

The role of extracellular matrix in vascular branching morphogenesis

Amel Mettouchi

INSERM, U1065; Centre Méditerranéen de Médecine Moléculaire, C3M; Université de Nice-Sophia-Antipolis; Nice, France

Angiogenesis requires the development of a hierarchically branched network of vessels, which undergoes radial expansion and anastomosis to form a close circuit. Branching is achieved by coordinated behavior of endothelial cells that organize into leading “tip” cells and trailing “stalk” cells. Such organization is under control of the Dll4-Notch signaling pathway, which sets a hierarchy in receptiveness of cells to VEGF-A. Recent studies have shed light on a control of the Notch pathway by basement membrane proteins and integrin signaling, disclosing that extracellular matrix exerts active control on vascular branching morphogenesis. We will survey in the present review how extracellular matrix is a multifaceted substrate, which behind a classical structural role hides a powerful conductor function to shape the branching pattern of vessels.

Introduction

Correct development of a functional blood vessel network necessitates coordinated signaling among adjacent cells, in balance with their environment, leading to hierarchical organization of a branched circuitry. Angiogenesis involves complex cellular events comprising sprouting, proliferation, migration, lumen formation, dynamic regulation of cell-cell contacts within endothelial cells; together with the establishment of connections with mural cells. Extracellular matrix (ECM) is known to contribute to the angiogenesis process by multiple ways. ECM is a source of anti-angiogenic peptides (reviewed in refs. 1 and 2), which will tune the angiogenic response in tissues. ECM proteins, via their binding and activation of integrin receptors trigger intracellular signaling pathways that regulate endothelial cell proliferation, survival and migration.^{3,4} ECM-integrin interaction is also crucial for the establishment of endothelial cell polarity and intracellular vacuole formation and coalescence, that are involved in lumen formation.⁵⁻⁷ We will focus in this review on the contribution of the ECM in the establishment of a branched pattern of endothelial tubes.

Correspondence to: Amel Mettouchi; Email: amel.mettouchi@unice.fr
Submitted: 09/17/12; Revised: 11/11/12; Accepted: 11/12/12
<http://dx.doi.org/10.4161/cam.22862>

Cellular Mechanisms Underlying Vessel Branching Out

The establishment of a ramified pattern requires the functional specialization of endothelial cells into “tip” and “stalk” cells, in response to vascular endothelial growth factor-A (VEGF-A).⁸ These cell populations are characterized by distinct phenotypes and positions in the nascent sprout and a hierarchical responsiveness to VEGF-A. Tip cells are distinguished by several features: (1) their leading position in the new vascular branch, (2) a high responsiveness to VEGF-A due to higher expression of VEGFR2, (3) a highly motile phenotype and (4) they extend numerous filopodia that sense the environmental composition in order to guide the outgrowth of the forming vessel toward the VEGF-A gradient and other attractive cues. Stalk cells, which follow the tip cells, have a higher proliferative capacity and therefore constitute the building elements of the vessel branch. They contribute to the sustained elongation of the branch and will establish the vascular lumen.⁸ Tip and stalk phenotypes are not permanent fate determinations but are rather dynamic states. In fact, tip and stalk cells constantly compete with each other and shuffle along the extremity of the growing sprout to occupy the leading position, thus transitioning from stalk to tip and later on back to stalk fates according to their advantage for VEGF-A sensing.^{9,10} An adequate ratio of tip and stalk cell number together with a regulated balance between stalk cell proliferation and tip cell migration are needed to generate an adequately shaped new vascular branch and the appropriate level of branching complexity in the forming network.

Molecular Mechanisms Controlling Tip and Stalk Fates during Endothelial Branching Morphogenesis

Endothelial tip and stalk cell specification is under the control of VEGF-A and Dll4-Notch pathways, which are intricately interconnected. This finding has been firmly established in different contexts such as loss-of-function studies in 3D endothelial cell cultures, tumor angiogenesis, zebrafish and mouse retina developmental angiogenesis and postischemic angiogenesis.¹¹⁻¹⁹ The Notch pathway involves interaction between adjacent cells, one presenting a ligand, either Jagged or delta, and the other exposing a Notch receptor.^{20,21} VEGF-A stimulates the tip cell fate, a response by default in endothelial cells, while Notch restricts this fate by a lateral inhibition mechanism and directs cells toward a stalk cell behavior. The VEGF-A present as a gradient in the angiogenic tissue binds to VEGFR2 receptors at

the surface of endothelial cells. VEGFR2 signaling increases the expression of Dll4, the endothelial specific ligand of Notch receptors, triggering maximal expression at the vascular front in the leading cells.^{16,18} Once exposed at the cell surface, Dll4 will ligate the Notch receptor expressed by adjacent cells and trigger its activation. Activation of Notch involves the proteolytic processing of its intracellular domain, which translocates to the nucleus and controls the expression of target genes.^{20,21} This transcriptional control ends up regulating the level of VEGF receptors, and therefore the capacity to respond to VEGF-A, in the signal-receiving cells. Indeed, Notch stimulation leads to a decrease in VEGFR2 and Dll4 expression^{18,22} and induction of VEGFR1 levels.^{18,23,24} VEGFR1 receptors bind VEGF with high affinity but have poor signaling activity and therefore antagonize VEGFR2 signaling. Such setting allows for the establishment of a hierarchical response to VEGF-A among endothelial cells, the tip cells expressing Dll4 and higher levels of VEGFR2 being highly responsive to VEGF-A and the stalk cells harboring poor attributes for VEGF-A response and expressing low levels of Dll4 ligand. Constant competition between these populations leads to cyclic changes in the balance of Dll4 expression, which drives competitive advantage, and therefore allows for switching in VEGF-A sensitivity and tip cell position throughout the growth of the vessel. Loss of Dll4 expression or Notch activity leads to “non-productive” angiogenesis characterized by anarchic development of the vascular network, which presents ectopic sprouting and excessive branching due to immoderate determination of tip cells and lack of a functional lumen.¹²⁻¹⁸

The emerging picture of regulatory pathways controlling branching processes that take place during sprouting angiogenesis converges on this notion of dynamic positional fate determination. Such determination relies on the setting of a hierarchical response to VEGF-A signals among activated endothelial cells, which is guaranteed by a complex and dynamic cell-cell signaling circuitry orchestrated by the Dll4-Notch couple. We will review here the many ways extracellular matrix influences VEGF-A actions and Notch signaling to modulate the branching pattern of the blood vessel network.

Extracellular Matrix Acts as a Solid-State Regulator of VEGF-A Diffusion and Gradient Shaping

Several studies point for a role of ECM proteins and proteoglycans in the tree-like patterning of vessels that is orchestrated by VEGF-A gradients. VEGF-A is the principal regulator of new blood vessels sprouting during developmental or pathological angiogenesis. VEGF-A is expressed as several isoforms from a single gene by alternative splicing. At least nine isoforms can be produced and the three more common ones differ by the presence of two different domains that confer to the molecule the ability to associate with cell surface heparan sulfate and with heparan sulfate proteoglycans in the extracellular matrix. The VEGF-A121 isoform (VEGF-A120 in mouse) lacks these domains, encoded by exons 6–7 of the *vegfa* gene, and is freely diffusible, VEGF-A189 (VEGF-A188 in mouse) possesses both domains and is found mostly on the cell surface and tightly associated with the

ECM, whereas VEGF-A165 (VEGF-A164 in mouse), which lacks the domain encoded by exon 6 of the gene, has intermediate diffusive properties as it contains one heparin binding domain.²⁵ The combinatorial expression of soluble and heparin binding VEGF-A isoforms allows the establishment of a concentration gradient in tissues, thereby forming a patterning cue for the branching of the nascent vascular network during angiogenesis.^{8,26} Mice expressing solely the VEGF-A120 isoform, lacking both heparin-binding domains, display a marked reduction in vascular branching complexity and harbor in several organs a vascular network with fewer branch points.²⁶ Analysis of the distribution of VEGF-A in these mice showed that VEGF-A120 appears widely dispersed in the tissue in comparison to the steep concentration gradient that is normally formed by VEGF-A isoforms in WT mice, as exemplified at the hindbrain midline region. As a consequence, endothelial cells at the front of the growing vascular network extend fewer and improperly orientated filopodia and form distended tips. These data indicate that deposition of secreted VEGF-A in the ECM is necessary for endothelial tip cells to extend filopodia, which would contact VEGF-A deposits. In an opposite situation, in mice expressing solely the VEGF-A188 isoform, which is sequestered at cell surface and in the surrounding ECM upon its secretion, the vascular plexus appears excessively branched, and tip cells extend numerous filopodia toward multiple directions. Interestingly, double VEGF-A120/188 heterozygotes have no overt vascular branching abnormalities, indicating that the combinatorial expression of a soluble and a heparin-binding VEGF-A isoform is sufficient to induce the formation of a normal branching pattern, even in absence of VEGF-A164.²⁶ This work therefore sets initial evidence that ECM contributes importantly to the branching pattern of angiogenic vessels. This role is achieved primarily by a sharp control of VEGF-A distribution in tissues, that allows correct orientation of tip cell filopodia for the guidance of the tip cell and the extension of a new sprout, rather than exposure of leading tip cells to a specific VEGF-A isoform or concentration.⁸ Interestingly, the proliferation rate of stalk cells is regulated by the local concentration of VEGF-A^{8,26} and it is precisely the coordinated actions of VEGF-A distribution in gradient and its local concentration that regulate a coordinated migratory response of tip cells and proliferative response of stalk cells to produce a correctly patterned vascular network.

A recent work has addressed the dual function of the ECM component fibronectin as a “VEGF-A organizer” and as an adhesion and migration ligand for endothelial cells.²⁷ To discriminate between these functions and determine which contribute to the guiding of sprouting tip cells, retinal angiogenesis was analyzed after compound deletion of astrocytic-produced fibronectin followed by reconstitution with mutant fibronectin, upon crossing the knockouts with a mouse line in which the RGD motif of fibronectin, in the Type III repeat 10, was replaced by RGE.^{27,28} This mutation in fibronectin impairs the site of interaction with α_v and $\alpha_5\beta_1$ integrins, involved in cell-driven fibronectin fibrillogenesis. Fibronectin^{RGE/RGE} mice die early in embryogenesis but assemble apparently normal fibronectin matrix, due to binding of α_v

integrins, but not $\alpha 5\beta 1$, to an alternate GNGRG motif in the Type I repeat 5.²⁸ Retinas deficient in astrocytic fibronectin production display slightly reduced vessel migration, measured by radial expansion of the vascular plexus, and tip cell filopodia appear short and excessively numerous. In addition, branch points were increased by 35.5% compared with control littermates. This indicates that fibronectin promotes tip cell migration capacity and/or persistency, and the orientation and elongation of filopodia. These effects could be due both to direct ligation to endothelial integrins and/or promotion of VEGF-A gradients and subsequent VEGF-VEGFR2 signaling. To test implication of integrins in the fibronectin function, fibronectin^{RGE} was re-expressed from one allele in fibronectin-deficient retinas. In such setting, mice showed only a small reduction in radial expansion of the vascular plexus, identically to astrocyte-specific heterozygous fibronectin knockout. This reveals a dose-dependent migration-promoting function of fibronectin, which would not be mediated by integrin binding to the RGD sequence of fibronectin. Interestingly, close examination of filopodia revealed their misalignment on the mutant fibronectin fibrils, disclosing a critical role of integrins in filopodia adhesion and stabilization on fibronectin matrix. Consistently, in mice lacking endothelial expression of $\alpha 5$ integrin, similar filopodial misalignments were also apparent and radial migration of vessels was also marginally affected.²⁷ To test the contribution of VEGF-A binding in the fibronectin function on retinal vessel patterning, peptides blocking VEGF-A binding to fibronectin²⁹ were injected postnatal in the eyes of wild type mice. This led to a 40% decrease in radial expansion of the vascular plexus over a 24 h period, illustrating the critical role of VEGF-A deposition in the ECM for the patterning of vessels. A similar decrease was obtained upon double deletion of fibronectin and heparan-sulfate proteoglycans in the retina. This suggests that both components of the ECM synergize to bind VEGF-A and support directed migration of tip cells,²⁷ although we cannot exclude that in this extreme setting, critical adhesive ligands are also missing. Altogether, this extensive study by the group of Dr. H. Gerhardt supports the notion that the ECM component fibronectin contributes to the vascular branching pattern, primarily through binding of VEGF-A and promotion of VEGFR2 downstream signaling, and in a more subtle way by allowing filopodia adhesion via $\alpha 5\beta 1$ integrins and therefore their orientation along the astrocyte-fibronectin network and the directionality of the outgrowth of a new sprout.

Altogether, these studies set the importance of ECM proteins and proteoglycans in the tree-like patterning of vessels that is initiated by VEGF-A gradient formation.

Extracellular Matrix Influences VEGF-A Signal Sensing by Endothelial Cells

Several components of the ECM can associate with VEGF-A, like fibronectin, perlecan and other heparan-sulfate proteoglycans, thereby contributing to control vascular branching pattern. A common feature arising from this property is that aside from organizing spatial deposit of the growth factor, association with

ECM ensures a control of VEGF-A bioavailability and modulation of the signal-sensing strength of endothelial cells.

Exons 6 and 7 of VEGF-A have been shown to encode the ECM binding domain.²⁵ As discussed in the above paragraph, affinity for the ECM is important to generate a morphogen VEGF-A gradient, but it also represents a storage mechanism to control its bioavailability. Apart from a regulation of VEGF-A association with ECM via alternative splicing, early in vitro studies have shown that processing of the matrix-bound isoforms by plasmin is required to release biologically active VEGF-A forms, able to induce cell proliferation and vascular permeability.^{30,31} Matrix metalloproteinases (MMPs) have also been implicated in the release of VEGF-A from extracellular matrix stores in vitro and in vivo. Such processing allows the release of the receptor-binding domain from the ECM-binding domain. Hence, VEGF-A fragments released from the matrix by MMPs were shown to promote formation of enlarged vessels, while an uncleavable form promoted the outgrowth of thin vessels with multiple branch points, suggesting a positive effect on tip cell functions.³² Interestingly, these two forms of VEGF-A were found to display different signaling properties.³³ Matrix-bound VEGF-A induces a prolonged phosphorylation of the VEGFR2 receptor, specifically at the Y1214 residue, and subsequently enhances activation of the downstream p38-MAP kinase pathway. The nature of the ECM to which VEGF-A was immobilized appeared to have no incidence, as similar effects were found if VEGF-A was bound to collagen type I, IV, V and VIII. When stimulated with ECM-bound VEGF-A, VEGFR2 was found to co-precipitate with $\beta 1$ integrins. Such regulated interaction of $\beta 1$ integrins with VEGFR2 promotes higher magnitude of VEGFR2 clustering and prolonged internalization, leading to enhanced phosphorylation on Y1214 residue and increased p38 stimulation. This modulation of VEGFR2 responses by ECM-bound VEGF-A is likely to be relevant to the establishment of the tip cell phenotype during the outgrowth of a vascular sprout.³³ Overall, these studies demonstrate that the affinity of VEGF-A for ECM confers specific signaling properties compared with the soluble form, demonstrating that beyond a gradient-shaping role, the ECM has a clear influence on the capacity of signal-receiving cells to respond to the ECM-presented vs. soluble form of VEGF-A and eventually engage different arrays of response. The binding of VEGF-A to fibronectin was mapped to the Heparin-II domain, corresponding to type III repeats 13–14 of the fibronectin molecule.^{34,35} In agreement with the notion of an ECM-promoted action of VEGF-A, the integrin-binding domain within Fn III 9–10 domain and the VEGF-A association domain, when present in the same fibronectin molecule, synergize to enhance VEGF-A-mediated activation of VEGFR2 in vitro.³⁵ Accordingly, in vivo deletion of astrocytic fibronectin or disruption of fibronectin-VEGF-A association leads to reduced VEGFR2 signaling.²⁷ Perlecan, a component of basement membranes, was also found to associate with VEGF-A via its heparan-sulfate side chains in the zebrafish model and to be required for VEGF-A turnover and proper localization of the VEGF-A protein in the embryo. Perlecan morphants display abnormal intersomitic vessels sprouting. The sprouts display reduced protrusive activity; they appear

thin and blunt-ended and often follow abnormal paths and fail to interconnect. Indeed, perlecan potentiates VEGF-A-induced phosphorylation of VEGFR2 in vitro, a function likely involved for correct patterning of the vascular sprouts.^{36,37}

Although the precise mechanisms involved in VEGF-A and extracellular matrix synergy are not very well defined yet, integrins were shown to play a crucial role. When endothelial cells are stimulated with collagen-immobilized VEGF-A, integrin $\beta 1$ coprecipitates with VEGFR2. Treatment of cells with integrin-specific blocking antibodies or genetic deletion of the $\beta 1$ gene attenuate phosphorylation and internalization of VEGFR2 and dampen its downstream signaling to p38 MAPK.³³ Interestingly, stimulation of cells with matrix-bound VEGF-A triggers $\beta 1$ integrin clustering into focal adhesions, suggesting a reverse effect of the growth factor on cell adhesion and/or integrin signaling.³³ Stimulation of endothelial cells plated on vitronectin with VEGF-A 165 was also shown to induce integrin $\alpha v\beta 3$ association with VEGFR2 and stimulation of VEGFR2 phosphorylation, signaling to PI3K and cell migration.³⁸ Several studies have investigated the molecular pathways involved in $\beta 3$ -mediated stimulation of the VEGF-A-VEGFR2 signaling axis. VEGF-A induces phosphorylation of the $\beta 3$ subunit of integrins on tyrosine residues 747 and 759, leading to integrin activation, interaction with VEGFR2 and further stimulation of VEGFR2 phosphorylation. Mutation of these tyrosine residues in $\beta 3$ integrin leads to loss of integrin-VEGFR2 interaction and impaired VEGFR2 phosphorylation, demonstrating the essential role of this phosphorylation event in the synergistic activation of VEGFR2 signaling.³⁹ VEGF-A 165 initiates a signaling pathway leading to a complex mechanism of cross-activation of the growth factor and integrin receptors, that leads to amplification of VEGF-A signaling and enhanced adhesion and migration via the activated integrin. Indeed, VEGF-A binding to VEGFR2 leads to its auto-phosphorylation, association and activation of the Src tyrosine-kinase. Src then also interacts with $\beta 3$ integrin and phosphorylates it on the Y747/759 residues, thereby triggering its conformational activation and its association with VEGFR2. High affinity binding of ECM molecules by $\beta 3$ integrin improves its outside-in signaling, leading to enhanced VEGFR2 phosphorylation.^{40,41}

Altogether, these studies emphasize a role of the ECM as a modulator of vascular branching and patterning, via its versatile roles on VEGF-A immobilization and gradient shaping, on duration of VEGFR2 activation and nature of its downstream signaling pathways, and on the support of a complex system of cross-activation of VEGF and integrin receptors. All these functions are likely involved in the coordinated migratory vs. proliferative response of endothelial cells and in the establishment of a tip cell phenotype with filopodial orientation and extension.

Extracellular Matrix Actively Controls the Process of Tip and Stalk Cell Selection

Apart from an indirect control on branching morphogenesis via modulation of VEGF-A action, recent works have demonstrated that ECM, and in particular laminin 411 (also known as laminin 8), directly signals via $\alpha 2\beta 1$ and $\alpha 6\beta 1$ integrins for specification of

the tip and stalk positional fates.^{42,43} Laminin 411 and laminin 511 (also known as laminin 10) are both vascular laminins.⁴⁴ Fine analysis of their cellular expression pattern in the retinal vessels indicates that laminin 511 (LN511) displays prominent expression in the nascent vascular plexus, while laminin 411 (LN411) harbors restricted expression at the vascular front with the most abundant expression in tip cells.⁴³ Interestingly, mice lacking LN411 or LN511 do not have the same vascular defects. LN511 knockout mice die during embryogenesis, before expression of this laminin is detectable in vascular basement membrane, and a majority of the embryo vasculature is unaffected. LN411 knockout mice have enhanced blood vessel formation, the vasculature being hemorrhagic, dilated and aberrantly branched (reviewed in ref. 44). LN411 knockout mice show increased retinal vessel density and excessive branching, together with increased filopodia extensions, suggesting that LN411 restricts tip cell formation.⁴³ Indeed, retinal vessels of LN411 knockout mice display reduced expression of Dll4 and Notch target genes Hey1, Hey2 and Nrarp. Endothelial cells cultured on LN411 adhere via integrins $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 6\beta 1$, and in the presence of VEGF-A have increased Dll4 and VEGFR2 transcription. LN511, ligated by $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins, but not $\alpha 2\beta 1$ integrin, does not support Dll4 expression. In order to analyze whether LN411-mediated control of the Dll4-Notch pathway involves integrins or alternate functions, retinal angiogenesis was studied in integrin knockout mice. Retinal vessels of integrin $\alpha 2$ or integrin $\alpha 3$ knockouts do not present a hypersprouting reminiscent of LN411 or Dll4 knockout. Interestingly, only the integrin $\beta 1$ knockout presented a hypersprouting phenotype, indicating complex contribution of distinct α - $\beta 1$ integrin heterodimers to mediate LN411 action on Dll4 expression and Notch pathway activation, and thus to regulate the tip/stalk endothelial patterning.⁴³ Mechanical studies in endothelial cells cultured on the prototype laminin 111, which engages $\alpha 2\beta 1$ and $\alpha 6\beta 1$ integrins similarly to LN 411, demonstrate that Dll4 is regulated at the transcriptional level by a cooperating mechanism triggered by these integrins.⁴² None of the other endothelial Notch ligands were regulated by integrins, and other ECM proteins composing the endothelial microenvironment as fibronectin, collagen I or collagen IV were unable to trigger Dll4 expression and Notch pathway activation. Selective knockdown of $\alpha 2$, $\alpha 3$ or $\alpha 6$ subunits shows that Dll4 expression is independent of $\alpha 3$ and requires both $\alpha 2$ and $\alpha 6$ integrins. Integrin $\alpha 2\beta 1$, when stimulated by its laminin ligand, triggers rapid expression of the FoxC2 transcription factor, which is required but not sufficient for Dll4 expression. Integrin $\alpha 6$ on another side triggers a signaling event required for Dll4 transcription that remains to be identified.⁴²

Together, these studies provide the demonstration that laminin 411, a component of the vascular basement membrane (a specialized ECM) triggers specific integrin signaling that controls expression of the Dll4 ligand and activation of the Notch pathway. These original findings provide the novel concept of ECM-mediated control of endothelial tip and stalk fates, and therefore show that ECM controls vascular branching pattern in parallel to canonical VEGF-A action, via integrin signaling.

Interestingly ECM, via its association with the vascular-specific secreted factor epidermal growth factor-like domain 7 (EGFL7), could also exert an indirect control on Notch signaling and tip/stalk fates. Indeed, EGFL7 is secreted by endothelial cells and associates abundantly with the interstitial ECM.⁴⁵ EGFL7 was recently shown to bind and antagonize Notch receptors in primary endothelial cells and in a transgenic mouse model.⁴⁶ EGFL7 overexpression under Tie2 promoter results in partial embryonic lethality accompanied by defects in cardiac morphogenesis, vascular hemorrhages, endothelial aggregates and vessel branching defects. Analysis of postnatal retinal angiogenesis reveals expanded vascular coverage, increased vascular branch points and a 50% elevation of the tip cell filopodia number in the transgenic retina. This retinal hyperangiogenic response is similar to situations where Notch function is disrupted. Accordingly, expression of Hes1, Hey1, Dll4 and NRARP Notch target genes was downregulated by 20 to 30% in the retina of P5 transgenic mice. Yeast two hybrid experiments and co-immunoprecipitation approaches in cells and E12.5 mice embryos show that EGFL7, which contains a putative DSL domain, interacts with NOTCH1 and NOTCH4 receptors. EGFL7 could exert its antagonist role on Notch signaling by binding to the receptor and eventually preventing endogenous Delta/Jagged ligand-Notch receptor interaction.⁴⁶ EGFL7 associates in particular to fibronectin and type I collagen whereas laminin and type IV collagen do not support its deposition.⁴⁵ It is therefore seducing to conceptualize that ECM could control endothelial tip and stalk fates in a complex balanced manner, laminin being able to induce Notch signaling via integrin-mediated Dll4 expression and fibronectin being able to antagonize Notch signaling via EGFL7 presentation.

Extracellular Matrix Provides Mechanical Cues that Control Branching Morphogenesis

Blood hemodynamic forces are known to represent a mechanical signal that contributes to differentiation of the arterio-venous system and patterning of the vessels during angiogenesis. ECM composition and topography influences tissue and organ development by generating physical forces causing cell deformation and reciprocal cellular tensile responses. ECM elasticity was shown for example to regulate mesenchymal stem cell fate.⁴⁷ Force transmission occurs via integrins at focal adhesions, that trigger actomyosin contractility and a subsequent transcriptional program that directs cells toward a specific lineage. Thus, sensing of soft, moderately stiff and rigid matrix by myosin II engages respectively neurogenic, myogenic and osteogenic lineages specification programs.⁴⁷ ECM may therefore contribute to control endothelial cells behavior and influence the branching pattern of vessels as a mechanical cue devoid of chemical specificity. Some examples arise from works based on modeled cultures on 3-D flexible substrates. Interestingly, expression levels of VEGF-A and VEGFR2 are higher in cultures subjected to contracting forces compared with situations where contraction was impaired.^{48,49} As a consequence, VEGFR2 shows distinct spatial patterns of expression, in adequation with substrate

deformations, while the release of mechanical forces is associated with homogenous VEGFR2 expression in cultured cells.⁴⁹ Moreover, cell-ECM generated forces are able to modulate the relative production of VEGF-A isoforms. Contraction was associated with increase in VEGF-A165 and VEGF-A189 mRNA levels, while VEGF-A121 expression remains unchanged. These data suggest that mechanical signaling originated from ECM controls VEGF-A alternative splicing, and that this, together with ECM-binding properties of each isoform, could reinforce the formation of VEGF-A gradients and the shaping of vascular structures in an *in vivo* situation.⁴⁹ The close exploration of mechanisms by which mechanical signals conveyed by ECM control transcription of the VEGFR2 gene revealed a complex regulatory pathway involving balance in the activities of two antagonistic transcription factors, TFII-I and GATA2.⁴⁸ These transcription factors compete with each other for occupancy of a common region in the VEGFR2 promoter, and GATA2 nuclear localization is under control of the p190RhoGAP, which interacts with the transcription factor and retains it in the cytosol. GATA2 stimulates the VEGFR2 promoter whereas TFII-I decreases its activity.⁴⁸ p190RhoGAP itself is controlled by mechanical distortion of the cytoskeleton associated with cell spreading on the ECM.⁵⁰ In the presence of growth factors, a stiffer adhesion substrate favors GATA2 nuclear translocation, while TFII-I nuclear levels are similarly high regardless of ECM stiffness. Thus, on soft matrix, cells respond to TFII-I and therefore express low VEGFR2 levels whereas on stiffer matrix, VEGFR2 expression is turned on by GATA2 action. This complex mechanism controls *in vivo* development and shaping of vessels, as shown in a system of subcutaneous grafting of matrigel plugs of defined elastic modulus and in the mouse retina vasculature, where intra-matrigel or intravitreal injection of TFII-I RNAi results in increased VEGFR2 expression and formation of highly branched and dilated vessels whereas GATA2 knock-down suppresses VEGFR2 expression and leads to decreased vascular network formation.⁴⁸

Endothelial tip cells experience important distortions during the formation of protrusive filopodia structures. Work by Fischer et al.⁵¹ shows that increasing ECM stiffness inhibits the extension of filopodia protrusions. Such inhibition is mediated by increased myosin-II contracting activity at the cell cortex. Indeed, cellular contractility via Rho-ROCK-mediated myosin II activation and ECM stiffness blocks initiation of filopodia structures and provokes retraction of the protrusive structures that successfully initiated. Time-lapse imaging of GFP-myosin-IIB shows that local breaches in cortical myosin density precedes filopodia initiation. Remarkably, creating local inhibition of myosin activity by application of blebbistatin with a microinjection pipet is enough to trigger protrusion and extension of filopodia. Such mechanosensory function of the ECM environment is likely to contribute to new branch extensions, migratory persistence and directionality, all events being important in vascular branching morphogenesis.⁵¹

Through these examples, it appears that the biomechanical properties of the ECM indubitably play an important role in the vascular branching processes.

Concluding Remarks

The shaping of a branched network of vessels requires a coordinated response to VEGF-A, achieved by a fine-tuning of endothelial cell receptiveness via the Notch cell-cell signaling pathway. ECM, by interacting with VEGF-A, modulates its availability, its gradient organization and its signaling properties. Also, by engaging a specific array of integrins, ECM can indirectly signal through the Notch pathway by controlling Dll4 ligand expression. Moreover, ECM mechanically influences cellular tension and cytoskeleton organization, which are key aspects for changes in cell shape and in the transcriptional landscape of cells that take place during sprouting.

As research on ECM contribution in vascular patterning is taking shape these last few years, the information it has generated thus far serves as a fertile ground for the basic challenges and therapeutic opportunities that lie ahead. The sharp comprehension of ECM modes of action will gain from the use of multidisciplinary approaches combining live cell biology and signaling techniques in 3D models of cultures with in vivo

loss-of-function approaches, systematic picturing of the panorama of ECM composition and biomechanical properties in zones of vascular front and plexus together with computational modeling of all these findings to assess their contributing role in the control of tip and stalk positional fates.

Investigating how the integration of multiple signaling networks determines the behavior of endothelial cells and the shaping of the vascular network will clearly be a source of valuable information in the years to come.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The author is supported by grants from the Agence Nationale de la Recherche (ANR A05135AS and ANR 11BSV300401), Association pour la Recherche sur le Cancer (ARC SFI20111203671) and institutional funding from the Centre National de la Recherche Scientifique (CNRS) and the Institut National de la Santé et de la Recherche Médicale (INSERM).

References

- Rhodes JM, Simons M. The extracellular matrix and blood vessel formation: not just a scaffold. *J Cell Mol Med* 2007; 11:176-205; PMID:17488472; <http://dx.doi.org/10.1111/j.1582-4934.2007.00031.x>
- Hynes RO. Cell-matrix adhesion in vascular development. *J Thromb Haemost* 2007; 5(Suppl 1):32-40; PMID:17635706; <http://dx.doi.org/10.1111/j.1538-7836.2007.02569.x>
- Avraamides CJ, Garmy-Susini B, Varner JA. Integrins in angiogenesis and lymphangiogenesis. *Nat Rev Cancer* 2008; 8:604-17; PMID:18497750; <http://dx.doi.org/10.1038/nrc2353>
- Mettouchi A, Meneguzzi G. Distinct roles of beta1 integrins during angiogenesis. *Eur J Cell Biol* 2006; 85:243-7; PMID:16546568; <http://dx.doi.org/10.1016/j.ejcb.2005.09.010>
- Zovein AC, Luque A, Turlo KA, Hofmann JJ, Yee KM, Becker MS, et al. Beta1 integrin establishes endothelial cell polarity and arteriolar lumen formation via a Par3-dependent mechanism. *Dev Cell* 2010; 18:39-51; PMID:20152176; <http://dx.doi.org/10.1016/j.devcel.2009.12.006>
- Davis GE, Bayless KJ. An integrin and Rho GTPase-dependent pinocytic vacuole mechanism controls capillary lumen formation in collagen and fibrin matrices. *Microcirculation* 2003; 10:27-44; PMID:12610662
- Iruela-Arispe ML, Davis GE. Cellular and molecular mechanisms of vascular lumen formation. *Dev Cell* 2009; 16:222-31; PMID:19217424; <http://dx.doi.org/10.1016/j.devcel.2009.01.013>
- Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, et al. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol* 2003; 161:1163-77; PMID:12810700; <http://dx.doi.org/10.1083/jcb.200302047>
- Jakobsson L, Franco CA, Bentley K, Collins RT, Ponsioen B, Aspalter IM, et al. Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. *Nat Cell Biol* 2010; 12:943-53; PMID:20871601; <http://dx.doi.org/10.1038/ncb2103>
- Arima S, Nishiyama K, Ko T, Arima Y, Hakozaki Y, Sugihara K, et al. Angiogenic morphogenesis driven by dynamic and heterogeneous collective endothelial cell movement. *Development* 2011; 138:4763-76; PMID:21965612; <http://dx.doi.org/10.1242/dev.068023>
- Sainson RC, Aoto J, Nakatsu MN, Holderfield M, Conn E, Koller E, et al. Cell-autonomous notch signaling regulates endothelial cell branching and proliferation during vascular tubulogenesis. *FASEB J* 2005; 19:1027-9; PMID:15774577
- Ridgway J, Zhang G, Wu Y, Stawicki S, Liang WC, Chantbery Y, et al. Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. *Nature* 2006; 444:1083-7; PMID:17183323; <http://dx.doi.org/10.1038/nature05313>
- Noguera-Troise I, Daly C, Papadopoulos NJ, Coetsee S, Boland P, Gale NW, et al. Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. *Nature* 2006; 444:1032-7; PMID:17183313; <http://dx.doi.org/10.1038/nature05355>
- Siekman AF, Lawson ND. Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries. *Nature* 2007; 445:781-4; PMID:17259972; <http://dx.doi.org/10.1038/nature05577>
- Leslie JD, Ariza-McNaughton L, Bermange AL, McArdow R, Johnson SL, Lewis J. Endothelial signalling by the Notch ligand Delta-like 4 restricts angiogenesis. *Development* 2007; 134:839-44; PMID:17251261; <http://dx.doi.org/10.1242/dev.003244>
- Lobov IB, Renard RA, Papadopoulos N, Gale NW, Thurston G, Yancopoulos GD, et al. Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. *Proc Natl Acad Sci U S A* 2007; 104:3219-24; PMID:17296940; <http://dx.doi.org/10.1073/pnas.0611206104>
- Hellström M, Phng LK, Hofmann JJ, Wallgard E, Coultas L, Lindblom P, et al. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature* 2007; 445:776-80; PMID:17259973; <http://dx.doi.org/10.1038/nature05571>
- Suchting S, Freitas C, le Noble F, Benedito R, Bréant C, Duarte A, et al. The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. *Proc Natl Acad Sci U S A* 2007; 104:3225-30; PMID:17296941; <http://dx.doi.org/10.1073/pnas.0611177104>
- Al Haj Zen A, Oikawa A, Bazan-Peregrino M, Meloni M, Emanuelli C, Madeddu P. Inhibition of delta-like-4-mediated signaling impairs reparative angiogenesis after ischemia. *Circ Res* 2010; 107:283-93; PMID:20508179; <http://dx.doi.org/10.1161/CIRCRESAHA.110.221663>
- Kopan R, Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell* 2009; 137:216-33; PMID:19379690; <http://dx.doi.org/10.1016/j.cell.2009.03.045>
- Phng LK, Gerhardt H. Angiogenesis: a team effort coordinated by notch. *Dev Cell* 2009; 16:196-208; PMID:19217422; <http://dx.doi.org/10.1016/j.devcel.2009.01.015>
- Williams CK, Li JL, Murga M, Harris AL, Tosato G. Up-regulation of the Notch ligand Delta-like 4 inhibits VEGF-induced endothelial cell function. *Blood* 2006; 107:931-9; PMID:16219802; <http://dx.doi.org/10.1182/blood-2005-03-1000>
- Harrington LS, Sainson RC, Williams CK, Taylor JM, Shi W, Li JL, et al. Regulation of multiple angiogenic pathways by Dll4 and Notch in human umbilical vein endothelial cells. *Microvasc Res* 2008; 75:144-54; PMID:17692341; <http://dx.doi.org/10.1016/j.mvr.2007.06.006>
- Funahashi Y, Shawber CJ, Vorontchikhina M, Sharma A, Outtz HH, Kitajewski J. Notch regulates the angiogenic response via induction of VEGFR-1. *J Angiogenesis Res* 2010; 2:3; PMID:20298529; <http://dx.doi.org/10.1186/2040-2384-2-3>
- Park JE, Keller GA, Ferrara N. The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. *Mol Biol Cell* 1993; 4:1317-26; PMID:8167412
- Ruhrberg C, Gerhardt H, Golding M, Watson R, Ioannidou S, Fujisawa H, et al. Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. *Genes Dev* 2002; 16:2684-98; PMID:12381667; <http://dx.doi.org/10.1101/gad.242002>
- Stenzel D, Lundkvist A, Sauvaget D, Busse M, Graupera M, van der Flier A, et al. Integrin-dependent and -independent functions of astrocytic fibronectin in retinal angiogenesis. *Development* 2011; 138:4451-63; PMID:21880786; <http://dx.doi.org/10.1242/dev.071381>
- Takahashi S, Leiss M, Moser M, Ohashi T, Kitao T, Heckmann D, et al. The RGD motif in fibronectin is essential for development but dispensable for fibril assembly. *J Cell Biol* 2007; 178:167-78; PMID:17591922; <http://dx.doi.org/10.1083/jcb.200703021>

29. Wijelath ES, Murray J, Rahman S, Patel Y, Ishida A, Strand K, et al. Novel vascular endothelial growth factor binding domains of fibronectin enhance vascular endothelial growth factor biological activity. *Circ Res* 2002; 91:25-31; PMID:12114318; <http://dx.doi.org/10.1161/01.RES.0000026420.22406.79>
30. Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N. Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. *J Biol Chem* 1992; 267:26031-7; PMID:1464614
31. Plouët J, Moro F, Bertagnoli S, Coldeboeuf N, Mazarguil H, Clamens S, et al. Extracellular cleavage of the vascular endothelial growth factor 189-amino acid form by urokinase is required for its mitogenic effect. *J Biol Chem* 1997; 272:13390-6; PMID:9148962; <http://dx.doi.org/10.1074/jbc.272.20.13390>
32. Lee S, Jilani SM, Nikolova GV, Carpizo D, Iruela-Arispe ML. Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. *J Cell Biol* 2005; 169:681-91; PMID:15911882; <http://dx.doi.org/10.1083/jcb.200409115>
33. Chen TT, Luque A, Lee S, Anderson SM, Segura T, Iruela-Arispe ML. Anchorage of VEGF to the extracellular matrix conveys differential signaling responses to endothelial cells. *J Cell Biol* 2010; 188:595-609; PMID:20176926; <http://dx.doi.org/10.1083/jcb.200906044>
34. Martino MM, Hubbell JA. The 12th-14th type III repeats of fibronectin function as a highly promiscuous growth factor-binding domain. *FASEB J* 2010; 24:4711-21; PMID:20671107; <http://dx.doi.org/10.1096/fj.09-151282>
35. Wijelath ES, Rahman S, Namekata M, Murray J, Nishimura T, Mostafavi-Pour Z, et al. Heparin-II domain of fibronectin is a vascular endothelial growth factor-binding domain: enhancement of VEGF biological activity by a singular growth factor/matrix protein synergism. *Circ Res* 2006; 99:853-60; PMID:17008606; <http://dx.doi.org/10.1161/01.RES.0000246849.17887.66>
36. Zoeller JJ, McQuillan A, Whitelock J, Ho SY, Iozzo RV. A central function for perlecan in skeletal muscle and cardiovascular development. *J Cell Biol* 2008; 181:381-94; PMID:18426981; <http://dx.doi.org/10.1083/jcb.200708022>
37. Zoeller JJ, Whitelock JM, Iozzo RV. Perlecan regulates developmental angiogenesis by modulating the VEGF-VEGFR2 axis. *Matrix Biol* 2009; 28:284-91; PMID:19422911; <http://dx.doi.org/10.1016/j.matbio.2009.04.010>
38. Soldi R, Mitola S, Strasly M, Defilippi P, Tarone G, Bussolino F. Role of alphavbeta3 integrin in the activation of vascular endothelial growth factor receptor-2. *EMBO J* 1999; 18:882-92; PMID:10022831; <http://dx.doi.org/10.1093/emboj/18.4.882>
39. Mahabeshwar GH, Feng W, Phillips DR, Byzova TV. Integrin signaling is critical for pathological angiogenesis. *J Exp Med* 2006; 203:2495-507; PMID:17030947; <http://dx.doi.org/10.1084/jem.20060807>
40. Mahabeshwar GH, Chen J, Feng W, Somanath PR, Razorenova OV, Byzova TV. Integrin affinity modulation in angiogenesis. *Cell Cycle* 2008; 7:335-47; PMID:18287811; <http://dx.doi.org/10.4161/cc.7.3.5234>
41. Mahabeshwar GH, Feng W, Reddy K, Plow EF, Byzova TV. Mechanisms of integrin-vascular endothelial growth factor receptor cross-activation in angiogenesis. *Circ Res* 2007; 101:570-80; PMID:17641225; <http://dx.doi.org/10.1161/CIRCRESAHA.107.155655>
42. Estrach S, Cailleteau L, Franco CA, Gerhardt H, Stefani C, Lemichez E, et al. Laminin-binding integrins induce Dll4 expression and Notch signaling in endothelial cells. *Circ Res* 2011; 109:172-82; PMID:21474814; <http://dx.doi.org/10.1161/CIRCRESAHA.111.240622>
43. Stenzel D, Franco CA, Estrach S, Mettouchi A, Sauvaget D, Rosewell I, et al. Endothelial basement membrane limits tip cell formation by inducing Dll4/Notch signalling in vivo. *EMBO Rep* 2011; 12:1135-43; PMID:21979816; <http://dx.doi.org/10.1038/embor.2011.194>
44. Hallmann R, Horn N, Selg M, Wendler O, Pausch F, Sorokin LM. Expression and function of laminins in the embryonic and mature vasculature. *Physiol Rev* 2005; 85:979-1000; PMID:15987800; <http://dx.doi.org/10.1152/physrev.00014.2004>
45. Schmidt M, Paes K, De Mazière A, Smyczek T, Yang S, Gray A, et al. EGFL7 regulates the collective migration of endothelial cells by restricting their spatial distribution. *Development* 2007; 134:2913-23; PMID:17626061; <http://dx.doi.org/10.1242/dev.002576>
46. Nichol D, Shawber C, Fitch MJ, Bambino K, Sharma A, Kitajewski J, et al. Impaired angiogenesis and altered Notch signaling in mice overexpressing endothelial Eglf7. *Blood* 2010; 116:6133-43; PMID:20947685; <http://dx.doi.org/10.1182/blood-2010-03-274860>
47. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell* 2006; 126:677-89; PMID:16923388; <http://dx.doi.org/10.1016/j.cell.2006.06.044>
48. Mammoto A, Connor KM, Mammoto T, Yung CW, Huh D, Aderman CM, et al. A mechanosensitive transcriptional mechanism that controls angiogenesis. *Nature* 2009; 457:1103-8; PMID:19242469; <http://dx.doi.org/10.1038/nature07765>
49. Rivron NC, Vrij EJ, Rouwkema J, Le Gac S, van den Berg A, Truckenmüller RK, et al. Tissue deformation spatially modulates VEGF signaling and angiogenesis. *Proc Natl Acad Sci U S A* 2012; 109:6886-91; PMID:22511716; <http://dx.doi.org/10.1073/pnas.1201626109>
50. Mammoto A, Huang S, Ingber DE. Filamin links cell shape and cytoskeletal structure to Rho regulation by controlling accumulation of p190RhoGAP in lipid rafts. *J Cell Sci* 2007; 120:456-67; PMID:17227794; <http://dx.doi.org/10.1242/jcs.03353>
51. Fischer RS, Gardel M, Ma X, Adelstein RS, Waterman CM. Local cortical tension by myosin II guides 3D endothelial cell branching. *Curr Biol* 2009; 19:260-5; PMID:19185493; <http://dx.doi.org/10.1016/j.cub.2008.12.045>