# Endothelial–stromal interactions in angiogenesis

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**Purpose of review**

Angiogenesis often occurs in the context of a wound or tumor stroma. This review will focus on the recent findings on the interactions between angiogenic endothelial cells and the other components of the stroma – fibroblasts, pericytes and extracellular matrix.

**Recent findings**

Large-scale gene expression arrays have provided a remarkable insight into the diversity of fibroblasts in different tissues and under different conditions. These somewhat neglected cells are now understood to play a critical role in tumor growth, regulating not only the phenotype of the tumor cells but also the angiogenic response that supports them. These advances are leading to an understanding of the soil and seed hypothesis at the molecular level. In addition, there is a new focus on the role of pericytes in regulating angiogenesis and their potential as targets for tumor therapy.

**Summary**

Initiation of new blood vessel formation requires metalloproteinases induction leading to the degradation of the basement membrane, sprouting of endothelial cells and regulation of pericyte attachment. Fibroblasts and their activated counterpart, the myofibroblast, play a large role in synchronizing these events through the expression of numerous extracellular matrix molecules, growth factors and morphogens, including fibroblast growth factors and transforming growth factor beta.

**Keywords**

angiogenesis, endothelial, extracellular matrix, myofibroblast, pericyte, tumor stroma

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## Introduction

A concept now gaining wide acceptance is that understanding the nature of the tumor microenvironment is as important in the long-term treatment of cancer as understanding the genetic underpinnings of the cancer itself. Although originally conceived over a century ago as the 'seed and soil' hypothesis (attributed to Stephen Paget [1]), we are only now beginning to understand the complex interplay between tumor cells (the seed) and the stroma, the tissue in which it grows (the soil). In the context of a tumor, stroma is defined as the surrounding extracellular matrix (ECM) and the mesenchymal cells within it including endothelial cells, fibroblasts, vascular smooth muscle cells (VSMC) and pericytes. In 1986, Dvorak [2] made the important observation that tumor stroma looked very much like a wound in that they shared active angiogenesis, numerous proliferating fibroblasts secreting a complex ECM, and all on a background of fibrin deposition. Recently, this observation was given a molecular basis through the global analysis of gene expression in tumors and serum-stimulated fibroblasts [3]. This study reported a strong correlation between the serum response of fibroblasts and gene expression in prostate and hepatocellular carcinoma, and a somewhat more variable correlation between the serum response and gene expression in breast, lung and gastric carcinomas. Many excellent reviews have been published on the role of stroma in promoting tumor growth [4–7], therefore, this review will focus specifically on the role of stroma in supporting angiogenesis, and particularly on the interactions between: endothelial cells, ECM and matrix metalloproteinases (MMPs); endothelial cells and fibroblasts; and endothelial cells and pericytes.

## Interactions between endothelial cells, matrix and metalloproteinases

Nonangiogenic endothelial cells are usually closely apposed to a complex basement membrane containing collagen IV, laminin, entactin/nidogen and perlecan – a heparan sulfate proteoglycan. Laminin exists in multiple isoforms and it is laminin 10 ($\alpha5\beta1\gamma1$) that is particularly enriched in endothelial cell basement membrane, and seems to correlate with quiescence as it is not present in developing, angiogenic vessels [8,9]. Endothelial cells interact with ECM proteins largely through surface-expressed integrins: $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$, all of which bind collagen and laminin; $\alpha_6\beta_1$, which binds laminin; $\alpha_4\beta_1$ and $\alpha_5\beta_1$, both of which bind fibronectin and fibrin;

and $\alpha_v\beta_3$ and $\alpha_v\beta_5$, which variously bind vitronectin, fibronectin, fibrin and laminin [10]. Collagen I and fibronectin are particularly enriched in the interstitium between vessels, and are usually engaged only during angiogenic sprouting. Similarly, fibrin is present only at wound sites and is strongly proangiogenic. Like wounds, tumors are also rich in fibrin. Engagement of integrins with ECM proteins induces two-way signaling: inside-out signaling, which is dependent upon talin, increases integrin affinity for ligand [11]; while outside-in signaling acts through focal adhesions and their associated adaptors and kinases to affect cytoskeletal rearrangements as well as gene expression [12]. Interestingly, *in vitro*, gels composed of either collagen I or fibrin are able to support angiogenic sprouting of endothelial cells, although different integrins are involved in each case – $\alpha_v\beta_3$ and $\alpha_5\beta_1$ in fibrin and $\alpha_2\beta_1$ in collagen [13,14]. Matrigel is a widely used ECM preparation derived from the mouse Engelbreth–Holm–Swarm sarcoma, and is enriched in collagen IV, nidogen and laminin, though the type of isoform has not been reported. Although endothelial cells will align into cords (tubules) within a few hours when plated onto Matrigel, true intercellular lumens are not formed, and the whole process is independent of RNA and protein synthesis. In addition, a number of nonendothelial cells, including fibroblasts and U87-MG glioblastoma cells, also align into similar cords on Matrigel [15].

Degradation and remodeling of the ECM is a critical component of angiogenesis, and MMP expression by each of the mesenchymal cell types in a tumor/wound is necessary. Endothelial cells must degrade the basement membrane to allow migration into the surrounding tissue and further proteolysis is required to make space for lumen formation [16••]. In an in-vitro assay using collagen gels, it was shown that lumen formation proceeds through the generation of intracellular vesicles that then fuse to form a vacuole, or proto-lumen. In turn, vacuoles in adjacent cells fuse, generating intercellular lumens. Knockdown by siRNA of MT1-MMP, but not MT3-MMP blocks lumen formation in this model [17]. An identical process of lumen formation has also been demonstrated during the development of the intersegmental vessels in zebrafish [18], although the role of particular MMPs has not yet been determined. MT1-MMP has been shown *in vivo* to localize to endothelial tip cells – the cells that lead the developing sprout [19,20,21••]. This restricted expression allows for precise matrix degradation at the advancing front, whereas matrix rebuilding can occur more distally when new basement membrane assembly is occurring. Interestingly, tip cell-restricted expression of MT1-MMP required the presence of VSMC; in their absence expression was widespread throughout the vessel. Gene deletion and other studies have also emphasized the important role of MMP2 and MMP9 in angiogenesis, and in particular a role for MMP9 in the angiogenic switch [22]. The activity of MMP2 and MMP9 is also spatially restricted, in this case, through their capture at the cell surface by $\beta_3$ integrin and CD44, respectively [23]. Given the important role of MMPs in angiogenesis and tumor cell mobilization, it is surprising that the numerous MMP inhibitors tested in clinical trials fared so poorly. A likely explanation is that MMPs can also generate antiangiogenic molecules, such as angiostatin and tumstatin, the loss of which could abrogate the positive effects of MMP inhibition [24].

Although an important role of MMPs is in the degradation of ECM, a perhaps equally important role is in the release of bound growth factors, such as vascular endothelial growth factor (VEGF), from the matrix. For example, a fraction of $VEGF_{165}$ in tissue is bound to heparan sulfate proteoglycans through its heparin-binding domain; however, active VEGF can be released through cleavage of the protein by MMP3 or MMP9 [25]. Release of VEGF allows for the growth of larger vessels, whereas a noncleavable form results in thinner vessels with multiple branch points.

MMP activity is controlled by a family of secreted inhibitors, the tissue inhibitors of metalloproteinases, (TIMP) 1–4 [17]. TIMP2 has additional actions in that it is required for the activation of MMP2 by MT1-MMP, and can also signal to the endothelial cells through binding to the laminin receptor $\alpha_3\beta_1$ [26]. This binding inhibits the activity of growth factor receptor tyrosine kinases such as FGFR or VEGFR through the action of the phosphatase Shp-1. In addition, de-novo synthesis of $p27^{Kip1}$ downregulates cdk2 and cdk4, resulting in cell cycle arrest [27].

## Interactions between endothelial cells and fibroblasts

Fibroblasts were originally described over 100 years ago and are still largely defined by their location and what they are not – nonsmooth muscle cells, nonendothelial cells, nonepithelial cells of the stroma. Only fibroblast activation protein appears to be somewhat specific, and this lack of good markers has hampered in-depth investigations. A recent gene analysis study revealed that fibroblasts are quite different cells depending on the tissue from which they are isolated [28], although all have the synthesis and maintenance of ECM as a primary purpose. Interestingly, however, myofibroblasts from multiple different breast carcinomas had similar gene expression profiles [29]. In response to wounding, and in the setting of tumor growth, fibroblasts become activated and highly synthetic, secreting for example, collagen I and IV, fibronectin, heparan sulfate proteoglycans, secreted protein acidic and rich in

cysteine (SPARC), tenascin and connective tissue growth factor (CTGF) [3,30]. These cells are recognized by their expression of α-smooth-muscle actin and are termed myofibroblasts in wound tissue, and have been called carcinoma-associated fibroblasts (CAFs) in tumors [7], where they may comprise up to 80% of the stromal fibroblasts in breast carcinomas [5]. Although tumor cells often secrete VEGF, the major source in most tumors is likely to be the fibroblasts [31]. VEGF, also called vascular permeability factor (VPF), is not only a morphogen for endothelial cells, but also an inducer of vascular leak. The subsequent deposition of fibrin provides a provisional matrix for angiogenic endothelial cells, and also acts as a chemoattractant for circulating endothelial progenitor cells, inflammatory cells, and resident fibroblasts.

TSP-1 is generally thought to be a negative regulator of angiogenesis, however it is released by platelets at fibrin-rich wound sites where angiogenesis is active, and a recent study suggests that it can stimulate VEGF expression by dermal fibroblasts. VEGF then drives the endothelial cell tubulogenesis [32]. In addition, TSP-1 also promotes endothelial cell migration by downregulating endothelial cell expression of PAI-1, a potent inhibitor of fibrinolysis.

Activation of fibroblasts is an important precursor to rapid tumor growth as can be seen in tumor xenograft experiments. When fibroblasts derived from tumor stroma (CAFs) are mixed with tumor cells, such as MCF7 breast carcinoma cells, and then injected into nude mice, they generate larger and faster growing tumors than MCF7 cells do when mixed with fibroblasts from normal, noncancer, tissue [33]. At least some of this effect is due to the expression of SDF-1 by activated fibroblasts, which recruits circulating endothelial precursor cells, thereby promoting tumor angiogenesis; however, fibroblast expression of hepatocyte growth factor, interleukin (IL)-8, SPARC, and transforming growth factor beta (TGFβ) is also likely to contribute [30]. In studies on pancreatic tumor stromal cells it was found that a nontumor-promoting phenotype correlated with low expression of CTGF, and that CTGF could be induced by TGFβ [34]. In admixing experiments, 3T3 fibroblasts expressing CTGF promoted faster growth of pancreatic tumor cells and an increased microvessel density when compared to injections of tumor cells and control 3T3 cells [34]. Fibroblast-expressed CTGF acts as a depot for VEGF that can be rapidly mobilized as a result of CTGF cleavage by either tumor-derived MMP7 [35•] or endothelial cell-derived MMP9 [25]. Fibroblasts also regulate endothelial cell activation and proliferation through expression of syndecans. Syndecan-1 is a heparan sulfate proteoglycan expressed on fibroblasts in more than 70% of human breast carcinomas. Its expression on fibroblasts in a tumor transplant model significantly increased microvessel density and vessel area [36], although the mechanism is not known.

Numerous in-vitro studies have examined the role of mesenchymal cells/fibroblasts in endothelial cell tubulogenesis [37–39], and in all cases their presence has been shown to promote sprouting and, especially, formation of intercellular lumens. A recent study directly examined the necessity for matrix deposition by fibroblasts in the promotion of endothelial cell tubulogenesis using reconstructed connective tissue [37]. It was found that reducing the fibroblast ECM synthesis by removing ascorbate from the medium reduced tube formation 10-fold. There was no reduction in fibroblast or endothelial cell number, and there was no decrease in fibroblast growth factor (FGF)2 synthesis. Remarkably, when the connective tissue was conditioned by fibroblasts, and the fibroblasts were then killed by repeated freeze–thaw cycle, there was still strongly diminished tubulogenesis in freshly added endothelial cells, even in the presence of fibroblast-conditioned medium. The implication is that living fibroblasts need to be in close association with the endothelial cells for tubulogenesis to occur, although it is not clear if the conditioned medium contained sufficient VEGF to promote tubulogenesis.

Earlier studies *in vitro* established a necessary role for fibroblast-derived factors in tubulogenesis [38], and these could be substituted by phorbol 12-myristate 13-acetate [40], suggesting a role for protein kinase C signaling in lumen formation. Recently, it has been reported that endothelial tube formation in fibrin gels is much more strictly dependent on the distance of the endothelial cells from the fibroblasts than the distance of the cells from the culture medium, suggesting that the fibroblast-derived factor(s) is poorly diffusible, either because of size or matrix interactions [41]. Indeed, there is evidence that collagen I synthesis by fibroblasts is a critical component of tube formation in fibrin, although it is not sufficient in the absence of fibroblasts (C.W. Hughes, unpublished observations). Bone marrow-derived mesenchymal stem cells can also support tubulogenesis, and this appears to be dependent on the expression of MT1-MMP [42]. Recent work has shed light on how cross talk between stromal cells regulates MMP induction. In response to VEGF and other angiogenic factors, fibroblasts, endothelial cells and tumor cells upregulate and release extracellular matrix metalloproteinase inducer (EMMPRIN), which not only induces MMP expression by mesenchymal cells but also drives differentiation of fibroblasts to myofibroblasts [43,44].

Finally, recent work has shown that endothelial cells can undergo an endothelial–mesenchymal transition to generate myofibroblast-like cells. In response to TGFβ, endothelial cells downregulate CD31 and upregulate

fibroblast-specific protein-1 [45••]. In-vivo studies using Tie2-CRE-mediated lacZ tagging of endothelial cells confirmed the endothelial origin of these myofibroblast-like cells in tumors.

## Interactions between endothelial cells and pericytes

Pericytes and VSMCs likely emerge from the same cell lineage and both ensheath blood vessels. While pericytes are embedded in the basement membrane and make intimate contacts with the endothelial cells, VSMC in contrast lie largely outside of the basement membrane and make only minimal direct contact with endothelial cells [46]. Similar to fibroblasts, there are few reliable markers for pericytes, although, they do express desmin, α-smooth muscle actin, regulator of G protein signaling 5 (RGS-5) and platelet-derived growth factor receptor (PDGFR)β [47,48]. Pericytes are essential for the stabilization of neovessels, and recent data suggest that this may involve regulation of MMP activity [16••]. In an in-vitro three-dimensional model of endothelial cell–pericyte interaction, cross talk between the cells led to sprout stabilization as a result of induction of TIMP3 in the pericytes and TIMP2 in the endothelial cells. Knockdown of TIMP expression led to vessel regression in a process dependent on MMP1, MMP10 and ADAM15. The major target of TIMP2 and TIMP3 in this assay was MT1-MMP [16••]. Interestingly, at the tip of developing sprouts, pericytes are absent and MT1-MMP expression is high, consistent with the need for matrix degradation at this location [21••]. Blocking Tie2 activity leads to reduced recruitment of VSMC/pericytes to the developing vessels and a consequent spread of MT1-MMP expression to the cells comprising the vessel trunk [21••]. Endothelial cell–pericyte interaction also leads to the activation of latent TGFβ, which subsequently signals to block both endothelial cell and pericyte proliferation.

Several factors have been implicated in the recruitment of pericytes to the vessel wall, including sphingosine-1-phosphate, angiopoietin-1 (Ang-1) and PDGFB [49]. Ang-1 is made by VSMC and pericytes and binds to the Tie-2 receptor on endothelial cells, leading to tight endothelial cell–pericyte interaction and vessel stabilization. Tie-2 signaling induces the expression and release of HB-EGF by endothelial cells, which binds to epidermal growth factor receptors and promotes migration of mesenchymal cells, including pericytes [50]. Ang-2 also binds to Tie2 and can act antagonistically to Ang-1. Systemic overexpression of Ang-2 had the unexpected result of rapidly regressing tumors as a result of pericyte loss and destabilization of the vessels within the tumor [51•]. As the relaxation of the pericyte-endothelial cell interaction is usually a prelude to further rounds of angiogenesis, this complete loss of vessel integrity, in the continued presence of VEGF, is hard to explain. Indeed, when Ang-2 was overexpressed in retina it led to an increase in the density of the vascular network [52], consistent with controlled angiogenesis. Loss of the PDGF pathway, by gene deletion of ligand or PDGFRβ, results in vascular aneurysms and multiple small hemorrhages, as a result of a loss of pericyte coverage [53]. Conversely, expression of a constitutively active form of the receptor PDGFRβ in embryonic stem cells leads to an abundant pericyte vascular coating when the embryonic stem cells are differentiated into embryoid bodies and induced to sprout vessels [54]. Surprisingly, overexpression of PDGFB in colorectal and pancreatic cancer cells led to reduced tumor growth, which was ascribed to an increase in mural cell coverage and increased vascular stability. Indeed, in follow-up experiments, VSMCs were found to decrease endothelial cell proliferation by 50% [55•].

MMP9 is expressed by pericytes and is important for migration of the cells to the vessel wall. In animals deficient for MMP9, vessels growing into transplanted tumors showed a paucity of pericyte coverage, which could be alleviated by transfer of wild-type (MMP9-positive) bone marrow [44]. Defective mural cell recruitment to vessels is also seen in the human disease cerebral autosomal dominant arteiopathy with subcortical infarcts and leukoenceohalopathy (CADASIL), which has been mapped to mutations in the *notch3* gene [46].

## Conclusion

Although endothelial cells have been the primary focus for angiogenesis research it is now clear that they function within a complex network of interactions. Endothelial cells receive activation and stabilization signals from the local matrix environment, with which they interact through integrins and MMPs, and from various mesenchymal cells, which, along with the ECM, comprise the stroma. Local variations, dependent on the tissue type, in the phenotype of the fibroblasts, pericytes, and endothelial cells are not addressed here. An important area for future study is determining the role of pericytes in stabilizing tumor vasculature. In some settings increasing the number of pericytes may stabilize and normalize blood vessels, allowing more effective delivery of tumor cell cytotoxics. Conversely, in other settings, limiting pericyte coverage may lead to destabilization and collapse of the vasculature, thereby promoting tumor regression. Only when we understand all of the tissue-specific cross talk between these components will we be able to rationally and successfully target angiogenesis in those tissues.

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Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
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Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 274–275).


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