

Article

Wnt5a Regulates Embryonic Müllerian Duct Development Through the Non-Canonical Wnt PCP Pathway

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Abstract

Müllerian anomalies are anatomical variations of the female reproductive tract resulting from the incomplete development of the embryonic Müllerian ducts. The molecular mechanisms driving Müllerian duct development are complex and poorly understood, resulting in the largely unexplained aetiology of these conditions. WNT5A is a critical regulator of key developmental processes, including patterning, cell proliferation, and migration. Mutations of *WNT5A* have been associated with Robinow syndrome, a congenital condition characterized by skeletal and genital anomalies. In the mouse, *WNT5A* is necessary for the posterior development of the Müllerian duct, and ablation of *Wnt5a* results in vaginal agenesis. However, *Wnt5a*^{-/-} uterine horns are hypoplastic and over 60% shorter than the wild type, suggesting specific functions in anterior Müllerian duct development. To better understand the role of *Wnt5a*, we performed single-cell RNA sequencing of developing Müllerian ducts. We found that the non-canonical Wnt PCP pathway was dysregulated in *Wnt5a*^{-/-} mice. In addition, *Wnt5a*^{-/-} Müllerian ducts were enriched in oviductal mesenchymal cells due to the transformation of the anterior uterine horns into oviducts. Our results indicate additional roles for *Wnt5a* during Müllerian duct development, prompting further investigations into uterine functions and anatomy in complex clinical cases of Müllerian anomalies including Robinow syndrome.

Keywords: Müllerian anomalies; female reproductive tract; uterus; oviducts; Robinow syndrome; Wnt5a; Wnt factors; PCP pathway



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1. Introduction

In mammals, the female reproductive tract consists of the oviducts, uterus, and cervix, as well as the upper part of the vagina, and provides the site for oocyte fertilization, implantation, and embryo development. The female reproductive tract rises from the Müllerian ducts, a pair of primordial genital ducts that form in the intermediate mesoderm during early embryogenesis. Müllerian duct development is complex and involves the coordination of several molecular and cellular events. In the mouse, this process occurs between 11.5 and 13.5 days post coitum (dpc) and is typically divided into three main phases [1,2]. First, cells within the anterior coelomic epithelium of the mesonephros become Müllerian progenitor cells and proliferate to form a placode thickening [3]. Although the specific molecular mechanisms involved remain unclear, critical pathways for the

specification of progenitor cells include BMP and FGF signalling [4]. These progenitors will form the Müllerian epithelium and—together with progenitors delaminating from the coelomic mesonephric epithelium and following epithelial–mesenchymal transition (EMT)—the Müllerian mesenchyme [5]. Subsequently, Müllerian progenitor cells start invading the mesonephric mesenchyme towards the neighbouring Wolffian ducts. Critical factors regulating this invagination process include LHX1 and WNT4, which are expressed in the epithelial and mesenchymal precursors, respectively [3,6]. In the third phase, the nascent ducts elongate posteriorly through cell proliferation and migration, using the Wolffian duct as a guide [3,7]. Müllerian duct elongation is regulated by inductive signals, including WNT9B and GATA3 from the Wolffian ducts, WNT4 from the mesonephric mesenchyme, and the PI3K pathway [2,5,8]. At 13.5 dpc, the two Müllerian ducts reach the urogenital sinus and fuse to form the utero-vaginal duct, which will give rise to the posterior portion of the uterus, cervix and upper vagina [3].

Subsequent to this stage of development, the Müllerian ducts start a differentiation process that will lead to the establishment of specialized cell populations and functionally distinct regions [9–11]. HOX, retinoic acid (RA), and WNT signalling are critical for the differentiation of the Müllerian ducts. Differential expression of *Hox* genes, which are expressed as early as 14.5–15.5 dpc, controls the segmentation of the Müllerian ducts [12–14]. *Hoxa9/d9* are predominantly expressed in the anterior region that will form the oviducts. *Hoxa10/d10* and *Hoxa11/d11* are expressed in the middle sections of the uterine horns, and *Hoxa13/d13* in the caudal region that will develop into the cervix and vagina [15]. In a similar way, starting from 14.5dpc, a concentration gradient of RA along the ducts induces oviduct, uterine, and vaginal differentiation [16]. Additionally, both the canonical and non-canonical Wnt pathways play critical roles throughout the formation, development, and differentiation of the Müllerian duct, regulating cell polarity, proliferation, migration, and EMT. Ablation of *Ctnnb1* in the Müllerian mesenchyme results in uterine hypoplasia, uncoiled oviducts, and a reduced number of uterine glands [17]. Conversely, constitutive stabilization of CTNNB1 leads to an increased number of glands, although uterine length is significantly reduced, suggesting a defect in Müllerian duct elongation [18]. In addition to Müllerian duct formation, *Wnt4* is involved in the differentiation of the Müllerian luminal epithelium and the development of uterine glands, as demonstrated by conditional ablation studies [6,19]. Ablation of *Wnt7a* also leads to uterine hypoplasia, uncoiled oviducts, and a lack of glands [20,21]. Interestingly, *Hoxa10* and *Hoxa11* are downregulated in *Wnt7a*^{-/-} uteri, which show a posterior homeotic transformation, with oviducts becoming uterus and the uterus differentiating to the vagina [20].

WNT5A is a critical factor regulating tissue morphogenesis, primarily through the non-canonical planar cell polarity (PCP) pathway [22]. Ablation of *Wnt5a* in the mouse causes the absence of a vagina and shorter uterine horns [23]. In kidney capsule graft experiments, *Wnt5a* was found to be postnatally necessary for uterine glandular genesis, independent of canonical factor *Lef1*. *Wnt5a* was also found to be required for the estrogen-directed regulation of *Wnt7a*, *Hoxa10*, and *Hoxa11*, which failed to be downregulated compared to controls [23]. In addition, conditional ablation of both *Wnt4* and *Wnt5a* in the Müllerian ducts resulted in variable phenotypes, including uterine aplasia, a reduced number of glands, and cervicovaginal agenesis [24]. In humans, WNT5A variants are associated with autosomal dominant Robinow syndrome, a condition characterized by skeletal, renal, and reproductive tract anomalies (OMIM #180700) [25–27]. Affected females display hypoplasia of the clitoris and labia minora, consistent with the abnormality of the posterior reproductive tract observed in *Wnt5a*^{-/-} mice [25,26,28].

However, the role that WNT5A plays in the development of the anterior reproductive tract remains largely uncharacterized. Given that WNT5A regulates several processes that are fundamental during embryogenesis, we sought to explore its involvement in Müllerian

duct development. We found that in *Wnt5a*^{-/-} Müllerian ducts, cell proliferation was significantly reduced due to inhibition of the PCP pathway. In addition, ablation of *Wnt5a* resulted in the anteriorization of the uterine horns, with downregulation of *Hoxa10* and upregulation of oviduct markers *Hoxa7* and *Foxl2*. These results suggest that WNT5A and the PCP pathway are necessary for proper anterior female reproductive tract development by regulating cell-fate decisions and patterning.

2. Materials and Methods

2.1. Mouse Strains

Wnt5a^{+/-} mice were obtained from Dr. Benjamin Hogan at the Institute for Molecular Biosciences of the University of Queensland and maintained on a C57BL/6 genetic background [29]. *Wnt5a*^{-/-} embryos were generated by crossing *Wnt5a*^{+/-} breeders. Genotyping was done according to the provider's protocol. Samples were collected at 18.5 days post coitum (dpc). All animal procedures were approved by the University of Queensland Animal Ethics Committee.

2.2. RNA Expression Analyses

Samples were stored in RNeasy lysis buffer (Qiagen, Crawley, UK), and total RNA was extracted using an RNeasy mini kit (Qiagen, Crawley, UK). For real-time PCR of the Wnt pathway, an RT2 Profiler PCR Array (Qiagen, Crawley, UK) was used, following manufacturer's protocol. Real-time PCR of additional genes was done using a SYBR Green PCR master mix (Applied Biosystems, Waltham, MA, USA) on cDNA generated by a high-capacity cDNA reverse transcription kit (Applied Biosystems). Expression was normalised to *Tbp*. Primers for the following genes were used: *Ror2* Fw: GGGAACCGGACTATTTATGTGG, *Ror2* Rev: AAGACGAAGTGGCAGAAGG; *Vangl2* Fw: GTGTGCTTTGGACAGGGAGC, *Vangl2* Rev: CACCTCCTTCAGCACAGGCT; *Rac1* Fw: TGCTTTTCCCTTGAGTCC, *Rac1* Rev: TCAGCTTCTCAATGGTGTCC; *Fos* Fw: TCCTTACGGACTCCCCAC, *Fos* Rev: CTCCGTTTCTCTTCCTCTTCAG; *Tbp* Fw: ACGGACAAGTGGCGTTGATTTT, *Tbp* Rev: ACTTAGCTGGGAAGCCCAAC. Three biological replicates were used for all real-time PCR assays, which were run on a QuantStudio3 system (Applied Biosystems, Waltham, MA, USA).

2.3. Histology and Immunofluorescence

Müllerian duct samples were fixed overnight in 4% paraformaldehyde phosphate-buffered saline (PBS), embedded in paraffin and sectioned at 5 µm. Müllerian duct sections were deparaffinized in xylene and rehydrated using decreasing alcohol concentrations. Heat-mediated antigen retrieval was performed in Tris-based buffer (Vector, Newark, CA, USA). Immunofluorescence was done as previously described [11] using the following primary antibodies: CDH1 (1:100, BD Biosciences, Franklin Lakes, NJ, USA, #610182), PH3 (1:100, Abcam, Cambridge, UK, #5176), LAMA (1:100, Sigma, St. Louis, MO, USA, #L9393), PAX2 (1:100, BioLegend, San Diego, CA, USA, #901001), SMA (1:100, Sigma #A2547), CTNBB1 (1:100, Millipore, Burlington, MA, USA, #05-665), VIM (1:200, Abcam #92547), JUN (1:100, Cell Signaling, Danvers, MA, USA, #9165), FOS (1:100, Novus Biologicals, Centennial, CO, USA, NBP1-89065), Phospho-JUN (1:100, Abcam ab32385), FOXL2 (1:100, Novus Biologicals, Centennial, CO, USA, #NB100-1277), and H2AX (1:200, Abcam ab26350). Images were taken on an LSM710 confocal microscope (Zeiss, Jena, Germany) and processed using Adobe Photoshop. Quantification of fluorescence intensity was done using ImageJ (version 1.53t).

2.4. Single-Cell RNA Sequencing

Uterine horns were dissected and incubated in DMEM/F12/Collagenase/Hyaluronidase/FBS (Stemcell) for 60 min at 37 °C. Tissues were subsequently centrifuged at 300× *g* for 5 min, and cells were resuspended in 0.25% Trypsin–EDTA before being incubated for 5 min in Modified Hanks' Balanced Salt Solution (HBSS, Stemcell) supplemented with 10% FBS. After centrifugation, cells were resuspended in 1 mg/mL DNaseI (Stemcell), washed three times in HBSS, resuspended in PBS, and counted to evaluate viability and concentration. The single-cell suspension was partitioned and barcoded using a 10X Genomics Chromium Controller (10X Genomics, Pleasanton, CA, USA) and the Single Cell3' Library and Gel BeadKit v3.1 (V3.1; 10X Genomics; Pleasanton, CA, USA, PN-1000121). Cells were loaded onto a Chromium Single Cell ChipG (10X Genomics; Pleasanton, CA, USA, PN-1000120) to target 10,000 cells. GEM generation and barcoding, cDNA amplification and library construction were performed according to the 10X Genomics Chromium User Guide. The resulting single-cell transcriptome libraries contained unique sample indices for each sample. The libraries were quantified on an Agilent BioAnalyzer 2100 using a High Sensitivity DNA Kit (Agilent, Santa Clara, CA, USA, 5067–4626). Libraries were pooled in equimolar ratios, and the pool was quantified by qRT-PCR using a KAPA Library Quantification Kit, (KAPA Biosystems, Wilmington, MA, USA, KK4824) in combination with a Life Technologies ViiA 7 real-time PCR instrument. Denatured libraries were loaded onto an Illumina NextSeq-500 (Illumina, San Diego, CA, USA) and sequenced over 2 runs using a 150-cycle High-Output Kit as follows: 28bp (Read1), 8bp (i7index) and 111bp (Read2). For additional sequencing, denatured libraries were loaded onto an Illumina NovaSeq 6000 (Illumina, San Diego, CA, USA) and sequenced using one SP100 cycle flow cell and one S1100 cycle flow cell as follows: 28bp (Read1), 8bp (i7index) and 91bp (Read2). Read1 supplies the cell barcode and UMI, i7 corresponds to the sample index, and Read2 sequences the 3' sequence of the transcript. Data preprocessing was performed using Cell Ranger 3.0.2 (10x Genomics, Pleasanton, CA, USA) and a mouse GRCm38 reference. The final read depth was ~18,000–26,000 reads per cell across all samples. Library preparation and NextSeq sequencing were performed at the Institute for Molecular Bioscience Sequencing Facility (University of Queensland). NovaSeq sequencing was performed at Microba (Translational Research Institute).

2.5. Analysis of Single-Cell RNA Sequencing

After data processing using Cell Ranger (v3.02), count matrices were loaded into R and analysed in a bioconductor/SingleCellExperiment environment using the *scran* (v1.20.1) [30] and *scater* (v1.20.0) packages [31] for QC and initial processing. Normalised log-scale counts were calculated with the deconvolution method described in [32]. Cells with fewer than 1500 UMIs or more than 10% of hemoglobin gene content were removed. Potential doublets were identified with *scrublet* (v0.2.3) [33] and removed (having a *scrublet* score over a manually defined threshold of 0.3). Cell-cycle phases were annotated with the *cyclone* method [34].

To create a reduced-dimensionality UMAP representation, first the 1000 most biologically variable genes were estimated with the *modelGeneVar* function in the *scran* package. To minimise the influence of the cell cycle on the layout, the log-count genes were 'corrected' for the cell-cycle stage with (*regressBatches*) before calculation of principal components. The first 20 principal components were used to create the UMAP layout.

Cell clusters were defined from these same corrected principal components using the *louvain* method (*k* = 20) [35] from the *igraph* packages (v1.2.6) [36]. Cluster marker genes were identified by looking for enriched genes in each cluster when contrasted with an average of all other clusters using a pseudobulk approach with *voom*, *limma* (v3.48.0) [37] and *topconfects* (*p* = 0.05, v1.8.0) [38]. Only genes expressed in at least 3 clusters/sample groups with at least 20 total UMIs were considered. Cell types were manually determined.

Clusterwise differential expression between *Wnt5a* knockout samples and the WT was calculated based on normalised counts of individual cells with limma, with sample and condition included in the model (v3.48.0) [37]. Each cluster was tested independently, and only genes with an average of 0.02 (1 per 50) reads per cell within the cluster were considered. For the analysis of differentially expressed genes, the fold change was set to ± 1.5 , and the false discovery rate was set to ≤ 0.05 . Pathway and bioinformatic analyses were done using DAVID Bioinformatics Resources, Ingenuity Pathway Analysis (IPA, Qiagen), and Enrichr [39,40].

2.6. Statistical Analyses

For gene expression using real-time PCR, statistical analysis was performed using an unpaired *t*-test (GraphPad). Data were represented as mean expression levels, along with the standard error of the mean.

3. Results

3.1. Loss of *Wnt5a* Disrupts Elongation and Differentiation of the Developing Müllerian Duct

By the end of embryonic development at 18.5 dpc, *Wnt5a*^{-/-} female reproductive tracts completely lacked a vagina, as previously reported (Figure 1A) [23]. *Wnt5a*^{-/-} uterine horns were over 60% shorter than wild-type controls, suggesting that Müllerian duct elongation was impaired, in addition to a posterior defect at the Müllerian tubercle, the site of vaginal formation (Figure 1A,B) [41]. To further characterize this process and assess if the defect in Müllerian duct development was due to reduced proliferation or increased apoptosis, we performed immunofluorescence staining using antibodies against proliferative marker PH3, and apoptotic markers cleaved CASP3 and H2AX at 13.5 dpc and 18.5 dpc. At both stages, PH3 expression was significantly reduced in *Wnt5a*^{-/-} samples, with a decrease in PH3-positive cells by 75% and 72% compared to the wild type, respectively (Figure 1C–E). Conversely, no differences in CASP3 and H2AX were detected (Supplementary Figure S1). At 18.5 dpc, when Müllerian duct development was completed, staining of epithelial marker CDH1 and basement membrane component LAMA1 did not show significant differences between *Wnt5a*^{-/-} and controls (Figure 2A). However, staining of epithelial marker PAX2 showed a more cuboidal than columnar epithelium in *Wnt5a*^{-/-} ducts (Figure 2B). This feature, in combination with reduced cell proliferation, was reminiscent of some phenotypic aspects of CTNNB1 stabilization [10]. In addition, staining of SMA showed a thicker smooth muscle layer in *Wnt5a*^{-/-} uterus horns, suggesting dysregulation of mesenchymal differentiation (Figure 2B). Further immunofluorescence analyses revealed that CTNNB1 was expressed in both epithelial and mesenchymal cells in *Wnt5a*^{-/-} uteri instead of only in the epithelium as in wild-type samples (Figure 2C), suggesting the dysregulation of *Ctnnb1* expression. Mesenchymal marker VIM was dramatically upregulated in *Wnt5a*^{-/-} compared controls, supporting dysregulated differentiation of the mesenchymal compartment or altered tissue remodelling, consistent with the observed increased in SMA expression (Figure 2C). Overall, these results suggest that WNT5A is necessary for the posterior elongation of the Müllerian ducts while maintaining the coordinated differentiation of epithelial and mesenchymal cells. Therefore, in addition to failure of vaginal formation as previously reported, loss of *Wnt5a* results in shorter uterine horns due to reduced cell proliferation and disorganized tissue architecture.

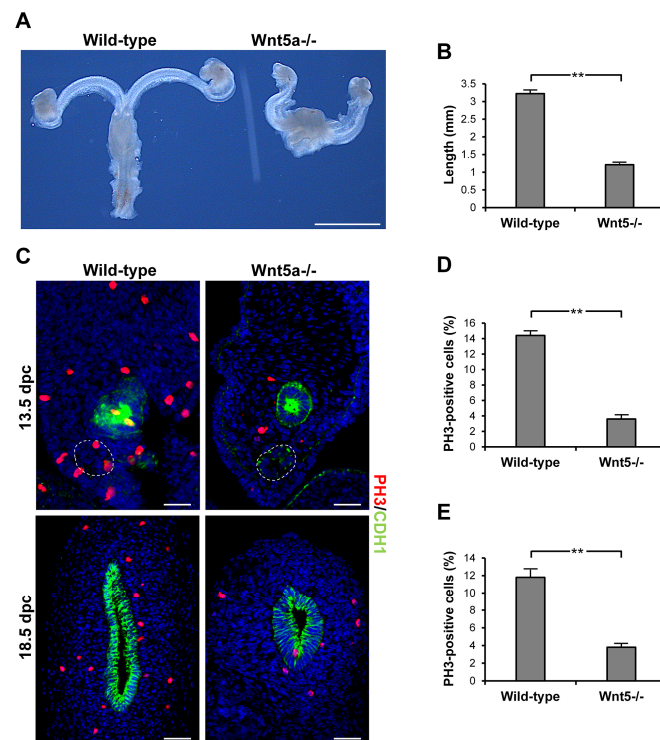


Figure 1. Phenotype of the Wnt5a^{-/-} reproductive tract. (A) Gross morphology of reproductive tracts shows reduced length of uterine horns and vaginal agenesis in Wnt5a^{-/-} mice compared to controls. (B) Measurement of the uterine horn length at 18.5 dpc. n = 5. (C) Immunofluorescence staining of proliferation marker PH3 (red) in samples at 13.5 and 18.5 dpc. At 13.5 dpc, epithelial marker CDH1 (green) stains the Wolffian ducts only; the Müllerian ducts are delineated with dashed lines. At 18.5 dpc, only the Müllerian ducts are present and are positive for CDH1. (D,E) Quantification of PH3-positive cells in 13.5 (D) and 18.5 dpc (E) samples. n = 3. Scale bars = 2mm (A) and 40mm (C). ** $p < 0.01$.

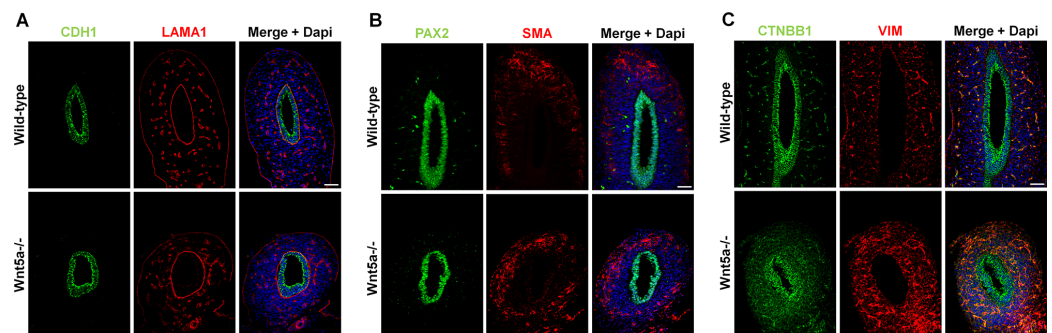


Figure 2. Dysregulation of mesenchymal markers in Wnt5a^{-/-} Müllerian ducts. (A) Immunofluorescence staining of LAMA1 (red) and CDH1 (green) at 18.5 dpc. The Müllerian duct had an unusual, rounded shape in Wnt5a^{-/-} mice. Expression of LAMA1 was normal in Wnt5a^{-/-} samples. (B) Immunofluorescence staining of PAX2 (green) and SMA (red) at 18.5 dpc. PAX2 staining revealed that the Müllerian epithelium of Wnt5a^{-/-} samples displayed a cuboidal morphology instead of the columnar morphology typical of wild-type ducts. In addition, SMA staining was stronger in Wnt5a^{-/-} samples. (C) Immunofluorescence staining of CTNBB1 (green) and VIM (red) at 18.5 dpc. While only the epithelium was positive for CTNBB1 in wild-type mice, Wnt5a^{-/-} samples expressed CTNBB1 in both the epithelial and mesenchymal compartments. In addition, mesenchymal marker VIM was upregulated in Wnt5a^{-/-} ducts. Scale bar = 40 μ m.

3.2. Wnt5a Regulates Müllerian Duct Morphogenesis Through the Non-Canonical PCP Pathway

To investigate the predominant signalling pathway regulated by WNT5A in Müllerian duct development, we used the WNT signalling pathway RT² Profiler PCR Array. At

13.5 dpc, the PCP gene *Jun* was significantly downregulated, as well as *Wnt5a*, as expected (Figure 3A). Upregulated genes included WNT inhibitor *Wif1*, as well as canonical factors *Wnt3a*, *Wnt7b*, *Wnt8a*, and *Wnt8b* (Figure 3A). These results suggest the activation of the canonical Wnt pathway, likely due to loss of WNT5A-inhibitory effects [42,43]. Given the complexity of signals regulated by WNT5A, we examined the expression of additional representative genes of the PCP Wnt pathway that were not represented in the PCR array. We found that *Fos* was also downregulated in *Wnt5a*^{-/-} samples, whereas *Ror2*, *Vangl2*, and *Rac1* did not show significant differences compared to wild types (Figure 3B). At 18.5 dpc, JUN and phospho-JUN localized in the Müllerian duct epithelium of wild-type controls, whereas FOS was expressed mostly in mesenchymal cells (Figure 3C,D). In *Wnt5a*^{-/-} samples, immunofluorescence confirmed the downregulation of JUN, FOS, and phospho-JUN (Figure 3C,D). These results are consistent with a morphogenic role of WNT5A during Müllerian duct development through the Wnt non-canonical PCP pathway.

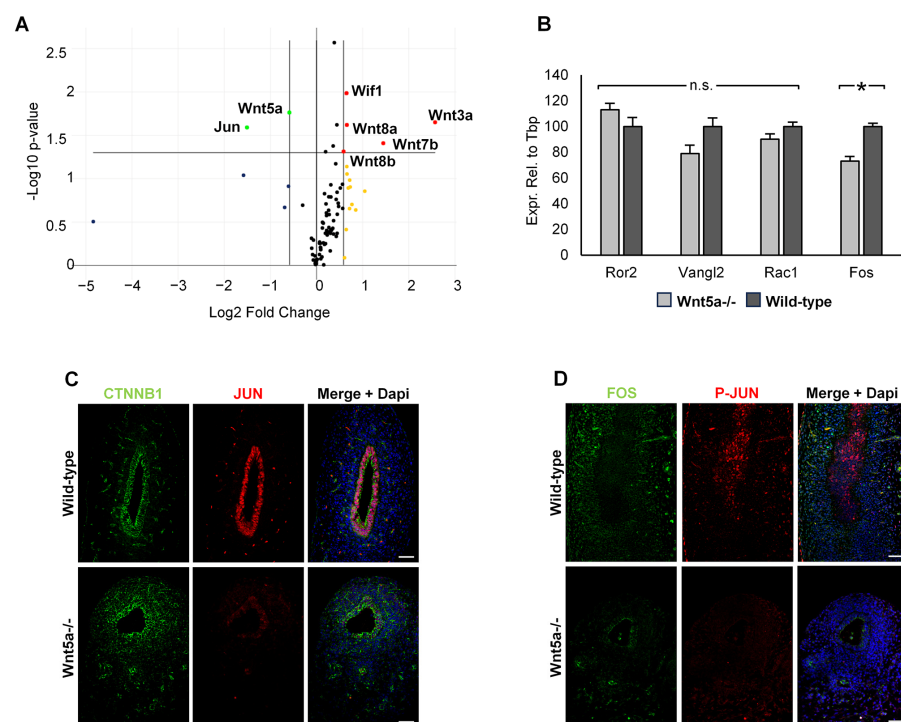


Figure 3. Downregulation of the PCP pathway in *Wnt5a*^{-/-} Müllerian ducts. (A) Volcano plot of the Wnt pathway RT2 Profiler PCR Array. Significantly upregulated genes are represented in red and downregulated genes in green. $n = 3$. (B) Expression profile of selected PCP genes. $n = 3$; * $p < 0.05$, n.s. not significant. (C) Immunofluorescence staining of JUN at 18.5 dpc, showing reduced expression in *Wnt5a*^{-/-} mice. (D) Immunofluorescence staining of FOS and P-JUN at 18.5 dpc. Both markers were downregulated in *Wnt5a*^{-/-} ducts. Scale bar = 40 μm .

3.3. Single-Cell Transcriptomics of the Developing Müllerian Ducts

To further understand the specific mechanisms involved in the disruption of Müllerian duct development following the loss of *Wnt5a*, we performed single-cell RNA-Seq analysis at 18.5 dpc, when elongation of the Müllerian ducts was complete. Single-cell profiling identified thirteen cell populations in wild-type and *Wnt5a*^{-/-} samples (Figure 4A). Cell identity was assigned to each cluster based on the expression of specific markers using the Mouse Cell Atlas database, as well as previous reports (Table 1 and Supplementary Table S1) [11,44–46]. Cluster C1 represents proliferative or progenitor cells that expressed mitotic genes *Cdc20*, *Cenpa*, *Cenpf*, *Kif20a*, *Ckap2*, *Ccnb1*, and *Ccnb2*, as well as stem cell markers *Sapcd2* and *Dlgap5*. Consistently, C1 was the most enriched in cells in the G2/M phase (Figure 4B). Cluster C2

expressed typical markers of the Müllerian mesenchyme, including *Amhr2* and *Hand2*. The expression of markers such as *Nkd2*, *Vcan*, and *Plac8* suggested cells form the inner mesenchyme, and *Hoxa10/11* and *Hoxd10/11* revealed localization in the uterine portion of the Müllerian ducts [45,47]. Cluster C3 represents smooth muscle cells, which expressed markers such as *Acta2*, *Tagln*, *Myocd*, and *Synpr*. These cells expressed *Hoxa10/11* and *Hoxd11*, localizing in the uterine portion of the Müllerian ducts. Cluster C4 represents mesothelial cells, expressing the typical *Upk1b*, *Upk3b*, *Tm4sf5*, *Msln*, *Muc16*, and *Lrp2* markers. Similar to C1, cluster C5 is representative of Müllerian mesenchyme cells positive for *Amhr2* and *Hoxa11*. The expression of replication and cell-division markers including *Cdc6*, *Top2a*, *Ccne1/2*, *Rrm2*, *Esco2*, and *E2f2* suggests cells undergoing active proliferation (Figure 4B, Supplementary Table S1). Cluster C6 is similar to C2 and also represents cells of the inner uterine mesenchyme, expressing genes such as *Amhr2*, *Nkd2*, *Vcan*, *Plac8*, *Hoxa10/11*, and *Hoxd10/11*. However, C6 uniquely expressed Wnt factors *Wnt4* and *Wnt6*, mesenchymal genes *Osr2* and *Pdgfra*, and ECM markers *Col6a2* and *Col13a1*, likely representing a subcluster with a different function or developmental state. Genes in cluster C7 include *Dpt*, *Lum*, *Postn*, *Slc26a7*, *Tbx18*, *Gata6*, and *Nr2f1*, indicating cells from the outer mesenchyme of the Müllerian ducts [45]. Cluster C8 represents epithelial cells that expressed *Wnt7a*, *Cdh1*, *Pax2*, *Pax8*, and *Hnf1b*. Cluster C9 is made up of myeloid cells, with characteristic expression of *Lyz2*, *Ms4a7*, *Ccl3*, *Ccl4*, *Tyrobp*, and several complement components. Cluster C10 expressed key endothelial markers including *Cdh5*, *Pecam1*, *Cldn5*, and *Icam2*. Cluster C11 is also representative of epithelial cells, although with a different expression profile compared to C8. Genes in C11 include *Wfdc2*, *Cldn3*, *Cldn7*, *Pax2*, *Elf3*, and *Ehf*. C8 and C11 may represent cell types at different stages of differentiation, with C8 showing a typical epithelial signature while C11 represents a seemingly transitional state with increased enrichment in genes involved in epithelial–mesenchymal transition, such as *Msx1*, *Msx2*, *Dlx5*, *Dlx6*, and *Ovol1*. Alternatively, these clusters may be located in different regions along the Müllerian ducts. Cluster C12 showed expression of pericytes markers, including *Rgs5*, *Heyl*, *Apold1*, *Cspg4*, and *Mcam*. Cluster C13 represents mesenchymal cells expressing genes such as *Dcn*, *Dpt*, and *Wfikkn2*. However, this cluster showed enrichment in factors that are expressed in the oviducts, including *Foxl2*, *Hoxa7/9*, and *Hoxc5/6/8/9* [48,49]. Interestingly, C13 is largely made up of cells coming from *Wnt5a*^{-/-} samples, suggesting that these cells are more abundant in Müllerian ducts lacking *Wnt5a* (Figure 4C).

Gene expression analyses of scRNA-Seq datasets revealed interesting insights into the cell-specific molecular mechanisms regulated by *Wnt5a* in the developing reproductive tract. When stringency was set to a fold change ± 1.5 and false discovery rate ≤ 0.05 , few but critical genes were found to be differentially expressed in several clusters, including C1 (proliferative cells), C3 (smooth muscle cells), C5 (proliferative mesenchyme), C6 (inner mesenchyme #2), and C7 (outer mesenchyme) (Figure 5 and Supplementary Table S2). The clusters with the largest numbers of differentially expressed genes were clusters C4 (mesothelium), C8 (epithelium #1), C11 (epithelium #2), and C13 (oviduct mesenchyme). Negligible changes were observed for clusters C2 (inner mesenchyme #1), C10 (endothelium), and C12 (pericytes), while C9 (myeloid cells) displayed no differentially expressed genes (Figure 5 and Supplementary Table S2). Functional annotation analysis for proliferative (C1), epithelial (C8 and C9), and mesenchymal (C5, C6, and C7) cells, which are the major cell types of the embryonic reproductive tract, revealed that the main biological processes affected by loss of *Wnt5a* were apoptosis for proliferative cells and differentiation, migration, and metabolic events for different subclusters of the epithelial and mesenchymal lineage (Table 2). These processes are regulated by master genes including *Fos* and *Jun*, as well as *Socs3*, *Gadd45g*, *Btg1*, and *Hspa1a* (Figure 5) [50–54]. We then interrogated Ingenuity Pathway Analysis (IPA) to identify the developmental and pathological processes that were associated with the observed changes in gene expression. The most represented molecular

and cellular functions were cellular development, growth and proliferation, and cell death and survival, confirming previous results (Table 3). Connective tissue development and function, as well as organismal, tissue and embryonic development, were the most enriched developmental categories (Table 3). Finally, the top three diseases and disorders were organismal injury and abnormalities, cancer, and reproductive system disease (Table 3).

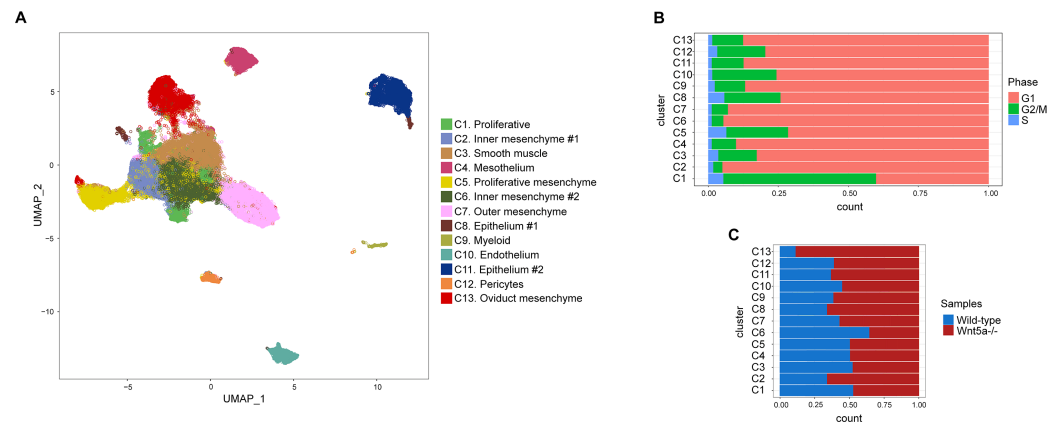


Figure 4. Cell cluster composition of 18.5 dpc Müllerian ducts. (A) UMAP plot from scRNA-Seq analysis of Müllerian duct samples at 18.5 dpc revealed 13 cell clusters (C1–C13). (B) Cell-cycle phases in each cluster. (C) Sample composition in each cell cluster.

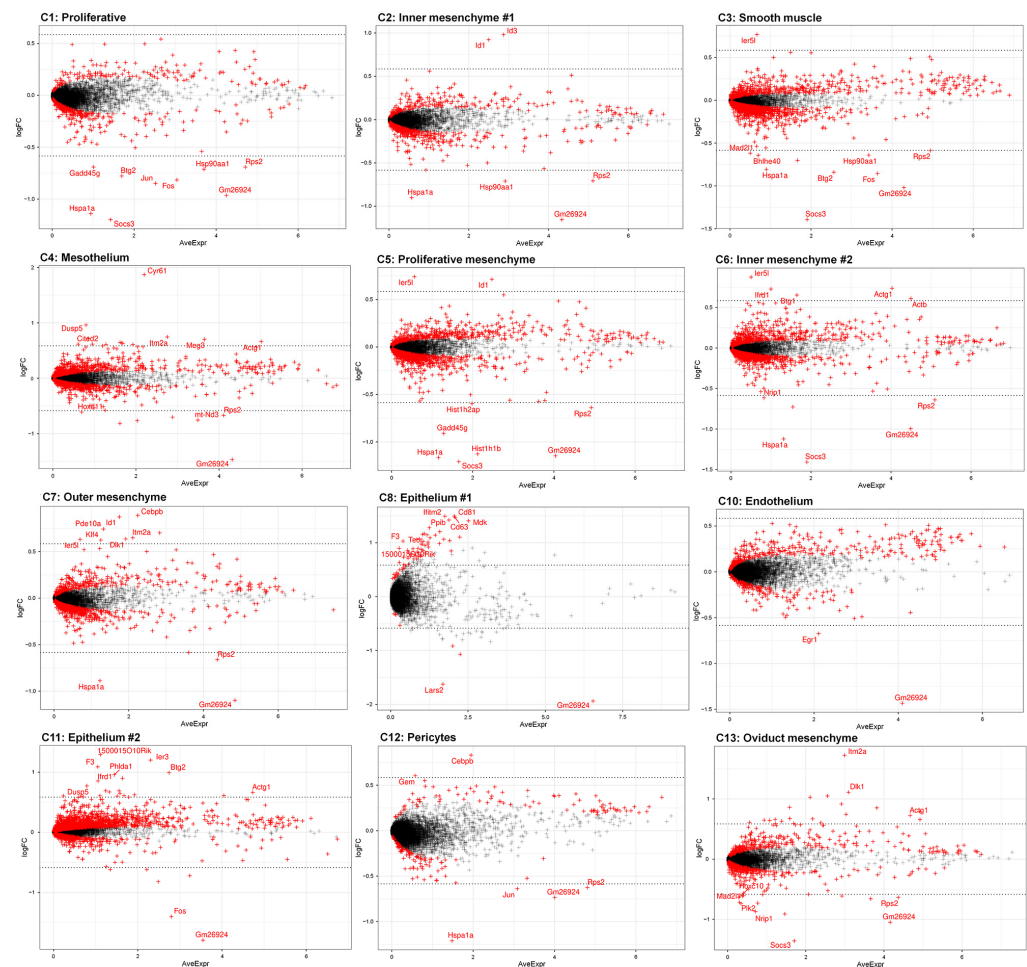


Figure 5. Cluster-specific differentially expressed genes. MA plots of gene expression in each cluster following scRNA-Seq. Dotted lines = fold-change cutoffs. Red = genes significantly different in expression, Black = genes not significantly different.

Table 1. Top 15 most enriched genes in each cell cluster.

c1	c2	c3	c4	c5	c6	c7	c8	c9	c10	c11	c12	c13
Proliferative	Inner Mesenchyme #1	Smooth Muscle	Mesothelium	Proliferative Mesenchyme	Inner Mesenchyme #2	Outer Mesenchyme	Epithelium #1	Myeloid	Endothelium	Epithelium #2	Pericytes	Oviduct Mesenchyme
Cenpa	Cnr1	Acta2	Upk3b	Hist1h2ap	Cnr1	Lum	Rmst	C1qa	Gpihbp1	Wfdc2	Higd1b	Foxl2
C330021F23Rik	Amhr2	Ldoc1	Tm4sf5	Hist1h2ae	Cpxm2	Cxcl13	Cdh16	Cpa3	Cldn5	Prap1	Rgs5	4833424O15Rik
Cdc20	Jakmip1	Cxcl14	Lrm4	Hist1h1b	Tfap2c	Sgcz	Cldn6	C1qb	Aplnr	Cldn3	Ndufa4l2	Sostdc1
Psrc1	Tmem178b	Olfml3	Upk1b	Hist1h1a	Gm13659	Cntn4	Stt4	Cma1	Cdh5	1600029D21Rik	Cpa1	Enpp2
Cenpf	Tfap2c	Synpr	Muc16	2810417H13Rik	Prlr	Gm26691	Atp2b2	C1qc	Pecam1	Urah	Aspn	Wnt16
Fam64a	Nkd2	Asic2	Msln	Hist1h2ab	Tex15	Rxfp2	Esrp1	Tpsb2	Icam2	Tacstd2	Ccl11	Dsc3
Kif20a	Tex15	Tagln	Krt14	Aunip	Vcan	Spon2	Slc1a1	Ccl4	Cd93	Rab25	Kcnj8	Aldh1a1
Ccnb1	Phactr3	Penk	Lrp2	Rrm2	Cck	Sntg1	Myh14	Tyrobp	Robo4	Cldn7	Vip	Foxl2os
Sapcd2	Cpxm2	Tac2	Myrf	Hist1h2ao	Amhr2	Postn	Cldn7	Ccl3	Plvap	Elf3	Gm13889	Hmgcs2
Ccnb2	C030034L19Rik	Ptger3	Podxl	Esco2	Tmem100	Pcdh10	Cdh1	Lyz2	Eltd1	Cdh16	Heyl	D3Bwg0562e
Cdkn3	9330154J02Rik	2610028E06Rik	Bnc1	Rad51ap1	Hoxa11	Cntn6	Wnt7a	Ms4a7	Fam167b	Pdzk1ip1	Fam162b	Ppp1r14c
RP23-45G16.5	Frem1	Npy2r	Krt7	Mybl2	Tulp2	Dpt	Slc14a1	F13a1	Myct1	Dlx5	Ebf1	Fgf10
Dlgap5	Tulp2	Mamdc2	A730046J19Rik	Tk1	Scube1	Cpa6	Wfdc2	Pf4	Mfng	Ehf	Reln	Osr1
Ckap2	Hoxa11	Zfp185	Fam180a	E2f2	Car14	Tbx18	Cldn3	Ctss	Ptprb	Pax2	Ace2	Hoxc8
Kif2c	Cck	Tcf21	Ildr2	Tcf19	Rxfp1	Csmd3	Chdh	Fcer1g	Esm1	Crb3	Fabp7	Atp1a2

Table 2. Top Gene Ontology (GO) classes for proliferative, mesenchymal, and epithelial cell clusters.

Cluster	GO Class	FDR
C1—Proliferatives	Regulation of apoptotic process	1.17×10^{-3}
C5—Proliferative mesenchyme	Regulation of apoptotic process	3.11×10^{-3}
C6—Inner mesenchyme #2	Positive regulation of cell differentiation	1.00×10^{-2}
C7—Outer mesenchyme	Negative regulation of transcription by RNA polymerase II	2.82×10^{-2}
C8—Epithelium #1	Response to interferon beta	1.90×10^{-4}
C9—Epithelium #2	Regulation of cell migration	8.84×10^{-3}

C2—inner mesenchyme #1 was excluded due to a small number of differentially expressed genes. FDR = false discovery rate.

Table 3. Pathway analysis of differentially expressed genes. Most enriched “Molecular and cell functions”, “System development” and “Diseases and disorders” categories.

Molecular and Cell Functions	Cell Cluster	p-value range
Cellular development	C1, C3, C4, C5, C6, C7, C8, C11, C13	8.92×10^{-3} – 2.86×10^{-15}
Cellular growth and proliferation	C1, C3, C4, C5, C6, C7, C8, C11, C13	8.78×10^{-3} – 2.86×10^{-15}
Cell death and survival	C1, C3, C4, C5, C6, C8, C11	9.19×10^{-3} – 1.81×10^{-17}
Cellular movement	C3, C4, C5, C7, C8, C11, C13	5.43×10^{-3} – 5.77×10^{-18}
Cell cycle	C1, C6, C7, C11, C13	7.89×10^{-3} – 2.35×10^{-16}
System development	Cell cluster	p-value range
Connective tissue development and function	C1, C3, C4, C5, C6, C7, C8, C11, C13	7.89×10^{-3} – 1.75×10^{-13}
Organismal development	C4, C6, C7, C8, C11, C13	9.19×10^{-3} – 5.98×10^{-17}
Tissue development	C3, C4, C6, C7, C8, C11, C13	8.92×10^{-3} – 6.63×10^{-16}
Embryonic development	C1, C7, C11	7.23×10^{-3} – 5.98×10^{-17}
Organismal survival	C3, C5	1.81×10^{-3} – 9.75×10^{-10}
Diseases and disorders	Cell cluster	p-value range
Organismal injury and abnormalities	C1, C3, C4, C5, C6, C7, C8, C11, C13	9.19×10^{-3} – 1.11×10^{-17}
Cancer	C3, C4, C5, C7, C8, C11, C13	5.44×10^{-3} – 1.01×10^{-16}
Reproductive system disease	C7, C11, C13	5.46×10^{-3} – 8.45×10^{-15}

3.4. *Wnt5a* Regulates Cell Fate of the Anterior Müllerian Ducts

One of the most striking differences between *Wnt5a*^{−/−} and control reproductive tracts is the C13 cluster. This cluster is made up of mesenchymal cells of the oviducts and was greatly enriched in the *Wnt5a*^{−/−} tracts. Therefore, we focused on the C13 cluster to obtain more insights into the underlying cellular and molecular mechanisms. Gene Ontology analysis identified apoptosis, proliferation, and transcription as top enriched categories for genes that were downregulated in C13, whereas endothelial cell migration, protein polymerization, and cell motility were associated with the upregulated genes (Table 4). Wnt signalling was the top pathway associated with the downregulated genes, as expected, followed by IL6 signalling and embryonic stem cell (ESC) pluripotency, whereas adipogenesis, myometrial contraction, and focal adhesion were associated with the upregulated genes (Table 5). The genes that were most upregulated included factors with critical roles in developmental patterning, cell migration, and adhesion, such as *Itm2a*, *Ier3*, and *Id1*, supporting the critical role of *Wnt5a* signalling in Müllerian duct elongation (Figure 6A) [55–57]. Like other clusters, key downregulated genes included *Jun* and *Socs3*. These factors, together with *Wnt5a*, are critical for cell-fate decisions, in addition to regulating developmental processes such as cell proliferation, differentiation, and apoptosis (Figure 6A) [11,58,59]. Strikingly, *Hoxa10* and *Hoxc10*, which are expressed in the portion of the Müllerian duct that will become the uterus, were downregulated in the *Wnt5a*^{−/−} cluster C13, whereas *Hoxa7*, a factor expressed in the oviduct, was significantly upregulated (Figure 6B) [60]. These results suggest that the C13 cluster of *Wnt5a*^{−/−} samples comprises cells that differentiated into mesenchymal cells of the oviducts rather than the uterus. Morphologically, *Wnt5a*^{−/−} reproductive tracts did not show an increase in the length of the oviducts (Figure 1A).

This raises the hypothesis of a possible transformation of the anterior uterus to oviducts in *Wnt5a*^{-/-} mice. Therefore, we performed immunofluorescence on the anterior portion of the uterus and stained for FOXL2, which, at this developmental stage, is a marker of the oviducts but not the uterus [61]. As expected, the oviducts of both control and *Wnt5a*^{-/-} mice were positive for FOXL2 (Figure 6C). However, only the *Wnt5a*^{-/-} anterior uterine horns expressed FOXL2, confirming that these cells had transformed into oviductal cells (Figure 6C). Overall, these results suggest that *Wnt5a* is involved not only in the elongation and directional patterning of the Müllerian ducts but also in regulating fate decisions of mesenchymal cells of the anterior reproductive tract to differentiate into oviducts vs. uteri.

Table 4. Top three Gene Ontology (GO) classes for downregulated and upregulated genes in cluster C13.

Gene Expression	GO Class	p-Value
Downregulated	Regulation of apoptotic process	9.94×10^{-4}
	Regulation of cell population proliferation	2.10×10^{-3}
	Positive regulation of transcription by RNA polymerase II	3.90×10^{-3}
Upregulated	Blood-vessel endothelial cell migration	3.36×10^{-3}
	Negative regulation of protein polymerization	3.90×10^{-3}
	Regulation of cell motility	7.88×10^{-3}

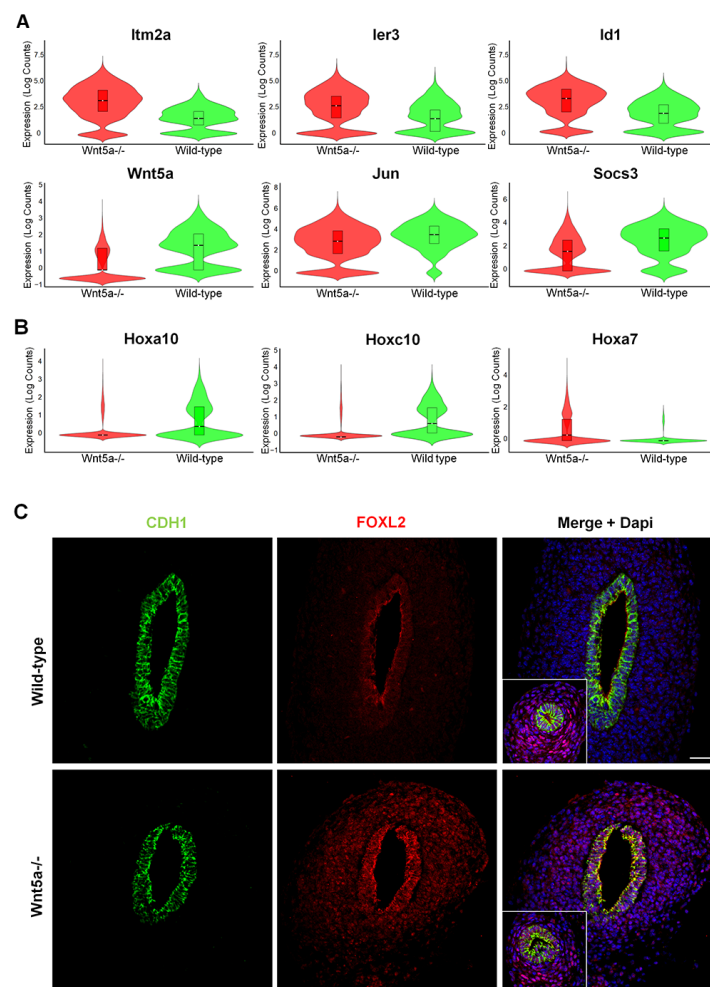


Figure 6. Anterior transformation of *Wnt5a*^{-/-} uterine horns. (A) Violin plots of dysregulated genes in cluster C13 of *Wnt5a*^{-/-} and wild-type ducts. (B) Violin plots of key Hox markers in cluster C13. For all genes in (A,B), $p < 0.001$. (C) Immunofluorescence staining of FOXL2 (red) in the anterior uterine horns, showing expression in *Wnt5a*^{-/-} samples. Inset: oviduct staining positive for FOXL2 in both wild-type and *Wnt5a*^{-/-} mice. CDH1 (green) was used as epithelial marker. Scale bar = 40 μm.

Table 5. Top three pathways for downregulated and upregulated genes in cluster C13.

Gene Expression	Pathway	p-Value
Downregulated	Wnt signalling	2.04×10^{-2}
	IL6 signalling	2.06×10^{-2}
	ESC pluripotency	2.90×10^{-2}
Upregulated	Adipogenesis	3.58×10^{-2}
	Myometrial relaxation and contraction	3.80×10^{-2}
	Focal adhesion	3.70×10^{-2}

4. Discussion

WNT5A plays critical roles in reproductive development. It is required for the formation of the posterior portion of the female reproductive tract, as well as the development of uterine glands and endometrial crypts [23,62]. WNT5A primarily signals through the non-canonical Wnt PCP pathway, which regulates both planar-cell and apical-basal polarity and is necessary for patterning, tissue outgrowth, and cell proliferation [63–65]. WNT5A binds to ROR2 and activates JNK, which, in turn, phosphorylates JUN, leading to the transcription of downstream targets including *Fos* and *Jun* itself [29,66–68]. Disruption of the PCP pathway through mutation of its members, including *Wnt5a*, results in a shortened apical-basal body axis and reduced elongation during organ development [69]. Although acting primarily through the non-canonical pathway, WNT5A can also modulate the Wnt canonical pathway, depending on receptor availability [43]. Activation of the PCP pathway by WNT5A binding to ROR2 has been reported to inhibit the canonical WNT pathway [22,43]. However, WNT5A can also activate the canonical pathway and lead to stabilization of b-catenin through binding of FZDs and RYK receptors [43,70]. Studies on zebrafish have shown that JUN and FOS could regulate the canonical pathway by physically interacting with b-catenin [71,72]. Moreover, Schambony et al. have shown that in *Xenopus*, WNT5A/ROR2 can activate PI3K and CDC42, in addition to JNK, leading to the transcription of *Papc* independent of the PCP pathway [73]. As such, the authors proposed WNT5A/ROR2 as an alternative, separate branch of the non-canonical Wnt pathway [73]. Overall, this body of work highlights the complexity of WNT5A signalling, capable of operating through different networks and depending on specific microenvironments.

This evidence could, at least in part, explain the heterogeneity observed in Robinow syndrome, which presents as autosomal dominant when associated with mutations in *WNT5A* and its mediators, *DVL1* and *DVL3*, and autosomal recessive if caused by biallelic mutations in *ROR2* [74]. A critical step in the activation of the Wnt PCP pathway by WNT5/ROR2 is signal transduction through DVL1/3, which phosphorylate JUN via RAC [74,75]. Additional candidate genes include *FZD2*, a WNT5A co-receptor; *RAC3*, which activates JNK/JUN phosphorylation; *NXN*, an interacting partner of DVL1; and *GPC4*—all factors involved in the Wnt PCP pathway [75]. Given the complexity of these networks and their dependency on combinations of ligands, receptors, and intracellular components, it is reasonable to assume that the pathogenesis of Robinow syndrome does not depend solely on the inactivation of one particular branch of the Wnt pathway but, rather, on the disruption of a delicate and environment-specific balance between canonical and non-canonical Wnt factors [75].

In this study, we showed that ablation of *Wnt5a* impaired murine Müllerian duct development during embryogenesis. Müllerian duct elongation was affected by a reduction in cell proliferation rather than increased apoptosis (Figure 1D,E, Supplementary Figure S1). GO analyses showed the downregulation of “regulators of apoptosis processes”, which include cell-cycle and stress-response modulators, suggesting a generally reduced engagement of upstream survival and stress-associated signalling rather than activation

of apoptotic execution pathways, consistent with the absence of detectable histological changes in apoptosis. Notably, reduced proliferation was observed in both the epithelium and the mesenchyme, indicating disruption of the paracrine cross-talk between these compartments, which is necessary for proper cell proliferation [17]. Several Wnt ligands exhibit distinct epithelial or mesenchymal expression patterns and functions. Thus, alterations in mesenchymal signalling can indirectly affect epithelial proliferation through paracrine mechanisms. We found that the Müllerian duct epithelium displayed cells of cuboidal instead of the typical columnar morphology, with increased production of mesenchymal markers VIM and SMA (Figure 2B,C). We also noted a change in the pattern of expression of CTNBB1, which, instead of remaining restricted to the epithelium like in wild-type mice, was expressed in both the mesenchymal and epithelial compartments of *Wnt5a*^{-/-} samples (Figure 2A) [10]. The coordinated interaction between mesenchymal and epithelial compartments is critical for the correct specification and differentiation of the reproductive tract [76]. Several inductive signals are exchanged between these cell types, including Wnt and Hox factors, and their inactivation results in a variety of Müllerian anomalies, from agenesis to homeotic transformation [1,76]. Using renal capsule grafting, *Wnt5a* was shown to be postnatally required in the Müllerian ducts for the estrogen-mediated inhibition of *Wnt7a*, *Hoxa10*, and *Hoxa11* [23]. It is possible that WNT5A could also be involved in the regulation of *Wnt* and *Hox* genes during embryonic development. Real-time PCR analysis revealed the upregulation of several canonical Wnt factors in *Wnt5a*^{-/-} mice, including *Wnt3a*, *Wnt8a*, and *Wnt8b*, as well as Wnt inhibitor *Wif1*, whose product can modulate the non-canonical pathway by binding to WNT5A (Figure 3A) [77]. Conversely, non-canonical PCP pathway effectors *Fos* and *Jun* were significantly downregulated at both the RNA and protein levels in *Wnt5a*^{-/-} Müllerian ducts (Figure 3). Adding to its ability to activate either the canonical or non-canonical pathways depending on receptor context, WNT5A was shown to compete with WNT3A, thereby inhibiting the b-catenin pathway [42]. In addition, reverse sections of *Wnt5a*^{-/-} Müllerian ducts revealed a rounded morphology that resembled the shape of reproductive tracts that are mutant for *Vangl2*, another PCP gene (Figures 2 and 3C,D) [78]. *Wnt5a* and *Vangl2* are transcriptionally independent, and *Vangl2* expression was unchanged in *Wnt5a*^{-/-} embryos (Figure 3B). In addition, WNT5A interacts with VANGL2 at the protein level, leading to the phosphorylation of the latter, which, in turn, regulates WNT5A activity [79]. Therefore, the rounded ductal morphology likely results from loss of planar-cell polarity and impaired tissue organization rather than altered *Vangl2* expression. Overall, these results suggest that during Müllerian duct development, WNT5A acts primarily through the PCP pathway while inhibiting the canonical WNT pathway and that its loss leads to the downregulation of *Fos* and *Jun* and ectopic expression of CTNBB1 in the mesenchyme, likely through post-translational regulations.

While *Wnt5a*^{-/-} mice lack the posterior portion of the reproductive tract, scRNA-Seq analysis revealed the presence of all other major Müllerian duct cell types, including mesenchymal, epithelial, smooth muscle, endothelial, and proliferative cells. Ablation of *Wnt5a* was found to impair critical developmental processes, including migration, differentiation, and apoptosis (Tables 2 and 3). A common denominator of gene expression changes in several of these clusters was the downregulation of PCP factors *Fos* and *Jun*, as well as the *Socs3* gene (Figure 5). Proper regulation of *Socs3* is necessary for the correct development of several organs, including the reproductive tract, kidneys, and bones [11,80]. Notably, FOS and JUN directly regulate *Socs3* expression by binding to its promoter, highlighting *Socs3* involvement in the PCP-directed establishment of Müllerian duct patterning [81]. Compared to controls, *Wnt5a*^{-/-} Müllerian ducts showed a significant enrichment in oviductal cells (i.e., cluster C13) (Figure 4). The role of the PCP pathway in regulating oviduct differ-

entiation is not well understood, and like other clusters, C13 showed reduced expression of *Jun* and *Socs3*, consistent with specific inhibition of the PCP pathway (Figure 6A) [5,17,21]. Because the length of the oviducts was not increased in *Wnt5a*^{-/-} mice, we hypothesized this phenotype could be explained by a transformation of the anterior portion of the uterus to oviducts rather than oviduct expansion. *Hox* genes play critical functions in regulating the segmentation of the female reproductive tract. In the chromosomes, these genes are physically clustered together, with *Hoxa7/9/10/11/13* arranged sequentially from the 3' to the 5' end. Their expression becomes localized in specific regions of the Müllerian ducts, with *Hoxa7/9* marking the oviducts, *Hoxa10* marking the uterine horns, *Hoxa11* marking the lower horns and endocervix, and *Hoxa13* marking the cervix and vagina [82]. In the *Wnt5a*^{-/-} C13 cluster, *Hoxa7* was upregulated compared to controls (Figure 6B). Ectopic expression of *Hoxa7* in Skov3 cells has been shown to induce tumours with oviduct-like structures [82]. Conversely, uterine markers *Hoxa10* and *Hoxc10*, which have similar expression patterns, were downregulated (Figure 6B) [14,60,83]. Finally, immunofluorescence of oviductal marker FOXL2 showed positive staining of the anterior *Wnt5a*^{-/-} Müllerian ducts, whereas controls remained negative (Figure 6C). There is currently no evidence that FOS and JUN directly regulate transcription of *Hox* genes during Müllerian duct development. Therefore, this phenotype could, again, arise from disruption of tissue polarity and architecture rather than a direct transcriptional effect mediated by FOS/JUN signalling, a mechanism observed in other systems [79]. In addition to *Hox* factors, another pathway that could account for the anteriorization of *Wnt5a*^{-/-} Müllerian ducts is the retinoic acid (RA) pathway. A fine-tuned gradient of RA is involved in the spatial determination of the oviduct–uterus borders [16,84]. When differential expression thresholds were relaxed to include differentially expressed factors with log₂FC = 0.40 (fold change = 1.32), RA activation was the top dysregulated canonical pathway (Supplementary Figure S2A). Predictive pathway analysis of cluster C13 in IPA showed a possible mechanism where *Wnt5a* ablation leads to the downregulation of *Jun* and *Socs3*, as well as other factors, including *Fn1*, which is involved in cell migration; *Nr2f2*, which regulates self-renewal, differentiation, and proliferation; and *Enpp2*, which is highly expressed in oviductal mesenchymal cells. These changes would result in loss of planar polarity; disruption of RA signalling gradients; and, ultimately, Müllerian anomaly (Supplementary Figure S2B) [85–87]. Overall, our findings are consistent with the transformation of the anterior *Wnt5a*^{-/-} Müllerian horns into oviducts. More specifically, proximal oviduct marker *Cepbp* was upregulated in cluster C13, whereas distal oviduct marker *Wt1* was downregulated, supporting the transformation of the anterior uterus to proximal rather than distal oviducts [46].

Although Robinow syndrome is believed to mostly affect the lower reproductive tract, this condition is also well known for its complex and highly variable presentations and a pathogenesis that remains only partly understood [74]. *WNT5A* variants have also been found in patients with Robinow syndrome but normal height, in cases with possible recessive transmission, and in Müllerian anomalies not associated with Robinow syndrome [27,74,88]. We have uncovered an additional function of *Wnt5a*, which seems to be involved not only in vaginal development but in the patterning and specification of the anterior reproductive tract. These insights may prompt further investigations when mutations of *WNT5A* are found or suspected, either in Robinow syndrome or other Müllerian anomalies, especially when associated with complex presentations affecting reproductive functions.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cells15040359/s1>, Supplementary Figure S1. Apoptosis in embryonic Müllerian ducts. Immunofluorescence staining of H2AX and cleaved CASP3 in wild-type and *Wnt5a*^{-/-} samples at 13.5 and 18.5 dpc. Scale bar = 40 µm. Supplementary Figure S2. Mechanism

of Müllerian anomaly in cluster C13. (A) Canonical pathway analysis of cluster C13. (B) Network representation of Wnt5a loss of function in cluster C13, resulting in the downregulation of key factors modulating cell polarity, RA signalling, and Müllerian duct morphogenesis. Supplementary Figure S3. Expression of representative markers. Quantification of fluorescence intensity for key markers as described in the main text. Data are represented as mean values normalised to the wild type $\pm$ SEM. (*) $p < 0.05$, (**) $p < 0.01$. Supplementary Table S1. Top 100 most enriched genes in each cell cluster. Supplementary Table S2. Differentially expressed genes in each cell cluster.

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