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Vasculogenic mimicry, a complex and devious process favoring tumorigenesis – Interest in making it a therapeutic target

Lucas Treppe⁽¹⁾, Sébastien Faure⁽²⁾, Nicolas Clere⁽²⁾

(1) Université de Nantes, CNRS, INSERM, CRCINA, F-44000 Nantes, France

(2) Micro et Nanomédecines Translationnelles, MINT, UNIV Angers, UMR INSERM 1066,
UMR CNRS 6021, Angers, France

Correspondence :

Nicolas Clere – Pharm D., Ph D

Inserm UMR 1066 CNRS 6021

IBS – CHU Angers

4, rue Larrey

F-49933 Angers Cedex

nicolas.clere@univ-angers.fr

Abstract

Tumor cell vasculogenic mimicry (VM), also dubbed vascular mimicry, describes the plasticity of aggressive cancer cells forming *de novo* vascular networks and is associated with the malignant phenotype and poor clinical outcome. VM is described in a plethora of tumors, including carcinomas, sarcomas, glioblastomas, astrocytomas and melanomas. The presence of VM is associated with a high tumor grade, short survival, invasion and metastasis. A variety of molecular mechanisms and signal pathways participates in VM induction and formation. Due to VM's contribution on tumor progression, more VM-related strategies are being utilized for anticancer treatment. After describing the main features of VM, this review will outline the importance of the tumor microenvironment during this process, and highlight the predominant molecular targets and signaling pathways involved. These data will make it possible to discuss the importance of VM-associated mediators in antitumor therapy and how it could allow to better understand the resistance to anticancer therapy.

Keywords: Cancer cells; tumor microenvironment; vessels; signaling pathways; endothelial cells; anticancer therapy

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79 **Abbreviations**

- 80 αSMA: alpha smooth muscle actin
81 CAF: cancer-associated fibroblast
82 COX2: cyclo-oxygenase 2
83 CSC: cancer stem cell
84 Dll: Delta-like ligand
85 EMT: epithelial-mesenchymal transition
86 EphA2: ephrin A2 receptor
87 EVs: extracellular vesicles
88 FAK: focal adhesion kinase
89 GSC: glioblastoma stem-like cell
90 HCC: hepatocellular carcinoma
91 HDAC: histone deacetylase
92 HIF: hypoxia-inducible factor
93 lncRNA: long non-coding RNA
94 LOXL2: lysyl oxidases like 2
95 miRNA: micro-RNA
96 MMP: matrix metalloproteinase
97 ncRNA: non-coding RNA
98 NSCLC: non-small cell lung cancer
99 PAS: periodic acid-Schiff
100 PGE2: prostaglandin E2
101 PI3K: phosphatidylinositol-3-kinase
102 TAM: tumor-associated macrophage
103 TGF-β: transforming growth factor beta
104 TME: tumor microenvironment
105 uORF: upstream open reading frame
106 VEGF: vascular endothelial growth factor
107 VE-PTP: vascular endothelial protein tyrosine phosphatase
108 VM: vasculogenic mimicry
109

1. Introduction

Since the studies of Judah Folkman (Folkman, 1971) supplemented by those of Napoleone Ferrara (Kim et al., 1993), tumor growth and metastasis have been explained by a change in the tumor vascular structure through angiogenesis and vasculogenesis. Formation of the tumor vasculature was largely thought to parallel physiological processes of neovascularization, with endothelial sprouting and proliferation being the principal routes of new blood vessel formation. However, this dogma was discussed after the demonstration by Maniotis *et al.* in 1999 (Maniotis et al., 1999) of vascular mimicry (VM) in malignant melanoma. In these tumors, the patterned vascular channels characteristic of aggressive primary and metastatic melanoma are different from angiogenic vessels in that vascular channels in aggressive melanomas are embedded in highly patterned matrix whereas angiogenic vessels are characterized by clusters of vessels and are not patterned. Furthermore, the patterned melanoma vascular channels were not found to be lined by endothelium whereas the contribution of an endothelium to angiogenic vessels is clearly identified. The hypothesis proposed to explain this new vascular architecture was a process of dedifferentiation of tumor cells into an endothelial-like phenotype whose clinical implications are clear: VM-forming tumors were more aggressive than their non-VM counterparts (Qin et al., 2019). Furthermore, VM-lined channels might not respond predictably to conventional anti-angiogenic therapies in melanoma (Schnegg et al., 2015) or other cancers (Angara et al., 2017; Hori et al., 2019).

A major controversial commentary has been proposed on the topic entitled “Vasculogenic mimicry: how convincing, how novel and how significant” (McDonald et al., 2000). Briefly, [the main criticisms raised from this commentary focused on different aspects of the original and rather descriptive VM study, which](#) led the authors to the following

questions: (i) Can the VM structures be considered as blood vessels and do they contribute meaningfully to blood flow? (ii) Can the presence or absence of endothelial cells and tumor cells surrounding the vascular lumen be established using unambiguous markers? (iii) Is there an interface between endothelial cells and tumor cells in blood vessel walls? Thus, the relevance of the conclusions from Maniotis *et al.* was questioned, considering the possibility that perhaps VM channels were not functional (Maniotis et al., 1999). Moreover, there were serious technical limitations in identifying VM channels using conventional immunohistochemistry which was prone to artifacts and not entirely objective. Despite these criticisms, it seems that VM has re-entered the spotlight in cancer research with several publications in journal with good scientific impact (Williamson et al., 2016; Xiang et al., 2018) and also with a significant publication rate in the last 10 years (940 hits on Pubmed with keywords “vascular mimicry cancer” or “vasculogenic mimicry cancer”). These last studies provide the basis to better define tumor VM which is the source of many resistance to anticancer therapies and also explain the aggressiveness of certain tumors.

2. Characteristics of vascular mimicry

During its vascular stage, tumor nutrition through diffusion is no longer sufficient and expansion of new vasculature is necessary for tumor growth (Folkman, 1990; Gimbrone et al., 1972). Presently, five vascularization modes are now recognized (Carmeliet and Jain, 2011): sprouting and intussusceptive angiogenesis, vessel co-option (Kuczyński et al., 2019), endothelial cell transdifferentiation and VM.

VM is defined as the formation of new blood vessels following the acquisition of vascular cell features or functions by tumor cells of a non-vascular origin. Two distinct VM types have been identified: tubular and patterned matrix VM. Patterned VM is composed of

an extracellular matrix of irregular thickness, that is positive for periodic acid-Schiff (PAS), laminin or heparan sulfate, and surrounding islets of cancer cells (Fig. 1A). Although patterned VM networks are shown to conduct fluids, the patterned matrix does not contain a continuous lumen and is morphologically different than blood vessels or their vascular matrix (Folberg and Maniotis, 2004; Maniotis et al., 1999). Furthermore, tubular VM is composed of tumor cells that mimic the normal endothelium to form hollowed and perfused tube-like channels (El Hallani et al., 2010) (Fig. 1B). Whilst blood and lymph vessels are formed by a monolayer of endothelial cells surrounded by a semi-continuous basement membrane, tubules from VM are formed by cancer cells resting on an inner glycoprotein rich matrix (Fig. 2) (Valdivia et al., 2019). In this review, unless specified, the term VM encompasses both tubular and patterned VM types.

Ongoing discussions in the field suggest a minimal set of experiments which needs to be performed in order to ascertain the occurrence of VM. Historically, both *in vivo* and *in vitro* models VM structures were positively stained with PAS, and they did not possess endothelial markers such as CD31 or CD34 (Maniotis et al., 1999). Although immunohistology and molecular studies have highlighted the expression of endothelial markers (CD31, CD34, VE-cadherin) in VM structures, PAS⁺/CD31⁻ staining is currently the most widely used marker for VM. *In vitro*, the molecular characteristics underlying VM are traditionally confirmed in models of highly invasive cell lines able to form tubes on Matrigel®. Finally, the functionality of hollow VM structures has been confirmed *in vitro* upon dye perfusion, and in tumor biopsies with the presence of red blood cells in VM structure's lumen (Ruf et al., 2003; Sood et al., 2001). To date, the occurrence of VM has been shown in various pre-clinical models of breast (Wagenblast et al., 2015), ovarian, prostate (Liu et al., 2012), astrocytoma, glioblastoma, hepatocellular carcinoma (HCC) and lung cancers. Depending on the cancer type, the

incidence of VM positive tumors (based on PAS+CD31- staining) varies considerably (5 to 65%) (Valdivia et al., 2019). However, these investigations need to be taken with a pinch of salt and backed-up by rigorous tissue examination (McDonald et al., 2000).

2.1. *Epithelial-mesenchymal transition and VM*

VM formation is a sophisticated process involving various mechanisms. While similarities have been shown between epithelial-mesenchymal transition (EMT) and VM, several studies have stressed the main role of tumor microenvironment (TME) during VM.

Initially, EMT has been recognized as a key step during embryonic morphogenesis (Nieto et al., 2016). In different pathophysiological conditions, including cancer, EMT involves various cellular changes in which epithelial cells loosen (i) their attachments to neighboring cells and (ii) their apico-basal cell polarity, while (iii) they start acquiring mesenchymal markers including vimentin, N-cadherin and fibronectin (Thiery, 2002). EMT is highly dynamic, implying transient (dubbed partial-EMT) and reversible states (Ribatti et al., 2020), as well as direct cell-cell interaction or secreted cues (reviewed in (Kim et al., 2020)). EMT implies different signaling pathways including (among other) TGF- β , Wnt, Notch and Hedgehog (Chen et al., 2016; Islam et al., 2016; Ma et al., 2018), as well as the expression of specific transcription factors (Snail, Twist1, SOX4 and ZEB1) acting as repressors of epithelial markers (Gil et al., 2016; Leskela et al., 2019; Ma et al., 2018). This transcriptional remodeling is accompanied by a cadherin switching (Wheelock et al., 2008), particularly from E-cadherin to a panel of mesenchymal cadherins (N-, P-, R-, T- and cadherin 11), that was correlated with tumor progression and invasiveness in squamous cell carcinomas (Miro et al., 2019) and endometrial adenocarcinoma (Fan et al., 2019). Thus, during EMT, cancer cells trade their epithelial characteristics to mesenchymal traits including cytoskeleton reorganization and higher

motility (Sannino et al., 2017). Altogether, initiating the first step of the invasion–metastasis cascade, detrimental for patient’s prognosis and raising cancer death-toll (Pastushenko and Blanpain, 2019).

Interestingly, several studies have shown that EMT is involved in VM formation (Fig. 2, inset i). As proof, it has been reported that ZEB1 small hairpin RNA inhibits both the formation of VM and EMT in triple negative breast cancer (Liang et al., 2018) and colorectal cancer models (Liu et al., 2012). Likewise, it has been found that Twist1 is able to regulate VM formation in astrocytoma, and that its expression is associated with the grade of astrocytoma, one of the most vascularized types tumor in human (Cao et al., 2019; Li et al., 2020). Thus, it suggests a non-negligible role of VM in glioma tumor vascularization. In HCC cell lines, chromatin immunoprecipitation and luciferase assays demonstrated that Twist1 binds the VE-cadherin promoter region and induces VE-cadherin transcription (a molecule which function is developed further below) (Sun et al., 2010). In breast cancer cell lines, Twist1 could mediate VM formation by transcriptional repression of claudin 15 (Zhang et al., 2020). Moreover, in HCC model, it has been confirmed that Twist1 regulates VM formation abilities, through the VE-cadherin/AKT pathway (Sun et al., 2010). Wnt signaling is involved in various physiological processes, such as tumor cell proliferation (Yang et al., 2017), endothelial cell differentiation, and angiogenesis (Shetti et al., 2019). Furthermore, several studies suggested that Wnt signaling induces EMT by repressing glycogen synthase kinase-3 β (GSK3 β)-mediated phosphorylation that subsequently regulates the phosphorylation/degradation of Snail, an important mediator of EMT (reviewed elsewhere in (Zhong et al., 2020)). Initially, it has been reported that Wnt/ β -catenin signaling was involved in VM formation in colon cancer (Qi et al., 2015). Recently, the mechanistic basis of osteosarcoma VM has been extended to 143B and

HOS cell lines [where](#) microarray analyzes showed enriched TGF- β and Wnt signaling pathway (Yao et al., 2020).

TGF- β is one of the key EMT driver in various pathologies including cancer (Wendt et al., 2009). Thus, it is not unfounded to assume that TGF- β signaling pathway may also regulate VM. As such, *in vitro* and clinical studies observed that (i) HCC patients with VM and ZEB2 nuclear expression have a shorter survival period than those without expression, and (ii) ZEB2 promotes VM by TGF- β induced EMT (Yang et al., 2015b). Moreover, in a model of SHG44 glioma cells transfected with TGF- β cDNA, EMT regulated by p38/MAPK signaling pathways could participate in VM formation, suggesting a role of this cytokine in the unfavorable evolution of glioma (Ling et al., 2016). [As an integral part of the EMT, TGF- \$\beta\$ drives the cadherin switching with a loss of E-cadherin and an increase of N-cadherin. Seemingly, VEGF-A plays a similar role during the hypoxia-induced VM in salivary adenoid cystic carcinoma \(Wang et al., 2019\) and various models of liver tumor \(Chen et al., 2019\). Thus, these findings suggest that the signaling pathways involved in EMT and VM formation are likely identical or perhaps complementary. A thorough transcriptomic meta-analysis of several VM-competent and TGF- \$\beta\$ -treated cancer cell lines could help clarifying this point.](#)

2.2. *Tumor microenvironment, team spirit in VM*

The tumor microenvironment (TME) is the [sophisticated](#) cellular milieu in which the tumor develops. Apart from the tumor cells, TME [comprises](#) blood [and lymphatic](#) vessels, the extracellular matrix and other non-malignant cells including cancer stem cells (CSCs), fibroblasts or immune cells (Wu et al., 2017). Several factors deeply influence the TME and are thus powerful modulators of cell's behaviors. In the following sections, we discuss new findings about how cells from TME contribute to the process of VM ([Fig. 2](#)).

253 *Cancer stemness properties and VM*

254 CSCs are **rare** subpopulations of neoplastic cells within a tumor, usually less than 5%, which
255 are able to generate new tumors in appropriate animal hosts. CSCs have been **first** identified
256 in human acute myeloid leukemia in 1994, **where authors showed that cancer** cells grafted
257 into SCID mice produce a large number of CD34⁺/CD38⁻ subpopulation of leukemic cells
258 (Lapidot et al., 1994). CSCs are also expressed in **several** solid tumor **entities** such as breast
259 (Yue et al., 2018), brain (Ren et al., 2018), colon (Lenos et al., 2018), gastric (Zavros, 2017) or
260 prostate (Gorodetska et al., 2019) cancers. Additionally, **evidence point out that these** CSCs
261 are the cause of tumor initiation, progression, invasion, metastasis, chemoradiotherapy
262 resistance, and relapse underscoring their paramount importance in tumorigenesis (Peitzsch
263 et al., 2017). Among the hypotheses behind the formation of VM, this process would be the
264 ability of VM tumor cells to adopt the pluripotent phenotype of embryonic cells (Seftor et al.,
265 2002). CSC marker expressions associated with VM in human malignant tumors suggest that
266 CSCs may participate in the formation of VM (**Fig. 2**, inset ii). Different biomarkers are used to
267 characterize and identify CSCs. CD133, also called prominin-1, is a common biomarker of CSCs
268 that was initially described as a biomarker in human hematopoietic stem and progenitor cells
269 (Yin et al., 1997). Currently, CD133 overexpression in various human cancers is considered a
270 significant (unifying?) marker of CSCs (Aghajani et al., 2020; Lathia et al., 2015; Wang et al.,
271 2020b). CD133⁺ tumor cells are able to induce tumor cell proliferation (Pavon et al., 2018),
272 metastasis (Liou, 2019) and to differentiate into other kinds of cells (Virant-Klun et al., 2019;
273 Zhang et al., 2020). Aldehyde dehydrogenase 1 (ALDH1) is another biomarker of CSCs in liver,
274 lung, ovarian, gastric, colorectal or breast cancer (Tomita et al., 2016), **and** described as a
275 potent inducer of tumor cell proliferation, differentiation and oxidative stress (Vishnubalaji et
276 al., 2018; Zhou et al., 2020). Different studies conducted on various tumor types have shown

a correlation between an increase of CD133 and ALDH1 expression, and VM formation. [For instance](#), in osteosarcoma, an aggressive malignant bone tumor in children and adolescents, positive rates of CD133, ALDH1 and VM are significantly higher in tumor tissues compared with control tissues, [and positively associated with lymph node and distant metastasis, as well as poor](#) patient overall survival (Bao et al., 2018). [In HCC, CD133 and VM are also](#) reported to be significantly higher in tumor tissues than in normal liver tissues (Xu et al., 2018a). Recently, in human and mice triple-negative breast cancer models, it has been confirmed that CD133⁺ and ALDH1⁺ cells form more VM channels *in vivo* (Sun et al., 2019). These results confirmed [CSCs as crucial modulators](#) in the process of VM formation.

Because human neural stem cells have been shown to transdifferentiate into endothelial cells (Wurmser et al., 2004), it is established that the differentiation of CSCs into endothelial cells represents one of the hypotheses explaining VM formation. Indeed, renal CSCs can generate endothelial cells *in vitro* and give rise to vessels with a human origin *in vivo* (Bussolati et al., 2008). Furthermore, human breast CSCs undergo endothelial differentiation to generate functional endothelial cells *in vitro* and *in vivo* (Bussolati et al., 2009). Subsequently, these data, obtained from *in vitro* studies, were supplemented by more robust [pre-clinical](#) and clinical studies. Thus, in glioblastoma, it has been reported that CSCs generate endothelial cells that form CD105⁺ (endoglin) tumor vessels (Wang et al., 2010). Moreover, Ricci-Vitiani *et al.* [showed that a broad range \(20-90%\)](#) endothelial cells in glioblastoma harbors the same chromosomal alterations as in tumor cells (Ricci-Vitiani et al., 2010). [Although controversial, these](#) studies demonstrate that CSCs in multiple tumors have endothelial differentiation abilities and could contribute to VM formation. [This fact is supported by a](#) study conducted on human glioblastoma samples, [showing](#) that the so-called glioblastoma stem-like cells (GSCs) may differentiate into CD31⁺/CD34⁺ endothelial cells and

promote VM formation (Mei et al., 2017). In another study conducted on HCC1937/p53 cells, derived from triple-negative breast cancer, cancer cell-transdifferentiated endothelial cells expressed endothelial markers such as VE-cadherin, matrix metalloproteinase (MMP)-2 and -9 and exhibited VM formation ability on Matrigel® (Izawa et al., 2018). Besides, in a mouse embryonic stem cell-induced model of lung CSCs, these cells were able to transdifferentiate into an endothelial cell-like phenotype expressing CD31 and capable of forming tubes on 3D matrix (Prieto-Vila et al., 2016). Taken together, cancer cell's plasticity and transdifferentiation clearly contribute to VM formation. However, presently the exact mechanism driving transdifferentiation and a common underlying signaling across cancer types are lacking but may be worth investigating for therapeutic purpose (see further below).

Hypoxia and VM

Intratumoral hypoxia, which is commonly observed in various solid tumors (Denko, 2008), could trigger numerous signaling pathways which may lead to adverse clinical outcomes including higher cancer invasiveness through promotion of tumor metastasis, EMT or angiogenesis (Chiu et al., 2019) (Fig. 2). Among different hypoxia-related pathways, hypoxia-inducible factors (HIFs) have been studied extensively (Wigerup et al., 2016). As major transcriptional regulators in response to hypoxia, HIFs consist of an oxygen-regulated HIF- α subunit (HIF-1 α or HIF-2 α) dimerizing with HIF-1 β under hypoxia. The dimer then complexes with CREB-cAMP-response element binding protein and activates the transcription of a panoply of target genes harboring hypoxia responsive elements (HRE). Various studies have evaluated the influence of oxygen decrease in the regulation of EMT and VM formation. For instance, Du *et al.* confirmed that hypoxia contributes to VM formation by inducing EMT. Interestingly, HIF-1 α expression, analyzed by immunohistochemistry in 71 specimens of

epithelial ovarian cancers, was correlated with loss of E-cadherin expression and up-regulated vimentin expression in 61% of VM-positive patients. Moreover, ovarian cancers with evidence of VM were significantly more likely to have high Twist1, Slug and VE-cadherin expression levels under hypoxia. *In vitro*, ovarian cancer cells presented morphological EMT-like changes under hypoxic conditions (Du et al., 2014). Furthermore, an association of HIF-1 α expression and VM formation has been reported in other solid cancers such as melanoma or HCC. An interesting study has confirmed that HIF-1 α promotes VM formation in HCC through lysyl oxidases like 2 (LOXL2, a secreted copper-dependent amine oxidase) up-regulation in hypoxic TME. Thus, from clinical HCC tissues, HIF-1 α , LOXL2 expression and CD31/PAS double staining were examined by immunohistochemical staining and correlated to poor prognosis. These data have been backed up by series of *in vitro* assays that confirmed a positive relationship between hypoxia, HIF-1 α and LOXL2 protein. Thus, HIF-1 α was found to induce EMT, HCC cell migration, invasion and VM formation by regulating LOXL2 (Wang et al., 2017). Hence, hypoxia appears as a critical player during EMT and VM formation, and could act on several intricate pathways.

Cancer-associated fibroblasts and VM

Cancer-associated fibroblasts (CAFs) are major components of stromal cells that surround cancer cells, and control proliferation, survival, angiogenesis, metastasis, immunogenicity and resistance to therapies (Su et al., 2018). CAFs constitute the main pool of collagen-producing cells, which directly communicate with cancer cells and various cells from the TME such as endothelial or inflammatory cells. However, the biological properties of CAFs are disparate with different types of CAF driving distinct functional contributions. Besides, previous studies indicated that CAFs arise from different sources of origin including local infiltrating fibroblast,

349 epithelial or endothelial cells, pericytes or adventitial fibroblasts of the vascular system, or
350 cancer cells themselves undergoing fibroblastic transformation, which may explain CAF's
351 morphological, phenotypical and functional variability (Chen and Song, 2019). In this context,
352 it has been proposed that CAFs could be determinant in VM formation (Fig. 2, inset iii). Thus,
353 in a recent study, VM-competent murine melanoma cells endogenously expressing the
354 matricellular protein CCN2 (also named CTGF), were injected in mice carrying a CAF-specific
355 CCN2 deletion. Interestingly, the absence of fibroblast-derived CCN2 reduced tumor
356 vasculature, including VM occurrence (Hutchenreuther et al., 2018). Furthermore, PAS⁺
357 tissues from human cutaneous melanoma were also positive for the vascular pericyte marker
358 α -smooth muscle actin (α SMA) within the extracellular matrix networks lined by tumor cells.
359 Moreover, when VM-competent tumor cells were co-cultured with pericytes, there was a
360 striking stabilization of the VM networks for up to 96 h (Thijssen et al., 2018). We could parallel
361 these findings with the fact that in glioblastoma, VM structures co-express α SMA and PDGFR,
362 another vascular pericyte marker (Francescone et al., 2012; Scully et al., 2012). The
363 importance of CAFs has also been found in gastric cancers where they promote tumorigenesis
364 through EphA2 (Hong et al., 2018; Nakamura et al., 2005). Pioneer studies in melanoma have
365 previously correlated the activation of the EphA2 receptor tyrosine kinase with VM formation
366 (Hess et al., 2001; Hess et al., 2006) (developed further below). Consequently, EphA2 was
367 significantly associated with VM formation in head and neck squamous cell carcinoma (Wang
368 et al., 2014) and gastric cancer cells (Kim et al., 2019b), which process was lowered in both
369 cases upon EphA2 knockdown. These findings were supplemented by a recent study in gastric
370 cancer AGS cells, showing that VM tube formation was significantly induced by CAF-derived
371 secretome, while conditioned media from normal fibroblasts derived from the same patient
372 had no effect. EphA2 silencing strategy in gastric cancer cells revealed that the CAF-mediated

VM promoting effect was mediated through the EphA2-PI3K pathway (Kim et al., 2019a). Altogether, these data suggest that CAFs, as powerful modeler of the TME, may have an underappreciated (yet) function in promoting VM formation.

Tumor-associated macrophages and VM

Both innate and adaptive immune cells are present and interact with the tumor cells *via* direct contact or through chemokine and cytokine signaling which shape the behavior of the tumor and its response to therapy. It is well established that tumor-associated macrophages (TAMs) are key regulators in the TME. In solid tumors, the main source of TAM is circulating monocytes rather than proliferating resident macrophages inside tumors. Macrophages can be categorized into M1 and M2 subtypes based on their polarization status. M1 macrophages can be activated by Th1 cytokine interferon gamma and microbial products, while M2 macrophages differentiate in response to Th2 cytokines such as IL-4, IL-10 or IL-13 (Qian and Pollard, 2010). In this context of TAMs, M1 macrophages are considered to exert tumoricidal properties whereas M2 macrophages promote tumorigenesis. Both M1 and M2 TAM phenotypes are plastic and reversible, and the TME plays a major role in the regulation of their functional polarization (Duluc et al., 2009; Hagemann et al., 2008). Since inflammatory microenvironment is involved in angiogenesis and plasticity of tumor cells, it has been assumed that inflammatory microenvironment might also promote VM formation (Fig. 2, inset iv). Thus, the infiltration of macrophages in VM-positive areas and the role and underlying mechanism of M2 macrophages in inducing VM formation have been studied in glioblastoma cells (Rong et al., 2016; Zhang et al., 2017). As such, the capability of channel formation in U87 cells with or without coculture with M2 (IL-4 stimulated) macrophages has been evaluated. The results revealed that M2 macrophages could enhance the ability to generate vascular

channels in U87 cells under normal oxygen condition. The high α SMA and barely detectable VE-cadherin expression in U87 cells proved that these vascular channels consisted of mural-like cells transdifferentiated from tumor cells. To gain additional insights into the mechanism by which M2 macrophages promote VM formation in U87 cells, the proinflammatory cyclooxygenase 2 (COX-2) signaling was investigated in the coculture model. Interestingly, COX-2 expression and the secretion of its downstream effector prostaglandin E2 (PGE2) were up-regulated in U87 cells after M2 macrophages coculture. Hence suggesting that M2 macrophages could induce VM via up-regulating COX-2 expression in glioblastoma cells (Rong et al., 2016). Seemingly, M2-like macrophages enhanced VM through amplification of IL-6 secretion by cancer cells (Zhang et al., 2017). These studies highlight intercellular communications (between M2-like macrophages and glioma cells) that induce VM formation via the involvement of different pro-inflammatory mediators.

Within the TME, several factors can influence the outcome and progression of tumorigenesis. For instance, hypoxia and inflammation contribute to cancer cell plasticity, which could reinforce both EMT and VM (Fig. 2). Concomitantly, tumor-associated macrophages are also important mediators of TME by altering tumor inflammatory status, and interacting with several stromal cells. Finally, growing evidences suggest that CAFs and mural cells are likely to contribute and stabilize VM network formation. In depth *in vivo* profiling would be required to properly assess cellular interactions occurring during VM. In particular, we envision that state-of-the-art single cell technologies and ligand-receptor prediction algorithms would allow a thorough understanding of VM, but such studies are currently lacking.

3. Molecular mechanisms in VM

In the following section, we discuss latest insights into the molecular mechanism and signaling pathways driving VM. Non-coding RNA molecules, acting as important epigenetics modifiers, will also be discussed in the context of VM. While a detailed molecular characterization driving VM is still lacking, this section primarily aims at presenting the most acknowledged mechanisms contributing to VM.

3.1. *VEGF signaling beyond angiogenesis, involvement in VM*

VEGF molecules and its receptors (VEGFRs) are key mediators in physiological (e.g. development and wound healing) and pathological angiogenesis including cancer. VEGF-A, the most studied VEGF member, regulates angiogenesis and vascular permeability by activating VEGFR-1 (FLT-1) and VEGFR-2 (KDR/FLK1), while VEGF-C/-D mostly regulate lymphangiogenesis *via* their receptor VEGFR-3 (FLT-4). In melanoma, the autocrine secretion of VEGF-A is shown to activate PI3K/PKC α and integrin-signaling pathways downstream VEGFR-1, thereby leading to VM (Vartanian et al., 2011). Endothelin 1, through its receptor endothelin-receptor type B (EDNRB), was identified as a melanoma progression marker and is thus associated to an aggressive phenotype (Bittner et al., 2000), and also appeared to play an important role in VM. Indeed, the endothelin 1/EDNRB axis could enhance the expression of VEGFR-3 and its ligands VEGF-C/-D *via* a cross talk involving the activation of c-SRC in a β -arrestin-1-dependent fashion (a pathway also linked to VE-cadherin signaling; see below) (Fig. 3A). This cooperative interaction between EDNRB- and VEGFR-3-mediated signaling resulted in the induction of melanoma cell migration and VM tube formation, and was further inhibited upon the concomitant blockade of these two receptors (Spinella et al., 2013). Peroxiredoxin-2 belongs to an important family of antioxidant proteins. In colorectal cancer, peroxiredoxin-2 expression was associated with VM formation and its knockdown reduced VEGFR-2

activation, allegedly due to an excessive level of oxidative molecules (Zhang et al., 2015a). Similarly, in Epstein-Barr virus-associated malignancies such as nasopharyngeal and gastric cancers where VM was reported, Epstein-Barr virus infection was shown to induce VEGF expression (Xiang et al., 2018; Xu et al., 2018b). However, the exact contribution of VEGF during nasopharyngeal cancer VM is still a matter of debate. Indeed, a first descriptive study indicated that transient silencing of VEGF-A or VEGFR-1 disrupted tubular structures from nasopharyngeal cancer cells, whereas inhibition of VEGFR-2 did not affect the process. Despite the fact LMP-1 and VEGFR-1 expressions were correlated to VM formation, in-depth investigation of the signaling involved was not further investigated by the authors (Xu et al., 2018b). Moreover, further study in the same Epstein-Barr virus-associated cancer outlined a VEGF-independent mechanism (demonstrated by the means of anti-VEGF blocking antibodies and specific anti-VEGFR inhibitors), which activates the PI3K/AKT/mTOR/HIF-1 α signaling cascade to induce VM (Xiang et al., 2018).

3.2. *CSC-related VEGF signaling in VM*

As previously discussed in this review, CSCs are discrete but critical sub-populations influencing tumorigenesis and therapy response. In line with their chemoresistance capacities, CSCs express stemness markers and high levels of drug efflux ATP-binding cassette (ABC) transporters. In melanoma, ABCB5-expressing CSCs co-express Tie-1 and VE-cadherin that are characteristic VM markers in aggressive uveal melanoma cells (Hendrix et al., 2001; Schatton et al., 2008). As compared to ABCB5⁻ cells, ABCB5⁺ melanoma cells overexpress VEGFR-1 and preferentially associate with VM structures. As such, VEGFR-1 signaling was important for laminin deposition as VEGFR-1 knockdown xenograft showed reduced VM occurrence and lower tumor growth (Frank et al., 2011). However, melanoma are heterogeneous with some

tumors that may initially respond to anti-VEGF therapy while other are intrinsically resistant. Mirroring this clinical observation, VEGF-A inhibition in anti-VEGF responsive xenografts leads to an increase in VM, CSC markers, as well as the induction of the HIF-1 α expression (Schnegg et al., 2015), reported to regulate VM and CSC phenotype in other cancers such as HCC (Ma et al., 2011), Ewing sarcoma (van der Schaft et al., 2005), melanoma (Zhang et al., 2009) and breast cancer (Mao et al., 2020). Examining whether HIF-1 α inhibition could prevent the increase in VM induction and CSC maintenance following VEGF-A inhibition in melanoma will be an interesting strategy. Further stressing the importance of cellular context and treatment response heterogeneity, Notch 3 knockdown in melanoma cells exhibiting high endogenous levels of this marker led to retarded tumorigenicity *in vivo* through depleting CSC fraction, corroborated with a reduction in VM. In contrast, Notch 3 silencing affected neither tumor growth nor CSCs in a melanoma cell line with relatively low endogenous Notch 3 expression (Hsu et al., 2017). In several cancers the zinc-finger transcription factor Slug is reported to be an essential mediator of Twist1-induced EMT and metastasis. As such, in HCC Slug is critical to maintain CSC subpopulation, VE-cadherin and mesenchymal markers expression, which further contribute to VM formation (Sun et al., 2013).

CSCs have been particularly well documented in brain tumors, where VM was reported and correlated to higher aggressiveness and shorter overall survival (El Hallani et al., 2010; Liu et al., 2011a; Liu et al., 2011b; Wang et al., 2013; Wang et al., 2012; Yue and Chen, 2005). Immunohistochemistry analysis showed that VM in glioblastoma is principally composed of mural cells co-expressing VEGFR-2, α SMA and PDGFR – the latter two representing vascular pericyte markers – whilst the endothelial marker CD31 was mostly absent (Francescone et al., 2012; Scully et al., 2012). Patient-derived glioblastoma CSCs, the so-called GSCs, were not capable to form any capillary-like structures *in vitro*. Conversely, this ability was retained by

GSC-derived differentiated cells expressing high levels of VEGFR-2 and α SMA, *via* a process dependent on VEGFR-2 and ERK signaling but not of its ligand VEGF (Francescone et al., 2012; Scully et al., 2012). Noteworthy, conflicting reports from another group indicate that VEGF, instead, could stimulate GSC-derived VM, albeit they also describe VEGFR-2 as paramount in inducing VM tube formation (Wu et al., 2017; Yao et al., 2013). These **discrepancies** could be attributed to the distinct GSC models used: fresh patient-derived *versus* U87 **cell line**-derived GSCs. Orthotopic mouse tumors with VEGFR-2-silenced GSCs showed decreased tumor growth and α SMA⁺CD31⁻ vessels without affecting the percentage of mouse CD31⁺ vessels (Scully et al., 2012). Thus, the authors hypothesized that a small population of GSC (<1,5%) could undergo EC transdifferentiation, which participates in tumor angiogenesis as previously described (Ricci-Vitiani et al., 2010; Wang et al., 2010).

3.3. *VE-cadherin, more than an endothelial marker*

VE-cadherin (also known as CD144 or cadherin-5) is a trans-membrane protein crucial for vascular development, as demonstrated by early lethal phenotype in **knockout mouse model** (Carmeliet et al., 1999). Endothelial VE-cadherin is a key player controlling cell-cell adhesion and barrier properties (see review in (Treppe et al., 2013)). *Via* its extracellular calcium domains, VE-cadherin forms cis- or trans-homodimers to support cell-cell adhesion. Besides, the extracellular calcium domain can bind the endothelial transmembrane receptor-type phosphatase VE-PTP (vascular endothelial protein tyrosine phosphatase) to poise endothelial barrier and keep endothelial permeability at low level (Orsenigo et al., 2012). **Of note, VE-cadherin's intracellular domain** is the target of numerous post-translational modifications including phosphorylation which orchestrate **different signaling cascades depending on the cell type considered (see below)**.

Although VE-cadherin is an archetypal marker of endothelial cells, a fundamental study reported its expression in highly aggressive human melanoma cells whilst being absent in poorly aggressive melanoma cells derived from the same patient (Hendrix et al., 2001). Highly metastatic melanoma cells had the ability to form vessel-like network in 3D matrix *in vitro*, but this was hampered upon VE-cadherin silencing or CRISPR-Cas9 knockout (Delgado-Bellido et al., 2019; Hendrix et al., 2001). In aggressive melanoma, the molecular pathways downstream VE-cadherin involve its phosphorylation on Y658 residue (pY658) by the focal adhesion kinase (FAK) (Delgado-Bellido et al., 2019) (Fig. 3B). Indeed, FAK silencing or pharmacological inhibition reduced pY658-VE-cadherin levels, even upon VEGF treatment, and decreased VM tube formation. Interestingly, a nuclear pool of pY658-VE-cadherin in complex with p120 was identified in the metastatic uveal cell line MUM2B (Delgado-Bellido et al., 2019). While this phenomenon was already described for p120 and other cadherins such as E-cadherin, this is a premiere for VE-cadherin and would require further validation in other cancer cell types prone to VM. FAK appears to be paramount in controlling the nuclear pY658-VE-cadherin relocalization, which can then associate in complex with Kaiso, a known transcriptional repressor. Upon interaction and binding with p120, Kaiso-transcription repression' activity is counteracted and the target genes induced. Thus, pY658-VE-cadherin could repress the binding of Kaiso to their target genes, induce the expression of CCND1 and Wnt 11, and thus poise the cells to induce VM *in vitro* (Fig. 3B). This recent report highlights how cancer cells such as melanoma cells could hijack existing pathways to induce VM. Recently, a study has developed an original model of culture by coating plates with a fusion protein consisting of the human VE-cadherin extracellular domain coupled to the immunoglobulin G Fc region (Fig. 3B). Strikingly, when the Bel7402 HCC cells were cultured on this substrate, they spontaneously adopted tube-like structures, reminiscent of VM. Concomitant with this

cytoskeleton rearrangement, the cells showed an increased expression in VE-cadherin, EphA2, MMP-2/-9 secretion and PAS staining. Phenotypically, these HCC cells demonstrated an increased migration and invasion, and higher levels of pFAK and pY658-VE-cadherin, associated to several EMT markers (Shuai et al., 2020). Altogether, this would indicate that VE-cadherin has a pivotal place in inducing the EMT, PI3K/MMPs and EphA2/FAK/p-VE-cadherin signaling, and thus trigger VM formation (Fig. 3B).

3.4. *VE-PTP, a VE-cadherin-associated receptor*

As mentioned above, the transmembrane receptor-type phosphatase VE-PTP associates with VE-cadherin to prevents its pY658 and thus maintains endothelial permeability at minimal level (Orsenigo et al., 2012). Similarly to VE-cadherin, VE-PTP is found expressed in aggressive melanoma cell lines, albeit absent in non-aggressive forms. Despite the fact that VE-PTP knockdown in HUVECs led to an expected increase in pY658-VE-cadherin (due to the absence of VE-PTP phosphatase activity), in aggressive melanoma cells it provoked a distinct outcome with a sharp reduction in pY658- and VE-cadherin total levels. Interestingly enough, immunoprecipitation experiments pointed out that, in this model, VE-PTP does not interact with VE-cadherin but rather with p120 catenin, which is important to prevent VE-cadherin degradation and to sustain the VE-cadherin nuclear pool (Fig. 3B). Noteworthy, the molecular interactions are different in HUVECs where VE-PTP appears linked to VE-cadherin rather than p120 (Delgado-Bellido et al., 2020). Altogether, this highlight that, albeit mimicking normal endothelial cell function, VE-cadherin and VE-PTP behave differently in aggressive melanoma cells to support VM.

3.5. *EphA2 signaling in VM*

565 The Ephrin family of receptor tyrosine kinases (Eph) and their membrane-tethered ligands
566 (ephrins) constitutes the vastest family of tyrosine kinase receptor. The Ephrin/Eph signaling
567 is involved in various biological processes throughout development, adulthood and
568 pathogenesis (Kania and Klein, 2016). For instance, EphA2, formerly called ECK (epithelial cell
569 kinase), and its ligand ephrin-A1 have been shown to contribute to pathological tumor
570 angiogenesis, albeit *via* an uncharacterized mechanism. Compared to poorly aggressive
571 melanoma cell lines, EphA2 is overexpressed in most advanced melanoma cell lines (up to 14.8
572 fold-change) (Easty et al., 1995; Hess et al., 2007; Hess et al., 2001) and metastatic melanoma
573 biopsies. Furthermore, it is associated with cancer progression and invasion (Easty et al., 1995;
574 Hess et al., 2007). In metastatic aggressive melanoma cell lines, VE-cadherin expression is
575 instrumental in regulating EphA2 localization at cell-cell adhesion complexes, and EphA2
576 tyrosine phosphorylation. Immunostaining and immunoprecipitation indicate that these two
577 transmembrane proteins form a complex *in vitro*, but this might be indirect and could involve
578 additional signaling molecules (Hess et al., 2006). However, even though VE-cadherin-EphA2
579 co-localization has been demonstrated *in situ* in human melanoma sections, a thorough
580 validation is currently lacking (e.g. tube formation). In gastric adenocarcinoma, a positive
581 association between EphA2 expression and VM is highlighted, and further validation studies
582 in gastric cancer cell line indicate that EphA2 knockdown led to a reduction in 3D tube
583 formation *in vitro* (Kim et al., 2019b). Opposingly, EphA2 overexpression in pancreatic
584 adenocarcinoma (Duxbury et al., 2004) and F10-M3 melanoma cells (Parri et al., 2009)
585 increases cell invasiveness in 3D assays, which is in line with the invasive features of VM cells.
586 The F10-M3 metastatic clone of the widely used B16 mouse melanoma cell line has been
587 shown to not express EphA2 and ephrin-A1. However, ectopic expression of EphA2 leads to
588 its tyrosine auto-phosphorylation and downstream signal transduction *via* FAK activation,

suggesting a ligand-independent activation (Parri et al., 2009) (Fig. 3B). We can note here that, as mentioned above in melanoma cells, FAK controls pY658-VE-cadherin and its nuclear localization as well as 3D tube formation (Delgado-Bellido et al., 2019). Therefore, although these studies were not performed using the same melanoma cell line, they indicate a possible link between VE-cadherin, EphA2 and FAK signaling in VM.

As with many other receptor tyrosine kinases, EphA2 auto-phosphorylation promotes its endocytic internalization, the first step of exosomal sorting (Tkach and Thery, 2016; Walker-Daniels et al., 2002). Such an autocatalytic EphA2 system needs to be poised by the opposing activity of protein tyrosine phosphatases (PTP) and particularly PTP1B located near the pericentriolar recycling endosomes (Nievergall et al., 2010). Another non classical EphA2 signaling mechanism was brought to light by studying the secretion profile of doxorubicin-induced senescent cells, and particularly their extracellular vesicle (EV) content. Upon ROS-induced PTP1B inhibition, EphA2 was shed among small EVs and promoted the ERK-dependent proliferation of recipient breast cancer cells through a EphA2/ephrin-A1 reverse signaling (Takasugi et al., 2017). Notwithstanding EphA2 phosphorylation has been linked to VM (Hess et al., 2001; Hess et al., 2006), to our knowledge no study has yet considered the EV communication during VM tube formation (although several studies carried out conditioned media treatments). Additionally, other EphA2 post-translational modifications such as ubiquitination, which balances the EphA2 membranous *versus* internalization remains uncharacterized regarding VM (Sabet et al., 2015).

3.6. *MMPs, powerful modelers of the tumor microenvironment*

These endopeptidases of the zinc-dependent family are implicated in a large variety of physiological processes including wound healing and organogenesis, as well as in pathological

conditions including tumorigenesis. All 28 MMPs are produced as proenzymes, and require a post-translational proteolytic cleavage to generate mature MMPs that have proteolytic activity against a broad range of substrates located on the extracellular matrix. As an example, the substrates from MMP-2/-9 include aggrecan, collagens, elastin, fibronectin, laminins, and glycosaminoglycans. MT1-MMP is a membrane-type collagenase which activates the MMP-2/TIMP-2 complex (Fig. 3A), and is considered as a key mediator of cancer progression, EMT and metastasis (Kessenbrock et al., 2010). Additionally, MMPs play a crucial role in reshaping cell-cell and cell-matrix adhesive characteristics, as well as the extracellular matrix, all processes occurring during VM. As such, first evidence came from the group of Mary J.C. Hendrix with microarray analyses revealing that the expression of several MMPs, MT1-MMP, TIMP-1 and laminin- γ 2 were fostered in highly *versus* poorly aggressive patient-derived cutaneous melanoma cell lines (Seftor et al., 2001). Beside, MMP-1/-2/-9, MT1-MMP and laminin- γ 2 immunostaining showed a preferential expression in 3D tubular structures formed by aggressive ovarian and melanoma cell lines (Sood et al., 2001) and confirm that MT1-MMP is required for VM tube formation (Fig. 3A).

The Rictor/mTORC2 complex can phosphorylate AKT at Ser473, thereby activating a central component in the PI3K-AKT pathway that regulates the expression of several MMPs. In melanoma cells, Rictor silencing decreased VM channels formation, cell migration and invasion, pSer473-AKT and the expression of MMP-2/-9 (Liang et al., 2017). Myoferlin, a type II membrane protein, is also a regulator of MMP-2 expression and vouch for adequate VM (tube formation and expression of EMT markers) in A375 human cutaneous melanoma cell line (Zhang et al., 2018). Likewise, supporting initial findings in melanoma and ovarian cancers, a recent study in large cell lung cancer cell lines indicates that recombinant MMP-2 could enhance tube formation ability, while recombinant MMP-13 (collagenase-3) or its ectopic

overexpression in H460 and H661 cell lines decreased VM formation (Li et al., 2017a). In melanoma and large cell lung cancer cell lines, the same group showed that while MMP-2 proteolytically cleaves laminin-5 into the 105 kD laminin-5 γ 2' and 80 kD laminin-5 γ 2x, MMP-13 can generate small laminin-5 fragments (20 to 40 kD). MMP-13-digested laminin-5 could decrease 3D tube formation but enhanced melanoma cell invasion in Transwell assay (Li et al., 2017a; Zhao et al., 2015). The authors hypothesized that MMP-13 proteolytic action may lead to the cleavage of the laminin-5 γ 2-containing EGF domain, thereby curbing EGFR downstream Raf/ERK/AKT signaling, as well as the rearrangement of F-actin, vimentin and α -tubulin (Li et al., 2017a). Overall, high MMP-13 expression was inversely correlated with VM occurrence in melanoma patients and H460 subcutaneous tumors, and associated to metastasis in patients (Li et al., 2017a; Zhao et al., 2015). MMP-13 expression was also correlated to poorer overall survival of melanoma patients but this could be attributed to endothelium-dependent tumor vascularization rather than VM (Zigrino et al., 2009).

Fine-tuning histone acetylation pattern by means of histone deacetylases (HDACs) is an important process controlling gene expression profile and frequently deregulated in cancers. This is the case for *HDAC3*, commonly upregulated in solid tumors (Karagianni and Wong, 2007). In glioma, HDAC3 silencing steers to an impaired 3D tube formation along with a decrease in MMP-14, laminin-5 γ 2 (the latter likely involved in AKT/ERK activation signaling) (Liu et al., 2015). More generally, pharmacological inhibition of class I and II HDACs (HDAC1-10) in glioma cell lines (with SAHA and MC1568), also affects vascular tube formation. The same holds true for GSCs, albeit at higher concentration (2 to 100-fold depending on the HDAC inhibitor), reflecting their well-known resistance to pharmacological treatments (Pastorino et al., 2019). Bromodomain and extra-terminal domain (BET) proteins are epigenetic decoders that recognize acetylated marks on proteins (including histones) and recruit proteins driving

transcriptional activation. JQ1, a BET inhibitor was effective in inhibiting VM of pancreatic ductal adenocarcinoma cells by decreasing the ERK1/2-MMP-2/-9 signaling pathway both *in vitro* and *in vivo* (Zhuo et al., 2019).

A large body of data report the interleukin-33 (IL-33) and its receptor ST2 (encoded by *IL1RL1*) to be important in tumor immune response and cancer cell invasion. As such the IL-33/ST2 axis is now considered as a promising new immunotherapy (Liew et al., 2016). Recently, IL-33 was shown to promote VM tube formation in aggressive melanoma cell lines. Mechanistically, downstream ST2 IL-33 could trigger the ERK1/2 phosphorylation and MMP-2/-9 expression (Yang et al., 2019). However, even though IL-33 was previously described to induce a migrating mesenchymal phenotype, this aspect was not investigated in the context of VM. LOXL2 also plays a key role in extracellular matrix remodeling and particularly in collagen and elastin fibers stabilization which is involved in several processes including cell adhesion, migration, invasion and the EMT. Particularly, LOXL2 interacts and cooperates with Snail to downregulate E-cadherin expression in metastatic carcinoma (Peinado et al., 2005). It is thus not surprising that LOXL2 was found correlated to VM (and to VE-cadherin expression in cancer cells), metastasis and poor patient prognosis in HCC (Shao et al., 2019).

3.7. *Semaphorins, potential guides in VM*

The Semaphorin family is composed of membrane-anchored and secreted guidance cues which provide a wide repertoire of signals, paramount for neuronal growth, angiogenesis and epithelial barrier integrity (Treppe et al., 2013). As such, semaphorins have been linked to EMT in pancreatic (Saxena et al., 2018; Tam et al., 2017), hepatic (Lu et al., 2018; Pan et al., 2016), breast (Yang et al., 2015a), cervical (Song and Li, 2017), ovarian (Tseng et al., 2011), head and neck (Wang et al., 2016) and oral cancers (Liu et al., 2018; Xia et al., 2019). Analogous to the

Ephrin guidance molecules described in the aforementioned section, the expression of the membranous Semaphorin-4D is correlated with VM in a cohort of non-small cell lung cancer (NSCLC). Downstream its receptor plexinB1, Semaphorin-4D triggers the RhoA/ROCK pathway thereby promoting cancer cell migration and invasion, and the occurrence of VM *in vitro* and in xenograft model (Xia et al., 2019). Accordingly, previous studies already reported the involvement of the RhoA/ROCK signaling in VM formation in melanoma, osteosarcoma and HCC (Xia et al., 2017; Xia et al., 2015; Zhang et al., 2014b; Zhang et al., 2015a). Semaphorin-4D silencing also led to a reduction in VE-cadherin, EphA2 and MMP-2/-9, suggesting an intertwined relationship between these partners during VM in NSCLC (Xia et al., 2019). In other models of lung adenocarcinoma, downstream the SAV1-MST1/2-LATS1/2-YAP/TAZ axis Semaphorin-4D was also shown to participate in VM formation (Zhao et al., 2020). Moreover, at the surface of head and neck squamous cell carcinoma cell lines, MT1-MMP proteolytically cleaves Semaphorin-4D and induces Semaphorin-4D-dependent pro-angiogenic response in surrounding endothelial cells (Basile et al., 2006). It is thus not farfetched to hypothesize that this phenomenon could also occur between cancer cells, allowing a directional migration during VM network formation.

3.8. *Notch signaling in VM*

The Notch pathway is a highly conserved intercellular signaling. Activated by the interaction of the trans-membrane ligands Delta-like (Dll1-4) and Jagged-1/-2 with Notch receptors (Notch 1-4) expressed on adjacent cells, signal transduction is propagated *via* the γ -secretase-driven proteolytic cleavage of Notch receptor intracellular domain (NICD) that is released and form a nuclear transcriptional activator complex (Bray, 2016). Initial work indicates that Notch 1/2, Jagged-1/-2 and Dll1 are upregulated in 'dysplastic nevi' and melanomas as compared to

709 common melanocytic nevi (Massi et al., 2006). Similarly, Notch 4 and Dll4 are overexpressed
710 in aggressive melanoma cell lines where Notch 4 is enriched in melanoma VM networks
711 (Demou and Hendrix, 2008), while Notch 4 blocking antibodies impair VM *in vitro* in a Nodal-
712 dependent manner (NICD can bind and drive Nodal gene expression) (Hardy et al., 2010).
713 Remarkably, Nodal was overexpressed in metastatic melanoma tissues, and curbing
714 downstream signaling pathway (*via* an ALK 4/5/7 inhibitor) could abrogate melanoma cell
715 invasion and tube formation capacity (Topczewska et al., 2006). In breast cancer,
716 overexpression and silencing strategies underline that Nodal promotes VM *via* the Smad2/3
717 pathway (Gong et al., 2016). Additionally, Notch 4 and Dll4 were also found to be associated
718 with VM, metastasis and poor prognosis in NSCLC (Wang et al., 2018). In aggressive human
719 melanoma cell lines, disruption of the Notch signaling with the DAPT γ -secretase inhibitor
720 leads to a seemingly more matured and stabilized *in vitro* tubular network, and melanoma
721 xenografted tumors displays larger and more branched VM channels, associated with an
722 increase in MMP-2 and VEGFR-1 expression (Vartanian et al., 2013). Choriocarcinoma, a rare
723 and highly metastatic gestational trophoblastic cancer, is reported to involve VM with
724 syncytiotrophoblasts lining pseudovascular channels (Shih, 2011), a process that is similar to
725 the embryonic microcirculatory networks (Rai and Cross, 2014). Forskolin is an inducer of
726 syncytialization, and Forskolin treatment in choriocarcinoma cells resulted in high expression
727 of the VM markers MMP-2/-9, EphA2 and VE-cadherin, tube formation, as well as the
728 activation of the Notch 1 signaling pathway. However, invasive ability and VM markers were
729 reversed by DAPT, suggesting a mechanism whereby Notch-signaling down tunes VM in
730 choriocarcinoma (Xue et al., 2020). Notwithstanding the behavior of trophoblasts is similar to
731 aggressive tumors during differentiation and implantation (Rai and Cross, 2014), Notch 1

signaling seems to have an opposing effect in melanoma and NSCLC (Vartanian et al., 2013; Wang et al., 2018), calling for further investigations before therapeutic applications.

3.9. COXs inflammation in VM

COX-2 is an enzyme responsible for catalyzing the conversion of arachidonic acid into prostaglandins such as PGE₂, of paramount importance during inflammation. Subsequently, PGE₂ binding to the prostaglandin E₂ receptor subtypes (EP1-4) activates the EGFR signaling, and PKC-dependent ERK1/2 activation, pathways described to be involved in VM (Fig. 3A). In breast cancer, COX-2 expression correlates directly with aggressiveness and metastasis. As such, *in vitro* VM tube formation is largely impaired by celecoxib treatment, a selective COX-2 inhibitor, but nearly completely rescued when cells are supplemented with prostaglandin E₂. Unbiased bulk RNA-sequencing approach was followed to disentangle the underlying regulatory mechanism. Interestingly, genes related to angiogenesis (e.g. MMP-1, fibronectin, LAMC2) and proliferation (STAT1, EGFR, MAPK) were diminished. Protein array further validated a reduction in major angiogenic proteins such as VEGF and TIMP-1/-2, making a parallel between VM and angiogenic pathways (Basu et al., 2006). Treatments with specific E₂ receptor antagonists further highlight the involvement of the EP3 and EP4 receptors to mediate the aggressive phenotype of SUM149 and SUM190 inflammatory breast cancer cells, and mediate VM 3D tube formation (Robertson et al., 2010). Of note, in glioblastoma, the presence of VM channel is associated with the expression of COX-2 and MMP-9 (Liu et al., 2011b). In an elegant *in vitro* coculture model of U87 glioma cell line and IL-4-activated M2 macrophages (as a model of TAMs), tube formation and COX-2 expression were increased. Downstream COX-2, the PGE₂/EP1/PKC pathway was of prime importance to induce VM

formation, and further confirmed *in vivo* with COX2-deficient U87 xenograft model (Rong et al., 2016).

3.10. Non-coding RNAs

Non-coding RNAs (ncRNAs) are not translated into proteins but bear a remarkable variety of biological functions such as RNA processing, transcription and translation regulations. ncRNAs can be grouped according to their length with long non-coding RNAs (lncRNAs) exceeding 200 nucleotides in length, small non-coding RNAs between 200 and 50 nucleotides, and tiny ncRNAs for the smallest. The latest group includes siRNAs, miRNAs and piRNAs. In this section, we summarized the mechanistic insights of ncRNAs involved during the process of VM (Table 1). For ease of reading, the different ncRNAs are mostly discussed and grouped by tumor type.

Similar to melanoma where VM was initially described (Maniotis et al., 1999), aggressive but not poorly invasive triple negative breast cancer cells, efficiently undergo matrix-associated VM under hypoxia. Upon transfection with miR-204, VM tube formation was highly impaired. The pleiotropic effect of the miRNA was assessed using ELISA-based phosphorylation antibodies arrays that revealed a reduced expression and phosphorylation levels of 13 proteins involved in PI3K/AKT, RAF1/MAPK, VEGF, and FAK/SRC signaling. Subsequent validation studies using inhibitors confirmed that PI3K- α and c-SRC signaling were a requisite for VM, and impeded by miR-204 (Lozano-Romero et al., 2020; Salinas-Vera et al., 2018). The lncRNA TP73-AS1 was found upregulated in VM positive tissues from triple negative breast cancer (Tao et al., 2018). Twist1, that accelerates tumor cell VM in breast cancer (Zhang et al., 2014a) harbors a potential miR-490-3p binding site. Tao et al. showed that TP73-AS1 inhibition could release the post-translational suppression of Twist1 induced

by miR-490-3p, and thus precluded VM formation (Tao et al., 2018). Interestingly, miR-490-3p was also identified to play a role in clear cell renal cell carcinoma. Particularly, the NR2C2 (nuclear receptor subfamily 2 group C member 2; aka TR4) could repress the miR-490-3p-dependent vimentin inhibition, and thus favor VM, tumor growth and metastasis (Bai et al., 2018). Dimethylarginine dimethylaminohydrolase 1 (DDAH1) is responsible for the conversion of asymmetric dimethylarginine (ADMA) to citrulline. ADMA is an endogenous inhibitor of the nitric oxide synthase (NOS) and plays a key role in angiogenesis (Eelen et al., 2018). In aggressive breast cancer cell lines, DDAH1 overexpression was counteracted by miR-193b which further disrupted VM (Hulin et al., 2017). The miR-124 was previously shown to inhibit EMT and breast cancer metastasis by targeting Slug (Liang et al., 2013). A report indicates that in cervical cancer, miR-124 down-regulates VM, migration and invasion, as a result from its interaction with AmotL1, a member of the angiomin family that regulates cell growth and motility.

A recent study identified 11 miRNAs that are differently expressed by the SKOV3 ovarian cancer cell line during tube formation in an hypoxic environment. Among the so-called “hypoxamiRs”, miR-765 was deeply downregulated upon hypoxia, and could decrease the levels of AKT/SRC axis and exert a negative regulation on VEGF-A by specific binding to its 3’UTR (Salinas-Vera et al., 2019). This time without any link to hypoxia, miR-200a expression was significantly lower in VM positive ovarian cancer. *In vitro* works indicate that miR-200a and miR-27b negatively regulates EphA2 and VE-cadherin expression, respectively, and thus inhibits VM (Liu et al., 2017; Sun et al., 2014).

Expression of the lncRNA LINC00312 is found to be higher in lung adenocarcinoma patients with metastasis. Seemingly, mice inoculated with LINC00312-overexpressing adenocarcinoma cells presented more lung metastatic tumor nodules and higher VM. RNA

pull-down assay identifies YBX1 as the sole interacting partner with LINC00312, that allows the increased expression of VE-cadherin, TGF- β , VEGF-A/-C levels putatively *via* AKT activation (Peng et al., 2018). Oncogenic signaling downstream estrogen receptor beta (ER β) play an important role in NSCLC progression and EMT. Particularly, ER β activation up-regulates MALAT1 expression and thus prevent miR-145-5p-mediated NEDD9 downregulation. In this setup, the oncogene NEDD9 seems primordial in inducing VM and cell invasion (Yu et al., 2019). MALAT1 expression was also tightly associated with VM in gastric cancer, via the induction of several signaling pathways including VE-cadherin/ β -catenin complex, ERK/MMP and FAK/paxillin signaling (Li et al., 2017b). Investigating whether these pathways are also affected during NSCLC-associated VM would be interesting.

It was shown that c-Myc and SOX2 are important to maintain the pool of liver CSCs, and also to induce VM (Sun et al., 2013). As such, when c-Myc and SOX2 were co-transduced in the HCC cell line HepG2, these cells displayed increased migration, invasion and 3D tube formation properties (Zhao et al., 2018). When compared to HepG2 parental cells, unbiased bulk RNA-sequencing identified 748 differentially expressed ncRNAs, among which lncRNA n339260 that was enriched in the c-Myc/SOX2 overexpressing cells. LncRNA n339260 overexpression in HepG2 was able to promote VM (higher VE-cadherin expression) and CSC markers, and VM tube formation. The miR-31-3p, miR-30e-5p, miR-520c-5p, miR-519c-5p, and miR-29b-1-5p were considered to be downstream the lncRNA n339260 (or regulated by the latter) as their expression increased/decreased following lncRNA n339260 upregulation/downregulation respectively (Zhao et al., 2018). In colorectal cancer, SOX2 is also important for maintaining CSC properties. Ectopic overexpression of miR-450a-5p was shown to suppress the expression of SOX2 by targeting its 3' UTR region and hence restrained SOX2-induced CSC properties, angiogenesis, and VM (Chen et al., 2020). Yang *et al.* studied

the mechanism of VM formation in the context of HCC tumor cells and CAF communication. While CAF-secreted TGF- β and SDF1 promote VM formation in tumor cells, miR-101 could attenuate TGF- β signaling by targeting its receptor TGF- β R1 and the downstream effector Smad2 in tumor cells, and simultaneously abrogate SDF1 expression in CAFs. miR-101 is thus pivotal to extinguish VM-promoting signaling in two different cell types (Yang et al., 2016). Zhao et al. found that miR-27a-3p expression was inversely associated with Twist1 expression. miR-27a-3p appears to mediate the inhibition of EMT and VM by downregulating VE-cadherin (upon binding to its 3'UTR), and miR-27a-3p down-regulation is associated with metastasis (Zhao et al., 2016a). miR-186 also suppresses Twist1 expression in prostate cancer, and hence thwarted EMT, cell motility and tube formation *in vitro* and metastasis in mouse model (Zhao et al., 2016b).

So far, glioma is the tumor entity with the greatest variety of miRNAs and lncRNAs described to play a role in VM. miR-Let-7f halted VM by disturbing periostin-induced migration of glioma cells (Xue et al., 2016). The levels of miR-141 (Li et al., 2018a), miR-26b (Wu et al., 2011) were inversely correlated with the grade of glioma, and both miRNAs were shown to directly target EphA2 an important regulator in VM. Recently, the group of Prof. Xue has studied two lncRNAs (LINC00339 and HOXA-AS2) that are upregulated in human glioma and positively correlated to VM. LINC00339 binds and prevents the action of the miR-539-5p, thereby activating the Twist1/MMP-2/MMP-14 signaling pathway to promote VM (Guo et al., 2018) (Fig. 3B). HOXA-AS2 acts via a different pathway, which first involves its binding to miR-373. Downstream EGFR signaling, miR-373 regulates the expression of VE-cadherin, MMP-2/-9 and thus VM (Gao et al., 2018). Stathmin 1 is a member of the microtubule destabilizing protein family that regulates microtubule dynamics during cell-cycle progression. Stathmin 1 is overexpressed in various cancers and its expression is

851 positively associated with higher grades of human glioma. In glioma cell lines, stathmin 1
852 appears to be the target of the (central nervous system) tissue specific miR-9 that refrains
853 growth and VM of ectopic xenografts (Song et al., 2013). Interestingly, over-expression of
854 miR-584-3p paralyses hypoxia-induced VM formation in human A172 glioma cells, by
855 antagonizing ROCK1-dependent stress fiber formation (Xu et al., 2017). As mentioned before,
856 the RhoA/ROCK signaling pathway has been described to participate in VM formation in
857 melanoma, osteosarcoma and HCC (Xia et al., 2017; Xia et al., 2015; Zhang et al., 2014a; Zhang
858 et al., 2015b). Recently, three studies from the same group identified several lncRNAs
859 regulating VM in glioma. Important translational regulations occur from the 3' and 5'
860 untranslated regions (UTR) of mRNAs. Particularly, upstream open reading frames (uORFs),
861 commonly found in the 5'UTR of eukaryotic genes, are important in the initiation of
862 translation. uORFs in a gene initiate nonsense-mediated RNA decay (NMD) that promotes
863 transcript degradation. An uORF was identified in the lncRNA ZNRD1-AS1, namely ZNRD1-
864 AS1-144aa-uORF. ZNRD1-AS1 is highly expressed in glioma and promotes VM formation,
865 while 144aa-uORF was down-regulated and VM hindered. Mechanistically, the lncRNA
866 ZNRD1-AS1 binds to miR-499a-5p, which in turn regulates the ELF1/EMI1 axis to hamper VM
867 formation (Wang et al., 2020a). Therefore, via an intricate regulatory process, the lncRNA
868 ZNRD1-AS1 could foster VM in glioma. The human splicing factor zinc finger, Ran-binding
869 domain containing protein 2 (ZRANB2) is a splicing protein that is part of the
870 supraspliceosome and mediates differential splicing of numerous primary transcripts (Yang
871 et al., 2013). The lncRNA SNHG20 is correlated with poor overall survival and tumor-node-
872 metastasis in several cancer types. In glioma cells, ZRANB2 promoted VM formation by
873 increasing SNHG20 stability. As a consequence, SNHG20 was able to promote Forkhead box
874 K1 degradation, thereby alleviating the transcriptional inhibition of VM-related proteins

MMP-1/-9 and VE-cadherin (Li et al., 2019). Hence, TZRANB2 and SNHG20 were powerful inducers of VM formation in glioma. Another recent report studied the regulation of the poly(A) binding protein cytoplasmic 5 (PABPC5), which binds to the 3' end of the poly(A) tail of most eukaryotic mRNAs. Interestingly, overexpression of the lncRNA HCG15 or PABPC5 promoted VM, while upregulation of the transcriptional repressor ZNF331 greatly curbed VM by directly repressing LAMC2 (coding for Laminin-5 γ 2, important for VM (Seftor et al., 2001; Sood et al., 2001)) and PABPC5 transcription. By targeting the VM-inhibitor ZNF331, the lncRNA HCG15 could thus promote VM formation in glioma (Jing et al., 2020).

In conclusion, these studies highlight the complexity and diversity of VM-related processes regulated by lncRNAs and miRNAs (Table 1). To our knowledge, presently no study reports the direct implication of EVs during VM. However, these EVs are recognized carriers of several ncRNAs and also modulators of extracellular matrix remodeling, a process greatly involved during VM. As a proof-of-concept, using the user-friendly online EVmiRNA database (Liu et al., 2019), we could identify miR-450a-5p and miR-101-3p as expressed in EVs from colon carcinoma (with a preferential enrichment in microvesicles). As such, in-depth investigations of the implication of EVs and EV-carried ncRNAs may certainly warrant interesting findings in VM formation.

4. VM and cancer therapy

4.1. Cancer resistance

For many aggressive tumors, current therapeutic strategies are insufficient. Thus, VM formation could explain the therapeutic resistances observed with cytotoxic or targeted chemotherapies: residual tumor cells may form VM channels, thereby providing oxygen and nutrients, which support cell proliferation and cancer progression. Not only has VM been

899 reported in different solid tumors, but tumor cell-originated neovascularization including
900 tumor-derived endothelial cell-induced angiogenesis along with VM have been suggested to
901 be involved in the development of resistance to anti-VEGF therapy as frequently observed in
902 glioblastoma. Anti-angiogenic drugs, mostly affecting VEGF-VEGFR pathway, cause dramatic
903 tumor size reduction and are largely used in glioblastoma therapy as adjuvants to control
904 abnormal vasculature. However, the benefits are transient since glioblastoma rapidly show
905 resistance to anti-angiogenic therapies upon prolonged treatment through the activation of
906 alternative vascularization pathways (Angara et al., 2017; Arbab et al., 2015). Moreover, it has
907 been noted that hypoxia associated with anti-angiogenic therapies may induce VM that
908 represents a mechanism whereby glioblastoma can escape anti-angiogenic therapies (Angara
909 et al., 2017). VM is also reported to represent a non-angiogenic pathway in breast-cancer
910 metastasis. In this last tumor, it has been reported that angiogenic primary breast carcinoma
911 can relapse not only as angiogenic, but also as non-angiogenic lung metastases. The authors
912 propose that this non-angiogenic pathway is a novel pathway of cancer progression and such
913 tumors are likely to be resistant to anti-angiogenic treatment. In addition, the role of anti-
914 angiogenic treatment in VM promotion has been confirmed in a study conducted in triple-
915 negative breast cancer cells. Thus, an enhancement of VM-positive cases has been observed
916 in sunitinib (a VEGFR tyrosine kinase inhibitor)-treated cells in comparison with control cells.
917 These effects were explained by an overexpression of HIF-1 α , VE-cadherin and Twist1 (Sun et
918 al., 2017). More recently, a study has been conducted with trastuzumab, a drug that target
919 the receptor tyrosine kinase HER2 in breast cancer cells. Several VM markers were
920 upregulated in trastuzumab-treated cells suggesting that HER-2 positive breast cancer cells
921 can exhibit VM in an angiogenic microenvironment after acquiring trastuzumab resistance

(Hori et al., 2019). Hence, VM may be regarded as one of the major causes of the development of resistance to anti-angiogenic therapy in solid tumors.

4.2. *Therapeutic targeting of VM*

If it is clearly established that VM participates in the therapeutic resistances of many solid tumors, in this regards it constitutes a promising reservoir for developing novel anticancer therapeutics, and an armamentarium of drugs [has](#) been investigated (Table 2). Recently, studies have been conducted with histone deacetylase (HDAC) inhibitors both in glioblastoma and in aggressive triple-negative breast cancer. [As previously described](#), altered expression and/or function of HDAC could play an important role in tumor initiation and progression (Li et al., 2018b) and [these enzymes are promising therapeutic targets in various cancers](#) (Sun et al., 2018).

Interestingly, *in vitro* and *in vivo* evidences [suggested the antitumor activity of HDAC inhibitors by preventing VM in different cancer models](#). For instance, it has been found that vorinostat, trichostatin A, entinostat (MS275) and MC1568 significantly decrease tube formation by U87MG and GSCs. These results were explained, at least in part, by a significant decrease of migration and invasion of glioma cells upon HDAC treatments (Pastorino et al., 2019). Furthermore, [it has been reported that](#) the specific HDAC inhibitor, entinostat, [is able to reduce VM structure formations in various triple-negative breast cancer cells](#). Thus, [this study revealed that treatment of MDA-MB-231, LM2-4, BT-549 or MCF-7 with entinostat](#) epigenetically led to the re-expression of the anti-angiogenic genes, serpin family F member 1 (SERPINF1) and thrombospondin 2 (THBS2), as well as the tumor suppressor genes, phosphatase and tensin homolog (PTEN) and p21. Otherwise, it has been found that entinostat downregulated the expression of VEGF-A, and that of the EMT-related genes,

vimentin and β -catenin (Maiti et al., 2019). These two studies offer interesting perspectives for the use of HDAC inhibitors against VM. However, these studies have limited clinical translation as they lack to evaluate the impact of these molecules on VM formation *in vivo*. No pre-clinical or clinical studies have yet been considered with HDAC inhibitors and it is still too early to seriously envision these molecules as potential therapeutic agents against VM formation.

As previously described, CSCs are **main actors** in VM formation and are also responsible for the low survival rate of patients with aggressive tumors. After chemo- and radiotherapy, only a small proportion of CSCs are capable to induce recurrence. Furthermore, EMT is also involved in the acquisition of CSC properties. Taken together, these findings suggest that a combination of EMT and CSC targeting may be beneficial for anti-VM formation therapy through a decrease of both invasion and metastasis and an improvement of patient's survival rate. It has been found that salinomycin, a potassium ionophore used as an anticoccidial drug, completely suppressed VM. The most prominent property of this drug is that it selectively kills CSCs (Gupta et al., 2009).

Furthermore, because VEGF-A enhances the ability of melanoma cells to form tube-like structures by activating VEGFR-1 and the downstream PKC- α pathway, it has been suggested to target VEGFR-1 to prevent VM (Fig. 3A). Interestingly, an anti-VEGFR-1 monoclonal antibody (D16F7) has shown antitumor activity by inhibiting chemotaxis, extracellular matrix invasion and VEGFR-1⁺ cancer cells VM in melanoma and glioblastoma, two aggressive tumors (Atzori et al., 2017; Graziani et al., 2016). Taken together, these findings highlight tumor type specificities and could explain the limited efficacy of sorafenib and thus suggesting a therapeutic benefit for tyrosine kinase inhibitors targeting preferentially VEGFR-1.

Various other strategies have been under discovery to inhibit VM formation. Because MMP-2- and MT1-MMP-mediated laminin- γ 2 cleavage reportedly induces VM formation, anti-MMP-2 or anti-MT1-MMP blocking antibodies treatment has been proposed to abrogate it (Seftor et al., 2001). A similar outcome was achieved when treating 3D culture of ovarian cancer cells at the onset of tube formation with incyclinide (*aka* Metastat), an inhibitor of MMP activity. However, this treatment could not affect an established network, suggesting a role of MMPs in early steps of tube formation rather than for their stability (Sood et al., 2001). A derivative of incyclinide has made it to the clinics with COL-3 (NSC-683551) to treat patients with refractory metastatic cancers (NCT00001683).

In addition, nanostructured functional drug-loaded liposomes, modified with an HIV peptide lipid-derivative conjugate and containing the chemotherapeutics epirubicin and celecoxib were tested for their effect on VM. The added value of this delivery system was to improve pharmacokinetic properties and bio-distribution of such anticancer agents. As such, tail vein injection of drug-loaded liposomes reduced VM expression via the inhibition of VE-cadherin, FAK, EphA2, HIF-1 α , and MMP-9 in invasive breast cancer xenografts in nude mice (Ju et al., 2014). Doxycycline has been used in combination with targeted drugs in clinical trials for patients with advanced cancer. Doxycycline is a semi-synthetic tetracycline which can inhibit MMP activation and cell proliferation, as well as interfere with tumor-related protein synthesis in mammalian cells. Doxycycline has a strong inhibitory effect on malignant cells especially NCI-H446 and A549 lung cancer cells (Cao et al., 2013; Ko et al., 2015). Interestingly, doxycycline treatment prolonged the mouse survival time and partly suppressed the growth of engrafted HCC tumor cells, with an inhibition rate of 43.39% (Meng et al., 2014). In HCC and lung cancer models, doxycycline is capable to inhibit E-cadherin degradation and down-regulates the expression of vimentin protein (Cao et al., 2013; Ko et al., 2015; Meng et al.,

2014). These findings show that doxycycline acts upstream of EMT-related signal transduction to inhibit a wide range of cellular function. As such, investigations in HCC revealed that (i) *in vitro* doxycycline promoted cell adhesion but hampered HCC cell viability, proliferation, migration and invasion; and (ii) *in vivo* higher amounts of VM and endothelium-dependent vessels were found in the control group than the treatment group (Meng et al., 2014).

Among the new molecules used in antitumor therapies, encouraging results have been obtained with galunisertib (LY2157299), a selective ATP-mimetic inhibitor of TGF- β R1, that improved glioma prognosis. Thus, from *in vitro* and pre-clinical studies, it has been found that VM was inhibited by galunisertib through a decrease in VE-cadherin and α SMA expression, as well as down-regulation of AKT and VEGFR-2 phosphorylation in galunisertib-treated glioma cells (Zhang et al., 2016).

Recently, several studies involving molecules extracted from plants have been tested in different tumor models. The properties of brucine, a traditional medicinal herb extracted from seeds of *Strychnos nux-vomica* have been tested in a triple-negative breast cancer cell line MDA-MB-231. The authors concluded that brucine inhibited VM through repressing EphA2 and MMP-2/-9 expression (Xu et al., 2019). Another study evaluated the effects of polyphyllin I, the main component of *Rhizoma paridis* in VM in HCC. It has been reported that polyphyllin I blocked VM through an inhibition of PI3K/AKT – Twist1 – VE-cadherin axis through a regulation of the transcriptional activity of Twist1 (Xiao et al., 2018). In a B16F10 metastatic melanoma model, Bhattacharyya *et al.* proposed lupeol as a substitute for dacarbazine at the onset of therapeutic resistance because it is able to block VM through a downregulation of the CSC marker CD133 (Bhattacharyya et al., 2019). If the abovementioned studies seem exciting for their authors, they remain extremely preliminary and do not constitute (yet) alternatives to recognized therapies. Indeed, no precise pharmacological data

has been demonstrated for the different molecules tested. The works do not specify the pharmacodynamic or pharmacokinetic characteristics of these molecules. For instance, brucine is weakly liposoluble (Li and Wang, 2017) and hardly crosses the barrier formed by the plasma and nuclear membranes. Also, if the pharmacological targets are intracellular, its availability may be limited. In addition, in the case of lupeol, the doses used to block VM are very high (40 mg/kg) and the published data did not provide clear evidence of a true antitumor activity of this molecule. According to our previous work (Pautu et al., 2019), the mice model B16F10 is extremely aggressive and, for us, it is not possible to think that lupeol is sufficient by itself to limit the progression of this metastatic melanoma.

5. Conclusion

As highlighted above, a plethora of signaling pathways and multiple growth factors and their receptors are described to promote VM. Therefore, one could predict that hindering a single signaling pathway by means of an inhibitor would have a limited suppressive effect on VM, since other signaling pathways would immediately compensate and eventually restore the process of switching to VM phenotype. In fact, inhibitors such as galunisertib, LY294002 (a PI3K inhibitor), doxycycline, everolimus or regorafenib showed only limited efficacy. For several months, it has been recognized that therapeutic combinations are essential to treat aggressive cancers. Therefore, it is essential to increase the number of studies combining different therapeutic approaches in order to limit tumor development by blocking significantly VM formation.

Declaration of Competing Interest

The authors declare no conflict of interest.

1042

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1046

1047 **Figure legends**

1048 **Table 1. Non-coding RNAs involved during vasculogenic mimicry**

1049 TNBC: triple negative breast cancer; HCC: Hepatocellular carcinoma.

1050

1051 **Table 2. Summary of therapeutics targeting vasculogenic mimicry**

1052 CSCs: Cancer stem cells; GSCs: Glioblastoma stem cells; HCC: Hepatocellular carcinoma.

1053

1054 **Fig. 1. The two types of vascular mimicry.**

1055 **A)** Patterned vasculogenic mimicry (VM) forms architectural patterns of loops and networks
1056 as revealed by periodic acid-Schiff (PAS)-stained phase contrast micrograph of metastatic
1057 cutaneous melanoma C8161 3D culture. Pictures acquire after 1 week on Matrigel;
1058 magnification x40. **B)** Tubular VM showing melanoma cancer cells that lined a vascular channel
1059 containing red blood cells, but without endothelial cells identified. Hematoxylin-eosin-
1060 staining; magnification x100. Reprinted from “Vascular Channel Formation by Human
1061 Melanoma Cells in Vivo and in Vitro: Vasculogenic Mimicry” by Maniotis et al., The American
1062 Journal of Pathology, 155/3, 739-752, 1999, with permission from Elsevier.

1063

1064 **Fig. 2. Partners in crime during vasculogenic mimicry in tumors.**

Tumor vascularization can be achieved by several processes such as the classical endothelium-dependent sprouting angiogenesis vasculogenic (bottom right), and endothelium-independent **tubular** vasculogenic mimicry (VM). Such blood conducting network is built by cancer cells themselves **resting on** an extracellular matrix (ECM) rich in laminin, stained by periodic acid Schiff (PAS; depicted as the red outline) **without endothelial markers such as** CD31 or CD34. In the tumor microenvironment, various cell types foster VM network formation. (i) The epithelial-mesenchymal transition (EMT) whereby cancer cells lose their polarity and acquire a migratory mesenchymal phenotype upon several signaling pathways (TGF- β , Wnt, Notch, etc.) and transcription factors (Snail, Twist1, ZEB1/2). (ii) Cancer associated fibroblasts (CAFs) are important player in ECM remodeling and their secretome contribute to VM formation. (iii) Tumor associated macrophages (TAMs) contribute to cancer cell transdifferentiation and pro-inflammatory cytokines. (iv) Cancer stem cells (CSCs) harbor pluripotent features allowing them to differentiate and form VM network, but also to do endothelial transdifferentiation contributing to tumor angiogenesis.

Fig. 3. Molecular highlights involved during vasculogenic mimicry formation.

A) VEGF-A, downstream the activation of VEGFR-1, lead to the activation of the PI3K/PKC pathway, converging to AKT with actin reorganization, and thus promoting vasculogenic mimicry (VM). COX-2 transforms the arachidonic acid into prostaglandin E2 (PGE2) that acts on its receptor E2 to foster the PKC/ERK1/2 axis, important during VM. Endothelin, via its receptor EDNRB, leads to β -arrestin-mediated c-SRC activation, which in turn cooperates with VEGF-C/-D to phosphorylate VEGFR-3. The VEGFR-3 activation leads to MMP2 transcription into its pro-MMP-2 form that require proteolytic activation from the MT1-MMP/TIMP2 complex. The active MMP-2 can then trigger the cleavage of laminin- γ 2 into laminin-5 γ 2',

which stimulates VM. **B)** Downstream VEGF-1/VEGFR-2 or EphA2 receptors, VE-cadherin can be phosphorylated on its tyrosine 658 (Y658) by FAK. Downstream PI3K/AKT and ERK activation, the expression of several MMPs (MMP-14/-2/-9) is induced and will activate the MMP-2-mediated VM functions in a similar manner as described for MT1-MMP. Via pY658, FAK mediates VE-cadherin nucleus translocation and the VE-PTP/p120 complex appears important to control this process. In the nucleus, upon binding to p120, the transcription repressor Kaiso can no longer repress the VM-inducible genes CCND1 and WNT11. When cancer cells are cultured on a matrix coated with the extracellular domain of VE-cadherin, the EphA2 and PI3K/AKT pathways are activated, and VM formation initiated.

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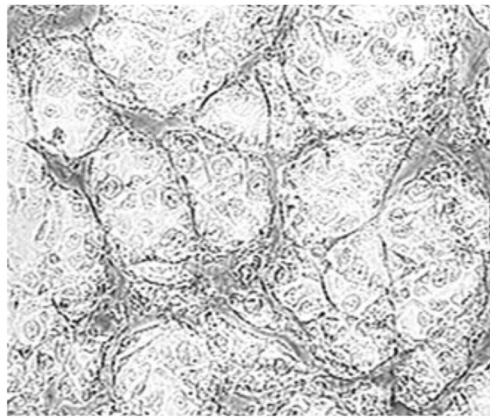
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Figure 1

A Patterned vascular mimicry



B Tubular vascular mimicry

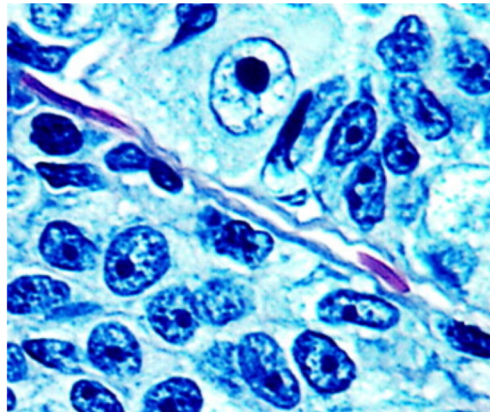


Figure 2

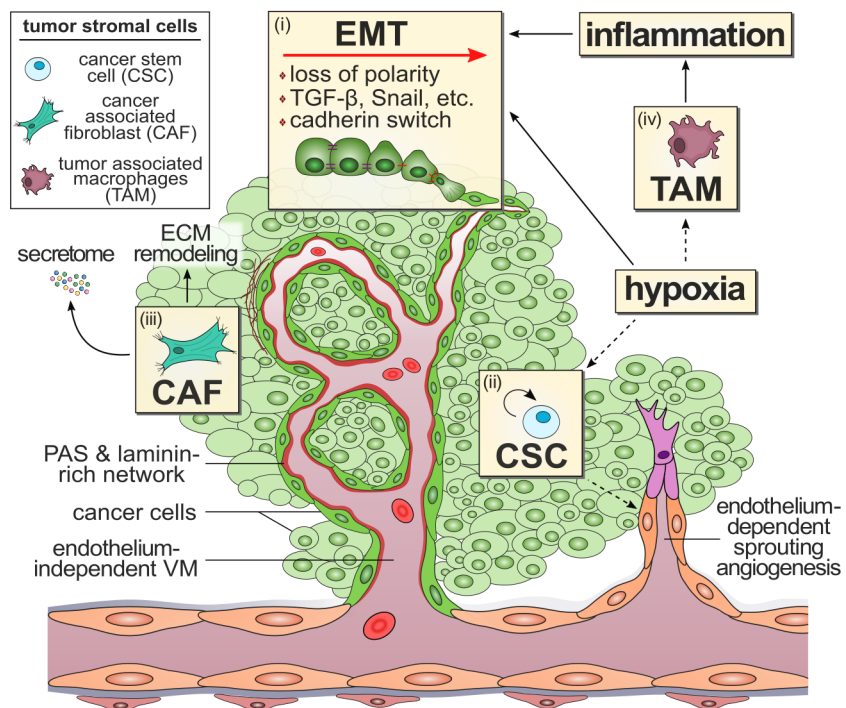
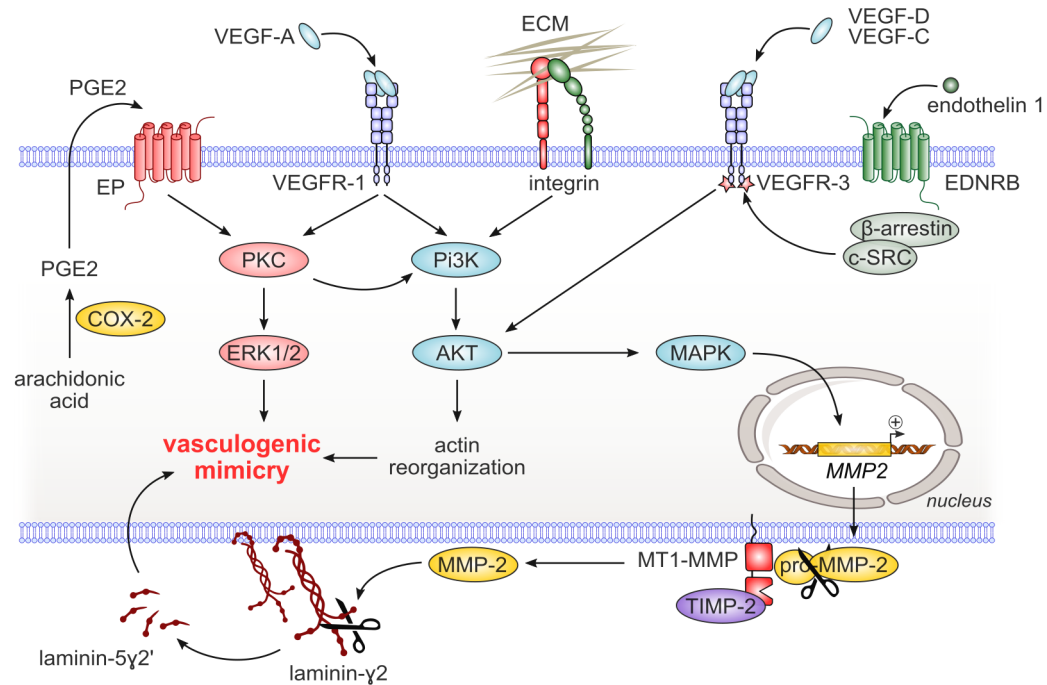


Figure 3

A VEGF/VEGFR-related signaling



B VE-cadherin-related signaling

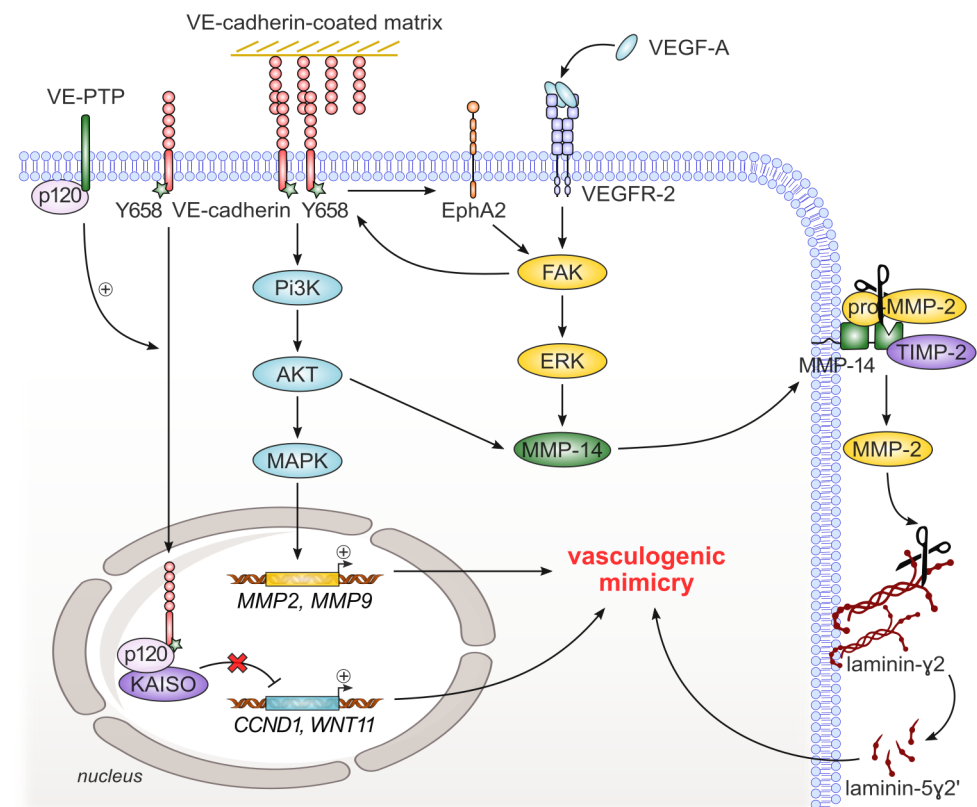


Table 1
Non-coding RNAs involved during vasculogenic mimicry.

ncRNAs	Cancer type	Signaling involved	miRNA involved	Effect on VM	References
miRNAs:					
miR-204	TNBC	PI3K/AKT, RAF1/MAPK, VEGF, and FAK/SRC signaling	n/a	suppress	(Salinas-Vera et al., 2018); (Lozano-Romero et al., 2020)
miR-490-3p	breast cancer; clear cell renal cell carcinoma	Twist1 and vimentin targeting	n/a	suppress	(Zhang, Li, et al., 2014b); (Tao et al., 2018)
miR-193b	breast cancer	DDAH1 targeting	n/a	suppress	(Hulin et al., 2017)
miR-124	breast cancer; cervical cancer	Slug targeting; interact with AmotL1 to reduce invasion and VM	n/a	suppress	(Liang et al., 2013)
miR-765	ovarian cancer	decrease VEGF-A and AKT/SRC signaling	n/a	suppress	(Salinas-Vera et al., 2019)
miR-200a	ovarian cancer	EphA2 targeting	n/a	suppress	(Sun et al., 2014)
miR-141	glioma				(Li, Huang, et al., 2018a)
miR-26b	glioma		n/a		(Wu et al., 2011)
miR-27b	ovarian cancer	VE-cadherin targeting	n/a	suppress	(Liu et al., 2017)
miR-27a-3p	HCC		n/a		(Zhao, Sun, et al., 2016a)
miR-450a-5p	colorectal cancer	suppress SOX2 expression	n/a	suppress	(Chen et al., 2020)
miR-101	HCC	target TGF-βR1 and Smad2 in tumor cells, and reduce SDF1 levels in CAFs	n/a	suppress	(Yang et al., 2016)
miR-186	prostate cancer	Twist1 targeting	n/a	suppress	(Zhao et al., 2016b)
miR-Let-7f	glioma	periostin targeting	n/a	suppress	(Xue et al., 2016)
miR-9	glioma	stathmin 1 targeting	n/a	suppress	(Song et al., 2013)
miR-584-3p	glioma	ROCK1 targeting	n/a	suppress	(Xu et al., 2017)
LncRNAs:					
TP73-AS1	TNBC	release the miR-490-3p's post-translational repression on Twist1	miR-490-3p	promote	(Tao et al., 2018)
LINC00312	lung adenocarcinoma	YBX1 as the interacting partner to increase VE-cadherin, TGF-β, VEGF-A/-C levels via AKT upregulated upon ERβ activation, prevent NEDD9 repression	unknown	promote	(Peng et al., 2018)
MALAT1	non-small cell lung cancer	ERK/MMP and FAK/paxillin signaling	miR-145-5p miR-376a	promote	(Yu et al., 2019) (Li, Wu, et al., 2017b)
n339260	gastric cancer	linked to c-Myc/SOX2 overexpression; increase VE-cadherin levels	miR-31-3p, miR-30e-5p, miR-520c-5p, miR-519c-5p, miR-29b-1-5p	promote	(Zhao et al., 2018)
LINC00339	glioma	activate Twist1/MMP-2/MMP-14 signaling	miR-539-5p	promote	(Guo et al., 2018)
HOXA-AS2	glioma	downstream EGFR signaling, increase VE-cadherin and MMP-2/-9 levels	miR-373	promote	(Gao et al., 2018)
ZNRD1-AS1	glioma	regulate ELF1/EMI1 axis	miR-499a-5p	promote	(Wang, Song, et al., 2020a, Wang, Yang, et al., 2020b)
SNHG20	glioma	increase SNHG20 stability, promote FOXK1 degradation and increase MMP-1/-9 and VE-cadherin levels	unknown	promote	(Li et al., 2019)
HCG15	glioma	ZNF331 targeting	unknown	promote	(Jing et al., 2020)

Table 2

Summary of therapeutics targeting vasculogenic mimicry.

Molecules	Experimental model	References
<i>HDAC inhibitors</i>		
Vorinostat	U87MG cells & GSCs	(Pastorino et al., 2019)
Trichostatin A		
Entinostat (MS275)		
MC1568		
Entinostat (MS275)		
	Triple-negative breast cancer cells (MDA-MB-231, LM2-4, BT-549, MCF-7 cells)	(Maiti et al., 2019)
<i>CSC inhibitors</i>		
Salinomycin	CSCs	(Gupta et al., 2009)
<i>Tyrosine kinase inhibitors</i>		
Anti-VEGFR1-mAb (D16F7)	Melanoma & glioblastoma	(Graziani et al., 2016); (Atzori et al., 2017)
Sorafenib	HCC patients	(Mao et al., 2020)
<i>MMP inhibitors</i>		
Incyclinide derivative (COL-3)	Metastatic cancers	NCT00001683
Doxycycline	NCI-H446, A549 cells & HCC	(Ko et al., 2015); (Cao et al., 2013); (Meng et al., 2014)
<i>TGF-βR1 antagonist</i>		
Galunisertib	Glioma	(Zhang et al., 2016)
<i>Molecules from plants:</i>		
Brucine	MDA-MB-231 cells	(Xu et al., 2019)
Polyphyllin I	HCC	(Xiao et al., 2018)
Lupeol	B16F10 cells	(Bhattacharyya et al., 2019)