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Review

The Metastatic Capacity of Melanoma Reveals Alternative Pathways of Cancer Dissemination

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Abstract: For many years the growth of solid tumors has been associated with their vascularization. The new vessels are needed to deliver oxygen and nutrients within the tumor mass. At the same time, these poorly stabilized vessels act as "Trojan horses" and open a way out for cancer cells. More recently, tumors have been identified whose growth appears to be independent of endothelial cell activity. Here we describe the ability of cancer cells to differentiate and reorganize themself in channels similar to blood vessels containing blood flow, overcoming the need for the angiogenic process of tumor vascularization. Together with the new vessels arising both from angiogenic and vasculogenic processes, these vessel-like structures can be exploited by tumor cells as a guide for migration and metastatic dissemination. In addition to classical intravascular dissemination, cancer cells can acquire pericytic features, interact with the endothelial basal lamina and migrate toward vessels or outside of the vessels. As expected, these alternative tumor behaviors assume greater importance if we consider that drugs with anti-angiogenic action directed against endothelial cells or their ligands are currently used in cancer therapy.

Keywords: metastasis; extravascular migratory metastasis; vascular mimicry; epithelial-to-mesenchymal transition; angiotropism

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1. Introduction

Metastasization is a multistep process in which cancer cells detach from the primary tumor (or other metastases) and spread to locoregional or distant lymph nodes, or to non-contiguous secondary sites. Here, if the tissue microenvironment allows them to survive, they generate a new tumor. Metastasis is responsible for about 90% of cancer-associated deaths [1]. The metastatic potential makes cancer highly lethal; indeed, early diagnosis before the metastatic stage forecasts a better prognosis. Despite the significant progress in cancer biology, it is still difficult to predict whether disseminated cancer cells are present at the moment of diagnosis or treatment and, if so, where they reside. About 1700 people die each day of cancer only in United States (GLOBOCAN2020, WHO https://gco.iarc.fr/databases (access date September 2021)), which further attests to the failure in managing the disease once it disseminates into the body. Classically, the metastatic process uses three ways to spread to distant organs: (i) blood circulation (hematogenous), (ii) lymphatic circulation, and (iii) spread through the abdominal and chest cavities (transcoelomic) [2,3]. While the circulatory system appears to be the most common route, the extent of lymphatic versus hematogenous spread depends on the origin and location of the primary tumor [4]. For instance, bone tumors and sarcomas spread primarily through the blood [5,6] while gastrointestinal, lung, and breast tumors spread through the lymphatic system [7–10]. Finally, transcoelomic spread appears to be restricted to mesotheliomas and ovarian carcinomas [11]. During tumor growth, cancer cells induce the formation and arrangement of a tumoral vascular bed, mainly through angiogenesis. The formation of new vessels contributes to the proliferation and the metastatic spreading of cancer cells [12]. Tumor

vasculature is usually disorganized, with thin and leaky walls and lack of complete pericytic coverage [13,14]; thus, cancer cells can easily penetrate the endothelial cell barrier. Cancer cells that enter circulation are called Circulating Tumor Cells (CTCs) [15]. CTCs are frequently found in the circulation of patients with primary tumors; however, the direct histological observation of tumor cells in vascular channels is infrequent. This "technical impasse" might be attributable to the short time the cells spend in the bloodstream, which is estimated to be in the span of minutes. In the bloodstream, CTCs are exposed to blood flow, to shear stress and/or to the action of immune cells. In this regard, it is important to remember that only 0.1% of the intravasate cells remain alive for more than 24 h, and only 0.01% produce metastatic foci [16,17]. Nevertheless, there is increasing evidence that some tumors, primarily melanomas and carcinomas, can adopt different strategies to grow and disseminate. Among these are vascular co-optation [18], vascular mimicry [19], and single or collective extravascular migration, also known as pericytic mimicry or perivascular invasion [19]. With the identification of vascular co-option and vascular mimicry, the paradigm within which tumor growth and dissemination are angiogenesis-dependent has been overcome [20,21], and non-angiogenic tumors have since been described in brain, liver, skin, and lymph nodes [22]. These alternative strategies increase the supply of nutrients into the tumor mass and increase its invasive and migratory capacities without forming new vessels. The lack of new blood vessels may have a bearing on the efficacy of anti-angiogenic therapies; thus, a detailed description of these processes becomes important in understanding cancer biology. On the other hand, extravascular migration still requires the presence of a well-developed vascular network. Here we will review the molecular mechanisms and the morphofunctional alterations described to date which led to these alternative modalities of metastasization.

2. Non-Angiogenic Intratumoral Vascularization

Tumor vascularization resulting from the growth of vessels from pre-existing ones or the recruitment of circulant endothelial cell precursors (EPC) is fundamental for tumor growth [23]. Furthermore, the angiogenic process is an essential component of the metastatic pathway. The vascular bed within the tumor provides the principal route by which malignant cells exit the primary tumor site and enter into circulation. For many tumors, vascular and lymphatic density can provide a prognostic indicator of metastatic potential, where highly vascular primary tumors have a higher incidence of metastasis than poorly vascular tumors [19]. The vascular structure acts as a physical and mechanical impediment to the passage of cancer cells into the bloodstream, affecting their fate and the new destination tissue. Over the years, the process of tumor vascularization has been the target of numerous studies that led to the characterization of the sprouting phenomena of new vessels from pre-existing ones (the angiogenic process), and to the identification of possible longitudinal partition that divided the vessel into, two identified as intussusceptive microvascular growth. These phenomena, well characterized in blood vessels [24], have been recently described into the lymphatic torrent as well [25,26]. In contrast with the angiogenic process, intussusception in blood and lymphatic vessels facilitates tissue vascularization without modifying vascular permeability. In addition, the identification of EPC suggests that vasculogenesis could be an alternative process in tumor vascularization. Indeed, EPCs, attracted into the tumor mass by the growth factors, leave the bloodstream to migrate into the tissues, finding a suitable microenvironment. Here they differentiate into mature endothelial cells and reorganize themselves into vascular structures. These new structures will reconnect to the pre-existing vessels of the tissue itself, allowing active circulation [27]. As already mentioned, the identification of these processes has for years supported the idea of tumor dependence on vessels, as suggested by Folkman in 1971. In the late 1990s, several studies suggested that brain and lung cancer growth proceeds independently from the neovascularization of the tissue, and that the tissue vascular network in fact persists with the structure and distribution of pre-existing vessels. In these tumors, cancer cells can hijack existing blood vessels for tumor growth,

survival and metastasis [28–30], or exploit their high plasticity to reorganize themself in functional channels. These processes are termed vessel co-option and vascular mimicry (VM), respectively.

Vessel co-opting tumors differ from angiogenic ones in their ability to preserve the vascular scaffold of the surrounding normal tissue instead of inducing a destructive wound healing-like reaction along with angiogenesis, fibrosis and inflammation [31,32]. Usually, vascular co-option occurs in solid tumors, such as in the brain, breast, kidney, and lung [30], with a well-organized vascular bed. In this process, pericytes play an important role in supporting and stabilizing ECs [33]. Even if the molecular mechanisms driving vessel co-option are poorly understood, pericytes physically interacting with cancer cells support cancer invasion [31,34]. The identification of vascular channels lacking ECs was introduced by Maniotis et al., who reported the plasticity of aggressive cancer cells forming de novo vascular networks in highly aggressive uveal melanomas [35]. VM has been described in a plethora of tumors, including carcinomas of the breast [36], ovary [37], bladder [38], lung [39] and prostate [40], as well as sarcomas [41], glioblastomas [42], astrocytomas [43] and melanomas [44]. The functional channels (that are not vessels) are composed of tumor cells with stem cell features, and present characteristic patterns in addition to the erythrocytes inside them. The presence of VM is associated with a high tumor grade, short survival, invasion, and metastasis. Although the molecular mechanisms of VM are not entirely clear, the hypoxia-inducible factor 2α (HIF2α)/vascular endothelial (VE)-cadherin axes are a key pathway [19]. Microarray analyses highlighted in melanoma cell formation channel the downregulation of several melanoma-specific genes, such as melanoma-cell adhesion molecule (MCAM), melan-A (MLANA), and microphthalmia-associated transcription factor (MITF), suggesting a regression in the differentiation state of the tumors. On the contrary, EC-related genes including the tyrosine kinase receptor 1 (TIE1), epithelial-cell kinase (EPHA2), VE-cadherin (CDH5), neuropilin 1 (NPR1) and hypoxia-inducible factor 1α (HIF1α) are up-regulated [45].

An exciting hypothesis associates the vascularization of non-angiogenic tumors with anti-angiogenic therapies (AATs). VEGF inhibitors such as bevacizumab and VEGFR tyrosine kinase inhibitors (RTK-Is) have been widely used in clinics. However, the benefits of AATs are only partial and do not last a long time. It has been demonstrated that the prolonged use of AATs can aggravate hypoxia, which in turn promotes revascularization. In experimental in vitro models of melanoma, the ability of vemurafenib-resistant cancer cells to organize themselves in perfused vascular-like channels has been described [46]. The RTK-I sunitinib increased VM under hypoxic conditions in renal carcinoma models [47] and triple-negative breast cancer cells through the overexpression of HIF-1, VE-cadherin, and Twist1 [48]. Again, in orthotopic glioblastoma models, bevacizumab or vatalanib administration upregulates the IL8/CXCR2 pathway and VM [49,50]. Thus, the non-angiogenic tumors show an altered drug response to AATs, bypassing the endothelial cell-dependent angiogenesis to supply themselves with oxygen and nutrients.

3. Epithelial to Mesenchymal Transition

During the metastatic process cells acquire distinctive mesenchymal properties, with a cellular transdifferentiation process defined as the epithelial to mesenchymal transition (EMT) [51]. EMT plays a central role, especially in carcinoma metastasization. Although during EMT cancer cells acquire their invasive capacity, the EMT switch is not enough to describe all migratory phenotypes of cancer cells. During EMT, cancer cells lose several epithelial markers, reduce adhesion molecules (i.e., the so-called "cadherin switch" from Epithelial to Neural cadherin), and up-regulate mesenchymal-related genes required to acquire invasive behavior. In this process, cells lose polarity and the expression of E-cadherin, and gain the expression of Smooth Muscle Actin and Fibroblast Specific Protein-1 [52], among others. The loss of E-cadherin expression is correlated with an invasive and undifferentiated phenotype in many epithelium-derived cancer cells. Meanwhile, the acquisition of N-cadherin is associated with heightened invasive potential [53,54]. The

intermediate filaments also switch; vimentin replaces keratin, which regulates multiple cellular processes in epithelial cells, for example during the slug dependent EMT [55]. This new cell arrangement induces a change in cell motility, causing the so-called "amoeboid" movement typical of mesenchymal cells. Carcinoma cells use transition mechanisms to detach from primary tumors and migrate to distal sites where they can form metastases. The amoeboid movement is characterized by a dominant cell side that interacts with the EMC and releases proteolytic enzymes, while the cytoskeleton contracts and the tail of the cell detaches. It is increasingly clear that the EMT program is a continuum of transitional stages between the epithelial and mesenchymal phenotypes, and does not involve a binary choice between full-epithelial and full-mesenchymal phenotype [56]. Furthermore, cancer cells expressing a mix of epithelial and mesenchymal phenotypes are more effective in circulation, colonization at the secondary site, and the development of metastasis. It is also important to point out that although EMT facilitates the dissemination of cells, the formation of metastases also requires the loss of the mesenchymal phenotype through the inverse Mesenchymal to Epithelial Transition process (MET) [57].

4. The Angiotropic Process: Walking on the Abluminal Side of Vessels

Recently, pathologists described, in melanoma samples, the presence of single cancer cells or cell aggregates near vessels on their outer side. Based on these histological observations, Lugassy and Barnhill have hypothesized, for the first time, the phenomenon of angiotropism. Cancer cells, acquiring the typical forms and positions of pericytes, can migrate extravascularly, returning to a neural crest cell migratory phenotype [58]. According to this concept, melanoma cells closely associated with the endothelium in a pericytic location, which are generally detected at the advancing front of the tumor and without evidence of intravasation, are defined as angiotropic melanoma cells [59,60]. Histological analysis has revealed that both micro and large vessels can be affected by the angiotropic phenomena, while data about neovessels or stabilized vessels are not conclusive yet. In this complex, the ECs show no signs of physiological damage or intravasation. Thus, in parallel to the classical metastatic dissemination, the migration of cancer cells outside the vessels has been referred to as extra vascular migratory metastasis (EVMM) (Figure 1, Table 1).

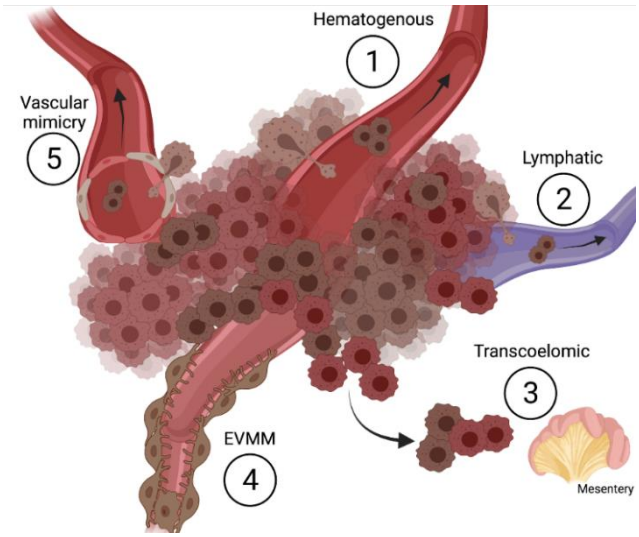

Figure 1. Schematic representation of metastatic routes (created with BioRender.com).

Table 1. Short description of the different routes of cancer dissemination.

Metastatic Process	Features
1. Hematogenous	Metastatic cancer cells detaching from primary tumor approach capillaries or angiogenic blood vessels, degrade the basal lamina, invade the endothelium, and intravasate into the flow as single cells or small groups. Finally, they colonize receptive distant organs.
2. Lymphatic	Metastatic cancer cells detaching from primary tumor degrade the basal lamina of lymphatic vessels and intravasate. Metastatic cancer cells enter into the lymphatic system by active movement, pass up the lymphatic flow, and colonize the lymph nodes and other organs.
3. Transcoelomic	Metastatic cancer cells detached or exfoliated from the tumor remain as individual or groups of cells in the cavities. The spread of cancer cells into body cavities occurs via penetrating the surface of the peritoneal, pleural, pericardial, or subarachnoid spaces. In the cavities, metastatic cancer cells proliferate in suspension, generate ascites, and/or adhere to other tissues.
4. Extra Vascular Migratory Metastasis (EVMM)	In EVMM, metastatic cancer cells detaching from the primary tumor approach capillaries or angiogenic blood vessels. Once in the vessel, cancer cells migrate along the abluminal side without intravasating, degrading the basal lamina or altering the structure of vessels.
5. Vascular mimicry (VM)	Metastatic cancer cells, under hypoxic pressure, can form vascular channels interconnected with the tumor vasculature. These leaky structures give nutrients and oxygen to the tumor and support the spread of metastatic cells.

In this view, EVMM can be considered as an important alternative to tumor dissemination. EVMM and pericyte mimicry, mainly described in melanoma [59], have been reported in various tumors including cutaneous squamous cell carcinoma, prostatic adenocarcinoma, carcinosarcomas of the ovaries and endometrium, glioma, liposarcoma and glioblastoma [61–63]. During EVMM, the perivascular localization of melanoma cells has been associated with the acquisition of stem-like plasticity and the expression of specific pericytic markers, including PDGFRβ, CD146, CD44, CD73, CD105, and CD144 [64]. All these observations resume the glioblastoma behavior where EVMM occurs, and cancer stem cells give rise to up to 80% of the pericytic compartment. Despite these similarities, further effort is required to understand whether all the pericytes in both glioblastoma and melanoma exert similar migratory and metastatic abilities as well as play a role in vessel stabilization. In vitro, in vivo, and clinical evidence of EVMM is summarized in Table 2. A recent study suggested that up to 37% of cases of melanoma exhibit EVMM [65]. Therefore, EVMM is probably a common phenomenon underestimated until now, likely due to technical limitations. Moreover, it has been observed that patients with progressive disease of melanoma that were sentinel lymph node-negative had progressive disease both in sentinel-basin and at distant sites. Therefore, it has also been hypothesized that EVMM, rather than hematogenous spread, might be responsible for the observed progressive disease with single organ involvement. For this, the understanding of the mechanisms of action of EVMM is of outstanding importance. For instance, differential analysis between angiotropic melanomas and non-angiotropic melanomas highlighted 15 critical genes involved in the modulation of EVMM [66]. Among these, KIF14 (kinesin family member 14), ECT2 (epithelial cell transforming sequence 2 oncogenes), and HMMR (hyaluronan-mediated motility receptor) seem to be implicated in cytokinesis. In addition, co-culture experiments demonstrated that the interaction of angiotropic melanoma cells with the abluminal vascular surface promotes the differential expression of genes related to cell migration (CCL2, ICAM1 and IL6), cancer progression (CCL2, ICAM1, SELE, TRAF1, IL6, SERPINB2 and CXCL6), EMT (CCL2 and IL6), stemness (CCL2, PDGFB, EVX1 and CFDP1), and pericytic recruitment (PDGFB) [67].

Table 2. Cancer types displaying perivascular migration.

Cancer Type	In Vitro Observations	In Vivo Observations	Clinical Observations
Melanoma/Uveal melanoma	In vitro co-culture system: C.Lugassy et al. 2013 [67] G. Fornabaio et al. 2018 [68]	In CAM assay: C. Lugassy et al. 2007 [69] In zebrafish model: G. Fornabaio et al. 2018 [68] In murine model: L.A. Bentolila et al. 2016 [70]	R.L. Barnhill and C. Lugassy 2004 [59] A.K. Rodewald et al. 2019 [71]
Glioma	Organotypic slice tissues: V. Montana and H. Sontheimer 2011 [72]	In murine model: S. Watkins et al. 2014 [73] F. Winkler et al. 2009 [74] M. Alieva et al. 2019 [75]	D. Zagzag et al. 2008 [76]
Sarcomatoid squamous cell carcinoma			K.M.H. Eleanor and S. Colin 2019 [77]
Cutaneous squamous cell carcinoma			F. Fedda et al. 2018 [78]
Prostatic adenocarcinoma	3D co-culture system: *C. Lugassy et al. 2005 [61]*	CAM model: C. Lugassy et al. 2005 [61]	C. Lugassy et al. 2005 [61]
Syringomatous carcinoma			E. Katayama et al. 2017 [79]
Gynaecological carcinosarcoma			J.M. Dyke et al. 2014 [80]
Well-differentiated liposarcoma			J. Shen et al. 2016 [81]
Pancreatic adenocarcinoma			M.J. Levy et al. 2009 [82]

5. Role of the Extracellular Matrix

Angiotropic tumors have been described as acquiring pericytic localization and being directly associated with the basal lamina of ECs. Ultrastructurally, the basal lamina is an amorphous matrix. Among the extracellular matrix (ECM) proteins, laminins are the principal non-collagenous components involved in cell migration, adhesion, and differentiation. Laminins are heterotrimeric glycoproteins composed of different combinations of alpha (five genetic variants), beta (four genetic variants), and gamma (three genetic variants) chains. In angiotropic melanomas, tumor microvessels showed minimal focal vascular positivity for α3, β3, and γ2 laminin, while β2 laminin positivity characterizes the vessels of the tumors. In this context, tumor cells spread on the abluminal surface of small vessels that are enriched in β2 laminin [83]. Alteration in the basal lamina is induced by, i.e., neutrophil elastase, which cleaves laminin-332 (α3, β3, γ2) and enhances the metastatic potential of melanoma cells [58]. Accordingly, in vitro and in vivo experiments clearly showed the ability of the C16 peptide (KAFDITYVRLKF), derived from the laminin γ1 chain, to enhance the angiotropic extravascular migration and pulmonary metastasization of melanoma cells [84]. The expression of LAMC2, LAMA4, and ITGB3 is increased in vascularized angiotropic melanoma areas with respect to the avascular ones in the same tumor [85]. Furthermore, the expression of laminin receptors represents an unfavorable prognostic factor in melanoma. CD36, a membrane glycoprotein involved in angiogenesis, supports vascular mimicry formation in human melanoma cancer cells, acting through α3 integrin-laminin interaction; in silico analysis of CD36 expression within the melanoma cohort of The Cancer Genome Atlas suggests that melanoma patients with high expression of CD36 have a poorer clinical outcome [86].

6. Approaches to Studying Angiotropism and EVMM

The emergence of new strategies through which cancer cells can metastasize is always accompanied by the need for new experimental plans to describe them. To this end,

different in vitro, ex vivo, and in vivo assays have been developed (Table 2). Here, we summarize the available models for the evaluation of angiotropism or EVMM of melanoma cell lines.

6.1. In Vitro 3D Models

In 2002 Lugassy and colleagues described the ability of primary human melanoma cells to migrate along the capillary-like structures of EC. Indeed, ECs plated onto a 3D Matrigel self-organize tube-like structures on which melanoma cells migrate with a velocity of 0.01 µm/min to 2 µm/min. Interestingly, this rate corresponds to a distance of 0.5 cm to 105 cm/year, agreeable with the diagnosis of metastasis [87]. Recently, we implemented this protocol and set up a long co-culture method. To this purpose, spheroids of human melanoma cells and human endothelial cells were embedded into 3D Matrigel scaffolds for ten days. As expected, ECs formed 3D tubular structures that came out of the spheroid. Melanoma cells used these structures to leave the spheroid, migrating in close contact with the outside of the sprouts over the next 4/6 days (Figure 2A).

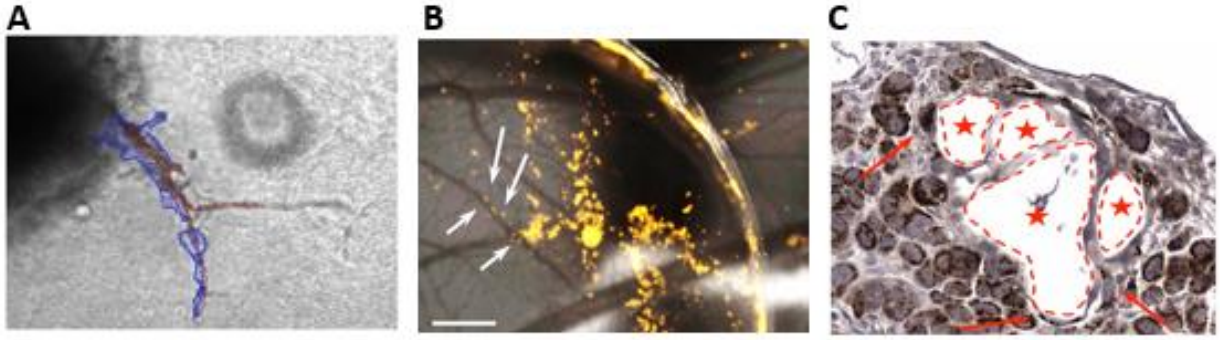

Figure 2. Experimental approaches for studying EVMM. (**A**) 3D matrigel-embedded spheroids of endothelial (highlighted in brown) and melanoma (highlighted in blue) cells; (**B**) Chick embryo chorioallantoic membrane (CAM). White arrows highlight melanoma cells (yellow) in close contact with the abluminal side of blood vessels. Scale bar 2 mm; (**C**) Histological image of metastatic melanoma cells in CAM section. Melanoma cells are stained with anti-human mitochondria (brown dots, DAB), and the lumen of the vessels are highlighted with red stars.

Although in vitro cell cultures hitherto represented an indispensable and reliable tool for biomedical research, 3D cell culture models are now closer to in vivo conditions. 3D cell culture favors the maintenance of cell polarity and more physiological cell–cell and cell–ECM complex interactions. Moreover, by using specific extracellular matrices and organic and/or inorganic scaffolds [88], researchers can modulate the mechanical supports and the availability of soluble factors potentially adsorbed on scaffolds. Setting up suitable 3D culture conditions usually remains time-consuming. Nevertheless, the long lasting culture of tumor cell spheroids or organoids allows the observation of tumor behavior and phenotypes that cannot be reached with 2D cultures [89,90]. For this, and even if 3D culture still lacks the microenvironmental complexity in which cancer cells reside in animal models (e.g., interaction with the immune system), the long term culture condition has proven to be widely useful for cancer research and drug screening [91].

6.2. In Vivo Models

The optical transparency of the zebrafish embryo makes it an excellent in vivo model to study tumor progression, tumor angiogenesis, and tumor metastasis, allowing high-resolution live imaging [92]. Briefly, labeled cancer cells are injected into the yolk of the zebrafish embryo two days post-fertilization. The migration of cancer cells outside the bloodstream can be followed in live time-lapse imaging for 2 to 3 days post-injection. Of course, the zebrafish embryo is a suitable model to evaluate the classic intravascular metastasization and the EVMM simultaneously. Alternatively, EVMM can be analyzed in the

chick embryo chorioallantoic membrane model (CAM). The CAM is a highly vascularized membrane that wraps the embryo and transports oxygen, calcium, and metabolites. In this context, tumor cells can be seeded on the CAM and followed over time in ovo [27]. The migration of cancer cells and their association with blood vessels can be monitored by using a stereomicroscope over 72 h (Figure 2B). Moreover, the samples can be fixed and analyzed by histology to visualize the angio-complex (Figure 2C). Specific approaches have been developed to study the angiotropic and EVMM processes. For this purpose, GFP-tagged human metastatic melanoma cells were stereotactically injected into the brain, and a vascular tracer was administered in the jugular vein of immunodeficient mice allowing the visualization of the interaction of melanoma cells with the brain vasculature [93]. The constant development of optically transparent chambers for live-imaging techniques may lead to new studies and new knowledge in the field of EVMM [94].

The in vivo models of zebrafish and chick embryo CAM have added complexity to the previously described in vitro models. Both these models share the advantages of being easy to handle, relatively cheap, highly suitable for imaging and, as substitutes for mouse models, perfectly reflective of the "Replacement" purpose of the 3R law. The zebrafish switches from embryo to larvae in a few days, while the chick embryo CAM hatches in 21 days. These relatively short time windows can influence the timing of the experiments and limit the drug screening strategy. Of note, the embryonic environments of CAM and zebrafish may inhibit tumor growth, reducing the expression of pro-oncogenes [95,96]. On the other hand, the same embryonic features can support the growth, differentiation, and migration of germline tumors, including teratomas [27].

The mouse model represents the "top-level model" in preclinical cancer research. Indeed, mice present several advantages, including their high conservation with humans in term of anatomy, physiology and genetics, which help to better model the metastatic process [97,98]. Moreover, they provide a good tool for drug discovery and verification. The variety of mouse strains, including humanized mice, are open to many cancer cell lines and patient derived xenograft (PDX) research [99,100]. However, obtaining and maintaining the animals can be very expensive, and requires highly specialized personnel.

7. Final Remarks

Solid tumors are characterized by the ability to support themselves in their growth and in the colonization of new body districts. However, the fastest route (hematogenous) may not be the best choice. Per this view, extravascular migration, although less characterized, assumes vital importance for cancer progression. In conclusion, more significant efforts should be devoted to elucidating these processes, as they could be potential and exciting therapeutic targets.

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