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REVIEW PAPER

Pericytes on the Tumor Vasculature: Jekyll or Hyde?

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Abstract The induction of tumor vasculature, known as the 'angiogenic switch', is a rate-limiting step in tumor progression. Normal blood vessels are composed of two distinct cell types: endothelial cells which form the channel through which blood flows, and mural cells, the pericytes and smooth muscle cells which serve to support and stabilize the endothelium. Most functional studies have focused on the responses of endothelial cells to pro-angiogenic stimuli; however, there is mounting evidence that the supporting mural cells, particularly pericytes, may play key regulatory roles in both promoting vessel growth as well as terminating vessel growth to generate a mature, quiescent vasculature. Tumor vessels are characterized by numerous structural and functional abnormalities, including altered association between endothelial cells and pericytes. These dysfunctional, unstable vessels contribute to hypoxia, interstitial fluid pressure, and enhanced susceptibility to metastatic invasion. Increasing evidence points to the pericyte as a critical regulator of endothelial activation and subsequent vessel development, stability, and function. Here we discuss both the stimulatory and inhibitory effects of pericytes on the vasculature and the possible utilization of vessel normalization as a therapeutic strategy to combat cancer.

Keywords Angiogenesis · Pericytes · Vascular normalization

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Introduction

Angiogenesis is a dynamic process that requires coordinated interactions between vascular cells and the extracellular matrix (ECM) to properly regulate the processes of capillary sprouting, lumen formation, and vessel stabilization. Unlike vasculogenesis, where blood vessels form *de novo*, new blood vessels are formed in angiogenesis by sprouting from the existing vasculature, a process which requires transient phenotypic plasticity of its participating cells. Mature normal vessels are composed of quiescent and stationary endothelial cells (EC), which form the inner vessel wall and conducting tubule of blood vessels, and perivascular mural cells, the pericytes and smooth muscle cells which cover the endothelial tubule and perform support functions necessary to maintain vascular stability and tissue homeostasis. Pericytes are embedded in the basement membrane of arterioles, capillaries, and postcapillary venules, as either single cells or as a discontinuous single cell layer surrounding the endothelial tubule, and coordinate signaling with endothelial

cells and other vascular components to help maintain vessel stability. In contrast, vascular smooth muscle cells (vSMC) form multiple concentric layers in association with arteries and veins and mediate vascular tone and contractility.

During the initiation of angiogenesis, the stable association between endothelial cells and pericytes in mature, normal vessels is disrupted, allowing transient phenotypic changes to occur in each cell type [1]. The pericyte coating of the vessel dissociates, followed by matrix degradation, vessel dilation and extracellular deposition of fibrin, to effectively abolish pericyte suppression of endothelial proliferation and migration in response to angiogenic signals. Assembly of a functional vascular sprout requires the selection of endothelial cells, with distinct phenotypic specifications (reviewed in [2] and [3]). Pro-angiogenic signals, such as vascular endothelial growth factor (VEGF), initiate a signaling cascade that enables an endothelial cell to become a tip cell, which guides the leading edge of the emerging sprout, whereas lateral inhibition via Dll4/Notch signaling instructs neighboring cells to become stalk cells, which follow the tip cell and proliferate to form the emerging stalk. The polarization of tip cells enables directed movement, so that the leading edge of the cell extends lamellipodia and filopodia to probe the microenvironment and detect guidance cues and repulsive signals, while the trailing edge maintains contact with stalk cells [3]. During physiological angiogenesis, mural cell precursors are then recruited to the newly developed sprout (discussed in detail below), where contact with endothelial cells results in their differentiation into mature mural cells. In this 'maturation phase', cell-cell junctions are established and basement membrane is reconstituted, resulting in quiescence of both cell types.

The Angiogenic Switch

It is well established that tumors must acquire the ability to stimulate capillary formation to progress from a small localized growth with a limited oxygen and nutrient supply to a well-vascularized enlarged tumor [4]. This conversion of the tumor from an avascular state to a vascular state has been termed the 'angiogenic switch' and occurs by local alteration of the balance of pro-angiogenic factors and the inhibitor molecules that maintain the quiescence of the vasculature [5]. This dogma has led to numerous investigations which have focused on the endothelial cell, as the main component of the vessel, and targeted its inhibition as a means to prevent pathological angiogenesis. However, the role of the mural cell, the other main component of the blood vessel, has received less attention. The importance of the initial pericyte dissociation step is underscored by the ability of breast cancer cells to increase initiation of angiogenesis without accelerating neovessel growth rate [6]. Hypoxia is thought to be an important early driver of the angiogenic switch (recently reviewed in [7]), likely by its ability to upregulate the vessel destabilizing factors VEGF [8] and Angiopoietin-2 (Ang2) [9]. Interestingly, macrophages have been demonstrated to contribute to the angiogenic switch in tumors [10]; this may result from both the ability of macrophages to produce VEGF [11] and/or Ang2 [12], as well as directly facilitate pericyte detachment from the vessel wall [1].

Abnormalities of Tumor-Associated Vasculature

Tumor-associated blood vessels exhibit structural and functional abnormalities which severely impact disease progression and the efficacies of therapies. Unlike the normal mature vasculature, which has a structural hierarchy of vessels with a characteristic size, shape and vessel wall structure, tumor vasculatures are frequently irregular and disorganized networks lacking conventional hierarchies, with abnormal branching and uneven basement membranes [13, 14]. These vessels are highly dysfunctional and promote the development of a microenvironment that stimulates non-productive angiogenesis and metabolic adaptations by tumor cells that favor metastasis. Oncogenic mutations in tumor cells that stimulate the production of angiogenic factors [15] and tumor co-option of macrophages to facilitate ECM breakdown and angiogenesis [16] can promote hyperactive vessel growth that lacks sufficient functionality to maintain tissue homeostasis. Inadequate or intermittent perfusion and oxygenation of these vessels renders the tumor hypoxic and acidic, which elicits additional pro-angiogenic adaptations, and creates a potentially self-perpetuating cycle of non-productive angiogenesis [17].

The defective normalization, or "abnormalization" (reviewed in [17]) observed in tumor vessels reflects altered associations and functions of both endothelial and mural cells. The endothelial cells of the vessel tubule are often gapped or have loosened junctions, along with increased vascular permeability and significant plasma extravasation (reviewed in [18]), resulting in both the formation of a pro-angiogenic fibrin/fibrinogen provisional matrix [19, 20] and increased interstitial fluid pressure in the tumor microenvironment [21–23]. Interstitial hypertension may cause several problems relating to the progression and treatment of tumors, including 1) the enforced flow of interstitial fluid bearing angiogenic growth factors and possibly metastasizing tumor cells into the surrounding microenvironment; and 2) compromised delivery of therapeutic agents across the vessel wall and into the tumor interstitium [14].

The immature functional state of tumor vasculature is further exemplified by the abnormal mural cell coverage and investment observed on tumor-associated blood vessels. Analysis of vascular morphogenesis and pathological

angiogenesis suggest that disruption of pericyte contact is required for initiation of angiogenesis. Studies analyzing mural cell investment of tumor vasculature suggest a decreased detection of pericytes [24]; however, this finding may partially reflect the choice of mural cell markers, as expression of differentiation markers may be altered in tumor-associated pericytes [25]. Studies using confocal or electron microscopy show that mural cells may be present on tumor-associated vessels but exhibit abnormal association with underlying endothelial cells (Fig. 1). In contrast to the tight association observed between pericytes and endothelial cells on normal capillaries, pericytes on tumor capillaries are loosely associated with endothelial cells and exhibit an abnormal shape, sometimes extending their processes away from the endothelium toward the tumor [25–27],

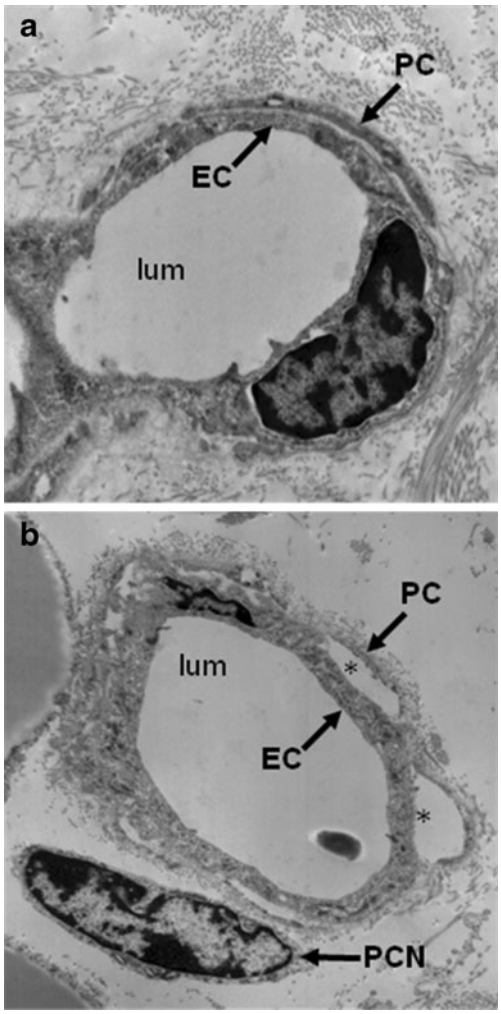

Fig. 1 Electron micrograph of endothelial-pericyte associations. **a**. A capillary of a normal vessel in mouse skin, showing tight association between endothelial cell and pericyte processes. **b**. A capillary of a tumor-associated vessel in an M1 chloroma tumor, showing dissociation of pericyte from endothelium with gaps (*) between both cell types. PC, pericyte; PCN, pericyte nucleus; EC, endothelial cell; lum, lumen. *(Image generated by Suleyman Ergun)*

as if the tumor is exerting a chemotactic effect that outcompetes physiological pericyte-endothelial interactions. As a result, the overall functional immaturity of these vessels allows for continued angiogenesis.

Little is known about the cause of the pericyte investment defect of tumor vessels. Co-injection of mouse embryo fibroblasts (MEF) with tumor cells results in MEF investiture of tumor vessels, suggesting that tumor endothelial cells may retain the ability to recruit mural cell precursors [26]. Thus, the defect may therefore lie in an inhibitory effect on mural cell proliferation or differentiation. It is interesting to note that the presence of the mural cell precursor C3H 10T1/2 can either stimulate endothelial tubule formation in response to VEGF-A, or inhibit tubule formation induced by bFGF [28], whereas differentiated vSMC inhibit endothelial sprouting in response to VEGF-A [29]. Interestingly, VEGF-A downregulates the expression of the differentiation marker smooth muscle actin (α-SMA) on vSMC in vivo [30]. Immature mural cells lacking the differentiation markers calponin and caldesmon do not confer vessel stability in vivo [31], and the mural cells on some tumor vasculature lack calponin expression [32–34]. It is possible that maintenance of mural cells in a less differentiated state may be a means by which tumors not only prevent mural cell-mediated vessel quiescence, but also facilitate angiogenesis.

As outlined below, many of the abnormalities of the tumor vasculature may result, either directly or indirectly, from the loss of the inhibitory, stabilizing properties of differentiated pericytes and/or the tumor-induced activation of pericytes (Fig. 2).

Modulators of Pericyte Recruitment and Investiture

Mural cell recruitment and blood vessel stabilization rely on multiple pathways including Angiopoietin/Tie2, Eph-Ephrin, PDGF/PDGFR, and S1P/$S1P_{1/2}$ signaling, both as independent pathways and modulation via crosstalk between the pathways. The angiopoietin family of growth factors are ligands for the Tie2 receptor. Angiopoietin-1 (Ang1) binds to Tie2, resulting in receptor phosphorylation and downstream signaling [35]. Although Ang2 can also bind Tie2, its canonical role is disruption of Ang1/Tie2 signaling in endothelial cells. However, Ang2 has been shown to stimulate Tie2 activation in a cell type- or context-specific manner [36–38]. The severe vascular defects and embryonic lethality of Tie2 receptor knockout [39, 40], Ang1 knockout [41], and Ang2 overexpressing mice [36] highlight the significance of these factors in proper development of the vasculature. Pericyte coverage of vessels is diminished and their interaction with endothelial cells is weakened in Ang1 knockout mice [41], although

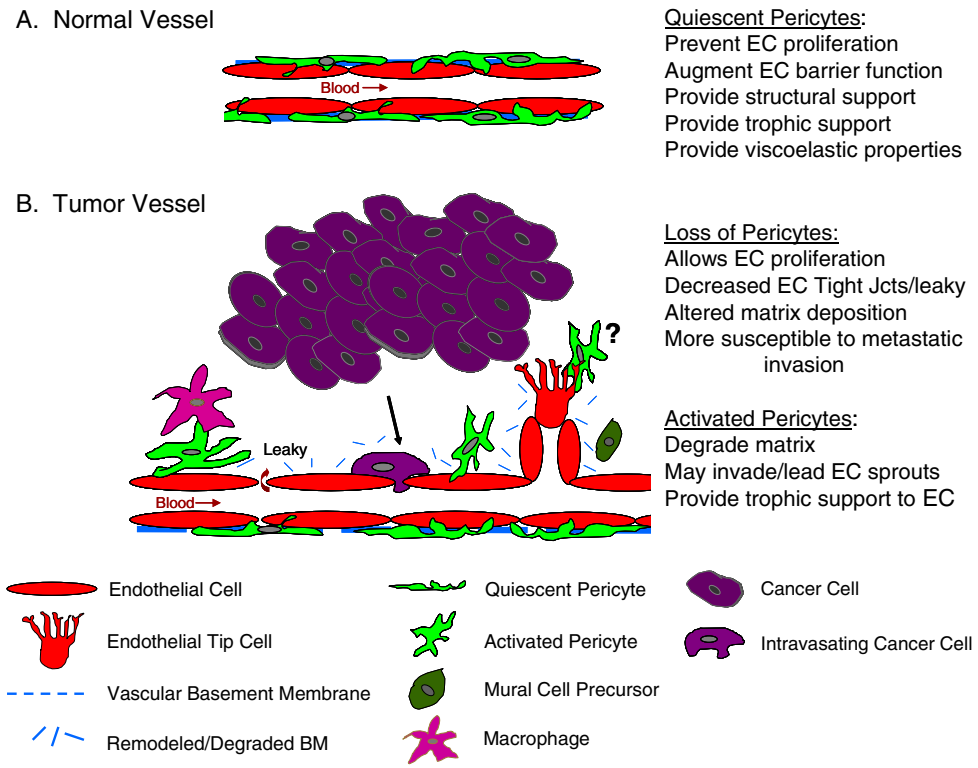

Fig. 2 **a**. Schematic representation of endothelial cells (EC) and pericytes on a normal quiescent vessel. Pericytes physically encircle the endothelial tubule, extending processes through the vascular basement membrane to physically contact the underlying endothelium. Following contact, pericytes participate in the deposition of the basement membrane (BM), enhance endothelial tight junctions (Jcts) to augment barrier function, prevent endothelial proliferation, provide structural support, and confer viscoelastic properties to the vessel to regulate blood flow. **b**. Abnormal association of pericytes with endothelium in a tumor vessel. At the initiation of angiogenesis, pericytes dissociate from the endothelial tubule in response to tumor-secreted factors and/or tumor-educated macrophages. The basement membrane undergoes remodeling or degradation, allowing endothelial cell proliferation to ensue. An invading endothelial tip cell leads the growth of the sprout. Alternatively, some evidence suggest pericytes may be present at the leading tip. Mural cell precursors may be recruited toward the new vessel, but in the tumor environment may fail to properly invest the endothelium. Under these conditions, the pericyte may not be able to exert inhibitory effects on endothelial cells, yet still provide trophic signals to facilitate their survival. Vessels that lack sufficient pericyte support are also commonly leaky, have abnormal basement membranes, and are more susceptible to metastatic invasion

loss of Ang1 expression after embryonic day E13.5 did not result in altered pericyte recruitment [42]. Likewise, Ang2 overexpression reduces capillary pericyte coverage during retinal development [43], while tumor models with Ang2 neutralization exhibit increased pericyte coverage of blood vessels [44–46]. Changes in Ang1/2 cytokine ratio alter mural cell recruitment to the vasculature. As proposed by Folkman and D'Amore [47], the recruitment of mural cells may be mediated by Ang1-induced endothelial secretion of mural cell chemoattractants. Indeed, endothelial cells stimulated with Ang1 secrete hepatocyte growth factor (HGF), enhancing mural cell migration towards endothelial cells in co-cultures [48]. Ang1-stimulated endothelial cells also secrete heparin binding EGF-like growth factor (HB-EGF) which activates mural cell ErbB1/ErbB2 receptors to induce mural cell recruitment [49, 50]. Additionally, increased recruitment of mural cells in aortic ring cultures is seen following Ang1 induction of MCP-1 and p38MAPK signaling, although the cellular source of the MCP-1 was not determined [51]. There is also in vivo evidence to suggest that mural cell precursors may express low levels of Tie2 [52], and that direct stimulation of mural cells with Ang1/2 may also influence recruitment. In vivo, the combination of Ang1 and VEGF induces an influx of mural cell precursors that is not observed with either factor alone [53]. In vitro, both Ang1 and Ang2 enhance mural cell precursor migration, with an additive effect rather than antagonism [54], while no significant chemotaxis was seen in differentiated mural cells [55]. Although Tie2 expression appears to be downregulated following mural cell differentiation [52], we and others have observed the induction of Tie2 expression on differentiated mural cells under angiogenic conditions such VEGF stimulation [56, 57] or in the presence of hypoxia [56], which may allow for direct recruitment of mural cells by Ang1. Following recruitment to neovasculature, mural cell secretion of Ang1 induces trans-association

of the Tie2 receptors between endothelial cells in close contact and enhances AKT related survival, vessel integrity, and quiescence [58]. Ang1-activated endothelial Tie2 also induces basement membrane deposition though AKT-induced Dll4/Notch signaling which further enhances the vascular barrier [59].

Platelet-derived growth factor (PDGF)/PDGFR is another pathway that strongly influences mural cell recruitment and investiture. Endothelial produced PDGF-B enhances mural cell proliferation, migration, and recruitment to growing vasculature through the mural cell PDGFR-β receptor [60]. An absence of mural cell investiture, hemorrhage, and embryonic lethality are found when either PDGF-B [61] or PDGFR-β [62] is knocked out, indicating their critical roles in angiogenesis. Similarly, reduced PDGFR-β signaling by receptor inhibition by imatinib [50], receptor hypomorphic mutations [63], and adenoviral expression of soluble PDGFR-β as a ligand decoy [64] all significantly decrease pericyte presence in the vasculature. Interestingly, endothelial, and not tumor, production of PDGF-B is needed for proper mural cell investiture, full coverage and formation of intimate mural-endothelial cell contacts. In the absence of the heparin sulfate proteoglycan (HSPG) binding motif of PDGF-B, pericytes are recruited but form only loose associations with endothelial cells and there is persistent microvascular dysfunction [65]. Ectopic expression of PDGF-B in tumor cells enhances pericyte recruitment, but is unable to improve pericyte investiture [66]. The endothelial production and subsequent retention of PDGF-B in the periendothelial space likely creates a gradient that promotes both pericyte recruitment and intimate association with the endothelial abluminal surface [66].

Recent studies suggest that PDGFR-β activity in mural cells may be modulated by VEGF [67]. In the presence of VEGF, VEGF-R2 and PDGFR-β complex, inhibit PDGFR-β phosphorylation, and subsequently reduce pericyte proliferation, migration, and incorporation along vascular sprouts. During angiogenic sprouting when VEGF is highly expressed, the inhibition of mural PDGFR-β may enable continued endothelial proliferation and growth of neovasculature. When angiogenic growth is sufficient in an area and VEGF levels drop, PDGFR-β activation would then be permitted, enabling mural cell recruitment, subsequent endothelial quiescence, and vessel stability. Enhancement of mural cell migration by PDGF-B also occurs indirectly by the stimulation of endothelial secretion of SDF-1α that then binds mural CXCR4 receptors [68]. PDGFR-α may also play a role in mural cell recruitment and maturation. In contrast to PDGFR-β, PDGF-B and PDGF-A can both bind PDGFR-α in mural cells, activating mural cell migration, recruitment to vasculature, and differentiation in a neuropilin-1 and $p130^{Cas}$-dependant mechanism [69].

Proper mural cell investiture is also influenced by Eph-ephrin interactions. Ephrin-B ligands and their Eph receptor tyrosine kinases are typically both membrane bound, requiring close cell-cell proximity for interaction. Eph-ephrins can signal bidirectionally, "forward" through the receptor and "reverse" through the interacting ligand's cytoplasmic domain. Similar to the knockouts in the Ang1/Tie2 pathway, knockout of mural cell ephrin-B2 is embryonic lethal, a result of edema and hemorrhage [70]. While total mural cell numbers are not compromised, ephrin-B2 knockout specific mural cells round up and poorly interact with the endothelium [70]. Not surprisingly, ephrin-B2 phosphorylation occurs at points where mural-endothelial contacts occur, and this reverse signaling in both endothelial and mural cells is required for their assembly into cord-like structures [71]. Furthermore, endothelial-EphB4 activation of ephrin-B2 induces mural Ang1 expression and increases Tie2 activation. As a result, pericyte investiture is enhanced and tumor blood vessel leakiness declines [72]. Additional reports suggest a requisite role for ephrin-B2 downstream of PDGF-B/PDGFR-β signaling and recruitment in pericyte-like hepatic stellate cells [73]. A possible role for EphA-ephrinA in pericyte physiology is less clear, although it has been shown that pericyte investiture is defective in EphA2-deficient mice [74].

Sphingosine-1-phosphate (S1P) and its family of membrane-bound G protein-coupled receptors $S1P_1$ – $S1P_5$ [75], previously known as EDG-receptors, also play an integral role in pericyte recruitment. Under varying conditions, mural cells express of all of these receptors, with highest expression of $S1P_{2/3}$ and lower levels of $S1P_1$ [76–80]. S1P regulates mural cell proliferation [76, 77, 81, 82], migration [77, 83], response to PDGF and EGF [79], and differentiation [82]. Defects in vasculature and discontinuous coverage of mural cells are seen following ablation of sphingosine kinases [84] or knockout of $S1P_1$ [85]. This effect is mediated by mural-endothelial interactions including N-cadherin trafficking [86] and adherens junctions [87] because endothelial-specific knockout mimics the phenotype of the full knockout [88]. In contrast, $S1P_2$ knockout enhances mural cell recruitment to tumor blood vessels, potentially through the production of angiogenic factors that promote vascular maturation like TGFβ or the loss of chemorepulsion mediated by $S1P_2$ on mural cell precursors [89]. While it is known that activation of $S1P_2$ by S1P inhibits mural cell migration and spreading through inhibition of Rac [90, 91], and the use of $S1P_2$ antagonist JTE-013 restores mural cell migration in response to PDGF [78], knockout of $S1P_2$ in mural cells could further aid in clarifying the $S1P_2$ direct versus endothelial-mediated effects. In summary, multiple systems are critical in driving the recruitment of mural cells, formation of tight mural-endothelial contacts, and subsequent stabilization of the vasculature. The mechanisms of these pathways and the factors that modulate their

activity during angiogenesis and disease remain an ongoing area of research.

Molecular Mediators of Junctional Interactions Between Pericytes and EC

Although separated by a basement membrane, pericytes and endothelial cells make physical contact with each other by extension of processes through openings in the basement membrane. This physical contact can be mediated by adherens junctions, gap junctions, and peg-and-socket junctions. In the latter, pericyte processes serve as the 'pegs' which insert into endothelial cell 'sockets' [92]. As these structures have been suggested to both be associated with more mature vasculature [93] and occur during angiogenesis [94, 95], the exact nature or function of these structures remains unclear. However, it has been suggested that these cell-cell contacts are also home to other stabilizing junctional interactions [1]; indeed, one study localized the expression of Ang1 to pericytes and Tie2 to endothelial cell membranes within such interdigitations [96]. Adherens junctions composed of N-cadherin play an important stabilizing role in pericyte-endothelial interactions, as blocking [97, 98] or endothelial loss [86] of N-cadherin results in decreased pericyte adhesion to endothelium.

Gap junctions are composed of proteins known as connexins, which couple with assembled connexins on an adjacent cell membrane to form a functional junction. Gap junction intercellular communication (GJIC) allows for the direct exchange of ions, second messengers, and small hydrophilic molecules (generally under 1 kDa) between neighboring cells to mediate organized growth and adaptive responses within tissues. Functional GJIC between endothelial cells and mural cells has been demonstrated in vitro using electron probe microanalysis [99], electrical resistance [100], and dye coupling studies [101, 102]; ex vivo by dye coupling studies [103] and in vivo by ultrastructural studies [104]. The presence of these gap junctions has been thought to facilitate the conductance of electrical signals along the vessels wall to control vascular tone [105, 106]. These gap junctions also play a critical role in vascular assembly during vessel formation. When mural cell precursors are recruited to a newly-developed vessel, they form Connexin 43 (Cx43)-dependent gap junctions with the endothelium which leads to activation of latent TGF-β to induce full mural cell maturation [107].

Another role for gap junctions in the vasculature has been recently proposed. Normal vessels undergo vascular remodeling in response to environmental, metabolic, and hemodynamic stimuli [108, 109], and proper gap junctions may be required for maintenance of normal responsive vascular structures [110]. Mathematical modeling suggests that, in the absence of properly integrated signaling along the vessels due to alterations in heterocellular gap junction communication, formation of arterio-venous shunts is favored [110]. Interestingly, an inactivating phosphorylation of Cx43, which is thought to disrupt junctional communication, has been reported on the capillaries of breast and other tumors [111]. In addition, VEGF decreases endothelial gap junction communication [112]. A loss of vascular gap junction activity could therefore explain both the decrease in detection of pericyte differentiation markers and the aberrant formation of arterio-venous shunts which plague the tumor vasculature.

Pericytes as Drivers of Angiogenesis

Pericytes May Remodel Matrix

Although the canonical view of pericyte function in angiogenesis holds that pericytes function primarily in the resolution of angiogenesis by contributing to vascular stabilization and maturation, circumstantial evidence suggests that pericytes play a permissive, if not stimulatory, role in early angiogenesis. The vascular basement membrane is a stabilizing physical barrier to soluble molecules and migrating cells that also serves as a scaffold for pericyte-EC interactions. Activation of matrix proteases and degradation of the basement membrane is necessary for angiogenesis to occur (reviewed in [113]) and there is evidence that tumors secrete factors that activate pericytes to degrade the basement membrane and liberate matrix-bound growth factors, thereby contributing to angiogenesis and possibly facilitating tumor growth and invasion. The basement membrane in the vicinity of pericytes is altered following stimulation of muscle angiogenesis in vivo, suggesting that matrix proteases have been activated [114]. Similarly, in hypoxia-induced angiogenesis in the brain, one of the earliest morphological changes observed was thickening of basement membrane between endothelial cells and pericytes, along with disintegration of the basal lamina at the leading edge of migrating cells [115] and upregulation of the urokinase plasminogen activator receptor (uPAR) on pericytes [116]. Immunostaining of sprouting tubules in human fetal brain angiogenesis shows that endothelial cells and pericytes associated with the basement membrane both express activated MMP-2, suggesting that these cells may cooperate to disassemble the basement membrane to allow cell migration and the emergence of a nascent sprout [117]. In breast carcinoma in situ samples, matrix metalloproteinase (MMP)-1 and MMP-9 were detected in both capillary pericytes and fibroblasts, and levels of these proteases were further increased in invasive carcinomas [118]. Increased MMP-9 expression by pericytes has been detected in

neuroblastoma xenograft tissues [119], human glioma samples [120], and human breast cancer tissues [121]. In vitro studies have shown that treatment with pro-angiogenic factors is sufficient to stimulate mural cells to secrete MMPs. Vascular smooth muscle cells treated with VEGF, an important angiogenic mediator in may cancers, secrete MMP-9 [122, 123], as well as MMP-1 and MMP-3 [122], resulting in the invasion of vSMC through Matrigel [122, 123] and a mixture of type I and type III collagen [123]. Similarly, MMP-2 and MMP-9 are activated in vSMC in response to bFGF [124] and IL-1β+PDGF-BB [125], respectively. In addition to facilitating sprouting angiogenesis, activated pericytes have also been implicated in the degradation of vascular basement membrane associated with the development of 'mother' vessels [126], a pathological subtype of angiogenic venule that divides to give rise to new 'daughter' vessels [127]. Interestingly, the degradation of basement membrane during this process results not from MMP production, but from an increase in pericyte-derived cathepsin activity which results in pericyte detachment and vascular enlargement [126]. These data, combined with reports that tumor cells induce invasion and migration of mural cells [128], suggest that tumor stimulation of mural cells may cause them to play an active role in the initiation and propagation of angiogenesis.

Pericytes May Lead Angiogenic Sprouts

Additional observations suggest that pericytes may have non-canonical roles in regulating vascular sprouting in certain tissues, possibly by helping to guide the emerging sprout [129]. Pericytes have been detected with endothelial cells at the growing vascular tip in the mouse retina. Combined immunohistochemistry and electron microscopy studies have detected pericytes during the earliest histological stage in the formation of granulation tissue during wound healing [130] and following the induction of angiogenesis in skeletal muscle by electrical stimulation [114]. Pericytes have similarly been reported to be located on sprouts emerging in mammary and other tumors [25, 131], although the mechanistic implications of this co-localization are not well understood. The guidance molecule Slit3 has been shown to be secreted by both endothelial cells and vSMC and interactions between Slit3 and endothelial Robo4 receptor stimulate endothelial proliferation, motility, chemotaxis, and tubule formation in vitro [132]. Since vSMC similarly express Robo1 and Robo4, the authors proposed that autocrine and paracrine Slit3/Robo signaling may help coordinate endothelial-mural cell interactions in vessel maturation, although additional studies are needed to determine the potential contributions of Slit/Robo signaling to earlier stages of angiogenesis [133].

Studies of angiogenesis in ovulation have observed that pericytes are located at the leading edge of endothelial sprouts in the collapsed follicle [134, 135], but become closely associated with EC in the mature corpus luteum (reviewed in [136]). Endothelial cells in luteal arterioles and capillaries produce nitric oxide and luteal pericytes express VEGF, suggesting that a hypoxia-driven paracrine loop may help coordinate sprouting [136]. In addition to contributing a VEGF gradient, pericytes may also degrade and remodel the ECM to form additional "guiding structures" that aid in the luteal invasion of endothelial sprouts, possibly by laying down a scaffold containing fibronectin that is permissive for endothelial migration [137]. A complementary or possibly alternative guidance structure is suggested by immunohistochemical analyses of growing microvessels in the developing human brain, where it was observed that pericyte markers extended the length of the growing microvessel, while endothelial markers were observed only in the initial segment of the vessel [117]. The immunohistochemical staining pattern was interpreted as illustrating endothelial cell-free segments in which leading pericytes were recruiting EC from the parental vessel, possibly by secreting factors such as VEGF or by cell-cell interactions mediated by molecules such as NG2 proteoglycan. Similar endothelial-free pericyte assemblies have been reported to regulate sprouting in retinal neovascularization, the adult mouse cornea [138], and mouse tumor models [25, 139], suggesting that pericytes may confer guidance to invading endothelial cells in a variety of contexts. Indeed, a clue to the reconciliation of these apparent discrepancies in the role of pericytes in sprouting angiogenesis is suggested by a study indicating that the positioning of pericytes at the leading front versus recruitment following nascent vessel formation is governed by the specific angiogenic stimulus [140]. Additional studies are required to determine (1) the extent to which pericyte assemblies contribute to neovascularization in other tissues and contexts; and (2) whether tumor-induced defects in pericyte functions pertaining to guidance of EC contributes to the altered architecture exhibited by tumor vasculature.

Pericytes Provide Trophic Support for Endothelial Cells

In addition to interactions promoting stability and quiescence, there are several reports indicating that pericytes provide paracrine survival support for endothelial cells. Immature tumor blood vessels which lack pericyte coverage are more vulnerable to VEGF withdrawal than vessels with more extensive pericyte coverage [141]. It has also been suggested that pericytes may play a role in the abundant microvascular proliferation found in glioblastoma [142]. Treatment with IL-1β [143], PDGF-BB, or media conditioned by colon cancer cells [144] induces VEGF production

in mural cells, suggesting that mural cells may provide paracrine trophic support in a variety of physiological and pathological contexts. Contact between endothelial cells and multipotent mesenchymal cells is required to induce mural VEGF [145]. Pericyte production of VEGF was observed in the developing retinal vasculature in vivo, further supporting the concept that heterotypic contact-induced pericyte differentiation upregulates local VEGF production as part of the regulatory program that stabilizes newly formed vessels. The functional consequences of trophic cross-talk between endothelial cells and pericytes is demonstrated by the observation that tumor vessels with pericyte coverage do not regress as efficiently following anti-vascular therapy as vessels lacking coverage [146–148], suggesting that a bi-compartmental strategy may be required to more completely target tumor vasculature. The combined use of VEGFR and PDGFR inhibitors has been investigated as a possible strategy to circumvent pericyte-mediated protection of tumor vessels by disrupting signaling pathways mediating functional endothelial-pericyte interactions to promote vessel regression [149–151]. Interestingly, combined inhibition of VEGF and PDGF signaling did not alter normal vessels [150], emphasizing the phenotypic differences between endothelial-pericyte interactions in normal and neoplastic tissues.

Pericytes as Inhibitors of Angiogenesis and Tumor Progression

Mural Cells are Necessary to Maintain a Quiescent Stable Endothelium

Several studies [152, 153] have demonstrated that actively proliferating endothelium lacks coverage by mural cells. In addition, mural cell-endothelial cell interactions are reduced following stimulation of angiogenesis by at least three different methods [154], and the arrival of pericytes coincides with the cessation of vessel growth during wound healing [155], suggesting that contact with mural cells leads to quiescence of endothelial cells. Diminished pericyte coverage in tumor vessels corresponds with increased endothelial proliferation. Whereas quiescent normal endothelia exhibit an average proliferation rate of only 0.1 % [156], breast tumor endothelial proliferation indices are elevated 20 to 50-fold [24, 157]. The negative correlation between mural cell investment and angiogenesis was further corroborated by a study showing that pericyte coverage in low vascular density areas of breast tumors is significantly higher than in areas with high vascular density [158]. A variety of in vitro studies culturing endothelial cells with vSMC or pericytes corroborate the decreased growth of endothelial cells under these conditions [159, 160]. Further, mural cells prevent endothelial cell migration [161] and sprouting [29], and activation of endothelial MT1-MMP [162]. Together, these data underscore the ability of mural cells to limit endothelial cell responsiveness to external angiogenic stimuli when they exhibit a normal, functional association with endothelium.

The molecular mechanisms regulating the functions of pericytes in stabilization and maturation of newly formed vessels are not well understood. Several in vitro studies using 3-dimensional (3D) collagen matrices suggest that crosstalk between endothelial cells and pericytes regulating the balance of MMPs and TIMPs is critical for vessel morphogenesis, sprout stabilization, and vessel regression. When grown in 3D matrices of type I collagen, endothelial cells arrange into networks and undergo lumen formation [163, 164]. During the formation of these networks, endothelial cells secrete high levels of the zymogens pro-MMP-1 and pro-MMP-10 [165, 166]. Activation of MMP-1 and MMP-10 correlated with collagen gel contraction and capillary tube regression. The addition of bovine retinal pericytes to the collagen matrix was sufficient to block MMP-1 and MMP-10-dependent capillary tubule regression in the presence of plasminogen, whereas other cell types were ineffective, suggesting that endothelial contact with pericytes inhibits vessel regression by limiting protease activity and/or function [167]. Endothelial cells were the predominant source of TIMP-1, TIMP2, and PAI-1, whereas pericytes were a strong source of TIMP-3 that was induced by EC-pericyte interactions. Targeting of TIMP-2 in endothelial cells and TIMP-3 in pericytes by siRNA resulted in capillary tube regression in a process dependent on MMPs. These results indicate that pericyte-dependent stabilization of capillary tubules is mediated, at least in part, by protease inhibition. Additional studies are required to determine whether tumors may alter regulation of protease activities contributed by endothelial cells and pericytes to subvert vessel morphogenesis and regression and distort normal vascular architecture.

Restoration of Pericyte Investment of Tumor Blood Vessels Leads to Tumor Inhibition

As discussed, Ang1 and its receptor Tie2 play an important role in mural cell recruitment and vessel maturation [39, 41]. Ectopic expression of Ang1 in breast, colon, and squamous cell carcinoma results in decreased tumor proliferation and angiogenesis in some xenograft models [168–170]. The blood vessels in Ang1-expressing breast [53] and other [170, 171] tumors demonstrated significantly greater association with α-SMA-positive mural cells compared to controls, suggesting that enforced maturation of the blood vessel functionally inhibited tumor angiogenesis. It is noteworthy that Ang1 can also potently stimulate angiogenesis via enhancing the migration, capillary formation, and survival of endothelial cells, which can lead to enhanced tumor

growth (reviewed in [172]); however, in xenograft models where Ang1 expression leads to enhanced pericyte investment, tumor growth is decreased [171]. Ang1 also decreased metastasis, tumor growth, and ascites formation in an experimental model of colon peritoneal carcinomatosis [173]. Recently, in an in vivo melanoma model it was shown that vessel stabilization is associated with a reduced tumor vessel density and slows the tumor growth significantly but it also results in a resistance to anti-angiogenic therapy [148]. These data, coupled with aforementioned studies demonstrating the anti-angiogenic effects of mural cell association, suggest that stabilization of tumor blood vessel by mural cells may be a desirable therapeutic goal by which new vessel formation may be inhibited and tumor growth thereby arrested. However, continued therapeutic success may require additional strategies to target a stabilized tumor vasculature.

Mural Cells Stabilize Vessels by Deposition of Matrix

The vascular basement membrane confers an additional level of structural stability to blood vessels. Not surprisingly, abnormal deposition of matrix is commonly observed in the unstable tumor vasculature. This may be manifested as discontinuous [174, 175] basement membrane, or abnormal morphology of the basement membrane including multiple layers and variable increased thickness [131, 175, 176]. Isolated pericytes have been shown to express the basement membrane components collagen IV and various laminin isoforms [177], as well as significant levels of fibronectin [178]. One recent study suggested that in vSMC-endothelial co-cultures the endothelial networks become ensheathed by collagen IV and collagen XVIII, while lamins and fibronectins localize to the vSMC layer [179]. Endostatin, a fragment of collagen XVIII and an endogenous inhibitor of angiogenesis [180] was shown to stabilize newly formed blood vessels by stabilizing the inter-endothelial contacts and by anchoring the nascent endothelial tubule to basement membrane which results in a better integration of pericytes into the capillary wall [181]. Another study demonstrated that while three-dimensional endothelial tubules cultured in the absence of pericytes showed some expression of basement membrane proteins, expression of collagen type IV, laminin, nidogens, perlecan, and fibronectin was substantially increased when pericytes are present [182]. This enrichment of matrix proteins results from the contribution of both pericytes (nidogen-1; laminin α_4, α_5, β_2, and γ_1 subunits; perlecan) and endothelial cells (fibronectin and laminin α_5). Changes in matrix deposition are associated with corresponding changes in integrin expression in both cell types, resulting in tighter adhesion to matrix and control of lumen diameter and endothelial behavior. Appropriate matrix deposition appears to be critically dependent on both fibronectin and the pericyte expression of TIMP-3 in co-culture, as inhibition of either results in altered collagen IV deposition and loss of tube maturation. Regulation of basement membrane deposition by pericytes appears to play an important role in not just structural support of the vessel but also provides the integrin-mediated cues which help to regulate vessel phenotype.

Mural Cells Enhance Endothelial Tight Junctions/Barrier Activity

The barrier function performed by a normal, mature endothelium results from the formation of tight junctions between adjacent endothelial cells. The abnormal tumor vasculature is hyperpermeable, due to activation of both transcellular and paracellular/intercellular pathways (reviewed in [183]). Increasing evidence suggests that pericytes may participate in regulation of vessel permeability. Pathological ‘mother vessels’, which are notably pericyte poor, are hyperpermeable via transcellular routes of extravasation [127]. In addition, mouse models with vessel maturation defects are reported to be more susceptible to leakiness [184] and/or have altered expression of endothelial tight junction proteins [185], suggesting a role in regulation of intercellular permeability. Studies using membrane co-culture models have demonstrated that mural cells enhance endothelial barrier function, as measured by in vitro permeability and electrical assays [97, 186–188], and heterotypic contact between pericytes and endothelial cells produces an endothelium that is more resistant to hypoxic injury [100]. Part of this may be due to physical location of pericytes, which preferentially situate themselves at endothelial cell junctions and cover gaps between endothelial cells [1]. Secretion of Ang1 by mural cells upregulates endothelial expression of tight junction proteins zona occludens (ZO)-1 and occludin [189, 190], suggesting a molecular mechanism by which mural cells enhance endothelial barrier function. Enhancement of barrier function may also result from pericyte-secreted S1P upregulation of VE-cadherin and downregulation of Ang2 in co-cultures [97]. Some studies suggest that barrier-enhancing effects are not observed in non-contacting co-cultures, leading to the hypothesis that intercellular communication through gap junctions is required for the enhancement of endothelial barrier function by pericytes [100]. Reports demonstrating the interaction of connexins with tight junction proteins ZO-1 and ZO-2 and the ability of ZO-1 to regulate the rate of undocked aggregations of connexins, known as connexons, to assemble into functional gap junctions [191, 192] suggest a potential mechanism by which crosstalk between endothelial cells and pericytes enhances both barrier function and vessel stabilization.

Vessels Lacking Mural Cells are More Susceptible to Tumor Metastasis

Hematogenous metastasis requires that tumor cells invade a blood vessel, spread via the bloodstream to a distal site, and extravasate out of the blood vessel into the new tissue. Several studies suggest that proper association between endothelial cells and mural cells may decrease susceptibility to metastasis. In colorectal cancer, the absence of mural cell coverage on tumor vessels correlates with metastasis [193] . Similarly, in a mouse model of prostate cancer, vessels stabilized by pericytes were less frequently invaded by tumor cells compared to regions with decreased pericyte coverage [194]. In addition, three different knockout animals with phenotypes exhibiting lack of mural cell stabilization of vessels are more susceptible to metastatic invasion [195, 196]. Further, the loss of pericyte-endothelial association observed in tumors overexpressing both bFGF and PDGF-BB is associated with increased metastasis [197], whereas anti-VEGF treatment, which elicits vessel normalization, prevents prostate cancer metastasis in a mouse model [198], suggesting vascular normalization may help prevent metastasis. Interestingly, normal venules display regions with decreased deposition of basement membrane proteins in regions that correspond to gaps between adjacent pericytes, sites which appear to be utilized by transmigrating neutrophils [199, 200]. One may therefore conjecture that the increased susceptibility of destabilized vessels may result, in part, from regions of altered matrix deposition in the basement membrane of tumor vessels.

Vascular Normalization as a Therapeutic Strategy

The dysfunctional tumor vasculature provides multiple barriers to the delivery and response to anti-tumor therapies. Recent work suggests that the use of anti-VEGF therapies may result in what Jain has termed a 'normalization' of the tumor vasculature. Some of the most structurally aberrant vasculature that lacks pericyte investment may be 'pruned' under these conditions [23], which can lead to increased regions of necrosis in the tumor [201]. Interestingly, the remaining vasculature may undergo a transient normalization period characterized by an Ang1-mediated increase in pericyte investiture and an MMP-mediated reduction of the abnormally thickened basement membrane [176]. During this 'normalization window', numerous preclinical models suggest that, due to an increase in vessel perfusion, oxygen to the tumor is increased (reviewed in [202]). Importantly, increased oxygenation allows for more robust response to radiation therapy, and may reduce the hypoxia-driven progression of tumors. The enhanced blood flow and decreased permeability of the 'normalized' vessels also results in a decrease in interstitial fluid pressure [23], resulting in enhanced delivery of systemic therapeutic compounds into the tumor (reviewed in [202]). In the brain, normalizing glioma vasculature via use of anti-VEGFR therapies results in decreased edema [203]. Administration of COMP-Ang1, an Ang1 variant, resulted in vessel normalization and enhanced delivery of 5-fluorouracil to achieve an enhanced therapeutic response in a lung cancer xenograft model [204]. Intriguingly, normalization of the vasculature by anti-angiogenic therapies [205] or in RGS5-knockout mice [206] leads to increased infiltration of cytotoxic leukocytes into the tumor, where they may facilitate immune rejection of the tumor [206]. Together these data suggest that vascular normalization may be a desirable therapeutic goal in the treatment of cancer. Ongoing studies aimed at identifying and optimizing the 'normalization window' should lead to improved strategies to effect tumor control.

Conclusions

Pericytes are receiving increasing recognition for the pivotal role they play in the development and function of the vasculature. While a primary function of the pericyte is to stabilize a vessel and render it quiescent, tumors can activate pericytes to participate in pathological angiogenesis. Both loss of the stabilizing functions of the pericyte and the acquisition of a pro-angiogenic phenotype facilitate the generation of a vasculature that is proliferative, leaky, and dysfunctional. Modulation of the two faces of the pericyte – the "Jekyll" and "Hyde" – may provide a therapeutic opportunity. The 'normalization' of the vasculature in response to anti-VEGF therapies, characterized by restoration of functional pericyte-endothelial associations, reverses the severity of many of the defects of the tumor vasculature. Judicial use of conventional chemo- and radiation therapy concurrent with this 'normalization window' shows promise in enhancing tumor response to therapy. A better understanding of the mechanisms by which tumors subvert pericyte function may lead to identification of means to disrupt this process, leading to a more sustained 'normalization' state that may be exploited to achieve better tumor control.

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