivo, non-invasive, 3D, and high-resolution images. In recent years, MRI has gained great importance due to the absence of ionizing radiation and its soft tissue contrast. Hence, MRI is used not only for its ability to define lesions with great spatial resolution but also to recover quantitative features that might be able to predict cancer progression.
- Positron emission tomography (PET) is a highly sensitive and specific imaging technique used to visualize the distribution and concentration of radiolabelled molecules injected into murine models. It is a form of quantitative whole-body imaging used for the in vivo monitoring of biological processes, such as enzymatic reactions, cellular metabolism, and cell proliferation and migration [41], which makes it an ideal tool for the imaging of cancer [32,42].
- Single-Photon Emission Computed Tomography is a technique that detects gamma radiation directly emitted by a radionuclide during decay and provides 3D information by acquiring multiple two-dimensional (2D) images while rotating around the imaged object. This technique is frequently used in oncology to visualize neuroendocrine tumours [43] and thyroid cancer [44,45] and to perform bone scintigraphy.
- Ultrasound is an ideal technique for detecting tumour growth in mice since it produces high-resolution images of small structures. It is a non-ionizing radiation technique, portable, easy to use, and quickly generates relevant images.
- Intravital microscopy is an optical imaging technique that enables highly sensitive in vivo imaging of tissue structure and function at high spatial resolution (cellular and sub-cellular) and temporal resolution. However, a surgical procedure is required to access the tissue/organ of interest for microscopy, having therefore the consequence that immediately after image acquisition the animal must be sacrificed. Intravital microscopy has been reported to be used in studies

involving a metastatic process [46,47] and the response of tumour blood vessels to vascular targeted therapy [48,49].

- Whole-body optical imaging is a sensitive technique based on fluorochromes excitation by an external light source (fluorescence) or by chemiluminescent enzymatic light emission reactions within the animal (bioluminescence). Despite the poor spatial resolution due to light scattering, this technique enables the integration of the light signal emitted to obtain a 2D planar image. Green fluorescence protein (GFP) has been widely used to measure in vivo tumour growth as well as the effect of metastatic spread and drug treatments on different types of cancer in mouse models [50,51].

Recently, Haldorsen et al. [32] described the use of a multimodal imaging platform based on a PET/CT scan and MRI in orthotopic EC cell-line-derived xenografts and PDX models and compared its relevance to bioluminescence imaging (BLI). In this study, they managed to monitor tumour growth, progression, and metastasis spread. Their findings bring forward the value of imaging techniques in the follow-up of orthothopic EC PDX models. Nevertheless, much research is needed to potentiate the use of imaging techniques for the follow-up of orthotopic EC PDX models. A comparative image of BLI and a PET/CT assay in mice models is shown in Figure 1.

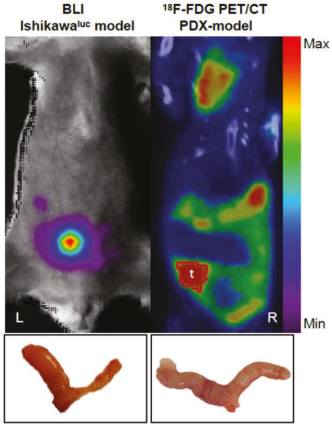

Figure 1. Comparison of imaging techniques for tumour evaluation. Bioluminescence imaging (BLI) enables the monitoring of cell-line-based orthotopic endometrial carcinoma, here demonstrated in a xenograft model generated from luciferase expressing Ishikawa cells (**L**). 18F-FDG PET/CT imaging is well suited for detection of endometrial carcinoma in PDX-models (**R**). Uterine tumours were confirmed by necropsy for each respective model (bottom).

2.3. Collaborative EC PDX Cohort

In a collaborative effort within the ENITEC consortium, we here describe in detail the PDX mouse cohort developed by the groups in Katholieke Universiteit (KU) Leuven, Haukeland University Hospital Bergen (HUHB), the Institute of biomedical research from Bellvitge–Institute Catalan of Oncology (IDIBELL-ICO), and the Vall d'Hebron Institute of Research (VHIR) in order to compile the information on EC PDX models available within this network. Altogether, we have generated a stable cohort of PDX models from primary tumour and metastasis from 124 EC patients recruited in different centres across Europe. This represents one of the most extensive and best-characterized EC PDX model cohorts available for research and is continuously increasing since we are generating new models from EC patients (Table 1).

Table 1. PDX models developed by European Network of Individual Treatment in Endometrial Cancer (ENITEC) consortium members.

Research Centre	Type of Sample	Type of PDX	Tissue	Engraftment Rate & Time		Mouse Strain	Number Models	Type of Models	Preclinical Drug Tested
IDIBELL-ICO	Primary tumor, metastases	orthotopic	small tissue fragment	75–90%	1–5 months	Athymic nude	64	60%EEC; 10%PS; 20%CS; 3%CC; 7%other types	Sorafenib, Chloroquine (61)
VHIR	Primary tumor, metastases, recurrences	heterotopic (s.c)	5–10 mm^3 tissue fragment	60–80%	2–3 months	Athymic nude	40	43%EEC; 32%PS; 10%CS; 2.5%CC; 5%undifferentiated 7.5%other types	Carboplatin Paclitaxel, Palbociclib (60)
KUL	Primary tumor, metastases, recurrences	heterotopic (s.c)	8–10 mm^3 tissue fragment	100%	3–5 months	Athymic nude	15	46%EEC; 13%PS; 13%CS; 7%undifferentiated 21%other types	Carboplatin, NVP-BEZ235, AZD 6244 (33)
HUHB	Primary tumor, metastases	orthotopic	Cell suspension	25–100%	3–13 months	NSG	5	60%EEC; 20%PS; 20%undifferentiated	

IDIBELL-ICO: Institute of biomedical research from Bellvitge–Institute Catalan of Oncology; VHIR: Vall d'Hebron Institute of Research; KUL: Katholieke Universiteit Leuven; HUHB: Haukeland University Hospital; EEC: Endometrioid endometrial cancer; PS: Papillary serous carcinoma; CS: Carcinosarcoma; CC: Clear cell carcinoma; s.c: Subcutaneous.

We have developed a heterogenous population of PDX covering almost every stage and grade of both EC histological subtypes. In particular, we have 63 endometrioid and 56 non-endometrioid EC PDX models, in which serous and carcinosarcoma histologies are the best represented with 22 and 19 models, respectively. Regarding staging, 62% of the endometrioid EC PDX models are represented by FIGO stage I and classified according to Morice et al. [25] as low to intermediate risk of recurrence, while the rest of the endometrioid EC PDX models are represented by advanced stage III (21%) and stage IV (3%) tumours classified as high risk. Among the non-endometrioid EC PDX models, almost 60% are advanced tumours represented by FIGO stages III and IV, whilst 32% of the models are early stage tumours (Table 1). Analysing in depth our data, we observed that approximately the same number of PDX models were developed from both histologies, showing that endometrioid EC and non-endometrioid EC tumours could grow as PDX. Similarly, we do not observe a trend towards the specific growth of any EC tumour subtype (Table 2).

Our PDX cohort was developed both by heterotopic transplantation of fresh tumour tissue fragments recollected from a surgery room and implanted subcutaneously into athymic nude mice and by orthotopic implantation throughout a laparotomic incision. The engraftment rate of subcutaneous EC PDX varied from 60–80%; however, once the tumour was developed, the engraftment rate increased to nearly 100% in subsequent passages. In addition, PDX models take approximately 3–5 months to engraft and develop the first generation, while subsequent passages take less time to engraft and progress. In contrast, the orthotopic PDX model engraftment rate varies from 75–90% and also takes 2–5 months to develop a palpable and transferable tumour. Additionally, a small cohort of five tumours from EC patients was also injected orthotopically into the left uterine horn of NSG mice. In this case, a primary tumour sample is manually dissociated, filtered, and centrifugated and then a cell suspension is injected by laparotomy in a 1:1 proportion with Matrigel [32]. For this type of model, the engraftment rate is lower, ranging from 25 to 100% in the first generation, and the time of engraftment is also slower: it takes on average 10 months to develop an orthotopic EC PDX model.

Table 2. ENITEC PDX models classified according to histology, stage, and differentiation grade.

		Endometrioid EC		**Non-Endometrioid EC**	
FIGO stage	I	39	62%	18	32%
	II	7	11%	4	7%
	III	13	21%	27	48%
	IV	2	3%	5	9%
Grade	1	20	32%		
	2	23	37%		
	3	20	32%	56	100%
Histology	Serous carcinoma			22	39%
	Carcinosarcoma			19	34%
	Clear Cell carcinoma			4	7%
	Others			11	20%

All of our models have been histologically characterized by H&E staining (Figure 2). Single-nucleotide polymorphism fingerprints analysis as well as the identification of gene mutations have been performed in some models. In the same way, we are working on the molecular characterization of PDX tumours by whole-exome sequencing and on the classification of those tumours according to the TCGA system. Finally, we have to mention that some of our PDX models have been used for preclinical drug-testing studies, described in the following section, with excellent results mimicking EC pathology and having a relevant response to treatment, demonstrating the high predictive value that PDX models could have in EC research.

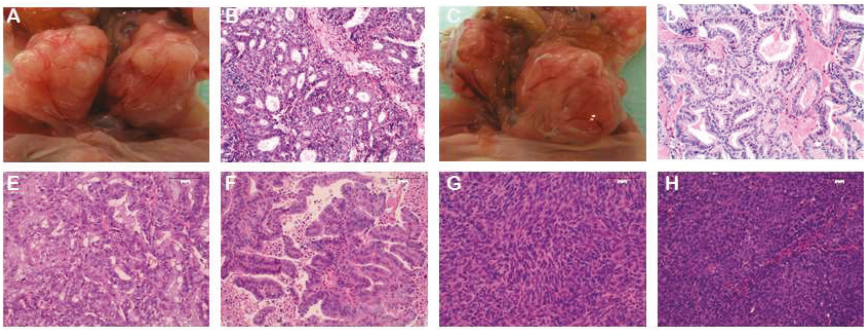

Figure 2. Representative PDX images from orthotopic (**A**–**D**) and heterotopic (**E**–**H**) models. (**A**–**D**) Orthotopic PDX from two different endometrioid EC patients. Panels A and C are a macroscopic image of the tumour growth in the uterus. Panels B and D represent images of the H&E staining of the PDX tumours. (**E**,**F**) H&E stainings from subcutaneous PDX models of two different endometrioid EC patients. (**G**,**H**) H&E stainings from subcutaneous PDX models of two different non-endometrioid EC patients. Panel G corresponds to a carcinosarcoma histology, and panel H corresponds to a serous carcinoma. Magnification 20×.

2.4. Use of EC PDX Models in Preclinical Studies

Personalized medicine refers to the discipline focused on treating patients individually with molecular-targeted therapies directed against the altered pathways of their own tumour. This is expected to maximize treatment efficacy and minimize side effects [52]. Based on this notion, PDX provides a powerful tool for personalized medicine as it retains the molecular profile of the individual tumour. Similarly, many reports have shown that response rates in PDX correlate with those observed in the clinic both for targeted agents and for classic cytotoxic drugs [53,54]. Thus, the potential for using these models for directing individualized therapy in patients is being increasingly recognized [55].

In EC, the PI3K/AKT pathway is constitutively active due to mutations, and so this pathway has been an attractive target for therapy in different EC preclinical studies. Winder et al. tested the effect of MK2206, an allosteric inhibitor of AKT, on the growth and invasion of three EC PDX models grafted under the renal capsule of NSG mice. They found that MK2206 treatment inhibited tumour growth as well as decreased invasion into the kidney and spread throughout the peritoneum in the three different types of PDX (endometrioid EC grade 2, endometrioid EC grade 3, and non-endometrioid EC serous) [56]. Similarly, Yu et al. reported the use of two EC PDX models to study the effect of a two-drug combination, which acts on AKT (ARQ092) and FGFR1/2 (ARQ087), to overcome AKT inhibitor treatment loss of efficacy and resistance. FGFR is also frequently mutated in EC, promoting tumour progression and treatment resistance. Based on this, they suggested that by combining AKT and FGFR1/2 targeted therapy, they would be able to overcome the resistance mechanism. Even though they had a synergistic effect in EC cell lines, they only obtained enhanced antitumour activity in one of the PDX models compared to the single-agent treatment. However, the authors suggested that it is necessary to define EC patient molecular signatures, i.e., with mutations of PIK3CA/PIK3R1 and FGFR, to design a suitable treatment strategy and predict patients' response [57].

The use of palbociclib against the cyclin-dependent kinases CDK4 and CDK6 has successfully been used in advanced breast cancer [58] and it is under evaluation in many other cancer types in phase II and III clinical trials [59]. In EC, Dosil et al. [60] performed the first preclinical study to test the therapeutic potential of palbociclib; specifically, this was tested in the endometrial malignancies driven by *Pten* deficiency. This work started with the assessment of palbociclib response in vitro and in a PTEN-deficient GEM model and was finally validated in a PTEN-mutated PDX model of endometrioid EC of FIGO stage IIIC and grade 2. This work evidenced that palbociclib has therapeutic potential as

an anticancer drug in the endometrium, since it reduces tumour cell proliferation and disrupts the tumourigenesis process [60].

Similarly, Dupreeuw et al. [33] tested the efficacy of NVP-BEZ235 (a dual pan-PI3K/mTOR inhibitor) and AZD6244 (an MEK1/2 inhibitor) in a PDX model harbouring a high-grade recurrent endometrioid carcinoma carrying PTEN, PIK3CA, and KRAS mutations. They showed that the treatment as a single therapy significantly reduced tumour growth compared to the control group. Moreover, when combining both therapies, NVP-BEZ235 and AZD6244, the treatment was as effective as Carboplatin, resulting in disease stabilization showing no increment of tumour growth.

All of the above-mentioned studies shared a similar approach to evaluating the efficacy of targeted therapies in EC: all of them relied on the use of PDX to validate a specific treatment which was first assessed in one or more in vitro and/or in vivo models. Moreover, PDX models could also be used to identify pathways responsible for therapy-resistant mechanisms and to identify new approaches to overcome any acquired resistance in EC tumours. Sorafenib, an antiangiogenic drug, has been proposed as a promising targeted therapy for EC, but a multicentre phase II clinical trial demonstrated moderate effects. In a recent work, Eritja et al. [61] studied the resistance mechanism of sorafenib in EC and demonstrated that autophagy acted as a protective mechanism against sorafenib. They developed in vitro assays and three different endometrial orthotopic xenografts (endometrioid EC grade 1, 2, and 3), and observed that the inhibition of autophagy by using cloroquine potentiates sorafenib effects in PDX orthotopic EC tumours. These results provided insights into the modest effects of sorafenib trials in EC patients and might open new avenues for the design of preclinical studies using sorafenib.

Equally important is the discovery of biomarkers to predict treatment-response, as this will help to tailor the treatment of EC patients. In this field, Groeneweg et al. [62] investigated the effectiveness of *HER2* inhibition in serous non-endometrioid EC. The combination of in vitro and in vivo cell-line and PDX models permitted the authors to demonstrate that lapatinib as a single agent and in combination with trastuzumab induced significant tumourstatic effects only in those tumours harboring *HER2* gene amplification. In the non-amplified tumour xenografts, a complete lack of response to any administered therapy was seen. Thus, this study unveiled that *HER2* gene amplification might be used a biomarker for response to *HER2* inhibition in uterine serous carcinoma, as has been shown in breast and gastric carcinomas. Similarly, in another study published by Groeneweg et al. [63], they demonstrated that the expression of nuclear Notch1 could be associated with tumour progression since it was expressed in a significant proportion of endometrioid ECs (12%) as well as in the majority of serous non-endometrioid EC analysed (58%). They showed that treatment with the gamma-secretase inhibitor MRK-003 decreased the proliferation of serous cell lines in vitro and restricted the growth of xenografts derived from serous cell lines and primary human serous tissue in vivo. Moreover, MRK-003 treatment augmented the anti-tumour activity of standard Paclitaxel/Carboplatin (P/C) therapy in one of the two primary human PDX models. The observed synergistic effect of MRK-003 with conventional P/C therapy in one primary model provides pilot data to suggest that the combination of a Notch inhibitor and standard chemotherapy may have promise in the management of serous carcinoma.

Based on these data, EC PDX models are currently playing an important role in defining new therapeutic options for the different EC subtypes and helping in the selection of populations of patients most likely to be sensitive to a new agent. However, all the studies performed up to date only include from a single to a few EC PDX models; thus, the results are hard to translate into larger populations.

3. New Perspectives on the Use of EC PDX Models

There exist interesting avenues for the exploitation of PDX models, which have been explored in other types of cancers but not in the field of EC research. A co-clinical trial is a concept, similar to personalized PDX models, which is based on the development of a PDX from a patient enrolled in a clinical trial and treated in the same way as the patient [30,64]. This strategy provides an interesting platform for the identification of predictive or response biomarkers and on which to assess the therapeutical benefit of novel combinations. Limitations of co-clinical trials include the limited ratio of

engraftment of PDX models and the extended time for PDX development of particular cases, which might impair the evaluation of the PDX response for all patients recruited in the clinical trial. Moreover, PDX models do not always faithfully represent primary tumour heterogeneity and, in these cases, PDX therapy response would not be relevant for the patient.

Another interesting approach is the use of PDX panels for preclinical studies as opposed to the traditional methods of assessing drugs in just a few models [65,66]. This approach uses PDX models as if those were patients participating in a phase II clinical trial, i.e., a cohort of different PDX models covering different types and stages of cancer are used, and only one or a few animals with specific characteristics are included per patient and receive a specific treatment. When analysing the responses to treatments, it is not the response of an individual mouse/tumour that matters, but the population response. It should be noted, however, that to completely capture the full inter-tumour heterogeneity of a particular cancer type, large panels of PDX are needed to cover different subtypes, stages, and grades of differentiation. Considering this, we have to mention that this approach is not feasible for tumour types that poorly establish as xenograft models.

Migliardi et al. [65] was the first to describe this mouse clinical trial approach. Later on, Gao et al. [67] proposed the use of just one tumour representing "a patient" to allow for even greater efficiency (the $1 \times 1 \times 1$ approach), determining that using just the one animal per cohort study design has outstanding reproducibility for the data collected. The utility of this approach is that it enables many more types of PDX and treatment groups to be assessed operationally, and the inter-heterogeneity of patients can be captured experimentally. This approach is closely related to a clinical study in patients and is being used by some researchers and pharmaceutical companies motivated to increase the success rate of drugs tested in preclinical phases that are finally approved by regulatory agencies.

4. Collaborative PDX Networks

At present, the cost and resources needed for PDX development and maintenance are a limiting factor for many researchers in order to develop their own models. For this reason, to facilitate working with PDX models, accessible collaborative networks between academic research groups have been established that closely cooperate with the clinic in order to associate preclinical experimentation and clinical activities. Such networks include the EurOPDX Consortium, the U.S. National Cancer Institute (NCI) repository of patient-derived models, the U.S. Pediatric Preclinical Testing Consortium (PPTC), the Children's Oncology Group (COG) cell culture and xenograft repository, the Public Repository of Xenografts (PRoXe), and the Novartis Institutes for Biomedical Research PDX Encyclopedia (NIBR PDXE) [68]. Some of the PDX models developed in EC in the ENITEC consortium are included in the EurOPDX network.

Besides academic collaborations, commercial companies have also started to provide PDX models, such as The Jackson Laboratory (Bar Harbor, ME, USA), Xenopat (Barcelona, Spain), CrownBio (Santa Clara, CA, USA), Oncotest GmbH (Freiburg, Germany), AVEO Oncology (Cambridge, MA, USA), Living Tumour Laboratory (Vancouver, BC, Canada), Urolead (Strasbourg, France), Experimental Pharmacology & Oncology Berlin-Buch GmbH (Berlin-Buch, Germany), and XenTech (Paris, France) [69].

5. PDX-Related Challenges

Although EC PDX models have been established for almost a decade, there are still several challenges that should be faced in the near future. First, immunocompromised mice are used so as to not reject human tumour, meaning that immunologically related aspects cannot be taken into account. Next, human stroma in mice is replaced by murine stroma over different generations. This has been shown for different types of tumours, but also more specifically for EC [33]. Both immunological and stromal cells are part of the tumour microenvironment, and it is known that they are implicated in cancer progression and metastasis [70]. Therefore, there is a need to investigate the tumour microenvironment [71]. Third, although tumour heterogeneity is maintained, only small pieces

of primary tumours are used, which means there is potential for loss of tumour information. Indeed, efforts to reproduce clear intra-tumour heterogeneity in different murine models should be addressed. Finally, it is becoming clear that, although the pharmacological and most of the original biological characteristics are maintained, tumours partially undergo mouse-specific evolution. More specifically, Ben-David et al. monitored the SCNAs of 1110 PDX models for 24 cancer types over time. They did find an accumulation of SCNAs over time that correlate with the primary tumour. However, several SCNAs observed in primary tumours disappear in PDX models and SCNAs acquired by PDXs differ from primary tumours [72]. Other studies suggest that, although there is indeed engraftment-associated selection, the majority of changes do not occur in oncogenic drivers and are therefore not affecting intra-tumour heterogeneity [33,68,73].

To overcome the lack of tumour microenvironment and immune cell interaction, humanized mice can be used [74]. Humanized models can be used to investigate cancer stem cells amongst other biological facets, such as tumour–microenvironment interactions and anti-tumour immune responses, and they can be used in immunotherapy research [75]. For endometrial cancer, no reports using humanized mice have been published yet. However, humanized mice have been successfully established for haematological malignancies [75] and many types of cancer [74,75]. Different methodologies can be used to generate humanized models; however, this subject exceeds the scope of this article and excellent reviews about humanized models can be found in the literature [68,73,75].

6. Conclusions and Future Perspectives

Over the last few decades, there has been increasing interest in developing more realistic and clinically relevant mouse models. In this context, PDX emerges as a promising preclinical mouse model mainly because it faithfully retains patient tumour characteristics and behaviour. Importantly, EC PDX models have already been used in an individualized approach to evaluate the efficacy of novel therapies and to identify biomarkers to predict treatment-response. Together with the advances of omics techniques, which allow us to increase our understanding of the molecular alterations of EC tumours and dysregulated EC pathways, EC PDX models are now an untapped source to improve the definition, consecution, and output of preclinical studies to increase the success ratio in further clinical phases. In this review, we compiled the information on EC PDX models that have been described in the literature and highlight the models that have been generated in the ENITEC consortium, from their generation to their use, and identify new perspectives and limitations of those models.

Author Contributions: Data collection: C.P.M., C.L.-G., S.C., A.G., T.V.N., D.A., T.F., A.V. (August Vidal), A.V. (Alberto Villanueva), X.M.-G., C.K., F.A., A.G.-M., and E.C.; Writing & Editing: C.P.M. and E.C.; Review: C.P.M., T.V.N., D.A., C.K., F.A., and E.C.

Funding: This work was supported by CIBERONC (CB16/12/00328), the "Fondo Europeo de Desarrollo Regional" FEDER (RTC-2015-3821-1), Grups consolidats de la Generalitat de Catalunya (2017 SGR-1661) and the Instituto de Salud Carlos III (PI14/02043; PI17/02071). An AGAUR grant funded CL-G (2018FI_B_00573), and a PERIS grant funded EC (SLT002/16/00315) from Generalitat de Catalunya. The present work has been also funded by the "Fonds National de la Recherche du Luxembourg" (FNR) via the PEARL-CPIL program to BD and an AFR grant to AL (PDR 2013-2, Project Reference 6835664).

Acknowledgments: The authors would like to acknowledge the work of all who have participated in the recruitment of clinical samples. We also thank the patients for their willingness to participate in the study. The KUL group (TVN and FA) acknowledges Trace, the KUL PDX Platform (www.uzleuven-kuleuven.be/lki/trace), through which the KUL EC PDX models are available.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

GEM	Genetically engineered mouse
PDX	Patient-derived xenograft
SCID	Severe combined immunodeficient mice
NOD	Non-obese diabetic
NSG	NOD/SCID gamma mice
FIGO	International Federation of Gynaecology and Obstetrics
EC	Endometrial cancer
TCGA	The Cancer Genome Atlas
SCNA	Somatic copy number alterations
POLE	DNA polymerase epsilon
ENITEC	European Network of Individual Treatment in Endometrial Cancer
PDTO	Patient-derived tumour organoids
CT	Computed tomography
MRI	Magnetic resonance imaging
PET	Positron emission tomography
GFP	Green fluorescence protein
BLI	Bioluminescence imaging
IDIBELL-ICO	Institute of biomedical research from Bellvitge–institute Catalan of Oncology
VHIR	Vall d'Hebron Institute of research
H&E	Hematoxylin and eosin
AKT	Alpha serine threonine kinase
FGFR	Fibroblast growth factor receptor
CDK4	Cyclin dependent kinase 4
CDK6	Cyclin dependent kinase 4
PTEN	Phosphatase and Tensin Homolog
NCI	National Cancer Institute
PPTC	Pediatric Preclinical Testing Consortium
COG	Children's Oncology Group
PRoXe	Public Repository of Xenografts
NIBR PDXE	Novartis Institutes for Biomedical Research PDX Encyclopedia

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International Journal of
Molecular Sciences

MDPI

Review

Modeling Endometrial Cancer: Past, Present, and Future

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Received: 30 June 2018; Accepted: 3 August 2018; Published: 9 August 2018

Abstract: Endometrial cancer is the most common type of cancer of the female reproductive tract. Although prognosis is generally good for patients with low-grade and early-stage diseases, the outcomes for high-grade and metastatic/recurrent cases remain poor, since traditional chemotherapy regimens based on platinum and taxanes have limited effects. No targeted agents have been approved so far, although several new drugs have been tested without striking results in clinical trials. Over the last decades, many efforts have been made towards the establishment and development of preclinical models, aiming at recapitulating the structural and molecular determinants of the disease. Here, we present an overview of the most commonly used in vitro and in vivo models and discuss their peculiar features, describing their main applications and the value in the advancement of both fundamental and translational endometrial cancer research.

Keywords: endometrial cancer; preclinical models; translational research

1. Introduction

Endometrial cancer (EC) accounts for 4.8% of all cancers diagnosed in women and is the fifth most common type of cancer in developed countries [1–3]. It is the most common malignancy of the female reproductive tract, with a cumulative risk of 1% by age of 75 years, while the death risk is 0.2% [1–3]. Around 320,000 new cases are diagnosed yearly worldwide, and 76,000 patients die every year from the disease [1–3]. Around 75% of all ECs are diagnosed as FIGO (International Federation of Gynecology and Obstetrics) stage I or II, corresponding with a 5-year overall survival that varies between 74% and 91%. Patients diagnosed as FIGO stage III or IV have a 5-year overall survival rate of 57–65% and 20–26%, respectively [3–5].

Recognized risk factors for the development of EC are (i) exposure to unopposed estrogens or to tamoxifen, (ii) diabetes, (iii) obesity, (iv) nulliparity, (v) early-onset menarche, and (vi) late-onset menopause, amongst others [6]. The increasing aging of the population together with higher frequencies of metabolic diseases and diabetes are possible explanations for the observed higher incidence rates of EC in the developed world (i.e., Western Europe and Northern America), compared to other regions [3]. Patients typically present with abnormal uterine bleeding and, in case of advanced disease, possibly abdominal and pelvic pain [7]. Standard diagnostic procedures consist of pelvic ultrasonography, endometrial biopsy, and hysteroscopy when the diagnosis is uncertain [3]. Treatment is primarily based on cytoreductive surgery, mostly total hysterectomy and salpingo-oophorectomy [3].

Depending on different prognostic factors such as tumor grade, histology, and myometrial and cervical invasion, patients can be divided into low-risk or high-risk groups, related to a long and short disease-free survival, respectively [8]. For high-risk patients and those with metastatic disease, surgery is combined with adjuvant radiotherapy and/or chemotherapy such as cisplatin, carboplatin, doxorubicin, and cyclophosphamide [6,8,9]. Since for recurrent and metastatic disease only limited treatment options are available [10–13], the last decade saw growing interest in novel targeted therapies.

Traditionally, EC has been divided into two subtypes with distinct clinical, pathological, histological and molecular behavior [14,15]. Type I EC are mainly low grade, estrogen-dependent, hormone-receptor-positive adenocarcinomas with endometrioid morphology and are often referred to as endometrioid endometrial cancers (EECs). EECs account for 85% of all ECs. Moreover, they are mostly diagnosed at an early stage and are generally characterized by a good prognosis [3,16]. Type I tumors often show alterations in the PI3K/Akt pathway, suggesting they could potentially respond to anti-PI3K/Akt therapies [13]. Commonly mutated genes include phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), KRAS proto-oncogene, GTPase (KRAS), fibroblast growth factor receptor 2 (FGFR2), and Catenin beta 1 (CTNNB1), amongst others [17]. The most frequently altered gene is the tumor suppressor Phosphatase and tensin homolog (PTEN), in approximately 50% of all cases, while the most common altered oncogene is KRAS, altered in 25% of cases [18]. Type II ECs are characterized by non-endometrioid histology and include carcinosarcomas, serous and clear cell carcinomas, and all tumors with different histology and molecular features [16]. Type II ECs are generally high grade, hormone-receptor negative, and have poor survival rates [3]. Serous carcinomas only account for 10% of all ECs, although they are responsible for 39% of the total EC deaths [19]. The overall survival rate for serous carcinoma and clear cell carcinoma is only 56% compared to the 86% reported for endometrioid carcinoma [20]. Type II ECs are characterized by high frequencies of Tumor protein p53 (TP53) mutations and other low-frequency genomic alterations, such as F-box and WD repeat domain containing protein 7 (FBXW7) and AT-rich interaction domain 1A (ARID1A) mutations and Erb-b2 receptor tyrosine kinase 2 (ERBB2) amplification [17].

The Cancer Genome Atlas recently identified four distinct EC molecular subtypes, i.e., the Polymerase e (POLE) ultramutated, the microsatellite instability hypermutated, the copy-number low microsatellite stable, and the copy-number high serous-like subgroups [17]. These subtypes show increasing grade, TP53 mutations, and somatic copy number alterations as well as decreasing mutation rates, respectively [17]. However, around 40% of all ECs belongs to a large nonspecific molecular profile (NSMP) subgroup, characterized by the absence of POLE or TP53 mutations and microsatellite instability. Recently, a somatic copy-number alterations (SCNA) analysis identified a different subgroup within the NSMP EC, refining the molecular classification of these poorly-characterized tumors. The proposed subgroup shows amplifications of 1q32.1, the locus where Double minute 4 protein (MDM4) is located, and, importantly, this type of amplification has been identified as a prognostic marker [21].

Our knowledge about EC biology has been increased during the past decades and continues to grow thanks to the use of many different preclinical models. With this review, we aim to discuss the general aspects of the different preclinical models available, their peculiar features and refinements, how they have been used to study EC, which progresses they enabled in our understanding of the disease, and their future challenges and applications, as highlighted in the graphical abstract (Figure 1).

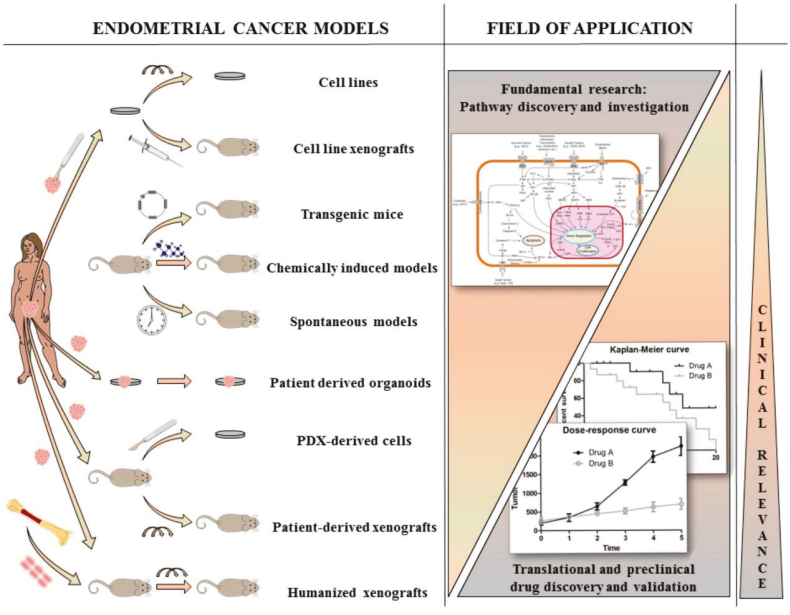

Figure 1. Presentation of the different available preclinical models present for endometrial cancer (EC) research. Cell lines and their derived xenografts are models for basic EC research however lack clinical relevance. Better, in vivo models are transgenic, chemically induced and spontaneous models, however they lack patient-derived properties. Patient-derived models (organoids, xenografts and humanized mice) have the highest clinical relevance and are useful for translational and preclinical drug discovery and validation, however they are less likely to be used for fundamental research.

2. Cell Lines and Cell Line-Derived Xenograft Models

Historically, in vitro cell lines have had a prominent role in anticancer drug development [22], although such models may lack clinical relevance due to the immortalization and adaptation processes induced by the continuous growth on plastic. The possibility of working under standardized conditions makes cell lines extremely useful for the discovery of molecular mechanisms and biological pathways related to an observed phenotype, while also allowing for cost-effective high-throughput screenings. However, it is worth noting that they are exposed to altered oxygen levels and nutrient composition, compared to the original tumors grown in the patients, and that they lack any sort of interaction with the microenvironment [22].

For endometrial cancer, multiple different cell lines have been established during the last decades (Table 1). The most commonly used cell lines—AN3CA, ECC-1, HEC1A, HEC1B, and Ishikawa—are type I tumor-derived cell lines, harboring alterations in the PI3K/Akt pathway, thereby representing the majority of EC tumors in the clinic. Short tandem repeat (STR) profiling of 10 of the most common EC cell lines showed that HEC1A, HEC1B, HEC50, AN3CA, KLE, and RL-95-2 have unique STR profiles, consistent with their originally derived tumors. Different variants of Ishikawa cell lines showed polymorphic genomic regions; however, high similarity profiles indicate that they originate from the same patient. Korch and colleagues genotyped different cell lines and found that the ECC-1 cell line does not match the original EnCa-101 tumor [23]. Therefore, the ECC-1 cell line has been discontinued and is no longer commercially available. This example points to the importance of proper annotation and to the need of a standardized authentication system for cell lines.

Table 1. Endometrial cancer (EC) cell line information. Genomic alterations of the most commonly used type I and type II EC cell lines.

Cell Line	Tumor Location	Type	PTEN	KRAS	TP53	PI3K/Akt Pathways Alteration(s)	Microsatellite Instability
AN3CA	Metastasis	I	Deletion	wt	Missense mutation	Yes	High
ARK1	Primary	II	n/a	n/a	n/a	Yes	n/a
ARK2	Primary	II	n/a	n/a	n/a	n/a	n/a
ECC-1 [1]	Primary	I	Missense mutation	wt	Missense mutation	Yes	High
HEC1A	Primary	I	wt	Missense mutation	Missense mutation	Yes	High
HEC1B	Primary	I	wt	Missense mutation	Missense mutation	Yes	Low
HEC50co	Metastasis	n/a	wt	Missense mutation	Deletion	n/a	n/a
Ishikawa	Primary	I	Deletion	wt	Missense mutation	Yes	High
KLE	Metastasis	n/a	wt	wt	Missense mutation	No	Low
MFE-280	Primary	I	wt	wt	Splice site mutation	Yes	Low
RL-95-2	Primary	I	Missense mutation	wt	Deletion	Yes	High
SPEC2	Primary	II	Not expressed	n/a	n/a	n/a	n/a

n/a, not available; [1] ECC-1 has been retracted from the market after the study by Korch et al. [23].

Tumor cells isolated from one single patient can lead to different cell lines, as illustrated by the HEC1A and HEC1B cell lines derived from the same surgical specimen [24], which differ in their microsatellite instability phenotype. This observation highlights that most of the info related to intra-tumor heterogeneity cannot be maintained in vitro by establishing only one cell line from one tumor. However, where available, paired cell lines originating from the same patient are extremely important to investigate this and other biological issues. Using the HECs cell lines, for example, Glaab and colleagues showed the significance of mismatch repair endonuclease PMS2 (PMS2) in the maintenance of genomic stability in human cells [24].

Nevertheless, peculiar molecular aberrations have been shown to be maintained when passaging a tumor in vitro. Specifically, alterations in the PI3K/Akt pathway have been observed in both EEC patients' tumors and EEC cell lines. Weigelt et al. analyzed 24 commercial EEC cell lines and described mutations in PTEN, PIK3CA, PIK3R1, and KRAS [25], with frequencies comparable to those seen in human EEC samples [25–28]. An analysis of gene copy number aberrations in the five most common EC cell lines—Ishikawa, HEC1A, HEC1B, EEC-1, and AN3CA—showed that the PI3K/Akt and Wnt pathways are commonly affected [29]. Furthermore, the PI3K/Akt/mTOR pathway has been validated as a potential target for novel targeted therapies [25], and Philip and colleagues recently showed that a combination of PI3K and Poly (ADP-ribose) polymerase (PARP) inhibition has synergistic effects in PTEN mutated cells [30]. Using EC cell lines, Lin et al. showed that cisplatin, which is a main therapeutic agent, exerts its effect by regulating autophagy through the PI3K/Akt pathway and that PI3K/Akt inducers could reverse cisplatin activity [31].

Chemoresistance and metastatic dissemination remain major hurdles for EC patients, and different chemotherapy resistance mechanisms have been described [12,32]. Recently, it has been shown that non-coding RNAs (e.g., of miR-139-5p and miR-143) might play a role in tumor growth, therapy resistance, and metastasis [33]. A different report indicated that the long non-coding RNA homeobox transcript antisense RNA (HOTAIR) contributes to platinum resistance in vitro [34] and that miR-205 is able to inhibit cell growth in progesterone-resistant Ishikawa cells [35].

Other fields of interest that make use of EC cell lines, often as starting models, are summarized in Table 2.

Table 2. Potential applications of EC cell lines for preclinical research.

Research Category	Field of Application	References
Fundamental Research	Molecular Biology	
	- Proliferation and migration - Tumorigenesis and dissemination mechanisms - Therapy resistance mechanisms - Pathways analysis and identification	[23,25,29,36,37]
	Epigenetics	
	- DNA/histones modifications - Post-translational protein modification - Non-coding RNAs	[33–35,38–44]
	Metabolism	[45–49]
	- Hormone metabolism - Glucose/glutamine metabolism - Fatty acid metabolism - Other	
	Functional analysis	[50]
	- New technologies development	
Translational Research	Drug discovery and validation	
	- Targeted therapies - Overcoming therapy resistance	[25,30,36,49,51,52]
	Biomarkers discovery	
	- Distinguish different EC types - Identification of signatures linked to treatment response	[36,43,53]

The use of large-scale omics technologies revealed marked intra- and inter-tumor heterogeneity in patients, which cannot be captured by single cell lines. Therefore, nowadays large cell line panels are often used to try to recapitulate as much as possible in vitro tumor heterogeneity and to identify genomic determinants of drug sensitivity. The most known panels are the National Cancer Institute 60 (NCI60) platform [54] and the Japanese Foundation for Cancer Research 39 (JFCR-39) [55], which do not, however, list EC lines, as well as the Center for Molecular Therapeutics 1000 (CMT1000), where ECs are represented by different cell lines [22,56].

Since the information about cell lines established decades ago is often scattered and lacks systematic annotations, the Broad Institute launched the Cancer Cell Line Encyclopedia (CCLE) initiative in 2012, with the aim of compiling genomic datasets and pharmacological response profiles of different cancer cell lines to selected compounds. Today, the CCLE counts more than 13,000 unique datasets (for gene expression, chromosomal copy number analyses, and mutational sequencing) from 1457 cell lines, of which 28 are ECs.

3. Organoids and Organs-on-a-Chip Models

The high attrition rates observed for novel compounds in oncology, due to the discrepancies between results obtained in preclinical and clinical settings, has been for long imputed to the use of suboptimal models, lacking predicting value in terms of therapeutic response. Two important factors that strongly limit the clinical relevance of the conventionally used cancer cell lines are the lack of interaction with the stromal compartment and the scarcity of normal tissue-derived counterparts. For this reason, significant efforts have been spent in the last decade to develop new ex vivo models that would better mimic the original tumors' physiology.

3.1. EC Organoids Models

The discovery that both healthy and tumor tissues can grow in vitro as self-organizing three-dimensional (3D) structures under specific growth conditions opened new perspectives for organoid cultures. Organoid models have been established from healthy human or mouse endometrium by Boretto et al. [57], as well as from endometrial cancer patients by Turco and colleagues, who adapted conditions used to grow adult human stem cells-derived organoids [58]. EC organoids maintained the architecture of the original tumors under a chemically defined medium, could be grown for extended periods of time (5 months), and showed genetic and molecular stability. Since the healthy endometrium is a dynamic and plastic tissue adapting and regenerating in response to hormonal cycles, the role of stem/progenitor cells is also being investigated during malignant transformation. Interestingly, it has been shown that endometrium progenitor cells display a high capacity to differentiate into cytokeratin-positive organoid cultures [59] and that cells highly expressing ALDH (alcohol dehydrogenase), a stemness marker for the endometrium, have a high organoid forming capacity [60].

EC patient-derived spheroids have been recently used to perform a pharmacological screening with 79 different targeted therapies by Kiyohara and colleagues, which showed that non-endometrioid carcinomas seem to be highly sensitive to survivin inhibition, while endometrioid cancers could be resistant [61]. Girda et al. established 15 patient-derived organoid cultures on which they screened multiple drugs. They observed that STAT3 inhibition does impede organoid formation in almost all cultures, confirming the key role of cancer stem cells in tumor growth and organoids establishment. Surprisingly, none of the cultures was affected by cisplatin or by different progestins. On the other hand, strong growth inhibition was observed in paclitaxel-treated organoid cultures, while moderate inhibition was described for tyrosine kinase inhibitors and fulvestrant treatment [62]. Because they are relatively fast to establish and easy to maintain in culture, organoids have also be used to provide proof of concept for drug repurposing in EC, as reported by Dasari et al. who found that Verteporfin, a photosensitizer normally used for photodynamic therapy in conditions such as macular degeneration, could potentially be effective in EC [63].

3.2. Organs-on-Chip Models

The so-called organs-on-chip (OOC) models are microfluidics systems where engineered biomimetic chambers containing cells or tissues are connected and continuously perfused by circulating medium, so as to simulate the physiological dynamics and functionality of tissues within one organ or the crosstalk between different organs [64].

Applied to the investigation of cancer tissues, such microfluidics technologies have high potential to become the future gold standard for drug testing in translational research. The approach to growing patient-derived tumor cells or tissues on chips proved to be feasible for lung and breast cancers and was shown to be capable of mimicking tumor growth, dormancy, invasion, and response to therapy [65,66]. In this view, the recent development of a multi-organ microfluidic system, called EVATAR, which simulates the human female reproductive tract and recapitulates its hormonally-controlled dynamics, paves the way for future applications related to gynecological pathologies, spanning from endometriosis to ovarian, cervical, and endometrial cancers [67].

4. In Vivo Models

Mouse and rat models are appreciated as standard animal models in translational cancer research, mainly because they are easily available and allow drug testing on a population scale, due to the short time needed to generate results, with the advantage of having tumor cells growing as 3D masses and in strict connection with the stromal compartment. However, they are also subject to caveats, when it comes to comparing the biology of murine and human tumors. A first point to consider is that humans live around 30–50 times longer compared to mice and rats, are ~3000 times larger,

and therefore undergo much more cell divisions. This implies that tumors might develop and evolve differently. It was indeed shown that rodent cells require at least two genetic alterations before gaining tumorigenic potential, while human cells are more difficult to transform [68]. Interestingly, the lifetime risk for developing cancer is comparable in rodents and humans. More specifically, 30% of laboratory rodents develop cancer by the end of their lifetime and 30% of humans develop cancer by 70–80 years of age [69]. However, it is known that at least some of the antineoplastic mechanisms that have been described (e.g., limited replicative potential and growth signaling self-insufficiency) are human-specific, implying possible differences in response to carcinogenesis and cancer chemopreventive agents [69,70]. Several excellent reviews are available on this subject [69–71].

4.1. Spontaneous EC Rodent Models

It is known that, just like humans, rodents can also spontaneously develop tumors if kept alive until their natural life end. However, what was surprising for researchers is that some specific rat strains have an abnormally high incidence rate of EC. In 1981, Deerberg and colleagues already noticed a 39% incidence rate of uterine tumors in female Han:Wistar rats [72,73].

Later, different spontaneous EC rat models were also described. Nagaoka et al. showed that in Donryu rats the incidence rate of endometrial adenocarcinoma was as high as 35.1% and that around 60% of all rats would develop proliferative lesions in the endometrium [74]. Tanoguchi et al. showed that KRAS mutation frequencies in tumors originating in Donryu rats are similar to those observed in human EC, suggesting potential relevance for the findings coming from this model [75].

Donryu rats have been historically used for the investigation of EC etiology and they helped to elucidate that hormonal imbalance, more specifically an increased estrogen:progesterone (E:P) ratio, can be linked to EC development [76,77]. Yoshida et al. and Kojima et al. showed that compounds decreasing the E:P ratio, such as bromocriptine and indole-3-carbinole, have a protective effect against EC [77,78], while compounds increasing the E:P ratio have the opposite effect. These observations explain why neonatal exposure to a high dose of the estrogenic compound p-t-octylphenol increases the likelihood of EC development [79]. Also, isoflavone aglycones, chemical compounds found in soy products that exhibit estrogen-like properties, have been shown to facilitate EC development in Donryu rats [80]. The notion that high-fat diets increase the E:P ratio, thus leading to an increased risk of EC incidence, came from studies in spontaneous rat models [81]. In addition, Nagaoka and colleagues showed that multiparity is linked with hormonal changes and leads to the suppression of EC incidence rate if compared to nulliparity in vivo [82].

Besides Donryu rats, other spontaneous EC rat models are available, such as DA/Han rats, BDII/Han rats, and the low spontaneous EC incidence rat strain F344. DA/Han rats are an inbred rat strain that exhibits high spontaneous tumor development (>60% if kept until the natural life end, 24–27 months) and has a high metastatic phenotype [73]. However, only limited information is present in the literature and these rats have not been widely used in EC research.

BDII/Han rats, on the other hand, are probably the better characterized spontaneous EC model, both at genomic and molecular levels. If kept to their natural life end (around 26–27 months), over 90% of the female rats die due to endometrial carcinoma [73,83]. Using gene sequencing and real-time PCR, Samuelson et al. showed that tumors in BDII/Han rats are molecularly similar to type I endometrial tumors. Amongst other alterations, they found allelic imbalance and altered expression of PTEN, and only limited aberrations in TP53 [84]. Of note, tumors in BDII/Han rats are hormone-sensitive, like type I human EC, since it has been shown that in rats ovariectomized prior to estrous cyclicity the tumor incidence rate decreased to 0% [73]. Another piece of evidence is that melengestrol acetate administration suppresses tumor growth [85]. Tumors from BDII/Han rats have been extensively genomically characterized, and the results suggest that the upregulation of CDK6 and/or Met could play a role in the development of cancerous lesions [86–94].

Recently, hormone receptor expression was evaluated in the Fischer 344 (F344) rat strain [95]. F344 is not a high-incidence EC rat strain; however, it is one of the most commonly used for

carcinogenicity testing. Tumors originating in F344 display high inter- and intra-tumor heterogeneity in terms of estrogen receptor (ER) and progesterone receptor (PR) expression, as observed in women, with the majority being ER^+PR^+. Of note, ER^+ tumors in F344 tend to be well differentiated, as reported in humans. Although in women EC tumors are mostly PR^+, F344 rats also develop PR^- tumors [95].

In conclusion, spontaneous EC rat models are excellent tools recapitulating some of the molecular and genomic features observed in human tumors. However, their value is generally underappreciated and they only have minimal use for drug testing in the preclinical setting. One of the drawbacks is that the timing of tumor development is difficult to predict since it is not possible to know if and when the lesions will develop and a long period of time may pass before they start to grow [69].

4.2. Chemically Induced EC Rodent Models

Although different spontaneous rat models for EC are available, tumors are still often induced by treatment with chemical compounds, such as artificial estrogens, in rat and/or mice models. Traditionally, researchers mainly use two related compounds to induce EC tumors in vivo, i.e., *N*-methyl-*N*-nitrosourea (MNU) [96,97] or *N*-ethyl-*N*-nitro-*N*-nitrosoguanidine (ENNG) [98,99]. These are alkylating agents which cause mutagenic and carcinogenic effects by alkylating DNA, RNA, and proteins [100], often in combination with estrogens [99,101,102]. Takahashi et al. showed that ENNG combined with estradiol, estrone, estriol, 16β-hydroxyestrone, 16α-hydroxyestrone, and 17-epiestriol significantly induces endometrial adenocarcinoma tumor formation and progression in ICR (Institute of Cancer Research) outbred mice [99]. All of these metabolites belong to the 16α-hydroxylation pathway or the upstream 16β-hydroxylation pathway of estrogen metabolism, while metabolites belonging to the downstream 16β-hydroxylation pathway and the 2-, 4-hydroxylation pathway, such as 2-hydroxyestriol, 2-methoxyestradiol, 2-methoxyestriol, and 16-epiestriol, have only limited to no effect on the growth of endometrial carcinomas [99].

Chemically induced EC animal models have been used as translational models to investigate the effects of chemopreventive agents. Niwa et al. used them to study the effect of danazol on endometrial carcinogenesis [97], while others investigated the effect of tamoxifen [103] and dietary indole-3-carbinole [98] on endometrial adenocarcinoma growth. A major drawback, however, is that exposure to the chemical compounds could have detrimental effects on the metabolism of specific tissues and organs. This strongly limits their use, since it implies that the metabolism of a novel drug could be somehow altered in animals that were exposed to chemicals [71].

4.3. Transgenic Mouse Models

Transgenic mice are mostly used for investigating biological mechanisms related to cancer development [71]. For EC, approaches based on different transgenes successfully led to the establishment of several models.

4.3.1. PTEN Knock-Out Mouse Models

Since PTEN is the most altered gene in EC, its knock-down has successfully led to the development of transgenic EC models [18]. Knock-out of one of two alleles ($PTEN^{+/-}$) is sufficient to generate hyperplasia, which develop to carcinoma in 20% of all cases, by the age of 10 months [104], while $PTEN^{-/-}$ homozygosity is embryonically lethal [73]. However, to study homozygous PTEN deletions in adult mice, different conditional systems have been recently developed, such as a tamoxifen-inducible transgenic system [105], an adenovirus-mediated Cre-lox system [106], or the isolation of $PTEN^{loxP/loxP}$ cells from the uterus of adult mice, followed by gene inactivation and re-implantation [107]. It has been shown that PTEN inactivation per se is sufficient to rapidly induce endometrial carcinoma [105]. Since microsatellite instability is a highly frequent event in endometrioid EC, Wang et al. established a transgenic mouse system that harbors a homozygote $MLH^{-/-}$ deletion next to the heterozygous PTEN loss ($PTEN^{+/-}$) and showed an accelerated onset of endometrial carcinoma [18,108], confirming the role of microsatellite

instability in EC. The importance of alterations in the PI3K/Akt pathway and microsatellite instability was also confirmed in vivo using transgenic mice [18].

Transgenic mice have also been used to study additional genes possibly related to EC development and their cooperation with each other or with PTEN. Contreras et al. showed that the inactivation of Serine/threonine kinase 11 (LKB1)—a master regulator of the Adenosine monophosphate-activated protein kinase (AMPK)-mTOR signaling—is sufficient to drive endometrial cancer development [109]. Cheng and colleagues also developed a model with a combined loss of PTEN and LKB1, with which they showed that loss of both genes leads to EEC and short survival, with a high dependency on the hyper-activated Akt pathway [106].

Another interesting approach is based on the establishment of primary cultures from the tumors developed in transgenic mice, as shown for the PTEN knock-out models [110,111].

4.3.2. TP53 Knock-Out Models

TP53 mutations are found in advanced type I EC and TP53 is the most commonly altered gene in type II EC. Daikoku and colleagues showed that conditional TP53 deletion alone does not lead to EC development, while a combined conditional PTEN$^{-/-}$TP53$^{-/-}$ deletion led to shorter survival and exacerbated disease state compared to PTEN$^{-/-}$ mice only, thereby confirming the importance of TP53 alterations in advanced type I EC [104]. Although type I EC is the most investigated subtype of EC because of its high incidence rate, type II EC is more aggressive and has a higher relative death rate [112]. Akbay et al. showed that the deletion of protection of telomeres protein 1A (POT1A)—a component of the shelterin complex stabilizing telomeres—combined with TP53 loss led to the development of type II-like EC in a mouse model by 9 months of age. In addition, it led to the insurgence of metastasis in 100% of the mice at 15 months. These results point to the importance of telomere instability and TP53 mutations in type II EC [112].

4.3.3. The Mitogen Inducible Gene 6 (MIG-6) Knock-Out Model

A different EC model has been established by knocking-out the Mitogen Inducible Gene 6 (MIG-6) [113], the expression of which is known to be regulated by mitogens and stress stimuli. MIG-6 is an immediate early response gene and acts as a negative regulator of EGFR signaling. It is a known progesterone receptor-regulated gene, and this can partially explain why a low E:P ratio is linked to low EC incidence. Using uterus-specific MIG-6 null transgenic mouse models, it was shown that MIG-6 has an estrogen-dependent tumor suppressive function [113]. Furthermore, it was shown that MIG-6 expression inversely correlated with the phosphorylation of ERK1/2 [114]. The estrogen-dependency of EC tumors was examined in PTEN deleted mice, leading to the conclusion that EC tumorigenesis is independent of estrogen in PTEN$^{+/-}$ mice [115] and the depletion of estrogen predominantly leads to neoplastic lesions, possibly explaining why endometrial carcinoma incidence is higher in peri- and postmenopausal women [116].

4.3.4. Transgenic Models: Remarks

In many cases, transgenic mice are used to investigate response to therapeutic agents. Different Akt and mTOR inhibitors have been tested in transgenic mice, showing good responses [106,109]. Recent preclinical studies using transgenic EC mice tested olaparib (PARP-inhibitor) [107], dienogest (fourth-generation progestin) [117], and palbociclib (CDK4,6 inhibitor) [118]. Such models have also been used to evaluate the effect of diet on EC tumorigenesis, showing that the elevation of ω-3-polyunsaturated fatty acids attenuates PTEN deficiency-induced EC development [119].

However, important caveats must be considered when these previously established transgenic mice are used in preclinical studies. First, the genetic insertion copy number and insertion site in the genome are mostly unknown, but they can have a major influence on treatment response. Temporal aspects of transgene activation should also not be neglected [71]. Finally, transgenic tumors lack naturally occurring heterogeneity and in this sense are not fully representative of human tumors.

4.4. Patient-Derived Xenografts (PDXs) and Humanized Mice

Patient-derived xenograft models (PDXs) are established by implanting a piece of freshly isolated tumor from a patient directly into immunocompromised mice [120]. Tumor pieces can be implanted heterotopically or orthotopically [120]. The orthotopic implantation has several advantages because the tumor develops within the same anatomic environment as the original one in the patient. However, this kind of implantation is technically challenging and implies the need for imaging systems to monitor tumor growth, which is why heterotopic implantations are often used to generate PDX models. Subcutaneously accessible implantation sites include the flanks, the mammary fat pad, the interscapular fat pad, and the renal capsule [121]. Different mouse strains can be used; athymic nude mice, non-obese diabetic/severe combined immune deficiency (NOD/SCID) mice, and NOD/SCID/interleukin-2 receptor common γ-chain (IL2-Rγ)-deficient (NSG) mice can be chosen based on the desired degree of immunosuppression [120,121]. The engraftment success rate ranges depending on the tumor type, the used mouse strain, and the specific implantation method. However, in general, it has been observed that engraftment is more likely to occur for metastatic tissue compared to primary tumor tissue, and can reach up to 90% [121]. Multiple studies have shown that PDX models maintain the original histological, molecular, and functional heterogeneity present in the patients' tumors over different cancer types [121–123]. What makes them an excellent model for translational cancer research is that PDXs can capture the complexity of the original human tumor (with high molecular and histological stability), they can predict the clinical response in patients [121,123–125], and they thus can be used as preclinical models for the validation of novel drugs and targeted therapies. PDXs can potentially be used for high-throughput drug screening [122,126]. In order to do so, Gao et al. showed the feasibility of the "one animal per model per treatment" ($1 \times 1 \times 1$) approach for drug screening [126]. Bruna et al. also showed that the use of PDX-derived short-term cell cultures (PDCs) are useful and are a better clinical model compared to conventional cell lines [122,126]. Furthermore, PDX models and their derived cells can be used for new biomarkers discovery and to investigate resistance mechanisms to treatments. They can potentially be used in xenopatient trials, co-clinical trials, and eventually in personalized medicine [123]. Recently, many efforts have been directed toward the development of humanized mice, in which a human immune system is (partially) restored, in order to investigate tumor interaction with the microenvironment and to investigate the role of the immune system in tumor growth and treatment response [127]. Although such systems are not available for EC yet, they might be interesting because immune blockade with immune checkpoint inhibitors are upcoming treatments for advanced and recurrent EC [128].

A detailed overview of the available EC PDX models and humanized mice can be found in the review article by Moiola et al. in this Special Issue [129].

5. Conclusions and Future Perspectives

During the last decades, our understanding of endometrial cancer biology increased mainly thanks to the advance of molecular techniques applied to the different available preclinical models.

Both in vitro and in vivo models helped to elucidate different aspects of the disease and paved the way for future preclinical and clinical investigations. Since a model is by definition imperfect in mimicking a real situation, and it naturally has concrete advantages for one aspect but disadvantages for another. Thus, the idea of employing integrative preclinical platforms with different models for one cancer type is gaining the interest of the scientific community. The exploitation of effective precision medicine platforms using different techniques and models, as has been shown for breast cancer and melanoma [122,130], is where the future of translational cancer research should point to for EC as well. In this view, initiatives like the recently established Models in Translational Oncology (MiTO) database will help to gather information about available models and help researchers in choosing the correct model to address a specific research question [131].

Author Contributions: Writing—review and editing: T.V.N., C.P.M., E.C., D.A., and F.A.

Funding: This research received no external funding

Acknowledgments: Frédéric Amant is a senior researcher for Research Foundation—Flanders (FWO).

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

3D	Three-dimensional
Akt	Protein kinase B
AMPK	Adenosine monophosphate-activated protein kinase
ARID1A	AT-rich interaction domain 1A
BDII/Han	Berlin-Druckrey II/Hannover
CCLE	Cancer Cell Line Encyclopedia
CDK4,6	Cyclin-dependent kinase 4,6
Cdk6	Cyclin-dependent kinase 6
CMT1000	Center for Molecular Therapeutics 1000
Cre-lox	Cyclization recombinase-locus of X-over P1
CTNNB1	Catenin beta 1
DA/Han	Dark Agouti/Hannover
E:P	Estrogen:progesterone
EC	Endometrial cancer
EEC	Endometrioid endometrial cancer
EGFR	Epidermal growth factor receptor
ENNG	*N*-ethyl-*N*-nitro-*N*-nitrosoguanidine
ER	Estrogen receptor
ERBB2	Erb-b2 receptor tyrosine kinase 2
ERK1/2	Extracellular signal-regulated kinase 1/2
F344	Fischer 344
FBXW7	F-box and WD repeat domain containing protein 7
FGFR2	Fibroblast growth factor receptor 2
FIGO	International Federation of Gynecology and Obstetrics
Han:Wistar	Hannover Wistar
HOTAIR	Homeobox transcript antisense RNA
ICR	Institute of Cancer Research
JFCR-39	Japanese Foundation of Cancer Research 39
KRAS	KRAS proto-oncogene, GTPase
LKB1	Serine/threonine kinase 11
loxP	Locus of X-over P1
MDM4	Double minute 4 protein
MIG-6	Mitogen Inducible Gene 6
MLH	MutL homolog 1
MNU	*N*-methyl-*N*-nitrosourea
mTOR	Mammalian target of rapamycin
NCI60	National Cancer Institute 60
OOC	Organ-on-chip
PARP	Poly (ADP-ribose) polymerase
PI3K	Phosphoinositide 3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PDX	Patient-derived xenograft
PDC	Patient-derived cell culture
PMS2	Mismatch repair endonuclease PMS2
POLE	Polymerase ε

POT1A	Protection of telomeres protein 1A
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
STAT3STR	Signal transducer and activator of transcription 3Short tandem repeat
TP53	Tumor protein p53
Wnt	Wingless/integration-1

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International Journal of
Molecular Sciences

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CTCF Expression is Essential for Somatic Cell Viability and Protection Against Cancer

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Received: 19 October 2018; Accepted: 11 November 2018; Published: 30 November 2018

Abstract: CCCTC-binding factor (CTCF) is a conserved transcription factor that performs diverse roles in transcriptional regulation and chromatin architecture. Cancer genome sequencing reveals diverse acquired mutations in *CTCF*, which we have shown functions as a tumour suppressor gene. While CTCF is essential for embryonic development, little is known of its absolute requirement in somatic cells and the consequences of *CTCF* haploinsufficiency. We examined the consequences of CTCF depletion in immortalised human and mouse cells using shRNA knockdown and CRISPR/Cas9 genome editing as well as examined the growth and development of heterozygous *Ctcf* (*Ctcf*$^{+/-}$) mice. We also analysed the impact of *CTCF* haploinsufficiency by examining gene expression changes in *CTCF*-altered endometrial carcinoma. Knockdown and CRISPR/Cas9-mediated editing of *CTCF* reduced the cellular growth and colony-forming ability of K562 cells. CTCF knockdown also induced cell cycle arrest and a pro-survival response to apoptotic insult. However, in p53 shRNA-immortalised *Ctcf*$^{+/-}$ MEFs we observed the opposite: increased cellular proliferation, colony formation, cell cycle progression, and decreased survival after apoptotic insult compared to wild-type MEFs. CRISPR/Cas9-mediated targeting in *Ctcf*$^{+/-}$ MEFs revealed a predominance of in-frame microdeletions in *Ctcf* in surviving clones, however protein expression could not be ablated. Examination of *CTCF* mutations in endometrial cancers showed locus-specific alterations in gene expression due to *CTCF* haploinsufficiency, in concert with downregulation of tumour suppressor genes and upregulation of estrogen-responsive genes. Depletion of CTCF expression imparts a dramatic negative effect on normal cell function. However, CTCF haploinsufficiency can have growth-promoting effects consistent with known cancer hallmarks in the presence of additional genetic hits. Our results confirm the absolute requirement for CTCF expression in somatic cells and provide definitive evidence of *CTCF*'s role as a haploinsufficient tumour suppressor gene. *CTCF* genetic alterations in endometrial cancer indicate that gene dysregulation is a likely consequence of *CTCF* loss, contributing to, but not solely driving cancer growth.

Keywords: CTCF; tumour suppressor gene; haploinsufficiency; zinc finger; CRISPR/Cas9; cancer; endometrial cancer; gene editing

1. Introduction

CTCF is a conserved multivalent transcription factor with diverse roles in transcriptional regulation and three-dimensional genome organisation such that it has been called the 'master weaver'

protein [1]. CTCF is essential during embryonic development, as *Ctcf* null embryos are unable to implant [2]. Tissue-specific deletion of this ubiquitous factor in mice using conditional *Ctcf* alleles has highlighted the importance of CTCF availability in somatic tissues. Conditional deletion of *CTCF* in thymocytes can hamper T-cell differentiation and cell cycle progression, but not ablate T cell function [3]. Conditional deletion of *Ctcf* in the limb mesenchyme induces extensive apoptosis during limb development highlighting CTCF's pro-survival role [4]. Similarly, deletion of *Ctcf* specifically during early mouse brain development, led to PUMA upregulation and subsequent massive apoptosis [5]. Of relevance for our studies, *Ctcf* heterozygous mice, however, are more prone to the formation of spontaneous cancers, as well those induced by radiation and chemical means [6].

CTCF links gene regulation to genomic architecture by co-ordinating DNA looping in concert with cohesin [7–9]. Within chromosomal territories, CTCF defines boundaries between sub-megabase-scale topologically-associated domains (TADs) [10–12] in a framework that is conserved [13]. These TADs themselves can serve as large gene regulatory domains establishing specific gene expression profiles [14]. TAD organisation is CTCF site orientation-specific [13,15] and rewiring of CTCF sites can significantly perturb gene expression by affecting promoter-enhancer interactions or boundaries between euchromatin and heterochromatin [16–18]. In cancer, hypermethylation or somatic mutation of CTCF binding sites has been shown to affect chromatin boundaries. This, in turn, can induce tumour suppressor silencing [19,20]; disruption of CTCF-dependent insulation leading to aberrant TAD formation and oncogene activation [21]; and cis-activation of genes implicated in cancer [22,23].

Our previous studies first demonstrated the growth inhibitory effects of CTCF in vitro [24] and subsequently confirmed that CTCF acts as a tumour suppressor gene in vivo by suppressing tumour growth [25]. Isolated *CTCF* mutations have been identified in breast, prostate and Wilms' tumours [26] and acute lymphoblastic leukaemia [27]. However recent cancer genome studies have revealed the extensive somatic mutations occurring in *CTCF* [28]. *CTCF* has been classified as a significantly mutated gene owing to its high frequency of mutation and deletion in endometrial cancer [29]. *CTCF* mutations are detected in 35% of endometrial carcinomas exhibiting microsatellite instability (MSI), and in 20% of MSI-negative tumours [30]. One report describing 17 oncogenic signatures in cancer, defines one signature, M5, as comprising MSI-positive endometrioid cancers and some luminal A breast cancers. In this subset of endometrioid and breast cancers, *CTCF* mutations were identified in 40% of samples including inactivation of specific zinc fingers (ZFs) of CTCF that would lead to altered DNA binding [31]. We since revealed that *CTCF* genetic alterations have a pro-tumourigenic effect in endometrial cancer by altering cellular polarity and enhancing cell survival [32].

Genetic lesions in *CTCF*, whether heterozygous deletion, nonsense, frameshift, or even missense ZF mutations, can all result in *CTCF* haploinsufficiency. In endometrial cancer, *CTCF* mRNA transcripts expressed from alleles containing nonsense or frameshift mutations are subjected to nonsense-mediated decay [30,32]. Somatic missense mutations in residues critical for CTCF ZF binding to DNA can result in selective loss of binding to some CTCF target sites, but not all [26], indicating the functional implications of incomplete loss of CTCF binding in cancer is unclear. Loss of heterozygosity (LOH) at 16q22 can lead to *CTCF* haploinsufficiency and *IGF2* up-regulation in Wilms' tumours [33]. To date, modelling the full impact of *CTCF* haploinsufficiency on CTCF's tumour suppressor function has not been previously examined.

In this study we assessed several genetic models of *CTCF* haploinsufficiency to reveal in detail the impact of heterozygous loss of *CTCF* in somatic cells, whole mice and human endometrial cancer. Depletion of CTCF expression in K562 erythroleukaemia cells using shRNA knockdown or CRISPR/Cas9-mediated targeting of *CTCF* decreased cellular proliferation. In vivo, *Ctcf* heterozygosity negatively impacted the growth and gross development of mice. However, p53 shRNA-immortalised $Ctcf^{+/-}$ mouse embryonic fibroblasts (MEFs) were functionally distinct from wild-type (WT) MEFs by exhibiting increased cellular growth and other known cancer hallmarks. Importantly, we were unable to generate *Ctcf* nullizygous MEFs after CRISPR/Cas9 genome editing confirming that CTCF is

absolutely essential for somatic cell viability. Finally, we examined curated human endometrial carcinoma genomic data and observed that *CTCF* haploinsufficiency contributed to the transcriptional dysregulation of specific loci as well as inducing a unique gene signature in human cancers.

2. Results

We used shRNA knockdown to model the cellular consequences of reduced CTCF expression in K562 cells. Western blots showed that CTCF protein expression was significantly knocked down by ~80% in the presence of doxycycline (dox) in sh.CTCF K562 cells compared to non dox-treated cells and sh.control cells (Figure 1Ai,ii). Cellular proliferation showed that CTCF knockdown resulted in a significant reduction of proliferation ($p < 0.0001$, Figure 1B). We similarly observed a significant reduction in sh.CTCF K562 colony number compared to non dox treatment and to sh.control (both $p < 0.0001$, Figure 1C). CTCF knockdown led to growth arrest with an increase in G1 phase ($p < 0.0001$), and a concomitant reduction of cells in S ($p < 0.0001$) and G2/M phases ($p = 0.0036$, Figure 1D). We next examined the response of sh.CTCF K562 cells after UV insult and observed CTCF knockdown in K562 cells resulted in an increase in cell viability after recovery from UV exposure ($p = 0.004$) and a decrease in apoptotic (Annexin V-positive) cells ($p = 0.0006$, Figure 1E).

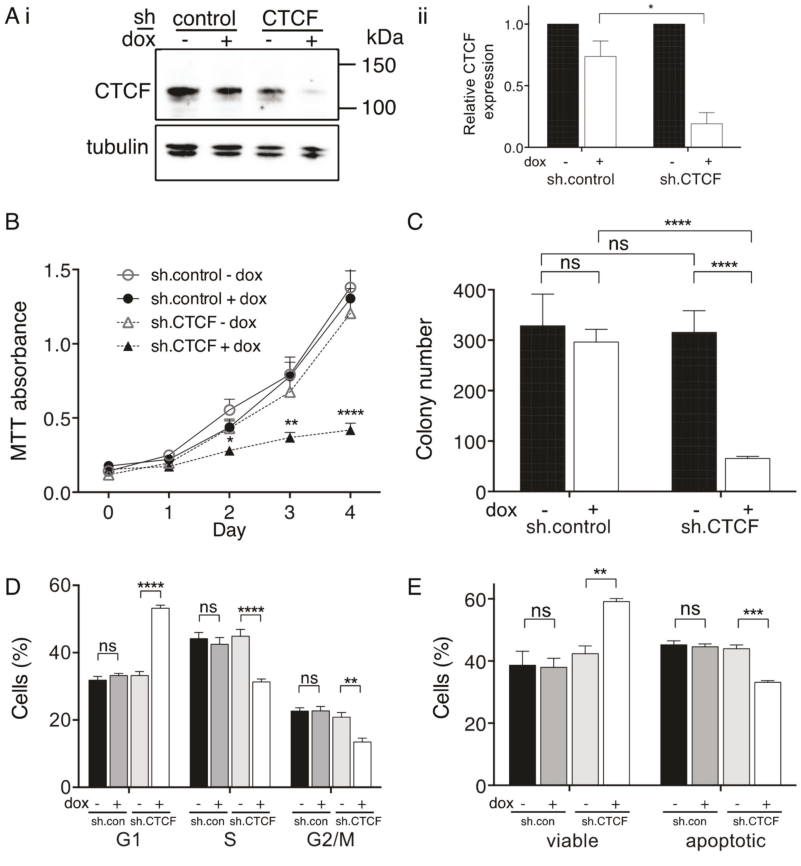

Figure 1. Inhibition of cell proliferation and clonogenicity following CTCF shRNA knockdown in K562 cells. (**A**) Immunoblot of CTCF shRNA knockdown in the presence and absence of doxycycline (dox) compared to control shRNA after 4 d: representative immunoblot (**i**); and relative CTCF expression normalised to α-tubulin confirmed by ImageJ densitometric analysis (**ii**). Functional assays performed

after 4 d knockdown including: (**B**) MTT proliferation; (**C**) colony forming assay; (**D**) cell cycle analysis; and, (**E**) apoptotic response after recovery from UV insult. Data represent the mean ± SEM for 3 experiments each performed in triplicate. Statistical analysis was performed using a Mann-Whitney U-test (ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

These data and our previous studies indicated that CTCF dosage is critical for its tumour suppressive functions [25,32], however *CTCF* haploinsufficiency has not been definitively modelled in vitro. To address this, we used CRISPR/Cas9-directed genome editing to induce genetic lesions in K562 cells (Supplementary Figure S1A), which we previously verified to contain wild type *CTCF* alleles using Sanger sequencing [25]. SgRNAs were designed to direct Cas9 nuclease cleavage on both strands of the critical third exon of *CTCF*, encoding the entire N-terminus of CTCF (Supplementary Figure S1B). All three *CTCF* sgRNA achieved efficient Cas9 cleavage of *CTCF* exon 3 (Figure 2A). A representative Western blot showing CTCF protein expression in clones isolated after CRISPR/Cas9-mediated targeting of *CTCF* using two sgRNAs (#3 and #5) is shown in Figure 2B. CTCF protein expression was decreased by approximately 50% in most surviving clones irrespective of the sgRNA used (Figure 2C). As each clone should contain at least one edited *CTCF* allele, we PCR-amplified the edited region in *CTCF*, cloned the PCR products and then sequenced them. We detected a mixture of *CTCF* alleles arising in clones including frameshifts induced by deletion or insertions near the protospacer adjacent motif (PAM) site or in-frame deletions leading to microdeletions in the CTCF protein (Figure 2D). In some clones, we observed three distinct edited *CTCF* alleles, consistent with K562 cells having a hypotriploid karyotype [34]. SgRNAs #2 and #3 induced 100% and ~96% gene editing efficiency respectively with a ~50:50 mixture of frameshifts and in-frame deletions (Figure 2D).

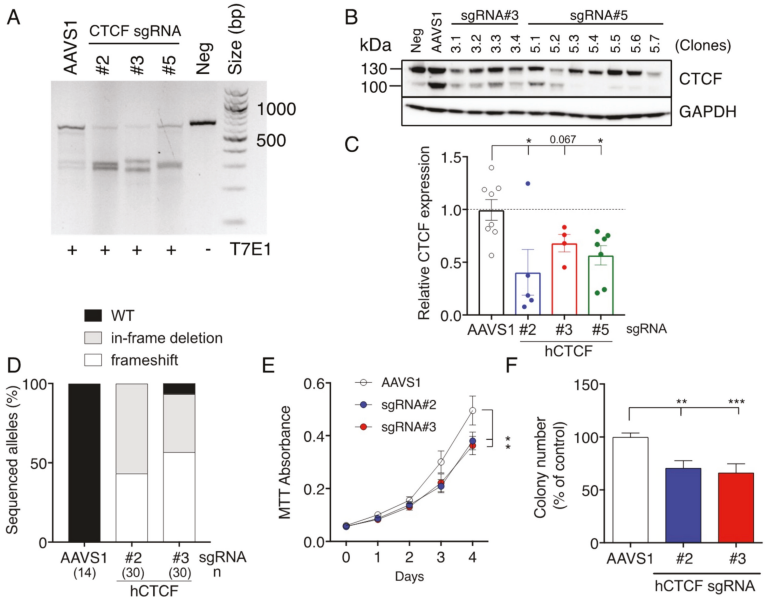

Figure 2. Inhibition of cell proliferation and clonogenicity following CRISPR/Cas9 targeting of *CTCF* in K562 cells. K562 cells were transduced with Cas9 and sgRNA-containing lentivectors (AAVS1 sgRNA = control; human *CTCF* exon 3 sgRNAs #2, #3, #5) and enriched for eGFP$^+$mCherry$^+$ cells using FACS; Neg = untransduced K562 cells. (**A**) *CTCF* exon 3 PCR amplification and T7 endonuclease I (T7EI) digestion: approximate expected sizes (in bp) for digested products #2 (310, 345), #3 (296, 359) and #5 (323, 332). Analysis of CTCF protein levels in K562 clones: (**B**) immunoblot;

and (**C**) densitometric analysis of upper 130 kDa band. CTCF protein expression normalised to GAPDH expression in each sample is shown relative to untransduced K562 cells. (**D**) Summary of results after sequencing of *CTCF* exon 3 PCR amplicons from individual clones; *n* = number of clones sequences (in brackets). Functional assays performed were MTT cell proliferation (**E**); and clonogenicity assays (**F**). Quantitative data represent the mean ± SEM for 3–4 experiments each performed in triplicate. Statistical analysis was performed using a Mann-Whitney U-test (ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

We next performed MTT cell proliferation assays on eGFP$^+$ mCherry$^+$ K562 cell pools (Supplementary Figure S1B) and showed that cells targeted using CTCF sgRNAs #2 and #3 exhibited reduced cellular proliferation ($p = 0.014$ and $p = 0.012$ respectively, Figure 2E). We also performed clonogenicity assays and confirmed that CRISPR/Cas9-directed genome editing of *CTCF* inhibited colony-forming ability by ~30 % for sgRNAs #2 and #3 (Figure 2F). Therefore, inducing genetic lesions in *CTCF* leading to haploinsufficient levels of CTCF in K562 cells had a negative impact on cellular growth.

We then examined *Ctcf* heterozygous mice to better determine what impact heterozygous deletion of the *Ctcf* locus has on post-natal growth and development. We backcrossed these *Ctcf*$^{+/\text{pgkneo}}$ mice (Figure 3A), which were originally described on a mixed 129SvJ:C57Bl/6J background and exhibited embryonic lethality as homozygotes [2], onto C57Bl/6J mice for at least 10 generations. Backcrossed C57Bl/6J *Ctcf*$^{+/\text{pgkneo}}$ mice bred with wild type C57Bl/6J (WT) mice had smaller mean litter sizes than from normal WT x WT mice (Figure 3B). This was explained by both female and male *Ctcf*$^{+/\text{pgkneo}}$ mice being born at sub-Mendelian ratios (~28% and ~24% respectively) compared to WT (*Ctcf*$^{+/+}$) mice (both $p < 0.0001$ *Chi*-square test; Figure 3C,D). After weaning at approximately day 21 we recorded mouse weights 3 times a week until 12 weeks of age. Female *Ctcf*$^{+/\text{pgkneo}}$ mice were smaller than WT littermates up to 7 weeks of age (~14% less body weight, Figure 3E), whereas male *Ctcf*$^{+/\text{pgkneo}}$ mice were consistently smaller than WT littermates in the first 12 weeks of age (~12% less body weight, Figure 3F). This reduced weight phenotype was maintained in male *Ctcf*$^{+/\text{pgkneo}}$ mice even beyond two years of age (Figure 3F). These data show *Ctcf* haploinsufficiency can significantly impact growth and development in mice. Examination of genetic variation occurring in CTCF in humans using the ExAC database [35] revealed that CTCF is extremely intolerant to genetic variation within the protein-coding region. CTCF exhibits significantly fewer nonsynonymous variants than expected (z score = 4.86) and can be classified as haploinsufficient due to intolerance to heterozygous loss-of-function variation (pLI score = 1.0; Figure 3G). Two genome-wide CRISPR screens in diploid cells [36,37] and a synthetic lethal gene trap screen in haploid cells [38] identified 916 core fitness genes essential for cell viability common to all 3 screens, including *CTCF* (Figure 3H). These data confirm CTCF as an essential gene in higher order eukaryotes.

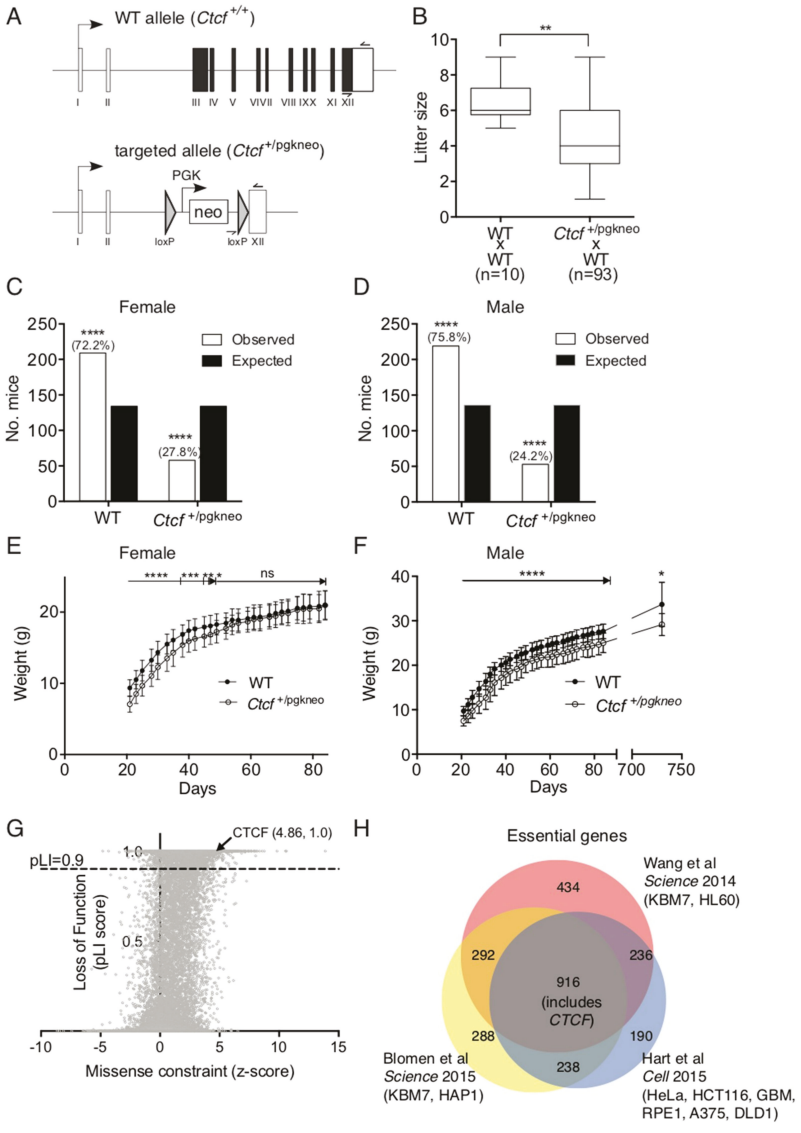

Figure 3. CTCF haploinsufficiency phenotype in mice and humans. (**A**) Schematic of targeted inactivation of *Ctcf* in mice. Open boxes represent untranslated regions, filled boxes represent coding region. Genotyping primers used to distinguish alleles are indicated with half-arrowheads. Litter sizes (**B**), and Mendelian ratios (%, in brackets) of female (**C**) and male (**D**) pups born from WT x $Ctcf^{+/pgkneo}$ intercrosses. Weights of pups during development following weaning at day 21 (mean ± SD): (**E**) for female; and, (**F**) aged male (>2 yo) mice. (**G**) Analysis of *CTCF* genetic variation in humans using the ExAC database with pLI > 0.9 indicating intolerance to heterozygous loss-of-function variation. The missense constraint is a measure of the deviation away from the observed variants in a gene versus the expected variants (high positive z-scores indicated intolerance to variation). (**H**) Venn diagram of essential genes identified in three independent genetic screens in human cells. Statistical analysis was performed using Fisher's exact test or Mann-Whitney U-test (ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

To examine the cellular consequences of *Ctcf* haploinsufficiency, we isolated mouse embryonic fibroblasts (MEFs) from a single litter containing 4 *Ctcf*$^{+/pgkneo}$ and 3 WT pups. These MEFs were immortalised with a retrovirus encoding stable shRNA knockdown of *p53* and then analysed by immunoblot for Ctcf protein expression (Figure 4Ai). Densitometric analysis of the *Ctcf*$^{+/pgkneo}$ and WT MEF samples confirmed Ctcf protein was reduced in *Ctcf*$^{+/pgkneo}$ MEFs to a mean of 58% of WT (p = 0.033, Figure 4Aii). We performed MTT assays and showed immortalised *Ctcf*$^{+/pgkneo}$ MEFs exhibited an increase in cellular proliferation compared to WT MEFs (p = 0.0028 day 2, p <0.0001 day 3, Figure 4B). *Ctcf*$^{+/pgkneo}$ MEFs also displayed an increase in colony-forming ability compared to WT (p < 0.0001, Figure 4C). We analysed cell cycle kinetics and showed that *Ctcf*$^{+/pgkneo}$ MEFs exhibited a decrease in G1 phase compared to WT MEFs (p = 0.0072) with a concomitant increase in G2/M phase (p = 0.0043) (Figure 4D). We next examined the cellular response to UV-induced apoptosis and found that immortalised *Ctcf*$^{+/pgkneo}$ MEFs exhibited a decrease in viability and concomitant increase in Annexin V-positive cells compared to WT cells (p = 0.0002 and p = 0.0005 respectively) (Figure 4E). Immortalised *p53*-deficient *Ctcf* heterozygous MEFs exhibit pro-tumourigenic characteristics, indicating that *Ctcf* is acting as a haploinsufficient tumour suppressor gene.

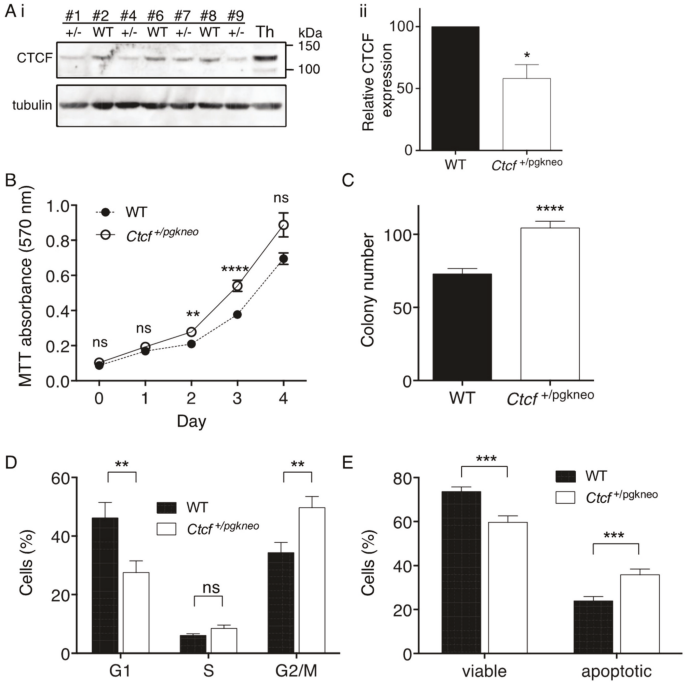

Figure 4. Functional characterisation of immortalised *Ctcf* $^{+/-}$ MEFs. (**A**) Immunoblot of whole cell lysates isolated from WT and *Ctcf*$^{+/pgkneo}$ (+/−) MEFs (clone number indicated), Th=thymus (**i**); densitometric analysis of Ctcf protein normalised to the β-tubulin loading control (**ii**). Functional assays including: MTT proliferation (**B**); clonogenicity (**C**) cell cycle analysis (**D**); and apoptosis assay following 18 h recovery from UV irradiation (**E**). Data represent the mean ± SEM for 3 experiments each performed with 4 *Ctcf*$^{+/pgkneo}$ and 3 WT cell lines. Statistical analysis was performed using a Mann-Whitney U-test (ns = not significant, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

We next used CRISPR/Cas9-mediated targeting of *Ctcf* in monoallelic hemizygous *Ctcf*$^{+/pgkneo}$ MEFs to assess the impact of inducing potentially deleterious *Ctcf* genetic lesions. Four sgRNAs were designed to target the first coding exon (exon 3) of *Ctcf*, as well as a control sgRNA targeting

the *Rosa26* locus (Supplementary Figure S1C). Efficient Cas9-directed cleavage of *Ctcf* exon 3 was observed using each sgRNA against *Ctcf*, whereas Rosa26 sgRNA had no detectable effect (Figure 5A). A clonogenicity assay was performed using *Ctcf*-targeted eGFP^{+}mCherry^{+} *Ctcf*$^{+/\text{pgkneo}}$ MEFs which showed that colony-forming capacity was significantly reduced to ~30–40% of control (Figure 5B). We isolated individual clones for each *Ctcf* sgRNA by FACS and then examined Ctcf protein expression. Ctcf expression in surviving clones was maintained despite attempts to inactivate the hemizygous *Ctcf* allele, however, in some clones lower molecular weight Ctcf species were detected, e.g., for sgRNA#4 (Figure 5C), sgRNA #1, #2, #3 (Supplementary Figure S2). These most likely result from in-frame deletions; or alternatively, frameshift mutations that occur in the first coding exon of *Ctcf*, such that alternative ATG start codons are utilised leading to N-terminal Ctcf protein truncations. Sequencing of CRISPR/Cas9 genome-edited surviving clones showed 44 out of 45 clones exhibited in-frame deletions or frameshift-inducing indels (Figure 5D). More than two-thirds of clones had in-frame deletions proximal to the PAM site leading to N-terminal microdeletions in Ctcf ranging in size from 1–62 aa (sgRNA#4; Figure 5E), some of which could be detected by Western blot (e.g., clones 4.2.3, 4.2.10, and 4.2.11; Figure 5C). These data confirm that CTCF is essential in somatic cells and that *CTCF* nullizygosity cannot be sustained in viable cells.

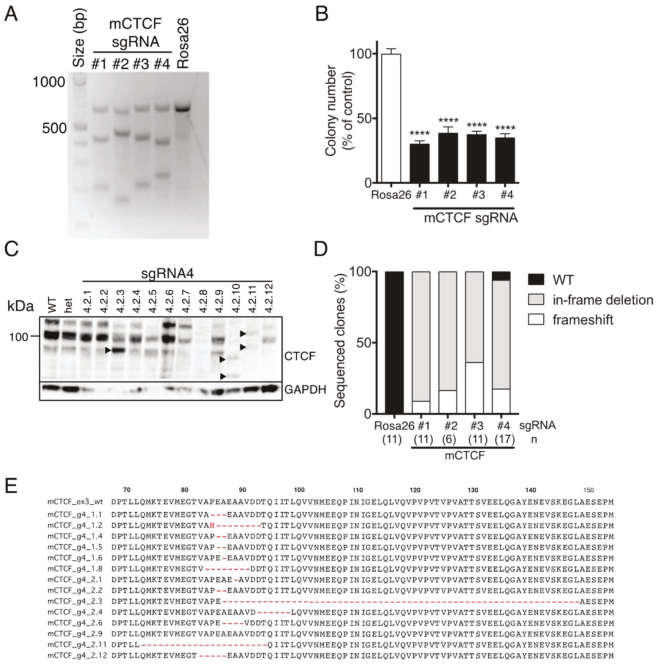

Figure 5. CRISPR/Cas9-directed editing of *Ctcf* in hemizygous MEFs. (**A**) *Ctcf*$^{+/\text{pgkneo}}$ MEFs transduced with Cas9 and sgRNA-containing lentivectors (mouse *Ctcf* exon 3 sgRNAs #1, #2, #3, and #4; *Rosa26* sgRNA) were FACS-enriched and subjected to T7EI digestion of *Ctcf* exon 3 amplicons amplified from isolated gDNA. Approximate expected sizes (in bp) for digested products #1 (427, 214), #2 (476, 165), #3 (428, 213), and #4 (399, 242). Clonogenicity assay (**B**); Western blot analysis of individual clones (from sgRNA#4, arrowheads indicate lower molecular weight Ctcf variants) (**C**); and molecular genetic analysis of individual clones; n=number of clones sequenced (in brackets) (**D**). (**E**) Examples of frequently occurring in-frame deletions in *Ctcf*$^{+/\text{pgkneo}}$ MEFs (from sgRNA#4). Quantitative data represent the mean ± SEM for three experiments each performed in triplicate. Statistical analysis was performed using Mann-Whitney U-test (ns = not significant, **** $p < 0.0001$).

To ascertain the impact of *CTCF* haploinsufficiency in the context of human cancers, we examined a uterine corpus endometrial carcinoma (UCEC) dataset from The Cancer Genome Atlas, which exhibits *CTCF* genetic alterations in 45 out of 232 patient samples (~19%) [28]. GISTIC analysis of this cohort assigned each patient sample into potential somatic copy number alterations based on relative CTCF expression level (Figure 6A). CTCF expression was decreased in a substantial proportion of endometrial cancer samples, some of which can be directly attributed to genetic deletion of *CTCF* (deep deletion). Many inactivating nonsense and frameshift mutations in *CTCF* were found in the notionally diploid population (40 out of 179, 22.3%; Figure 6A). Samples with inactivating mutations and confirmed deletions are classified herein as '*CTCF*-altered'. We then analysed other gene mutations that co-occurred with or were mutually exclusive in *CTCF*-altered endometrial cancers. *TP53* mutations (66 out of 68) occurred with mutually exclusivity to *CTCF* mutations ($p = 9.28 \times 10^{-6}$, Figure 6B,C); whereas mutations in *MED13L*, encoding a subunit of the Mediator transcriptional co-activation complex, co-occurred with *CTCF* mutations in 13 out of 23 cancers ($p = 2.64 \times 10^{-5}$, Figure 6B).

We analysed RNAseq data available for these endometrial cancer samples and showed that *CTCF* gene expression was not significantly decreased in *CTCF*-altered cancers despite the presence of inactivating mutations (Supplementary Figure S3A). We next examined the chromosomal distribution of all expressed genes detected above threshold in endometrial cancers (~13,000) and found an enrichment for genes expressed on chromosomes 11, 16, 22 and X (Figure 6D). However, in *CTCF*-altered cancers there was enrichment for genes expressed on chromosomes 1, 7, 9, 17, and 20 (Figure 6C). Further analysis of enriched chromosomal regions with altered gene expression highlighted multiple loci on the short arm of chromosome 17 including 17p13.1 (which contains the *TP53* locus), and the long arm of chromosome 20 (Figure 6D). *TP53* gene expression was significantly decreased in *CTCF*-altered cancers ($p = 0.0437$, Supplementary Figure S3B), however, there was no significant difference after the exclusion of samples containing *TP53* mutations from the analysis (Supplementary Figure S3C). To gain further insight, gene ontology analysis of biological processes in *CTCF*-altered endometrial cancers indicated *CTCF* mutation may impact predominantly on transcriptional regulation, cell signalling pathways, such as p53, and DNA methylation (Figure 6E). Closer examination of genes that were dysregulated in *CTCF*-altered cancers showed expression of the *CTCF* paralog *CTCFL* was decreased ($p = 0.0167$; Supplementary Figure S3D), the exemplar CTCF-regulated gene *H19* was decreased ($p = 0.0087$; Supplementary Figure S3E), whilst no change was observed in *ZFHX3* expression which is located adjacent to *CTCF* on chromosome 16q22 (Supplementary Figure S3F). Importantly, expression levels of the tumour suppressor genes *CDKN2A* and *PIK3CA*, which are deleted or mutated in endometrial cancer [28,39], were decreased in *CTCF*-altered samples ($p = 0.0006$ and $p = 0.0007$, respectively; Figure 6G, Supplementary Figure S3G,H). Putative tumour suppressor genes *CDH6* and *IGF2BP2* were two of the most significantly fold-decreased genes ($p = 0.0003$ and $p = 5.71 \times 10^{-5}$ respectively; Figure 6F, Supplementary Figure S3I,J). Furthermore, the expression of estrogen-responsive genes *KIAA1324, MLPH, MSX2, SPDEF*, *TFF3*, and *PIGR* were all significantly up-regulated in *CTCF*-altered endometrial cancers ($p = 0.0004$, 0.0007, 0.0032, 0.0009, 0.0122, and 0.0028 respectively, Figure 6F, Supplementary Figure S3K–P). Lastly, differentially expressed genes in *CTCF*-altered cancers were significantly overrepresented in a 19 gene signature that classifies endometrial cancers into endometrioid and serous subtypes (8 out of 19, $p = 1.46 \times 10^{-6}$) [40] as well as a 320 gene classifier that distinguishes endometrioid and serous endometrial cancers from uterine carcinosarcomas (94 out of 320, $p = 2.96 \times 10^{-44}$) [39].

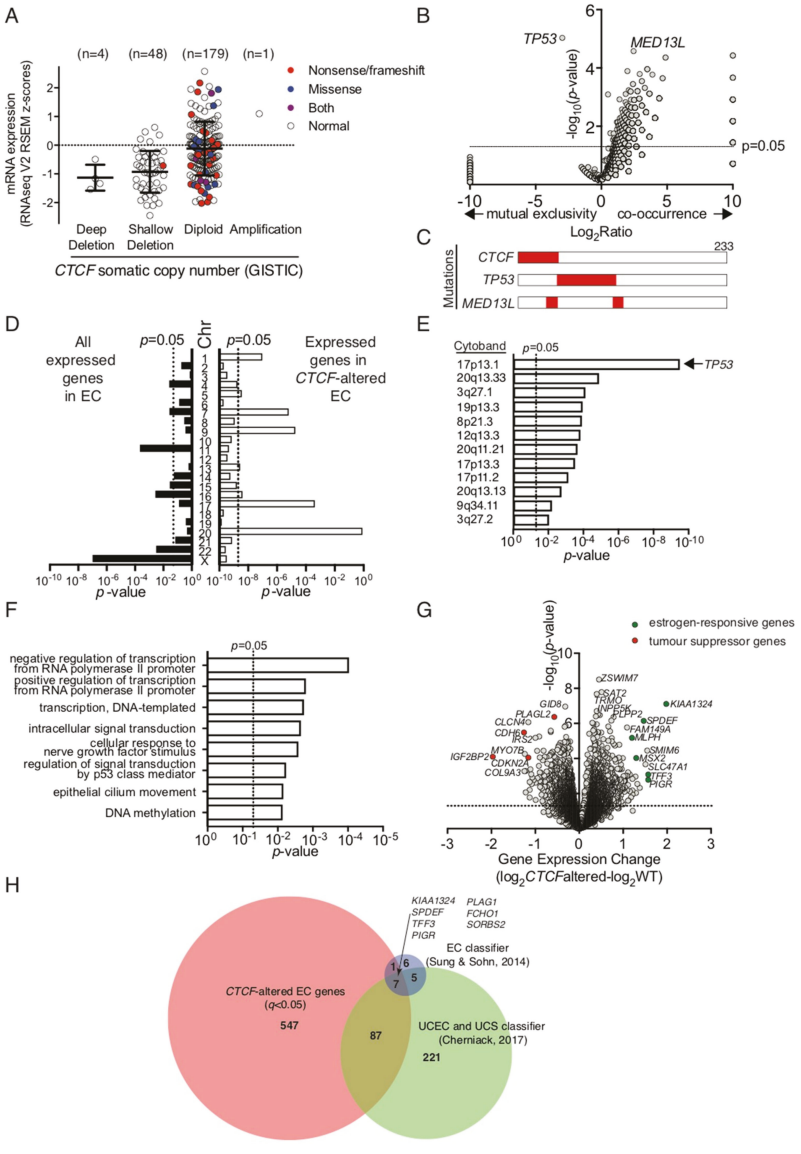

Figure 6. The molecular genetic landscape of *CTCF*-altered endometrial cancers. Gene expression and DNA sequencing data was analysed from the TCGA endometrial carcinoma patient cohort [28]. (**A**) GISTIC analysis of mRNA expression indicative of somatic copy number alterations in endometrial carcinomas. Filled symbols indicate cancers with *CTCF* coding region mutations. (**B**) Plot of significantly co-occurring or mutually exclusive mutant genes with *CTCF*-altered cancer. (**C**) Schematic showing the co-occurrence between *CTCF*, *TP53* and *MED13L* mutations in endometrial cancer (n = 233 patient samples). (**D**) Chromosomal distribution of all expressed genes in endometrial carcinoma (n = 13,271) vs those differentially expressed (n = 642; $q < 0.05$) in *CTCF*-altered cancers (data is normalised for gene density). (**E**) Chromosomal location (cytoband) of genes differentially expressed in *CTCF*-altered endometrial cancers ($q < 0.05$). Data for (**D**) and (**E**) were analysed using the Fisher's exact test. (**F**) Biological process terms enriched in gene ontology analysis of *CTCF*-altered endometrial cancers

($q < 0.05$). (**G**) Plot of most significantly differentially regulated genes in *CTCF*-altered cancers compared to *CTCF* WT cancers, genes of particular interest are highlighted. (**H**) Genes common to *CTCF*-altered differentially regulated EC genes (642 total; $q < 0.05$) and two gene classifiers used to distinguish uterine corpus endometrial cancers (UCEC) from serous cancers as well as uterine carcinosarcomas (UCS) [39,40].

3. Discussion

Haploinsufficiency arises when only a single functional copy of a gene is inadequate for normal cell function [41]. *CTCF* was identified as one of nearly 300 haploinsufficient genes in humans based on published literature or a clear association with genetic disease [42]. Herein we empirically demonstrate that CTCF can be classified as haploinsufficient due to its intolerance to loss-of-function polymorphisms in humans. CTCF haploinsufficiency resulting from germline or de novo genetic mutations in *CTCF* (including genetic deletion, frameshift mutations or missense mutation) causes intellectual disability in humans; now classified as autosomal dominant mental retardation (MRD21; OMIM #615502) [43–45]. The impact of CTCF mutations on human gene expression manifested as a predominant downregulation of genes involved in the cellular response to extracellular stimuli [43] and hypermethylation of CTCF-binding sites [45]. The mechanisms that connect *CTCF* haploinsufficiency with cancer have yet to be elucidated.

Numerous studies over more than a decade using siRNA or shRNA knockdown of CTCF have only been suggestive of CTCF's essential role in normal cell function. Typically, such experiments are short term, do not fully ablate CTCF expression and cells remain viable. Similarly, our shRNA knockdown of CTCF in K562 cells showed that cell proliferation and clonogenic capacity was decreased, cell survival after UV insult was increased, and cells underwent growth arrest. Paradoxically, after CTCF knockdown here, and in corroboration of our previous CTCF overexpression study [25] we observed tumour suppressive phenotypes for CTCF in K562 cells. These data reveal that physiological CTCF expression levels are critical for normal cellular function and reveal the functional importance of maintaining physiological CTCF expression levels.

More recently CRISPR/Cas9 mediated genome editing techniques have given tremendous insight into CTCF's roles in higher-order chromatin organisation. Most studies have focussed on rewiring CTCF-mediated chromatin interactions such as disrupting TAD boundaries [16] and switching the orientation of CTCF target sequences to alter genome topology [17]. Unbiased genetic screens using CRISPR/Cas9 sgRNA libraries or synthetic lethality in haploid cells have identified CTCF as part of an 'essentialome' containing ~900 core fitness genes required for cell viability [36–38]. However, only two studies to date have directly focused on targeting CTCF in mammalian cells using CRISPR/Cas9 editing [46,47], but with disparate outcomes. *CTCF* heterozygous MCF10A clones generated by CRISPR exhibited similar proliferation rates compared to control, though cells were slower to repair double-stranded DNA breaks [46]. Silencing of CTCF in the RS4;11 acute lymphoblastic leukaemia cell line increased colony numbers in soft agar overlays compared to control, though CTCF protein appeared to be reduced to minimal levels [47].

Our strategy was to examine the essentiality of CTCF in somatic cells by CRISPR/Cas9 targeting of *CTCF* in K562 cells and *Ctcf* hemizygous MEFs. It was clear that despite efficient editing of *CTCF* alleles in hypotriploid K562 cells, haploinsufficient levels of CTCF protein expression were still maintained in surviving clones. Edited cells exhibited reduced cell proliferation and colony-forming ability consistent with our shRNA knockdown of CTCF in K562 cells. However, in *Ctcf* hemizygous MEFs that only contain one coding allele of *Ctcf*, we were unable to completely abolish Ctcf protein expression using CRISPR. Accordingly, in surviving clones we detected a high incidence of in-frame microdeletions in *Ctcf*. As these microdeletions occur in the intrinsically disordered N-terminus we do not expect them to significantly impact Ctcf function. Induced frameshift deletions were also likely to produce truncated CTCF proteins initiating from alternate in-frame ATG start codons within the N-terminus. These results

confirm that CTCF is absolutely required for somatic cell viability and that CTCF cannot be completely inactivated in cells. Interestingly, residual CTCF protein levels can be depleted to minimal amounts in the cell before viability is significantly impacted. Targeted degradation of CTCF in mouse embryonic stem (ES) cells using an auxin-inducible degron system highlighted that endogenous CTCF protein levels could be decreased by up to 99% for at least 2 d duration without a significant impact on cell proliferation or viability [48]. Acute depletion impacted on CTCF looping and insulation of TAD regions, but genomic compartmentalisation was maintained [48]. *CTCF*-null ES cells can progress to the blastocyst stage (E3.5) purely via retention of maternal *CTCF* mRNA, but exhibit peri-implantation lethality by E4.5–E5.5 [2].

We have also shown that CTCF has a dose-dependent impact on embryonic development, as even haploinsufficient levels of Ctcf protein affected embryonic development in mice. We observed heterozygous *Ctcf* mice being born at sub-Mendelian rates (24–28%) compared to WT littermates, which was previously suggested by a study using mice with a conditionally targeted *Ctcf* allele, but was not thoroughly quantified or statistically verified [3]. Interestingly, the same *Ctcf* hemizygous mice are born at normal Mendelian rates on a mixed C57Bl/6J:129SvJ background [6], indicating that the strain background is important factor to consider in any *Ctcf* genetic deficiency studies in mice. *Ctcf* heterozygosity also impaired normal mouse weight gain during adult development for up to seven weeks, and which then remained constant in aged male mice. As these mice were fed a normal chow diet, we could not determine whether *Ctcf* heterozygosity impairs body weight control, metabolism or nutrient signaling pathways. In future studies we will examine glucose and insulin levels in plasma after feeding-fasting cycles, body tissue composition using dual energy X-ray absorption as well as studying the impact of different chow compositions on *Ctcf* heterozygous mouse development.

CTCF hemizygous mice are more susceptible than WT mice to spontaneous cancer development, as well as radiation- and chemically-induced cancers [6]. Tumours in $Ctcf^{+/-}$ mice compared to WT mice also exhibit increased aggressiveness in terms of invasion, metastatic dissemination and mixed epithelial/mesenchymal differentiation, confirming CTCF as a haploinsufficient tumour suppressor [6]. Our current findings showing an increase in cell proliferation, colony forming ability and numbers of cycling cells in p53-shRNA immortalised $Ctcf^{+/-}$ MEFs support these conclusions. Furthermore, CTCF depletion can increase genomic instability by hindering homologous recombination repair of DNA double-stranded breaks and cause hypersensitivity to DNA damage [46]. As a result, our observation of an increase in DNA damage after UV treatment of $Ctcf^{+/-}$ MEFs is consistent with impaired DNA repair. These data may explain why *CTCF* haploinsufficient MEFs in the context of additional genetic hits to p53, exhibited a number of cancer hallmarks.

The TCGA UCEC cohort consisting of low- and high-grade endometrioid carcinomas and serous tumours were genetically defined into four categories [28]. The majority of *CTCF* somatic mutations occurred in POLE ultramutated, MSI hypermutated and copy-number low cancers, whilst copy-number high cancers with a serous-like pathology harboured *TP53* mutations [28]. This was consistent with our analysis showing mutually exclusivity between *CTCF* and *TP53* mutations in endometrial cancer. *CTCF* haploinsufficiency due to *CTCF* copy loss results in poorer survival outcomes in patients with endometrioid UCEC [6], as well as serous UCEC [32]. Our analyses provide insight into the molecular pathophysiology underlying these observations. Since CTCF is known to co-ordinate higher-order chromatin architecture to facilitate interactions between transcription regulatory sequences, our data reinforces the impact that *CTCF* haploinsufficient loss imparts in endometrial cancer via transcriptional regulation. *CTCF* haploinsufficiency results in differential regulation of genes located at specific loci, particularly on chromosomes 17 and 20, including cytoband 17p13.1 containing the *TP53* locus. Whilst this is not reflected in a significant change in *TP53* mRNA expression once accounting for the *TP53* mutation status of patient samples, genes involved in p53-mediated signal transduction are impacted. Genes involved in DNA methylation were also differentially regulated in *CTCF*-altered endometrial cancers. Molecular genetic analysis of

Ctcf$^{+/-}$ mice showed DNA methylation instability compared to wild type mice [6]. Divergent CpG methylation due to *Ctcf* hemizygosity was restricted to specific loci with regions within a 2 kb window surrounding divergent CpGs exhibiting a generalised pattern of DNA hypermethylation [6]. In humans with heterozygous *CTCF* mutations exhibiting an intellectual disability, specific CTCF sites exhibited DNA hypermethylation [45]. This epigenetic dysregulation may offer an explanation as to why differential gene expression was observed at particular chromosomal loci in *CTCF*-altered endometrial cancers.

One possible hallmark of *CTCF*-altered endometrial cancers is the downregulation of tumour suppressor genes including *PIK3CA*, *CDKN2A*, *CDH6* and *IGF2BP2*. The tumour suppressor *PIK3CA* is ranked fifth after *CTCF* in the most frequently mutated genes in UCEC [6] whilst *CDKN2A* is downregulated in POLE, MSI, and copy-number low cancers compared to high-copy number cancers [28]. CDH6, which helps maintain epithelial integrity in the endometrium [49], has been shown to be a putative tumour suppressor in cholangiocarcinoma [50]. *IGF2BP2*, which was the most down-regulated gene in *CTCF*-altered endometrial cancer, was identified as a candidate tumour suppressor gene in a pan-cancer screen for homozygously deleted genes [51]. Loss of IGF2BP2 staining, which is a feature of endometrioid cancers, but not serous cancers, has been proposed as a biomarker for distinguishing endometrial tumour pathology [52].

A second hallmark of *CTCF*-altered endometrial cancers is the upregulation of estrogen-responsive genes, which includes *KIAA1324*, *MLPH*, *MSX2*, *SPDEF*, *TFF3* and *PIGR*. *CTCF* mutations do not occur in a tumour type-specific manner, but rather they define a subset of hormone-responsive cancers [31]. CTCF is a negative regulator of the pioneer factor FOXA1, which facilitates estrogen receptor interactions with chromatin in response to estrogen [53,54]. Therefore, in *CTCF* haploinsufficient endometrial tumours, FOXA1/ER interactions with chromatin may increase leading to upregulation of estrogen-responsive genes. KIAA1324, which is a positive regulator of the autophagy pathway and may protect cells from cell death, was the most upregulated gene in *CTCF*-altered endometrial cancer [55]. KIAA1324 is a marker of grade I endometrial cancer which decreases with increase in tumour grade and disease stage [56] and is a key member of gene signatures classifying histological subtypes [39,40]. Other estrogen-responsive genes upregulated in *CTCF*-altered cancers, namely *SPDEF*, *TFF3* and *PIGR*, are also components of these gene signatures, indicating that loss of *CTCF* could be an important factor determining endometrial cancer progression and pathology.

4. Materials and Methods

4.1. Cell Lines

Human erythroid leukaemia (K562) cells were grown in RPM1 1640 medium while human embryonic kidney (HEK293T) and mouse embryonic fibroblast cells were cultured in DMEM. Basal media were supplemented with 10% FCS (*v*/*v*), penicillin (100 U/mL) and streptomycin (100 µg/mL). All human cell lines were authenticated by short tandem repeat profiling (Cellbank, Westmead, Australia).

4.2. Expression Vectors and Antibodies

CTCF shRNA knockdown was performed using the pFH1-UTG-CTCFshRNA lentivector and the corresponding control shRNA vector expressing *Arabidopsis thaliana* mir-159a [32]. This lentiviral vector contains eGFP and a doxycycline-inducible shRNA. For CRISPR/Cas9 genome editing: plasmid 52628-Bsd-T2A-H2B-mCherry was used to express single guide RNAs (sgRNAs) as a lentivector and was a kind gift from Yifei Liu (Yale Fertility Centre, New Haven, CT, USA). Sense and antisense oligonucleotides encoding the sgRNAs (Supplementary Table S1) were phosphorylated with T4 polynucleotide kinase (New England Biolabs, Ipswich, MA, USA), annealed and then cloned into 52628-Bsd-T2A-H2B-mCherry following *BspMI* digestion. Plasmid 53190-pLV-hUbC-Cas9-T2A-eGFP used for stable expression of a human-codon

optimised Cas9 nuclease [57] was obtained from Addgene (Watertown, MA, USA). For immortalisation of MEFs, pMSCVp53.1224, a retroviral vector encoding a p53 shRNA, a kind gift from Ross Dickins (Walter and Eliza Hall Institute, Melbourne, Australia), was used. Primary antibodies include: rabbit polyclonal antibody against CTCF (1:1000) [24], mouse monoclonal antibodies against CTCF (1:1000) [58], α-tubulin (1:5000; sc-23948, Santa Cruz, Dallas, TX, USA) and GAPDH (1:5000; ab8245, Abcam, Cambridge, MA, USA). Secondary antibodies include: rabbit or mouse antibodies conjugated to horseradish peroxidase (Abcam, Cambridge, MA, USA; 1:5000).

4.3. Retroviral and Lentiviral Transduction

Viral supernatants were produced by calcium phosphate transfection of HEK293T cells: with pJK3, pCMVTat and pL-VSV-G packaging plasmids used to produce retroviruses; and pRSV-Rev, pMDLg/p.rre and pMD2.VSV-G used to package lentiviruses. Viral supernatants collected after 24–48 h were 0.45 μM-filtered and snap-frozen or concentrated by ultracentrifugation for 2 h at 20,000 rpm in a SW28 Beckman rotor. Viral supernatant was resuspended on ice in 10% (*v*/*v*) FCS/DMEM at 1/100 th of the original volume. Adherent cells (1–5 × 10^5/well) were seeded in six-well plates before addition of fresh medium containing viral supernatant (~5 × 10^5 transducing units) and Polybrene (4 μg/mL; Sigma, Zwijndrecht, The Netherlands) and 'spin-oculated' for 90 min at 1500 rpm. The supernatant was replaced with medium 12 h post-transduction and fluorescent cells were purified 24 h later by fluorescence activated cell sorting (FACS; >95% purity on re-analysis) using a FACS Influx (Becton Dickinson, BD, Mountain View, CA, USA). K562 cells (~5 × 10^5/mL) in 1 mL medium with 4 μg/mL Polybrene were placed in a 5 mL capped FACS tube and transduced with viral supernatant for 90 min by 'spin-oculation'. The cells were resuspended and incubated at 37 °C for 4 h before removal of viral supernatant. For in vitro assays, cells were either plated out immediately or allowed to recover after sorting for 48–72 h in medium containing 100 μg/mL Normocin (InvivoGen, Toulouse, France).

4.4. CRISPR/Cas9 Genome Editing, Validation and Molecular Genetic Analysis

SgRNAs targeting the first coding exon of human *CTCF* and mouse *Ctcf* (exon 3) were designed using the Zhang lab CRISPR design tool (crispr.mit.edu). SgRNAs targeting the adeno-associated virus integration site 1 (AAVS1) and the *Rosa26* locus were used as negative control guides in human and mouse cells respectively. We used lentiviral vectors to co-express a sgRNA with mCherry, as well as a 3XFLAG-tagged Cas9 nuclease 2A-peptide linked to eGFP. Transduced cells were FACS-enriched for $eGFP^+mCherry^+$ cells after 48 h from which gDNA was extracted from pools after 6 d for a T7 Endonuclease I assay to detect Cas9-directed DNA cleavage. We also isolated single $eGFP^+mCherry^+$ cells by FACS into 96-well plates and expanded them before isolation of genomic DNA and whole cell lysates. Genomic DNA was isolated using the Purelink Genomic DNA Extraction kit (ThermoFisher) and PCR primers were used to amplify across the targeted region (see Supplementary Table S1). PCR amplicons were denatured and re-annealed to allow heteroduplex formation, then digested with T7 Endonuclease I (New England Biolabs, Ipswich, MA, USA) according to manufacturer's instructions and then resolved using DNA gel electrophoresis. We PCR-amplified *CTCF* exon 3 from genomic DNA isolated from K562 and $Ctcf^{+/-}$ MEF clones, which had been subjected to CRISPR/Cas9-mediated gene editing, using Platinum Taq (Thermo Fisher Scientific, Waltham, MA, USA). Amplicons were ligated into pGEM-T-Easy (Promega, Madison, WI, USA) and then transformed into *E. coli*. Each clonal amplicon was then confirmed using Sanger sequencing in both directions.

4.5. Isolation of Mouse Embryonic Fibroblasts

All animal experiments were performed in accordance with an approved institutional animal ethics protocol from the Royal Prince Alfred Hospital Animal Welfare Committee (SSWAHS #2013/046 approved 5 August 2013). $Ctcf^{+/-}$ mice were obtained on a mixed C57Bl/6:129SvJ

background from the Fred Hutchinson Cancer Research Centre (Seattle, WA, USA) [2]. These mice have had the complete coding region of one *Ctcf* allele replaced with a loxP-flanked cassette containing a *pgk* promoter and *neo* gene, designated $Ctcf^{+/pgkneo}$ (Figure 3A). Mice homozygous for this allele ($Ctcf^{pgkneo/pgkneo}$) exhibit embryonic lethality prior to embryo implantation [2]. Mice were backcrossed at least 10 generations onto C57Bl/6 mice from the Animal Resources Centre (Perth, WA, USA) before beginning phenotyping studies. Timed matings were performed with $Ctcf^{+/pgkneo}$ male mice and C57Bl/6 females and female mice were checked daily for vaginal plugs. At 13.5 dpc, pregnant females were euthanised by CO_2 asphyxiation. The uterine horns were removed and the foetuses released whilst immersed in PBS. Each pup was removed from its amniotic sac, decapitated and fetal liver removed. The carcasses were minced with a scalpel and then incubated in trypsin/EDTA solution (Invitrogen, Basel, Switzerland). The tissue fragments were triturated to break up clumps, and then concentrated using centrifugation to remove trypsin. Fresh trypsin was added to create a homogeneous solution of cellular material. The trypsin was inactivated in excess DMEM medium containing 10% (v/v) FCS and then the centrifugation step repeated. The MEFs were plated in 15 cm plates and allowed to grow for 2–3 d until there were sufficient adherent cells for cryopreservation. The remaining MEFs were transduced with MSCVp53.1224 retroviral supernatant for immortalisation. Cells were selected in 50 µg/mL hygromycin (Roche Life Science, Mannheim, Germany) to enrich for immortalised MEFs and then frozen down after 1–2 passages (P2-P3 MEFs). $Ctcf^{+/pgkneo}$ mice and MEFs were genotyped according to primers listed in Supplementary Table S1.

4.6. Western Blot Analysis

Protein extracts were prepared with cell lysis buffer containing 20 mM Tris-HCl (pH 8), 150 mM NaCl, 1% (v/v) Triton X-100, 0.1% (v/v) SDS, 0.5% (w/v) sodium deoxycholate, and EDTA-free protease inhibitor cocktail (cOmplete, Roche Life Science, Mannheim, Germany), prior to separation using denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto PVDF membranes in a semi-dry transfer apparatus before immunoblotting. Membranes were blocked in PBS/0.1% (v/v) Tween 20 containing 20% (v/v) BlokHen (AvesLab, Portland, OR, USA) or PBST containing 0.3% (w/v) BSA, 1% (w/v) polyvinylpyrrolidone and 1% (v/v) PEG (mw 3350). Protein expression was detected using primary antibodies followed by washing and staining with appropriate secondary antibodies conjugated to horseradish peroxidase (HRP). The HRP substrate SuperSignal® Chemiluminescent Substrate (Pierce) was detected on a Kodak Imagestation 4000R Pro (Woodbridge, CT, USA) or BioRad Chemidoc Touch (Hercules, CA, USA). Blots were stripped with ReBlot Plus (Merck Millipore, Guyancourt, France) prior to re-probing with protein loading control antibodies. Densitometric analysis of bands from three independent blots was performed using ImageJ (National Institutes of Health, University of Wisconsin, USA).

4.7. Cell Assays

Cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Merck Millipore, Guyancourt, France). Adherent cells (1000/well) or suspension cells (5000/well) were plated in triplicate in a 96-well plate and proliferation was assessed over 4 d by the addition of MTT at 37 °C overnight. The reaction was quenched with isopropanol/HCl and absorbance measured at 572 nm using a Polarstar Omega plate reader (BMG Labtech, Durham, NC, USA). The clonogenic capacity of adherent cells was measured by plating 1000 cells/10 cm plate in triplicate and incubating for 8–10 d. Cells were washed with PBS, fixed with ice-cold methanol and stained with Giemsa Stain (Sigma, Zwijndrecht, The Netherlands) diluted 1:20 in triple-distilled water before scoring. The clonogenic capacity of K562 cells was measured by plating 5000 cells diluted in Iscove's Modified Dulbecco Medium (Life Technologies, Rockville, MD, USA) containing 3 mL Methocult GF H4230 (Stem Cell Technologies,

Vancouver, BC, Canada) onto 35 mm gridded plates in triplicate and incubating for 8–10 d. To assess UV-induced apoptosis, cells (1×10^5/well in a 12-well plate) were plated in triplicate. The following day, medium was removed from attached cells and replaced with PBS. Plates with lids removed were placed in a Stratalinker UV Crosslinker (Stratagene, La Jolla, CA, USA) and exposed to UVC irradiation (2000 μJ for MEFs, 4000 μJ for K562 cells) and allowed to recover for 18 h. Cells were harvested and stained with anti-Annexin V-APC (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol and with propidium iodide (PI) solution (5 μg/mL, Sigma, Zwijndrecht, The Netherlands). Cells were analysed on a Fortessa flow cytometer (BD Biosciences, San Jose, CA, USA) with analysis performed using FlowJo 9.7.6 software (Treestar, Ashland, OR, USA). Cell viability was measured after addition of PI and then analysed by flow cytometry. The viable population represents the Annexin V^-PI^- cells; the apoptotic population represents the Annexin V^+PI^- and Annexin V^+PI^+ cells combined.

4.8. Cell Cycle Analysis

For cell cycle analysis by DNA content, cells were washed with PBS prior to fixation in ice-cold 70% ethanol and stored overnight at 4 °C. Post-fixation, cells were washed twice with PBS to remove all traces of ethanol prior to staining with a solution containing PI (20 μg/mL), 0.1% (*v*/*v*) Triton X-100 and RNase-A (200 μg/mL). Cells were incubated for 15 min in the dark at room temperature and analysed on a Canto-II flow cytometer (BD Biosciences, San Jose, CA, USA). Cell cycle analysis was undertaken using FlowJo 9.7.6 cell cycle modelling software (Treestar, Ashland, OR, USA) by applying the Dean-Jett-Fox algorithm.

4.9. Bioinformatics Analysis

Gene expression and somatic mutation data from the uterine corpus endometrial carcinoma dataset [28] was downloaded from cBioPortal. Of the 500 samples described, 240 contain matched sequencing and copy number alteration data. Statistical tests already conducted on these data were also downloaded, including Student's test (*p*) and Benjamini-Hochberg adjusted *p*-values (*q*). *CTCF*-altered cancers included those with somatic mutations (missense, nonsense, frameshift) and deep deletions ($n = 45$). Normal *CTCF* included shallow deletions and non-mutant samples ($n = 178$). The *CTCF*-altered gene signature used in subsequent analysis includes all differentially expressed genes ($q < 0.05$).

5. Conclusions

We examined CTCF essentiality and haploinsufficiency in somatic cells and mice using various molecular genetic techniques and models. Despite achieving efficient genome editing of *CTCF* using CRISPR the inability to obtain complete ablation of CTCF expression reinforces its requirement. In all cases, cellular fitness in CTCF-targeted cells was compromised leading to surviving cells compensating with reduced CTCF protein expression or truncated CTCF protein variants. Consequently cell proliferation, colony-forming ability and cycling cells were reduced. However, in the presence of additional genetic hits, such as in p53, *CTCF* haploinsufficient cells exhibited known cancer hallmarks, namely increased proliferation and reduced cell cycle control. In human endometrial cancer datasets, we identified a unique gene signature in *CTCF* haploinsufficient cancers arising from differential gene expression at specific loci. Downregulation of tumour suppressor genes and upregulation of estrogen-responsive genes may be a molecular feature of *CTCF*-altered endometrial cancers. Our study clearly demonstrates that CTCF is a haploinsufficient tumour suppressor gene that is essential for somatic cell viability and protects against cancer. As the master of weaver of the genome CTCF plays an essential role in chromatin organisation, the full impact of *CTCF* haploinsufficiency on three-dimensional chromatin architecture remains to be elucidated.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/19/12/3832/s1.

Author Contributions: C.G.B. conceived the study, performed mouse experiments, designed the CRISPR/Cas9 targeting strategy, analysed data and wrote the manuscript. J.R. supervised research and governance and provided scientific guidance; C.M. and Y.F. performed cellular biology experiments; K.B. genotyped mice and performed phenotypic characterisation of mice; C.S. assisted in drafting the manuscript; and G.F., V.V.L., and D.I.L. provided reagents and discussion.

Funding: Financial support was provided by Tour de Cure (Scott Canner Research Fellowship) to C.G.B. and for research grants to C.G.B. and J.E.J.R; Tour de Rocks project support to C.G.B.; National Health and Medical Research Council grants (#507776 and #1128748 to J.E.J.R); Cancer Council NSW project grants (RG11-12, RG14-09) to J.E.J.R. and C.G.B.; and support grants from Cure The Future Foundation and anonymous foundation.

Acknowledgments: The authors wish to thank the Centenary Institute Advanced Cytometry Facility for FACS and the Centenary Institute animal house staff for animal husbandry.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

CTCF	CCCTC-binding factor
CRISPR	Clustered regularly interspaced short palindromic repeats
MSI	Microsatellite instability
MEF	mouse embryonic fibroblast
FACS	fluorescence-activated cell sorting

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International Journal of
Molecular Sciences

MDPI

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Receptor Activator of Nuclear Factor Kappa B (RANK) and Clinicopathological Variables in Endometrial Cancer: A Study at Protein and Gene Level

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Received: 17 May 2018; Accepted: 17 June 2018; Published: 22 June 2018

Abstract: The system integrated by the receptor activator of nuclear factor kappa B (RANK) and its ligand, RANKL, modulates the role of hormones in the genesis and progression of breast tumors. We investigated whether the expression of RANK was related with clinicopathological features of primary endometrial tumors. Immunohistochemistry was used in an endometrial cancer tissue array containing samples from 36 tumors. The amount of RANK mRNA was examined in a tissue scan cDNA array containing cDNA from 40 tumors. Normal endometrium was examined for comparison. Immunohistochemical analyses showed that RANK expression was higher in malignant than in normal endometrium ($p < 0.05$). RANK expression was related to histological grade (Pearson correlation index = 0.484, $p < 0.001$), but not to tumor stage or to age of the women. The gene expression was similar in malignant and normal endometrium. The study of RANK isoforms confirmed that the overall relative abundance of the three clearly identified transcripts was similar in normal and pathological endometrium. RANK protein expression increased from normal to malignant endometrium, and the expression level was related with tumor grade but not with stage or the age of subjects in endometrial cancer. In contrast, similar comparisons showed no change in RANK gene expression.

Keywords: RANK; endometrium; endometrial cancer; prognosis; immunohistochemistry; gene expression

1. Background

Endometrial tumors are members of the big family of endocrine-related cancer. This is certified by their frequent expression of estrogen and progesterone receptors and by their hormonal sensitivity [1]. Moreover, the long-term exposure to estrogens is a risk factor whose impact is reduced by progestogens [2,3]. Tumors from other reproductive-related organs, like the breast, are also endocrine sensitive.

Studies published in the latter years have shown that the system integrated by the receptor activator of nuclear factor kappa B (RANK) and its ligand, RANKL, may have a role in breast cancer oncogenesis (reviewed in [4]). RANK and RANKL are members of the tumor necrosis factor (TNF) family of cytokines [5]. Activation of members from the TNF family has a multifunctional

role, including inflammation, organogenesis, apoptosis, or immunological functions [6]. Of interest, malignant breast cancer cells express RANK and RANKL, and an association between the expression of RANK and parameters of aggressiveness of breast tumors has been found in experimental studies [4]. Furthermore, recent data suggest a role for RANKL/RANK in unstable mammary cell populations of BRCA1 mutation carriers (already bearing a molecular signature similar to that of basal breast cancer) [7]. Parallel clinical data have found that RANK expression is related with some important prognostic parameters, like survival or metastatic potential [8,9].

The data on RANK/RANKL in breast cancer raises questions about a potential role in endometrial cancer. Recent work has confirmed that RANKL/RANK are expressed in human endometrium and that the expression is increased in endometrial cancer. The immunohistochemical (IHC) levels of RANK seemed to relate with patients' survival or metastatic status of the tumor, but not with prognostic clinicopathological parameters, such as tumor grade of differentiation [10,11]. To clarify that apparent paradox, we have designed a comprehensive methodological approach in which both IHC and quantitative gene expression were used to investigate RANK in primary endometrial tumors from which clinicopathological features were available. In a more particular analysis, we explored whether an association exists with gene expression of specific RANK isoforms.

2. Results

2.1. Immunohistochemical Analyses

RANK-specific staining was widely observed in most endometrial samples, but higher intensity was found in malignant areas. Faint positive staining extended across luminal and glandular epithelium and stroma in benign endometrium. Positivity clearly accumulated at the cellular membrane, showing a typical honeycomb pattern, but cytoplasmic staining was common, particularly in malignant areas. Staining intensity was higher in epithelium than in stroma. Illustrative images of endometrial samples from healthy and malignant tissue representing the mentioned pattern of expression are shown in Figure 1.

Quantitative analysis of IHC revealed that staining intensity of RANK was higher in malignant than in healthy tissue (Figure 2A, $p < 0.05$,) and correlated (Pearson) with histological grade ($r = 0.484$, $p < 0.001$). In separate comparisons, values of RANK staining in grade III tumor samples were significantly higher than in grade II ($p < 0.05$) or grade I ($p < 0.01$, Figure 2B) tumors. Differences in RANK expression between histological grade II and grade I tumors were of borderline significance ($p = 0.094$).

Contrary to tumor grade, we did not detect any significant correlation between tumor stage and protein RANK values ($r = 0.246$, $p = 0.103$). Indeed, the expression of RANK did not seem to be discriminative of tumor stage because the average RANK values were similar between stage groups (Figure 2C).

No correlation was detected between the expression of IHC RANK and age ($r = 0.171$, $p = 0.327$, Figure S1A). The result did not change when tumors were grouped by age range (10-year intervals, $r = 0.220$, $p = 0.205$, Figure S1B) or when analyses were performed within sets of tumors that were grouped by histological grade or by tumor stage.

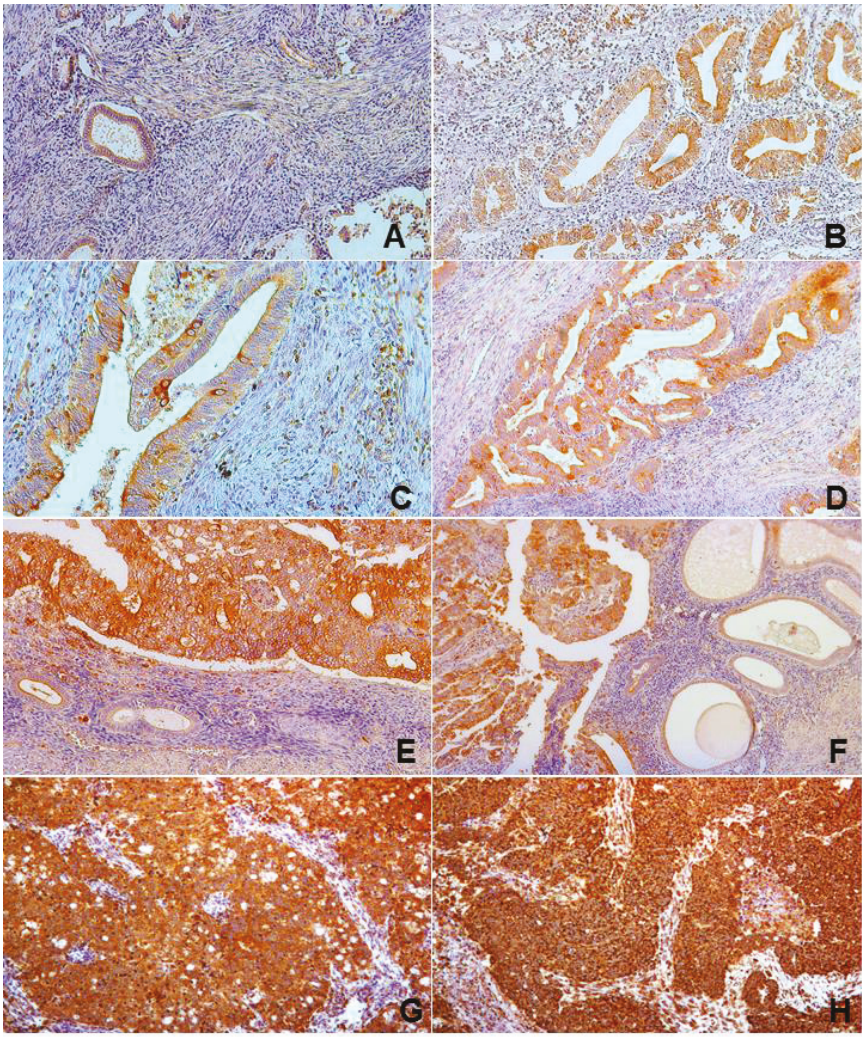

Figure 1. Representative patterns of RANK immunohistochemical staining (**brown** color) in normal (**A**), hyperplastic (**B**), and malignant (**C**–**H**) endometrium. Illustrative examples of the histological grade I (**C**,**D**), II (**E**,**F**), and III (**G**,**H**) tumor tissues are shown. Intense staining in epithelium of all samples with widely increased signal in undifferentiated areas of malignant tissues (**C**–**H**) was observed. Signal augmented gradually with the increasing histological grade. Note clear cytoplasmic as well as nuclear staining in epithelial and stroma cells of malignant tissue (**C**). A typical honeycomb staining pattern for RANK was observed in malignant tissue of different histological grade tumors (**D**,**E**). Note increased signaling in RANK staining in the upper (malignant) vs. lower (normal) areas of the tissue in (**E**). Also, note wider distribution of RANK signaling in histological grade III tumors (**G**,**H**) vs. lower grade (**C**–**F**) and normal (**A**) endometrium. Magnifications, ×100 (**A**,**B**,**D**–**H**) and ×200 (**C**).

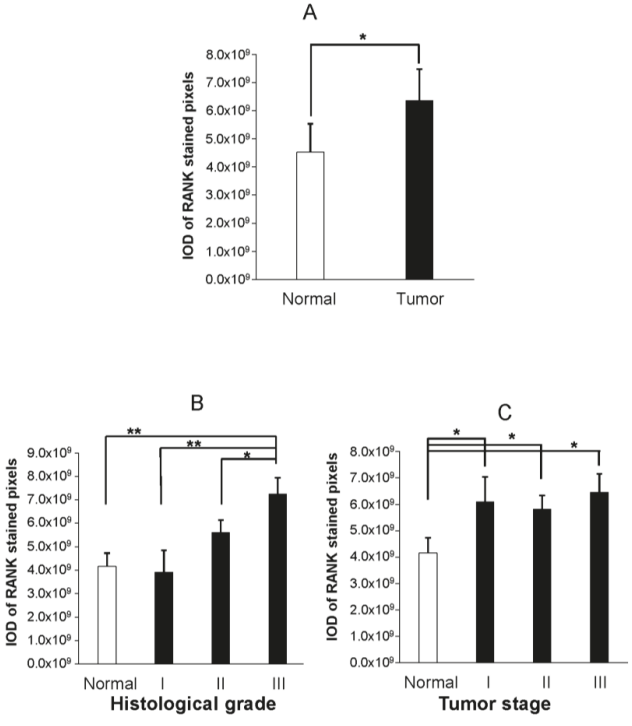

Figure 2. Graphs show mean ± SEM RANK protein expression as detected by quantitative immunohistochemistry in endometrium from normal and endometrial cancer samples. A Student's *t*-test was employed for comparisons of RANK IHC staining between normal and tumor endometrial samples (**A**). An ANOVA followed by LSD post hoc analysis was performed to detect mean differences between normal and tumor samples grouped according to their histological grade (**B**) or tumor stage (**C**). Asterisks in (**B**) denote significant differences between histological grade III and the remaining (lower histological grade and normal endometrium samples) groups. Higher but not statistically significant RANK values were noted in histological grade II when compared to histological grade I or to normal samples ($p = 0.105$, $p = 0.094$, respectively). Asterisks in (**C**) denote statistically significant differences between normal and each of the groups resulting from grouping pathological samples according to their tumor stage. No differences in IHC staining values were detected when tumor stages groups were compared to each other. * $p < 0.05$, ** $p < 0.01$.

2.2. PCR Analyses

RANK mRNA expression was consistently detected in all but one control and five tumor endometrial samples. When normalized to β-actin, relative values of RANK mRNA expression were not different between normal and malignant endometrium (Figure 3A). Analyses restricted to malignant samples could not detect significant correlation (Spearman's Rho, r_s) between mRNA RANK expression and histological grade ($r_s = 0.117$, $p = 0.461$), tumor stage ($r_s = 0.098$, $p = 0.439$), age ($r_s = 0.190$, $p = 0.274$, Figure S2A), or stratified age ($r_s = 0.229$, $p = 0.187$, Figure S2B). Similarly, no significant differences in mRNA RANK expression were found between groups when samples were stratified attending to histological grade (Figure 3B), tumor stage (Figure 3C), or age. Additionally, we observed that there were no differences in QF-PCR RANK mRNA when tumor cases were grouped according to the presence/absence of affected nodes (Figure S3A) or the confirmed existence of metastases (Figure S3B).

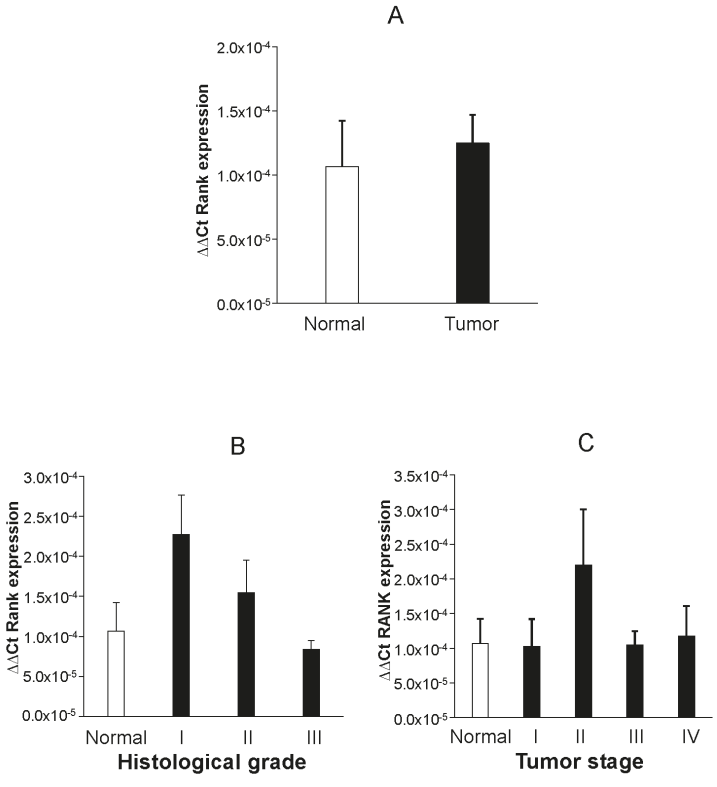

Figure 3. Graphs show mean ± SEM RANK mRNA expression as detected by RT-QF-PCR in endometrium from normal and endometrial tumor cases. A Mann–Whitney *U* test was employed for comparisons of RANK mRNA values between normal and overall endometrial tumor cases. (**A**) A Kruskal–Wallis followed by Mann–Whitney analysis were performed to detect differences between normal and tumor cases grouped according to their histological grade (**B**) or tumor stage (**C**). No statistically significant differences were detected.

Amplification of RANK isoforms revealed different levels of expression as follows: *TNFRSF11A* > *TNFRSF11A_Δ8,9* > *TNFRSF11A_Δ7,8,9* > *TNFRSF11A_Δ9*. The *TNFRSF11A* was the highest expressed isoform, detected at Ct 28–31 and showing a 3–5-fold higher expression than *TNFRSF11A_Δ8,9*. Also, *TNFRSF11A_Δ7,8,9* showed 5–7-fold lower values than *TNFRSF11A* and thus was detected above a valid limit of detection in 50% of the cases. Finally, *TNFRSF11A_Δ9*, the lowest expressed isoform, was only detected in 6 samples and with Ct values ranging from 35 to 40, a limitation that made us discard them for subsequent analysis. The relative abundance of the other two detected transcripts, *TNFRSF11A_Δ8,9* and *TNFRSF11A_Δ7,8,9*, was also similar in normal and pathological endometrium (Figure S4). Grouping the tumor samples according to histological grade or clinical stage did not affect the relative abundance of transcripts. Overall, these results suggest that RANK mRNA expression is not altered at the transcriptional level in endometrial tumors.

3. Discussion

Overall, our findings of a direct relationship between RANK expression and tumor histological grade partially differ from the only published study on primary tumors, and although consistent with that study, no association of RANK was found with tumor stage or with age [12]. The absence of

metastases in our IHC series prevented us from investigating the relationship of RANK protein with that feature.

We have also investigated RANK expression at gene level in another set of tumors and have found that there were no differences between normal and malignant endometria. Accordingly, no relationship could be detected with clinicopathological parameters of tumors or when tumors were grouped according to the presence of nodal or distant metastases. The differential expression of RANK isoform genes did not change the conclusion.

There is not an obvious explanation for the difference, despite the fact that IHC and RT-PCR data were obtained in different tumor series. The possibility that post-transcriptional upregulation might have an influence seems a possible option.

More advanced work in breast cancer may help to accommodate our apparently paradoxical findings. Immunohistochemical data have shown that RANK expression was associated with lower overall survival and with lower disease-free survival in breast tumors [8,9]. Another study using a publicly available microarray dataset of primary breast cancer patients found an association of RANK expression with skeletal metastases [13]. However, debate also exists in what refers classical clinicopathological markers in breast cancer. High RANK expression was associated with higher grade and cell proliferation, as well as with negative hormone receptors in one study [8], but the findings were not confirmed in another study [9].

Less information exists in what refers to more sophisticated molecular biology technology. The assessment of RANK mRNA levels found a preferential association with high tumor size and grade, and with negative estrogen receptors in one study [13], but another study using RT-PCR found that the RANK transcript levels were lower in tumors than in normal tissue samples, and reduced expression of RANK was associated with general and bone metastases or death because of the disease [14]. Despite the need for more clarifying work, the existing data have moved investigators to propose RANK expression as a prognostic marker in breast malignancies. Additionally, new work keeps providing data that add consistency to a key role of RANK/RANKL in the management of breast cancer [15]. Whether the value of RANK in endometrial cancer will reproduce that in breast cancer is still uncertain. Even so, the value of RANK as one useful marker in endometrial cancer deserves attention. The effort will be worthwhile because, should a role for RANKL/RANK be found in the genesis or progression of endometrial cancer, the possibility of using ad hoc anti-RANKL proteins might open a new area in prevention and treatment [6,16]. Looking again at the breast, the phase III clinical trial (D-CARE) is already examining the potential of denosumab, an anti-RANKL antibody, to increase the bone-metastasis-free survival [17].

4. Methods

4.1. Tissue Samples and Experimental Design

To investigate the prognostic value of RANK protein expression, specimens of normal and tumorous endometria were obtained through a tissue microarray (TMA) commercially available (catalogue number ab178155) from Abcam (Cambridge, UK). The TMA contained 48 cases of paired normal and malignant tissues with different histological grades and TNM stages. Tissues in the TMA had been arrayed according to the grade of malignancy and were all ready for IHC processing. Each case was represented by 2 formalin-fixed, paraffin-embedded 4 μm gross, 1.5-mm diameter spots on the TMA. The age (years) of cases by histological groups was (N, mean ± standard deviation, SD): normal (12, 46.75 ± 10.08); endometrial adenocarcinoma (36, 51.64 ± 8.59). The postmenopausal status of the cases was unknown. Clinicopathological features of the endometrial tumors are detailed in Table 1.

Table 1. Clinicopathological features of the 36 endometrial carcinoma cases investigated with immunohistochemistry.

Parameter	Variable	N	%
Age	<50	14	38.88
	≥50	22	61.12
Grade	I	4	11.11
	II	17	47.22
	III	13	36.11
	Unreported	2	5.56
Stage	I	23	63.89
	II	9	25.00
	III	3	8.33
	Unreported	1	2.78

To investigate whether the amount of RANK mRNA was related to prognostic factors of endometrial cancer, a commercially available (catalogue number EDRT305) TissueScan^{TM} cDNA array (TcDA) plate, containing cDNA from endometrial tumors at different histological grades, clinical and TNM stages, was purchased from OriGene (Rockville, MD, USA). The endometrial cancer array contained cDNA (N, age in years, mean ± SD) from primary endometrial adenocarcinoma (40, 62.68 ± 12.74). The postmenopausal status of the cases included in the cancer was unknown. Clinicopathological features of the tumors included in the supplemented TcDA are detailed in Table 2.

Table 2. Clinicopathological features of the 40 endometrial carcinoma cases investigated with RT-QF-PCR in the tissue cDNA microarray.

Parameter	Variable	N	%
Age	<50	5	12.50
	≥50	35	87.50
Grade	I	2	5.00
	II	21	52.50
	III	17	42.50
Stage	I	12	30.00
	II	7	17.50
	III	13	32.50
	IV	8	20.00
M-Stage *	M1	7	17.50
	MX	33	82.50
N-Stage **	N0	19	47.50
	N1	11	27.50
	Nx	10	25.00

* N0, N1, NX respectively denote that nodes are negative, positive, or could not be assessed. ** M1, MX respectively denote that distant metastases are positive or could not be assessed.

Added to the PCR array plate were 8 exogenous cDNAs from normal proliferative endometrium that was retrieved from women (aged 30–35) undergoing voluntary sterilization at our center. The Human Ethics Review Committee of INCLIVA approved on 19 December 2013 the whole protocol and a signed consent form was obtained from women subjected to endometrial biopsies. Samples were obtained with an endosampler (Pipelle, Cornier, Neully-en-Telle, France) at the time of surgery, as previously described [18]. Tissue was stored frozen at −80 °C until subsequent mRNA extraction, and RT procedures were achieved to obtain cDNA following routine methodology [19].

Approximately 2 µL containing 100 ng of equivalent cDNA from each endometrial biopsy were pipetted on the original array plates from OriGene. Once the plates had been set up, RANK isoforms

were amplified by PCR and its relative expression normalized as described below so as to explore for the existence of quantitative and qualitative differences (i.e., alteration of the pattern of RANK isoforms) associated to the analyzed parameters of the endometrial tumors.

4.2. IHC Detection of RANK in TMA

Immunohistochemical staining of RANK was performed on arrayed tissue by using a human RANK/TNFRSF11A antibody (MAB6831—R&D Systems Inc., Minneapolis, MN, USA) coupled to a Dako (Glostrup, Denmark) REAL© EnVision© Peroxidase/DAB+, Rb/Mo Detection System (catalogue number K500711). Initial pilot experiments were conducted with samples of normal endometrium in order to optimize the concentration of RANK antibody and related IHC step procedures. In brief, sections were dewaxed by incubation in an oven at 60 °C for 60 min and rehydrated routinely. Antigen retrieval was performed by heating at 120 °C (under 1.9 bar pressure) for 2 min and submerging slides into Dako Low Retrieval Solution (i.e., citrate buffer at pH = 6). Endogenous peroxidase was quenched by incubation of the slide with 3% H_2O_2 in PBS at room temperature for 15 min. Subsequently, slides were incubated overnight at 4 °C with RANK primary antibody diluted 1:150 in Dako antibody diluent. On the next morning, the slides were washed with PBS and incubated for 20 min at RT with the HRP-link included in the Dako kit following manufacturer instructions. Detection of peroxidase was revealed using 3,3′-diaminobenzidene and subsequently the TMA was counterstained for 30 s with Harris haematoxylin, rehydrated, and mounted for visualization. Negative controls were included in each experiment by incubating tissue sections with antibody dilution buffer instead of the primary RANK antibody. Positive control slides consisted of triple-negative breast cancer and giant cell tumor of bone.

4.3. Quantification of IHC RANK Signalling in TMA

Spots in the array were photographed at different magnifications (10× or 20×) for descriptive and quantitative analysis of RANK signaling. Specifically, for quantification purposes, four random high-power fields (20×) were photographed per core/spot in the TMA with the use of an image analysis system linked to a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan). Due to the reduced size of each spot (1.5 mm), the number of images taken (4) was sufficient to embrace and represent the extension of the whole tissue in each core. The density of RANK staining was estimated as a function of RANK distribution and intensity signaling. For such purposes, the brown-stained (RANK) area of interest in each image was initially outlined and highlighted by using the segmentation tool included in Image Pro Plus 6.0 (Media Cybernetics Inc., Silver Spring, MD, USA) software analysis. Subsequently, the optical density of each individual pixel included in the outlined area of interest was obtained so that RANK density was expressed as the summation of the optical densities (Integrated Optical Density (IOD) parameter) provided by outlined pixels.

4.4. QF-PCR Amplification and RANK and Quantitative Analysis

OriGene endometrial cancer array supplemented plates were loaded into each well with 30 μL of a master mix stock solution containing 2× SYBR green mix (Life technologies, Carlsbad, CA, USA), water, and specific primers at 0.05 μm final concentration. The sequence of primers for β-actin, RANK (TNFRSF11A) and its variant RANK isoforms lacking exon 9 (*TNFRSF11A_Δ9*), exons 8–9 (*TNFRSF11A_Δ8,9*), and exons 7–9 (TNFRSF11A_Δ7,8,9), has been previously described [20]. Real-time PCR was performed using an ABI PRISM 7500 Sequence Detection System (Perkin Elmer Corp., Norwalk, CT, USA) according to the manufacturer instructions with a heated lid (105 °C), an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative expression level of RANK was calculated with the comparative $2^{\Delta\Delta Ct}$ method, where $\Delta C_t = C_{t(target)} - C_{t(control)}$, $\Delta\Delta C_t = C_{t(target)} - C_{t(calibrator)}$ and all samples were normalized to the *β-actin* gene.

In order to quantify alterations in profiling pattern of RANK isoforms associated with cancer parameters, the expression of each RANK isoform per case was normalized by dividing its specific

$2^{\Delta\Delta Ct}$ value by the summation of $2^{\Delta\Delta Ct}$ values from the four RANK isoforms. Results were expressed as percentage.

4.5. Statistical Analysis

Statistical analysis was performed using SPSS 23.0, and the data were expressed as mean ± SD or standard error of the mean (SEM), where appropriate. For single comparisons (i.e., presence/absence of affected nodes or metastases and differences in RANK protein and mRNA expression between normal and pathological cases), a Student's *t*-test or a nonparametric Mann–Whitney *U* test were employed. For multiple comparisons, cases were grouped according to age, histological grade, and tumor stage, and one-way ANOVA followed by a post hoc (Fisher's LSD) analysis or a nonparametric Kruskal–Wallis followed by Mann–Whitney tests were employed to detect specific differences between groups. A bivariant Pearson or Spearman's Rho correlation test for normally and nonnormally distributed samples were respectively employed to detect correlations between RANK expression and age of patients, histological grade, and tumor stage. Statistical significance was defined as $p < 0.05$.

5. Conclusions

We found that the IHC expression of RANK was higher in malignant than in normal endometrium and that RANK IHC score correlated with tumor grade. Gene expression, however, did not change between normal and malignant tissue. Future work should investigate whether the difference is reproduced in larger tumor series and, in such cases, which are the molecular determinants. Also, further work should clarify whether RANK expression relates to metastatic disease and survival.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/19/7/1848/s1. Data supporting the results reported in this article are kept in an ad hoc created data-base stored in the Department of Pediatrics, Obstetrics and Gynecology of the School of Medicine, University of Valencia, Spain. Access to those data is available under request.

Author Contributions: A.C., V.R.-G. and J.M. were responsible of performing experimental work, data acquisition and statistical analysis. O.B. was responsible of pathological analyses. R.G. and J.J.T. participated in study concept and design, in the supervision of experimental work, and in manuscript preparation. A.C. was responsible of study concept and design, interpretation of data, and writing of the manuscript. All authors reviewed and approved the manuscript.

Funding: This work was supported by Spanish Ministry of Economy and Competitiveness through the Miguel Servet Program [CP13/00077] cofounded by FEDER (European Regional Development Fund) and awarded to Raúl Gómez as well as by Carlos III Institute of Health grants awarded to Raúl Gómez [PI14/00547 and PI17/02329] and to Antonio Cano [PI12/02582].

Conflicts of Interest: The authors declare no conflict of interest.

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International Journal of
Molecular Sciences

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Development of an Image-Guided Orthotopic Xenograft Mouse Model of Endometrial Cancer with Controllable Estrogen Exposure

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Received: 1 August 2018; Accepted: 22 August 2018; Published: 28 August 2018

Abstract: Endometrial cancer (EC) is the most common gynaecological malignancy in Western society and the majority of cases are estrogen dependent. While endocrine drugs proved to be of insufficient therapeutic value in the past, recent clinical research shows promising results by using combinational regimens and pre-clinical studies and identified potential novel endocrine targets. Relevant pre-clinical models can accelerate research in this area. In the present study we describe an orthotopic and estrogen dependent xenograft mouse model of EC. Tumours were induced in one uterine horn of female athymic nude mice using the well-differentiated human endometrial adenocarcinoma Ishikawa cell line—modified to express the luciferase gene for bioluminescence imaging (BLI). BLI and contrast-enhanced computed-tomograph (CE-CT) were used to measure non-invasive tumour growth. Controlled estrogen exposure was achieved by the use of MedRod implants releasing 1.5 μg/d of 17β-estradiol (E2) in ovariectomized mice. Stable E2 serum concentration was demonstrated by LC-MS/MS. Induced tumours were E2 responsive as increased tumour growth was observed in the

presence of E2 but not placebo, assessed by BLI, CE-CT, and tumour weight at sacrifice. Metastatic spread was assessed macroscopically by BLI and histology and was seen in the peritoneal cavity, in the lymphovascular space, and in the thoracic cavity. In conclusion, we developed an orthotopic xenograft mouse model of EC that exhibits the most relevant features of human disease, regarding metastatic spread and estrogen dependency. This model offers an easy to manipulate estrogen dosage (by simply adjusting the MedRod implant length), image-guided monitoring of tumour growth, and objectively measurable endpoints (including tumour weight). This is an excellent in vivo tool to further explore endocrine drug regimens and novel endocrine drug targets for EC.

Keywords: endometrial cancer; orthotopic xenograft model; estrogen dependent; bioluminescence imaging; contrast-enhanced CT scan

1. Introduction

Endometrial cancer (EC) is the most common gynaecological malignancy in the Western world with over 60,000 new cases estimated in 2017 (U.S.; https://seer.cancer.gov). About 70% of ECs are diagnosed at an early stage and are treated with surgery and with selected cases also receiving adjuvant chemo/radio therapy. Nevertheless, about 25% of EC patients will develop recurrent diseases. In patients with recurrent EC, and in EC cases with primary advanced stage and distant metastatic disease, treatment options are limited. The y consist of aggressive chemotherapy, which however yields low efficacy, poor patient prognosis, and is accompanied by significant side effects [1,2]. Hence, the re is a need for additional treatment possibilities.

EC is an estrogen dependent disease, but in contrast to other hormone-dependent conditions like prostate or breast cancer where endocrine therapy is successfully used, treatment of EC with hormonal drugs showed little efficacy [3–6]. However, recent clinical trials indicate that the efficacy of hormonal drugs is improved if combined with other targeted treatments (see Section 3) [7]. Since ongoing pre-clinical research has already identified a number of additional novel endocrine targets [8–10], it can be envisaged that in the near future several endocrine targets and novel drugs will be available for various combinational regimens in EC clinical trials.

To accelerate research in this field, relevant pre-clinical models need to be developed that better mimic the human situation, in comparison with the current models. In the case of EC, pre-clinical models are based on in vitro 2D monolayer cell cultures, 3D spheroid cell cultures, and in vivo models using the chorioallantoic membrane assay (CAM) or by a subcutaneous injection of human EC cell lines in athymic mice. However, the se models are suboptimal and often lack estrogen dependency and clear estrogen responses (see Section 3) [10–12].

Orthotopic xenografts models are relevant pre-clinical tools because the tumour is induced at the same location and similar microenvironment where it occurs in humans. Recently, different research groups reported successful orthotopic EC xenografts in mice that mimicked the human condition including metastatic spread [13–16]. In particular, Haldorsen and co-workers optimised the orthotopic EC xenograft using the estrogen dependent Ishikawa cell line. However, tumours were grown in the presence of the natural endogenous source of estrogens, the ovaries, and the authors did not explore the possibility to control estrogen exposure [14]. To be able to control and modulate steroidal exposure and mimic conditions like post menopause, in collaboration with the same authors, we here further advanced this orthotopic EC xenograft model to control steroid exposure and induce estrogen dependent tumour growth—as is the case in the majority of ECs in humans. This model offers the possibility to manipulate estrogen (and other steroid) dosage, image-guided monitoring of tumour growth, and easily determined objective endpoints to measure estrogen response. Moreover, it exhibits clinical features similar to human EC like metastatic spread and the presence of lymphovascular

invasion (LVI), thus being a highly relevant model of the disease. We believe this is an excellent in vivo tool to further explore endocrine drug regimen and novel endocrine drug targets for EC.

2. Results

2.1. Orthotopic EC Xenograft: Cell Titre Optimization

We first determined the optimal number of Ishikawa-derived cells required for tumour induction using three different titres (1×10^6, 3×10^6 and 5×10^6 cells/mouse; Table 1) that were injected in gonad-intact mice with no further steroid supplementation (see scheme in Figure 1a). Ishikawa clone 1 was used in this optimization. The lowest dose (1×10^6 cells) gave a weak bioluminescence (BLI) signal and tumour engraftment did not occur in one-out-of-three injected mice. Although all mice receiving 5×10^6 cells developed a tumour, it was technically challenging to inject such a large number of cells into the uterus. Orthotopic injection of 3×10^6 cells appeared to be the optimal dose since no technical difficulties were experienced and all mice developed a tumour.

Table 1. Optimization of cell titre for tumour induction.

Injected Cells (Number)	Number of Mice	Mice with Tumor (%)	Peritoneal Metastases *	Distant Metastases #
1×10^6	3	2 (67%)	I	0
3×10^6	3	3 (100%)	I, L, K, S, St	1 (T + LVI)
5×10^6	3	3 (100%)	I, L, K, S, St	0

* All mice developed peritoneal spread as assessed by ex-vivo BLI (bioluminescence). # Number of mice with metastases. I = intestine, L = liver, K = kidney, S = spleen, St = Stomach, T = thoracic cavity; LVI = lymphovascular invasion.

2.2. Ovariectomy and Estrogen Supplementation (MedRod Implants)

In order to control the levels of circulating E2 and measure estrogen dependent EC growth, the ovaries, which are the main endogenous sources of female sex steroids, were removed by ovariectomy (OVX) and E2 was provided exogenously by E2 releasing MedRod implants. MedRods consisted of 1–4 mm diameter cylinder-shaped elastic rods containing E2 and were implanted subcutaneously on the back of the mice, in between the scapulae. The serum E2 concentrations in mice with E2 MedRods with daily releases of 1.5 µg/day (5.5 nmol/day) resulted in constant average serum concentrations of E2 being between 338 and 443 pM (Figure 1b). Accordingly, implants releasing another kind of estrogen (estrone) had similar performances, i.e., constant serum levels of the test compound at different doses (Supplementary Figure S1).

2.3. Estrogen Responsiveness of the Orthotopic EC Xenograft

To assess estrogen dependency of tumour growth, cancer cells (3×10^6) were injected, mice were ovariectomized, and E2 or placebo releasing MedRods were implanted (Figure 1a and Table 2). Three Ishikawa derived clones (clone 1, clone 2 and clone 3) were used. The se clones were obtained through genetic modification and clonal expansion of the parental Ishikawa cells (see Section 4) and behave similarly in terms of E2 responsiveness based on previous characterisation [10]. Three clones were nevertheless used to avoid any clonal effect biasing the results.

Previous studies on estrogen dependent cancer models showed that estrogens are required for initial tumour engraftment (lag phase) [17–19]. The refore, complete OVX was performed approximately two weeks after tumour cell injection, i.e., when tumours were successfully engrafted, as assessed by positive BLI signals. However, at the time of tumour cell injection, the ovary at the same horn-side of the injection was removed (ipsilateral OVX) to avoid that, after the lag phase (two weeks later), tumour overgrowth would impede the complete removal of the ovary or that part of the tumour would be removed together with the ovary. Following complete OVX, MedRod implants releasing either placebo or 1.5 µg E2/day were implanted (see timeline in Figure 1a and the procedure in Supplemental Figure S2).

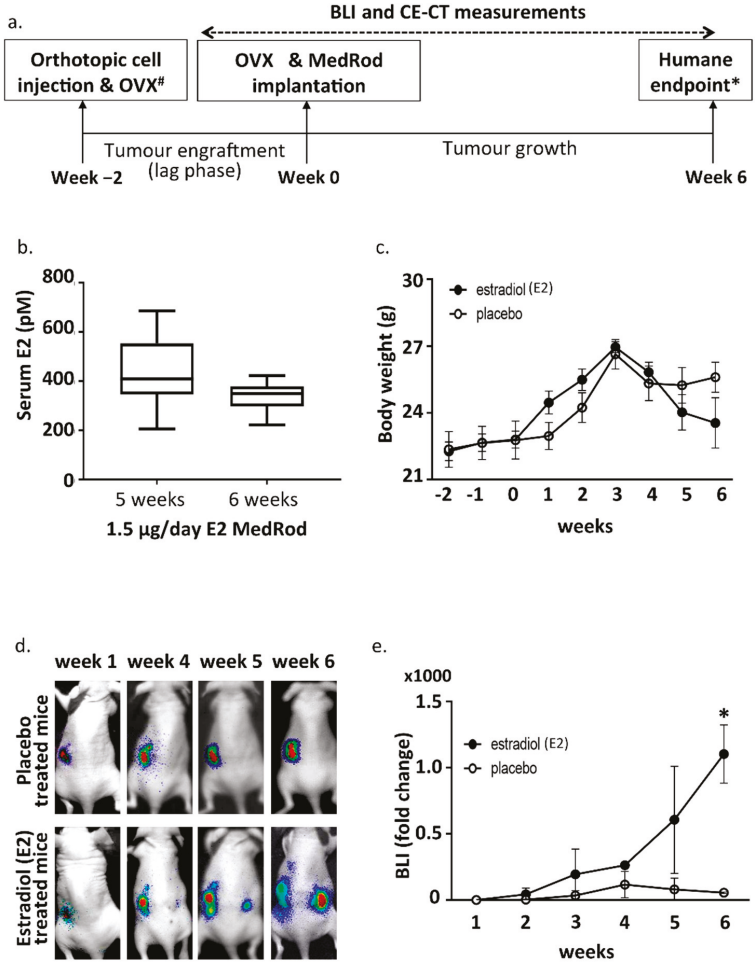

Figure 1. Experimental design and bioluminescence (BLI) results. (**a**) Timeline of the mouse model. Tumors were induced by orthotopic cell injection in one horn of the uterus (week 2). At the same time, ipsilateral OVX was performed. Two weeks later (i.e., at the end of the lag phase; week 0) OVX was performed at the other uterine horn as well and the MedRod delivery system for E2 or placebo supplementation was implanted subcutaneously. Tumor growth was monitored weekly by BLI until the humane endpoint was reached. Before euthanasia CE-CT was performed. # Ipsilateral OVX and OVX was not performed in the first experiment testing the cell titer. * Humane endpoint: signs of discomfort due to large-sized tumours. (**b**) Serum E2 concentration (LC-MS/MS) in mice implanted with MedRod devices releasing 1.5 µg/day of E2. Boxplots indicate the median and the lower and upper quartiles. Blots represent data from nine (5 weeks) and 12 mice (6 weeks) per time point. (**c**) Body weight. Mean values and standard deviations are shown (placebo: 8 mice; E2: 8 mice). (**d**) Representative images of in vivo sequential BLI. Note that the BLI signal from the location of primary tumour induction tends to decrease at late time points due to necrosis of the tumour tissue (as assessed histologically). (**e**) BLI fold change during the experiment. Shown data refers to the experiments with the Ishikawa clone three. Similar data was obtained with the other cell clones (data not shown). Mean values and standard deviations are shown (placebo: 3 mice; E2: 2 mice). Asterisk (*) indicates a p-value < 0.05 (t-test).

Tumors were grown for an additional six weeks (eight weeks in total; i.e., two weeks for tumour engraftment/lag phase and six weeks for E2-stimulated tumour growth) and growth was monitored weekly by BLI. Tumors developed in all sixteen mice (Table 2). Three weeks after OVX and E2/placebo supplementation, weight loss started to be observed in all mice, most likely because of the side effects and initial cachexia due to tumour growth, and this was more pronounced in the E2 group (Figure 1c). At week six after OVX and E2/placebo supplementation, mice presented signs of discomfort due to large-sized tumours and mice were euthanized (humane endpoint). During the six weeks following OVX and E2/placebo supplementation, tumour growth (assessed by BLI) was clearly E2 responsive and in the E2 arm, the BLI signal was not limited to the tumour induction area (as in the placebo group), but it spread to other locations in the abdomen (Figure 1d,e). As expected, within the same treatment group (E2 or placebo), no differences were observed in the growth of tumours induced with different Ishikawa clones.

Table 2. Overview of the experimental animals used to develop the E2-dependent endometrial cancer model.

Ishikawa (3×10^3 Cells)	OVX	Placebo/E2	Number of Mice	Mice with Tumor	CE-CT	Peritoneal Metastases *	LVI # (No.)
Clone 1	+	Placebo	3	3 (100%)	3	I, L, K, S (P, A)	0
	+	E2	3	3 (100%)	2	I, L, K, S, St (P, A)	3
Clone 2	+	Placebo	2	2 (100%)	2	I, L, K, St (A)	1
	+	E2	3	3 (100%)	3	I, L, K, S, St (P, A)	2
Clone 3	+	Placebo	3	3 (100%)	0	(A, P)	0
	+	E2	2	2 (100%)	0	(A)	2

* All mice developed peritoneal spread as assessed by ex vivo BLI. Location is indicated as follow: I = intestine, L = liver, K = kidney, S = spleen, St = stomach, P = psoas, A= adipose tissue (by brackets, histologically confirmed); # LVI = lymphovascular invasion; number of mice with metastases.

2.4. CE-CT, Endpoint and Tumor Characteristics

Prior to sacrifice (six weeks after OVX and E2/placebo supplementation), 11 animals were investigated by CE-CT scans (Table 3 and Figure 2a). Tumor volume estimation confirmed a more sustained tumour growth in the E2 group (mean volume 0.5 mL) compared with the placebo group (mean volume 0.2 mL). Tumor density was very variable between mice (Table 3) and was not significantly different between the groups (E2 group: 28 ± 26 vs. placebo group: 92 ± 67). Mice were euthanized and uteri, tumours, and other organs were isolated and examined. As expected, the uteri of the E2 group were substantially larger compared with the placebo, due to estrogen stimulation (Supplemental Figure S2). Both uterine wet-weight as well as tumour wet-weight were significantly higher in the E2 treated group compared with placebo (Figure 2b). The weight of surgically removed tumours correlated with the tumour volume estimated by CE-CT (Figure 2c). Histologic evaluation of uteri confirmed the presence of epithelial gland proliferation in the E2 treated group and the absence of growth in the placebo group (Supplemental Figure S2). Tumors grew throughout the whole uterine wall and expanded locally beyond the uterus. Histologic evaluation confirmed the orthotopic localization of EC, embedded in the endometrium/myometrium (Figure 3a). As tentative discrimination between mouse and human (i.e., induced tumours) tissues, we used immunohistochemistry and antibodies able to recognise, predominantly, the mouse ERα (MC-20) or both human and mouse ERα (HC-20; Figure 3b). While some cross reactivity between species of these antibodies is present, the y indicate that the tumour is most likely of human origin. Tumor histology showed no clear glandular organization, but still it recapitulated the human situation and nested or trabecular histology structures with hypochromatic nuclei and several mitotic figures were seen (Figure 3c,d). Large tumours showed signs of necrosis (Figure 3d). No major histologic differences were seen between the used clones (not shown).

Table 3. Overview of the CE-CT scan data.

Ishikawa	Placebo/E2	x (cm) [1]	y (cm) [2]	z (cm) [3]	Tumor Volume (mL) [4]	Tumor Density ± SD [5]	Tumor Weight *
Clone 1	placebo	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Clone 1	placebo	0.27	0.23	0.27	0.01	131.7 ± 15.6	20
Clone 1	placebo	0.63	0.53	0.41	0.07	−9.3 ± 4.9	118
Clone 2	placebo	0.14	0.14	0.23	0.002	137.3 ± 25.9	18
Clone 2	placebo	1.18	0.9	1.47	0.78	107.8 ± 4.5	568
Clone 2	E2	0.68	0.74	0.93	0.23	24.8 ± 4.7	333
Clone 1	E2	0.57 + 0.80 + 1.0	0.51 + 0.94 + 0.76	0.63 + 0.95 + 0.85	0.77	57.3 ± 8.0	1123
Clone 1	E2	0.78	0.98	0.97	0.37	31.2 ± 3.2	353
Clone 2	E2	n.d.	n.d.	n.d.	n.d.	n.d.	2147
Clone 2	E2	1.25	1.04	1.58	1.03	47.8 ± 15.2	1347
Clone 2	E2	0.49	0.45	0.43	0.05	−6.3 ± 10.4	81 [6]

[1] x: Maximum transverse tumour diameter at the axial image depicting the largest tumour diameter. [2] y: Maximum anterioposterior tumour diameter at the axial image depicting the largest tumour diameter. [3] z: Maximum sagittal tumour diameter at the sagittal image depicting the longest sagittal tumour diameter. [4] Tumor volume (mL): $= x \times y \times z/2$. * Tumor weight: real wet-weight (in mg) of surgically removed tumours after sacrifice. n.d.: non-detectable/determinable. [5] Tumor density is indicated as mean values ± standard deviation (SD). [6] This mouse (indicated by the red dot in Figure 2) belongs to the E2 treated group but initially there was no tumour engraftment (i.e., no BLI signal at the moment of E2 supplementation, week 0, probably due to sub-optimal cell injection). This mouse was nevertheless kept in the experiment and showed BLI signals, though strongly delayed, during the next measurements.

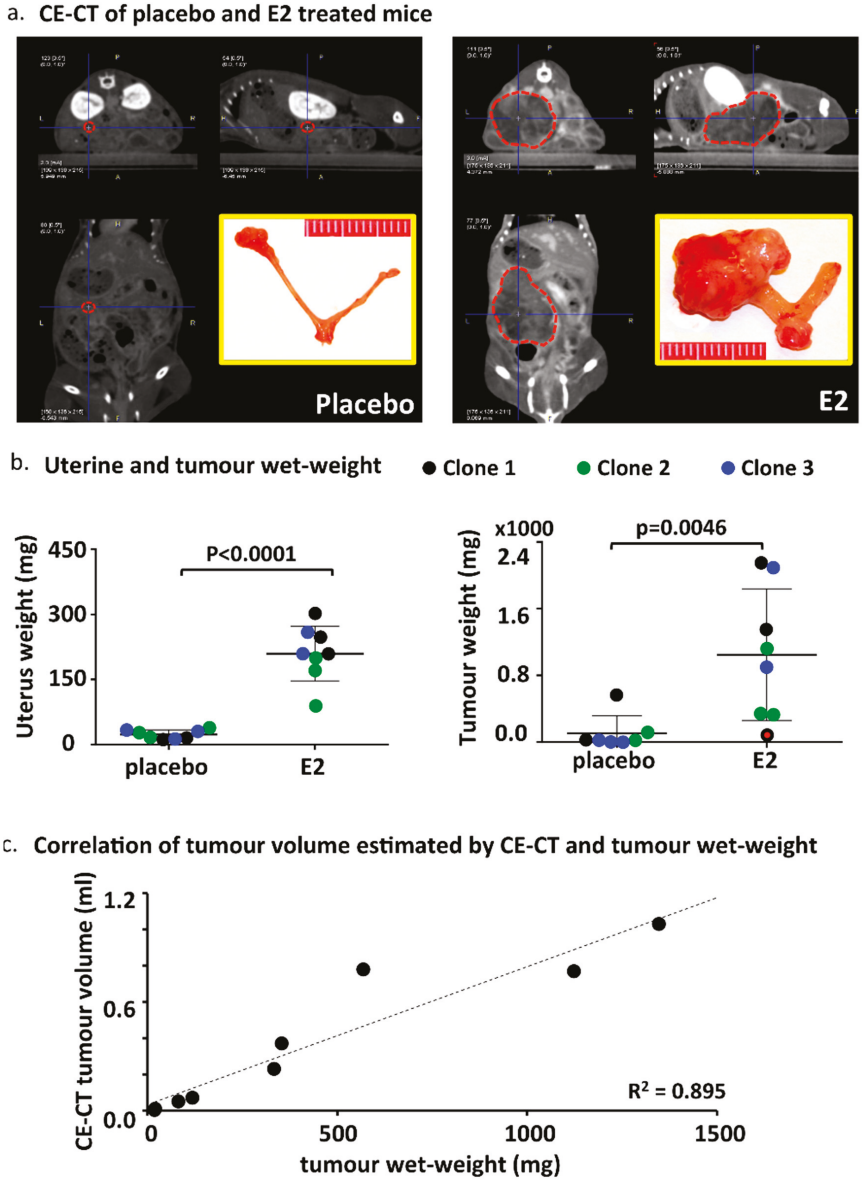

Figure 2. Tumor growth determined by CE-CT and wet-weight at sacrifice. (**a**) Representative CE-CT scan images centered by the blue cross at the center of the tumour (indicated by the red dotted line). The yellow-bordered inset shows the size of the uterus and the tumour surgically removed at sacrifice. (**b**) Uterine and tumour wet-weight in the E2 treated and placebo groups. The color codes indicate the different cell clones used. The red dot in the right panel shows the tumour weight of an E2 treated mouse from clone 1 with no proper tumour engraftment (no BLI signal at the moment of E2/placebo supplementation, week 0) probably due to sub-optimal cell injection (see also Table 3). *p* values are computed using *t*-test (outlier included). (**c**) Correlation between tumour volume estimated by CE-CT and the wet-weight of surgically removed tumours at sacrifice.

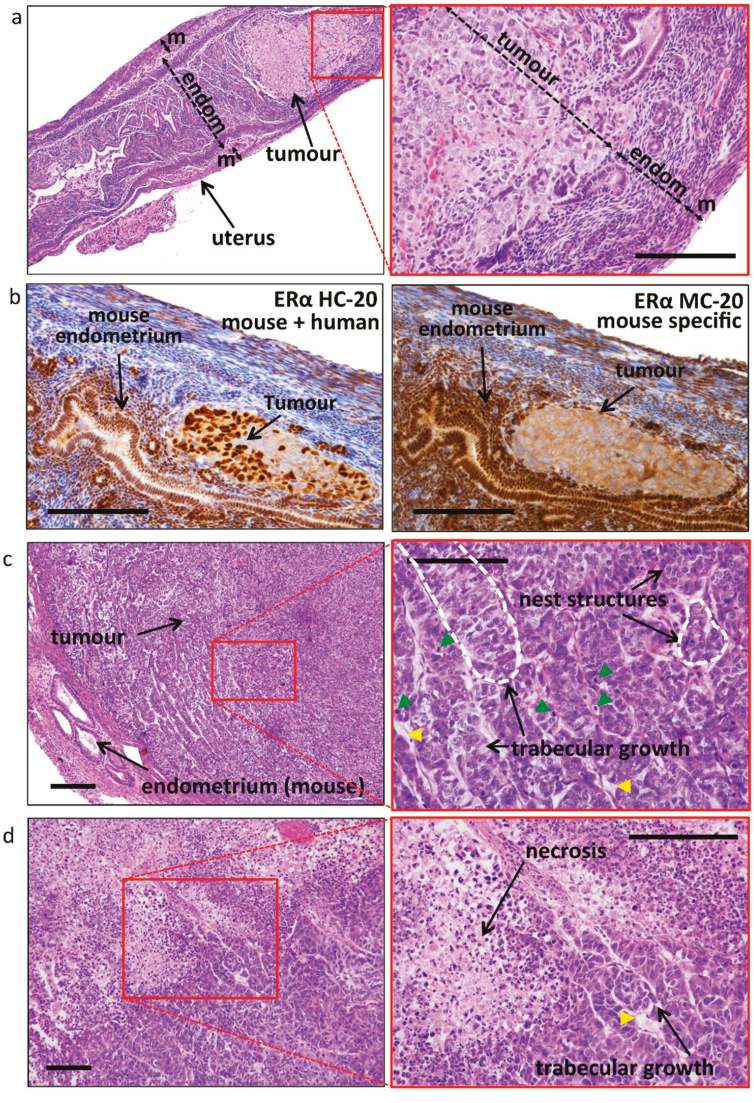

Figure 3. Tumor histology and estrogen receptor expression. (**a**) Representative histology showing the orthotopic location of the induced tumour. The mouse shown is from a placebo treated mouse, where tumour growth remained confined inside the uterus until the end of the experiment. The thickness of the endometrium (endom.) and the myometrium (m) (and the tumour, in the right image only) is indicated by the double-headed arrow. Bar scale: 200 μm. (**b**) Analysis of ER-α expression using antisera directed against human receptor but cross-reacting with the mouse ER-α (left, HC-20), where both tumour (most likely of human origin) and mouse endometrial tissues have nuclear staining, and using antisera directed against mouse ER-α (right, MC-20), with nuclear staining only in mouse tissues and showing cytoplasmic background in the tumour. Bar scale: 200 μm. (**c**) Representative image of the tumour histology (E2 treated sample), with nest and trabecular structures (indicated by the dotted white line on the enlargement, right image). Mitotic figures (green arrowheads) and blood vessels (yellow arrowheads) are visible. Bar scale: 200 μm. (**d**) Representative image of a large tumour with a large necrotic core. Yellow arrowheads: blood vessels. Bar scale: 200 μm.

2.5. Metastases

Metastatic spread was assessed post-mortem by ex-vivo BLI, and it was observed in all mice (Tables 1 and 2 and Figure 4a), the extent being related to the tumour size. Metastatic spread was also assessed histologically in all peritoneal organs (see Tables 1 and 2 and Figure 4b). Slides were prepared for histology every 200 µm of thickness in order to cover the complete depth of the tissue. In most cases, metastatic peritoneal lesions were superficial on various organs in the abdomen, and with some exceptions, did not infiltrate the organs (Figure 4b). Careful histologic assessment was performed by an experienced pathologist (LK; blinded for other characteristics and origin of the samples) for the presence of lymphovascular invasion (LVI) in the tissue surrounding the uterus and in the tumour. Seven out of eight E2 treated animals (87%) developed LVI, whereas only 1/8 (12%) developed LVI in the placebo group (Tables 1 and 2). Representative sections depicting metastases at different sites are displayed in Figure 4b.

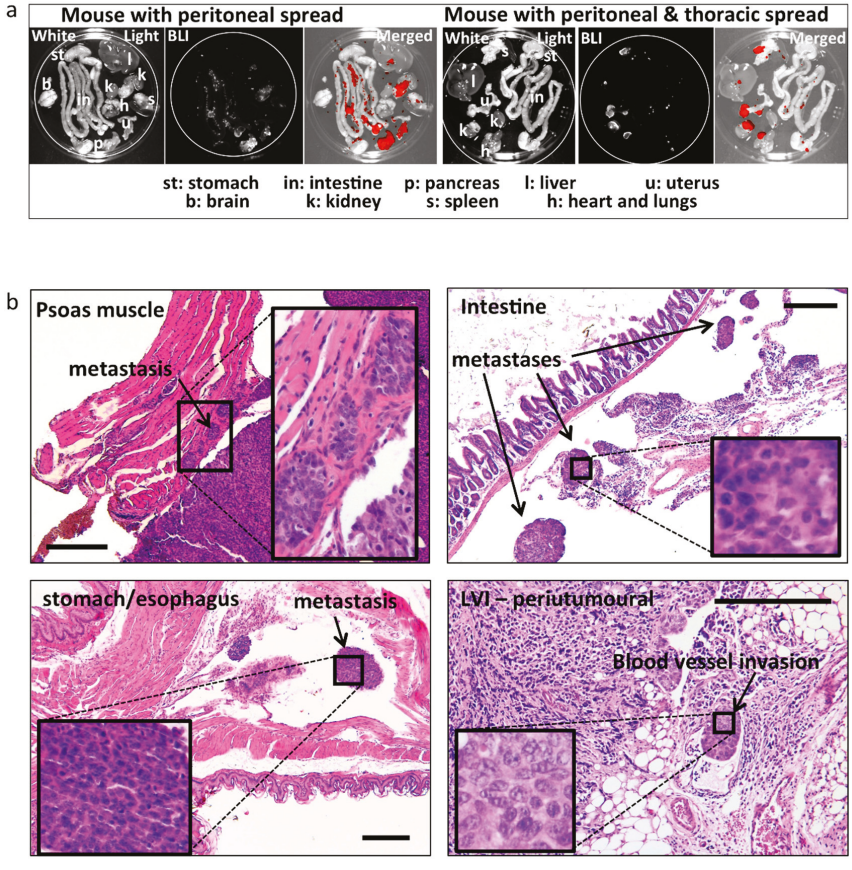

Figure 4. Ex-vivo BLI and histologically confirmed metastases. (**a**) Representative images of post mortem ex-vivo BLI used to assess the metastatic spread to different peritoneal and extra peritoneal organs. Left: example of spread restricted to peritoneal organs. Right: example of spread to the heart/lungs (thoracic cavity). Strong BLI signal (white in the BLI images and red in the merged images) was associated with fat tissue adjacent to peritoneal organs (spleen, kidneys). (**b**) Representative images and histologic confirmation of tumour spread to the psoas muscle, intestine, stomach/oesophagus, LVI, and infiltrating tumour cells in the (anatomic location/organs are indicated in the images). Bar scale: 300 µm.

3. Discussion

In this study we describe the development of an orthotopic endometrial cancer (EC) mouse model where estrogen exposure was controlled and estrogen dependent tumour growth was measured. Tumors were induced using the well-differentiated human endometrial adenocarcinoma Ishikawa cell line, modified to express a luciferase reporter gene [10] for bioluminescence imaging (BLI). Moreover, a novel MedRod delivery system was used in which subcutaneous implants provided a constant release of 17β-estradiol (E2) after ovariectomy (OVX).

Various in vitro and in vivo models of EC exist. A few EC cell lines are available and those authenticated and deposited in cell banks are the estrogen sensitive Ishikawa—the less estrogen sensitive Hec1A, Hec1b, RL95.2, KLE, and An3CA (www.atcc.org). The se cells can be grown in vitro as monolayers or as spheroids [20]. It is however challenging to measure direct cell proliferation (e.g., increased cell number, BrdU incorporation) in response to E2 stimulation using these cells (own experience and [21]) and it is frequently necessary to assess cell growth using immunohistochemical markers like Ki-67 or cyclin expression. One of the simplest in vivo models is based on EC cells xenografted on the chorioallantoic membrane (CAM) of fertilised eggs [10]. This model is very suitable to test drugs with strong cytotoxic effects, thus allowing measuring differences in tumour volumes/wet-weight as endpoints [22], but due to the short time of xenograft growth (approximately five days), long-term experiments (such as the E2-dependent growth) need to rely on immunohistochemical markers as endpoints [10]. EC models based on rodents are also available in which tumours develop in mice because of genetic manipulation or chemical exposure or tumours are induced via xenotransplantation of tumour cells subcutaneously or in the fat pad [21].

A subcutaneous EC xenograft model in OVX mice receiving estrogen supplementation was also developed and showed estrogen dependency [17]. The induction of tumours orthotopically, however, represents an important advancement in oncology research, and orthotopic EC models were recently developed using Hec1a and Ishikawa cells [13–15]. The orthotopic model is a more biologically relevant model than the subcutaneous or fat pad models since tumours are grown in the same microenvironment and the same physiologic conditions as these occur in humans. In addition, as shown in the present study and in previous publications [13,14], the orthotopic model has clinical relevance because tumour cells infiltrate the myometrium and invade the peritoneal cavity and the vascular and lymphatic systems as well, thus mimicking the progression of human disease. In our study, we advance this orthotopic mouse model of EC to optimise and control its estrogen dependency upon OVX. Since most ECs are diagnosed and treated after menopause, when the ovaries no longer produce estrogens, correctly dosing the levels of these steroids is of extreme importance to best mimic the human EC.

In order to exogenously dose and control estrogen levels, we used a recently optimised delivery system consisting of subcutaneous implants called MedRods. This release system provides cost effective and a constant dosage of the immobilised compound by diffusion in the body for a long period (minimum 10 weeks), the refore, in most cases, only one implantation for the whole duration of the experiment is required. Different delivery systems for exogenous administration of steroids and other compounds exist, which include oral administration via drinking water, regular injections, subcutaneous pellets, or osmotic mini pumps [23–25]. The se different delivery systems have obvious advantages and disadvantages with lab-to-lab preferences. Administration via drinking water is cost effective but dosing is difficult to control. Injections require continuity in the application, resulting in concentration peaks and causes discomfort to the animals. Subcutaneous pellets are easy to apply but often the delivery rate is not constant, whereas osmotic minipumps are efficient in terms of delivery rates, full customisation of the compound to deliver is feasible (minipumps are assembled by the investigators themselves) but are relatively expensive, large in size, and have a limited elapse time, which may require pump replacements in the course of the experiment. Using the MedRod implants, our model proved to be E2 dependent with clear differences in tumour growth and wet-weight between the E2 and placebo groups. Thus, MedRod implants provide a valid alternative to other

existing delivery systems. In our experiments we used a relatively high level of E2, i.e., 300–400 pM, which is about 10 times more concentrated compared with the peak E2 levels in cycling female mice (proestrus, E2 level is about 40 pM). This E2 dose was chosen on the one hand because it was used in previous studies [16,17,19,26], and on the other hand to secure both fast tumour growth and clear and measurable differences between placebo and E2 groups. Nevertheless, lower estrogenic doses (e.g., the low active E1 at concentrations lower that 100 pM, comparable or lower than 40 pM of E2—Supplemental Figure S3, but also the experiment on cell titre performed in gonad intact mice, Table 1) elicited tumour growth and uterine responses that were indistinguishable from those induced by the positive control E2.

Since most EC in humans are estrogen dependent, it is extremely relevant that the present orthotopic EC xenograft model exhibits this feature of the human disease. The estrogen dependency of EC is currently not exploited in the clinic as is in breast or prostate cancer treatment, due to the reported low efficacy of endocrine drugs for EC patients [3–6]. While single-endocrine drug treatments have failed to show good efficacy, a recent trial of dual regimen including an aromatase inhibitor (AI) and an mTOR inhibitor yielded promising results with good efficacy [7]. It is generally agreed upon that there is room for improvement in the current use of hormonal drugs in EC, and that endocrine treatment may represent an important approach for EC in the future [27]. This is demonstrated by the numerous, currently ongoing phase II trials that are testing various combinational treatments, e.g., mTOR inhibitor in combination with AI and metformin (NCT01797523: https://clinicaltrials.gov); AI and ribociclib (NCT03008408); dual mTORC1/mTORC2 inhibitor and AI (NCT02730923); mTOR and AI inhibitors compared with progestogens and tamoxifen (NCT02228681); ribociclib and AI (NCT02657928); sodium cridanimod in conjunction with progestin (NCT03077698); and AI combined with palbociclib (NCT02730429).

In addition, a number of novel potential endocrine drug targets are being discovered [8–10,28,29] and therefore the establishment of proper tools for pre-clinical testing of these novel endocrine targets is of foremost importance.

The protocols and methods described in this study can help future research in endocrine drug discovery and accelerate the translation from bench to bedside. We use E2 stimulation as the reference of positive tumour growth, but the model is amenable to mimic different clinical situations, like post menopause (over 90% of all EC cases), recapitulated by OVX and ad-hoc steroid supplementation, or pre menopausal EC, using gonad intact mice with cycling E2 and progesterone. We used BLI for tumour growth monitoring, but we also demonstrated a good correlation between BLI and CE-CT findings, making our method also suitable for systems lacking an endogenous reporter gene (e.g., luciferase/BLI), such as patient derived EC tumour xenografts (PDX), the most relevant animal model in cancer research available today [30].

In conclusion, we present here an orthotopic xenograft mouse model of EC that mimics the human condition in terms of tumour localization, estrogen dependency, and metastatic spread. This model will be useful for future pre-clinical studies testing the efficacy of novel drugs and of combinational regimens of novel and existing treatments.

4. Materials and Methods

4.1. Ethics Statement

All animal procedures were approved by The Netherlands National Committee for the protection of animals used for scientific purposes and the Central Authority for Scientific Procedures on Animals or by the National Animal Experiment Board of Finland (DECNR: 2012_079, September 2012). All procedures were performed according to the European Convention for the Protection of Vertebrates Used for Scientific Purposes.

4.2. Cell Lines and Tumor Graft Preparation

The human endometrial adenocarcinoma cell line Ishikawa (ECACC, Sigma-Aldrich, Zwijndrecht, The Netherlands) was previously modified in our laboratory to express firefly luciferase fused with green fluorescent protein (GFP) for non-invasive bioluminescence imaging (BLI). The established Ishikawa clones were thoroughly characterized for the maintenance of markers of the parental Ishikawa cell line and were authenticated by Short Tandem Repeat (STR) profiling [10]. Since different cell lines/clones may behave differently (clonal effects), three different Ishikawa clones (Ishi-M3-HSDA, Ishi-M1-HSDB, and Ishi-M3-EVC, from now on referred to as clone 1, clone 2, and clone 3) were used in the present animal experiments. Cells were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with sodium-pyruvate, L-glutamine, penicillin-streptomycin, and 10% foetal bovine serum at 37 °C with 5% CO_2 in humidified air, and tested negative for mycoplasma (MycoAlert, Promega, Madison, WI, USA). For orthotopic injections, cells at no more than 70% confluency were detached with Accutase (Invitrogen), pelleted and resuspended in 30 µL ice-cold Matrigel (Basement Membrane Matrix; Becton Dickinson, Vianen, The Netherlands).

4.3. Optimization of the MedRod Steroid Delivery System

To control estrogen exposure (for estrogen dependent tumour growth), the endogenous source of estrogens (ovaries) was removed and an exogenous estrogen supply was provided (estrogen supplementation) by using the recently optimised MedRod implants (PreclinApps Ltd., Raisio, Finland). MedRods are polydimethylsiloxane cylinders covered by a silicone membrane that have a constant release of matrix embedded 17β-estradiol (E2) for at least 10 weeks. The serum estrogen levels in MedRod bearing mice were determined by LC-MS/MS—as previously described using approximately 1 mL of blood collected at sacrifice by heart puncture [31].

4.4. Orthotopic Estrogen Dependent Endometrial Cancer Mouse Model

Eight-week-old female athymic nude mice (Crl:NU(NCr)-Foxn1nu) were purchased from Charles River ('s-Hertogenbosch, The Netherlands) and housed in groups of 3–4 animals in individually ventilated cages and under specified pathogen-free conditions. Food and water were both sterilised and provided ad libitum. Complete diet with moderate energy density and very low nitrosamine content (Mouse maintenance diet, V1534-703, ssniff Spezialdiäten GmbH, Soest, Germany) was used.

For tumour cell injections, mice were anaesthetised with isoflurane (Forane, Abbott laboratories Ltd., Maidenhead, UK) using 4–5% isoflurane in pressured air for induction of sedation and 1.5% isoflurane in pressured air for maintenance. Mice were placed on a heating pad in ventral decubitus. The skin was disinfected, an incision was made laterally at the lower dorsum, and one uterine horn was exteriorised (in most cases, the left uterine horn was operated). If ovariectomy (OVX) was performed (see text), ipsilateral OVX was first performed at the uterine horn side of tumour induction. To this end, a ligature was placed in the fallopian tube and the ovary was removed above the ligature. The uterine horn was clamped at the two edges just before the fallopian tube at the top and just before the uterus body at the bottom in order to prevent leakage. Re-suspended cells (30 µL of ice-cold Matrigel; 1–5 million cells as indicated in the text) were slowly injected into the endometrial cavity with a 25 G needle. The needle was retracted after waiting for a few seconds to allow the polymerisation of the Matrigel cell-suspension and prevent leakage in the abdominal cavity. Muscle and skin incisions were sutured with 6–0 absorbable sutures (Supplemental Figure S2). The un-resected ovary was removed approximately two weeks after tumour cell injection, when the tumours had successfully engrafted (determined by BLI; next paragraph).

For the implantation of the MedRod implants containing either placebo or E2 (releasing 1.5 µg E2/day; 5.5 nmol/day), a small incision was made in the loose skin of the animal neck. A pocket was bluntly dissected under the skin and the MedRod was placed using forceps. The incision was closed with a 6–0 absorbable sutures. For analgesia, mice received 7.5 mg/kg Carprofen (Norbrook

Laboratories Ltd., Newry, UK) subcutaneously, pre and post-operatively, for the following two days. The time-line of the orthotopic xenograft model is illustrated in Figure 1a.

4.5. Imaging by BLI

Tumor growth was visualised weekly by sequential BLI using the Andor iXon Ultra 897 camera in the X-RAD 225Cx system (Precision X-ray Inc., North Branford, CT, USA). Mice were anaesthetised with isoflurane and injected intraperitoneally with 150 mg/kg D-Luciferin (Becton Dickinson) approximately 10 min prior to imaging. Six planar images were obtained at 0, 45, 90, 180, 270, and 315° angles (angle 0° corresponding to image from the top, dorsal side of the mouse). Images obtained from angles 270°–315° were used to compute the BLI signal intensity for mice where the tumour was induced on the left uterine horn (45°–90°, in the case of right horn tumour induction). BLI data was analysed using ImageJ (v.1.48, National Institute of Health—NIH—Bethesda, MD, USA) [32], and the total photon flux was determined in the Region of Interest (ROI) located in the abdominal area corrected for background signal.

4.6. Micro Contrast Enhanced-Computed Tomography (CE-CT)

Prior to euthanasia, micro CE-CT imaging was performed using the small animal micro-IR (X-RAD 225Cx, Precision X-ray Inc., North Branford, CT, USA). Mice were anaesthetised with isoflurane, and to enhance soft tissue contrast, 150 µL of iodinated Omnipaque 350 (GE Healthcare, Little Chalfont, UK) was injected in the tail vein immediately prior to imaging. Images were acquired as described earlier [33]. In short, an 80 kVp, 2.5 mA imaging protocol with an acquisition rate of 5 frames/s, a spatial resolution of 100 microns, and a gantry rotation of 0.5 revolution/min was used to image the abdominal region of all animals. The imaging dose was 39 cGy, spot size was approximately 1 mm, and the imager mode was in low gain (2 × 2 binning). The beam was filtered with 2 mm aluminum to remove the low energy photons that do not contribute to the imaging.

The micro CE-CT images were analysed using the PMOD v.3.7 software (PMOD Technologies LLC, Zürich, Switzerland). The maximum tumour diameter was measured in three orthogonal planes (x, y and z) and the tumour volume was estimated using the following equation; tumour volume = $x \times y \times z/2$. Tumor density was measured in a volume of interest (VOI) placed in a representative part of the tumour, avoiding necrotic or haemorrhagic areas if present. The volume of the tumour VOIs had a median (range) of 0.02 (0.002–0.39) mL, the wide range is explained by the variable tumour size.

4.7. Histological Examination and Immunohistochemistry

Tissue biopsies were fixed in 3.7% formaldehyde, embedded in paraffin and processed for histological examination. Histology was determined by a pathologist (LK) from 4 µm haematoxylin & eosin (Sigma-Aldrich, Zwijndrecht, The Netherlands) stained sections. Detection of estrogen receptor-α (ERα) of human origin was performed with immunohistochemistry using the antibody HC-20 (Santa Cruz Biotechnologies, Heidelberg, Germany), whereas for mouse ERα, antibody MC-20 (Santa Cruz Biotechnologies) was used. In brief, sections were subjected to deparaffinization followed by rehydration. Heat-induced antigen retrieval was performed and slides were blocked in 1% BSA/PBST and subsequently incubated overnight at 4 °C with 500 times diluted ERα antibody, as described earlier [10]. The EnVision detection system was used according to the manufacturer's manual followed by visualization with Diaminobenzidine (DAB).

4.8. Statistics

Statistical analyses were performed using KaleidaGraph version 4.1.3. (Synergy Software, http://www.synergy.com). Variance was analysed using parametric student's t-test whereas the Pearson's correlation coefficient was used to evaluate the relationship between two variables. Differences and correlations were considered statistically significant at $p < 0.05$.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/19/9/2547/s1.

Author Contributions: Conceptualization, A.R., S.X., P.K., C.K., R.F.P.M.K. and G.F.J.K.; Methodology, G.F.J.K., N.S., B.D., A.Z., N.L.; Pathology assessment, L.K.; Radiology and image analysis: G.F.J.K., K.E.F., I.S.H., L.D., F.V., L.E.J.R.S. and C.K.; Steroid profiling by LC-MS/MS, S.A., M.R.H., P.K. and N.S.; Writing-Original Draft Preparation, A.R., S.X. and G.F.J.K.; Writing-Review, Figures & Editing, A.R., S.X., K.E.F., N.S. and G.F.J.K.; Supervision, A.R., S.X. and R.F.P.M.K.; Funding Acquisition, A.R.

Funding: The present study was sponsored by the Dutch Cancer Society ('KWF Kankerbestrijding': www.kwf.nl), contract number UM-13-5782 granted to A.R.

Acknowledgments: The authors are grateful to Karlijn Cornel (Department of Gynaecology) for helping with animal protocol optimization, A. Vaniqui and S. van Hoof (Department of Radiotherapy, Maastricht University, Maastricht, The Netherlands) for assisting with the small animal micro-IR/SmART device; to J. van de Laak (bachelor student Zuyd Hogeschool, Heerlen, The Netherlands) for assisting in the wet laboratory work. The authors are grateful to the members of the animal care units of Maastricht University, University of Turku and TCDM personnel.

Conflicts of Interest: Pasi Koskimies and Niina Saarinen are employers of Forendo Pharma Ltd. No additional conflict of interest is declared.

Abbreviations

BLI	Bioluminescence imaging
CAM	Chorioallantoic membrane assay
CE-CT	Contrast-enhanced computerised axial tomography
E2	17β-Estradiol
EC	Endometrial cancer
GFP	Green fluorescent protein
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LVI	Lymphovascular invasion
OVX	Ovariectomy
ROI	Region of Interest

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ISBN 978-3-03921-496-9