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Crosstalk Between Vascular Endothelial Growth Factor, Notch, and Transforming Growth Factor-β in Vascular Morphogenesis

Notch and Vascular Smooth Muscle Phenotype

Notch Signaling in Cardiac Development

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Crosstalk Between Vascular Endothelial Growth Factor, Notch, and Transforming Growth Factor-β in Vascular Morphogenesis

Matthew T. Holderfield, Christopher C.W. Hughes

Abstract—Vascular morphogenesis encompasses a temporally regulated set of morphological changes that endothelial cells undergo to generate a network of interconnected tubules. Such a complex process inevitably involves multiple cell signaling pathways that must be tightly coordinated in time and space. The formation of a new capillary involves endothelial cell activation, migration, alignment, proliferation, tube formation, branching, anastomosis, and maturation of intercellular junctions and the surrounding basement membrane. Each of these stages is either known or suspected to fall under the influence of the vascular endothelial growth factor, notch, and transforming growth factor-β/bone morphogenetic protein signaling pathways. Vascular endothelial growth factor is essential for initiation of angiogenic sprouting, and also regulates migration of capillary tip cells, proliferation of trunk cells, and gene expression in both. Notch has been implicated in the regulation of cell fate decisions in the vasculature, especially the choice between arterial and venular endothelial cells, and between tip and trunk cell phenotype. Transforming growth factor-β regulates cell migration and proliferation, as well as matrix synthesis. In this review, we emphasize how crosstalk between these pathways is essential for proper patterning of the vasculature and offer a transcriptional oscillator model to explain how these pathways might interact to generate new tip cells during retinal angiogenesis. **(*Circ Res*. 2008;102:637-652.)**

Key Words: angiogenesis ■ endothelial cell differentiation ■ transcriptional regulation ■ vascular endothelial growth factor ■ vascular endothelial growth factor receptors

The vascular system is dynamic in that it constantly responds to metabolic, immunologic, and growth demands. Because tissues expand during organismal growth, or in response to higher metabolic demands, a state of hypoxia may develop in tissues as growth outstrips the available supply of oxygen. In response, those tissues release vascular endothelial growth factor (VEGF), which then triggers the activation of local endothelial cells (ECs) and initiates the angiogenic response, thus restoring an adequate blood supply and returning local oxygen tension to an acceptable level.[1] Angiogenesis, which proceeds with input from several signaling pathways, including transforming growth factor (TGF)β and notch, is a cascade of events that results in the generation of new blood vessels that anastomose to provide a new vascular supply to that tissue. The coordinated events of EC activation, proliferation, migration, alignment, tube formation, branching, and anastomosis are here collectively termed vascular morphogenesis.[2] Formation of a mature

Original received November 19, 2007; revision received January 11, 2008; accepted February 7, 2008.

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Circulation Research **is available at http://circres.ahajournals.org** **DOI: 10.1161/CIRCRESAHA.107.167171**

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blood vessel from these immature capillaries may also include development of a larger diameter vessel as the result of further EC proliferation,[3] the construction of a permanent basement membrane (the structure of which can differ in different vascular beds)[4] and the recruitment of perivascular cells, such as pericytes and smooth muscle cells. However, these later events are not the focus of this review; rather, we focus on the early events that induce and pattern new and remodeling vasculature.

By considering vascular morphogenesis as a series of connected, but overlapping, events, it becomes clear that a strict temporal and spatial regulation of cell signaling pathways and downstream gene expression are required within a developing vessel for proper assembly to occur. Thus, at different times within the same vessel, ECs may be required to migrate or remain stationary, proliferate, or become quiescent, extend filopodia or retract them, and form or not form lumens. In this review, we first discuss the individual roles that 3 major signaling pathways (VEGF, notch, and TGFβ) have in vascular morphogenesis, and then we review how they act coordinately in space and time to regulate this process.

Overview of Vascular Morphogenesis

Vascular morphogenesis/angiogenesis in adult tissues may differ in important ways from the similar process in development. For example, vessels in the adult are contained within a substantial basement membrane, whereas in development, the basement membrane is at best thin or even absent.[4] Thus, in development degradation of basement membrane before sprouting is not limiting, whereas in adult, this is an important initiating event. In adult, angiogenesis often occurs in the context of inflammation and coordination of these 2 events is likely to be critical. Furthermore, it is possible that different members of a given family of angiogenic regulators play the same role in different contexts: for example, we have evidence that jagged-1 plays a similar role in inflammatory angiogenesis as dll4 does during development (Sainson RCA, Johnston DA, Chu HC, Holderfield MT, Nakatsu MN, Crampton SP, Davis J, Conn E, and Hughes CCW, submitted manuscript, 2008). In all settings, however, a critical early step is the selection of a tip cell: the first cell to emerge from the parent vessel and the cell destined to lead the sprout into the tissue and anastomose with other vessels to complete a vascular loop.

Much of our present understanding of angiogenic sprouting at the cellular level derives from 4 model systems: rabbit cornea,[5] the developing mouse retina,[6] intersegmental vessel (ISV) growth in zebrafish,[7] and in vitro modeling using ECs embedded in collagen or fibrin gels.[8,9] In the normally avascular cornea, new vessels can be induced to grow by transplantation of tumor tissue[5] or by implantation of growth factor-containing pellets. Sprouting vessels are led by a tip cell that emerges at approximately day 4. Once capillary loops have formed, these then extend toward the tumor. In mouse embryos, the retina is avascular, but soon after birth vessel growth initiates at the center, around the head of the optic nerve and proceeds outward as a 2D planar network following a gradient of VEGF laid down by astrocytes migrating ahead of the nascent vasculature. The planar nature of the vascular network greatly facilitates the visualization of angiogenic sprouting.[6] Sprouts are composed of a tip cell and 1 to 2 trailing trunk cells. Once this sprout anastomoses to form a loop, a new tip cell may emerge to continue the outward expansion of the plexus.[6]

In zebrafish, the ISVs stereotypically sprout from the dorsal aorta and extend between the somites, meeting on the dorsal side where they then anastomose to form the dorsal longitudinal anastomotic vessel (DLAV). The ISV is usually composed of 3 to 4 cells: a base cell that connects to the dorsal aorta, 1 to 2 connector cells that extend across the width of the somite, and a dorsally positioned T-shaped cell that connects to the DLAV. Branching within the somites is never seen.[10] Because the embryos are transparent, vessel growth can be followed by time-lapse videomicroscopy and transgenic lines carrying vascular-specific green fluorescent protein have aided in the tracking of vessels as they grow.[7] In vitro assays provide a unique opportunity to study sprouting as the ECs are readily manipulated genetically and can be easily harvested for analysis of gene expression.[9,11–13] Sprouting of ECs in fibrin gels models pathological and wound-healing angiogenesis,[9] whereas sprouting in collagen gels may model developmental and physiological angiogenesis, where collagen is more prevalent.[14] Recent findings in both of these systems suggest that the basic processes of sprout initiation, migration, proliferation, and tube formation involve the same sets of genes and the same morphogenetic processes in vitro as they do in vivo.[8,10,15–23]

Tip cells in all of these models have a similar and distinct phenotype: they are migratory and express numerous filopodia and lamellipodia that interrogate the surrounding tissue. They do not form lumens and only in certain tissues, such as the developing DLAV in zebrafish, are they ever seen undergoing division.[24] It has been suggested that ECs can undergo a form of epithelial-to-mesenchymal transition (EMT)[25]; however, tip cells never fully separate from the trailing cell and VE-cadherin–positive adherens junctions remain intact (Sainson RCA, Hughes CCW, unpublished observations, 2005). Tip cells do, however, lose the strict polarity that they had when part of the vessel wall, because once they have sprouted, they no longer exhibit a luminal face. Tip cells can also be distinguished by their gene expression profile, showing enriched expression of VEGFR2,[15,23] platelet-derived growth factor (PDGF)B,[15,16,23] Unc5b,[23,26] dll4,[16,19,23] and jagged-1 (Sainson RCA, Johnston DA, Chu HC, Holderfield MT, Nakatsu MN, Crampton SP, Davis J, Conn E, and Hughes CCW, submitted manuscript, 2008).

Trailing the tip cell is a trunk cell. These have fewer and shorter filopodia and are often seen undergoing division, and, most distinctively, they form lumens and are thus fully polarized.[15,17,22] Several mechanisms have been proposed for lumen formation including cord hollowing and intracellular vacuole formation and fusion.[27,28] At the root of any mechanism of tubulogenesis is the establishment of cell polarity: the defining of an apical and basal face of the endothelium. Recently, elegant work in vitro and in zebrafish has provided strong support for both of these models.[8,17] Using time-lapse

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videomicroscopy, it was shown that ECs in 3D cultures form large intracellular vacuoles by fusion of pinocytotic vesicles in an integrin and cdc42/rac1-dependent process.[8] The vacuoles of adjacent cells then fuse to form an intercellular lumen. By following the movement of quantum dots injected into the bloodstream of zebrafish, it was then shown that a similar process of pinocytosis followed by vacuole fusion occurs during sprouting of the intersegmental vessels from the dorsal aorta.[17] The resolution of sprouting includes the maturation of complex junctions and the modification of the provisional basement membrane to form a mature, laminin-rich structure. EGFL7 has also been suggested to regulate EC lumen formation; however, its role may be more indirect than originally suggested, involving chemoattraction[29] and promotion of EC–matrix crosstalk.[30]

Role of VEGF in Vascular Morphogenesis

There are 4 members of the VEGF gene family in mice and humans (VEGF-A to -D) and a related protein, placental growth factor (PlGF).[1] They are homodimeric, secreted glycoproteins whose precise regulation is essential for correct specification, assembly, and patterning of the vasculature. Indeed, loss of even a single allele of VEGF-A results in embryonic lethality.[31] VEGF-A is expressed in several isoforms by alternative splicing, which differ by the presence or absence of two domains with affinity for heparin and heparan sulfate proteoglycans. VEGF121 (VEGF120 in mice) lacks both heparin-binding domains and is freely diffusible, generating a shallow gradient away from the source.[32] VEGF189 (VEGF188 in mice) has both heparan sulfate–binding domains and binds tightly to extracellular matrix (ECM) and the cell surface and, therefore, generates a very steep gradient. VEGF165 (VEGF164 in mice) has a single heparin-binding domain, has intermediate affinity for matrix, and generates a gradient of intermediate steepness. The importance of gradient shape was elegantly shown by generating mice expressing only a single VEGF isoform.[32–34] Mice expressing only VEGF164 are viable and show no obvious vascular defects. Conversely, mice expressing only VEGF120 display postnatal angiogenesis defects in several organs. A comprehensive analysis of the VEGF120 mice, focusing on the developing subventricular plexus revealed a deficit in vascular branching and an increase in lumen diameter.[32] Mice expressing the nondiffusible VEGF188 isoform, on the other hand, showed a complementary defect with ectopic branching and unusually thin vessels.[32] In-breeding to generate mice expressing VEGF120 and VEGF188, but not VEGF164, abrogated both of these phenotypes and generated normal mice. As predicted, the VEGF gradients in the VEGF120 mice were quite shallow compared with wild type.[32] And consistent with this, expression of VEGF120 in the retina is sufficient to drive EC proliferation but not to guide tip cells efficiently.[15] Importantly, however, VEGF121 and VEGF165 are equally potent at driving EC proliferation in vitro, suggesting that they signal similarly.[3] This implies that it is the gradient shape that conveys a branching signal to the ECs, rather than qualitative differences in signaling. Indeed, in tissue culture in which ECs are provided VEGF, but not in a gradient, sprouts are long (up to 1000 μm) and show relatively minimal branching compared with, for example, developing retina.[9,22]

The role of matrix-binding, and specifically heparan sulfate–binding, growth factors in the patterning of branching structures has been noted in other systems. For example, in drosophila, branching of the tubular tracheal system is regulated by binding of the fibroblast growth factor (FGF)-like molecule branchless to a receptor tyrosine kinase (RTK) called breathless.[35] The activity of breathless in this context is also dependent on the genes sugarless and sulfateless, both of which regulate heparan sulfate biosynthesis.[36] In tumors, the expression of matrix-degrading matrix metalloproteinases (MMPs) can also alter the VEGF gradient. Cleavage of matrix-bound VEGF to produce a soluble form resulted in dilation of vessels but no neoangiogenesis, whereas cleavage-resistant forms induced extensive sprouting of long thin vessels.[37] These findings are reminiscent of those obtained from the subventricular plexus and described above.[32]

The importance of a VEGF gradient has been further elucidated in the retinal model in which a gradient is required for the generation and polarization of tip cell filopodia.[15] In this setting, expression of the major VEGF receptor VEGFR2 (KDR/Flk1) is enriched in tip cells and specifically in a punctate pattern along the filopodia.[15] Engagement of VEGFR2, but not VEGFR1, on tip cells promotes cell migration toward the source of VEGF but not cell proliferation. In contrast, VEGF signaling to trunk cells promotes proliferation. The importance of the VEGF gradient in extension of tip cell filopodia was apparent in VEGF120 mice, in which flattening of the VEGF gradient emanating from the astrocytes resulted in filopodia that were fewer in number, shorter, and also misdirected out of the plane of the vascular network. These data have been interpreted in the context of a model in which tip cells respond to the gradient of VEGF by extending filopodia and migrating, whereas trunk cells respond to the local concentration of VEGF by proliferating.[15] An alternative way of putting this is that the combination of a higher affinity receptor complex found in tip cells, as the result of the coexpression of VEGFR2 and neuropilin-1 (Nrp1),[15] along with the higher concentration of VEGF at the tip, acts to drive a signaling pathway downstream of the receptor that favors migration. In trunk cells, on the other hand, the combination of a lower-affinity receptor (VEGFR2 in the absence of Nrp1) and a lower concentration of VEGF results in activation of the proliferative pathway. Although the presence of Nrp1 in tip cells will help to functionally sharpen the gradient over the length of the cell, the mechanism underlying the switch in response to higher or lower concentrations of VEGF is not known. Perhaps somewhat perplexingly, studies in vivo have implied, and in vitro work has confirmed, that the diameter of vessels is set, at least in part, by a graded proliferative response of ECs to the local concentration of VEGF.[3,33,38–40] Once the lumen of a new vessel has formed, VEGF-driven proliferation of ECs can result in circumferential growth and enlargement of the lumen[3] or lengthening of the vessel.[41] So again, how the cells select a response that matches the local VEGF concentration is not known.

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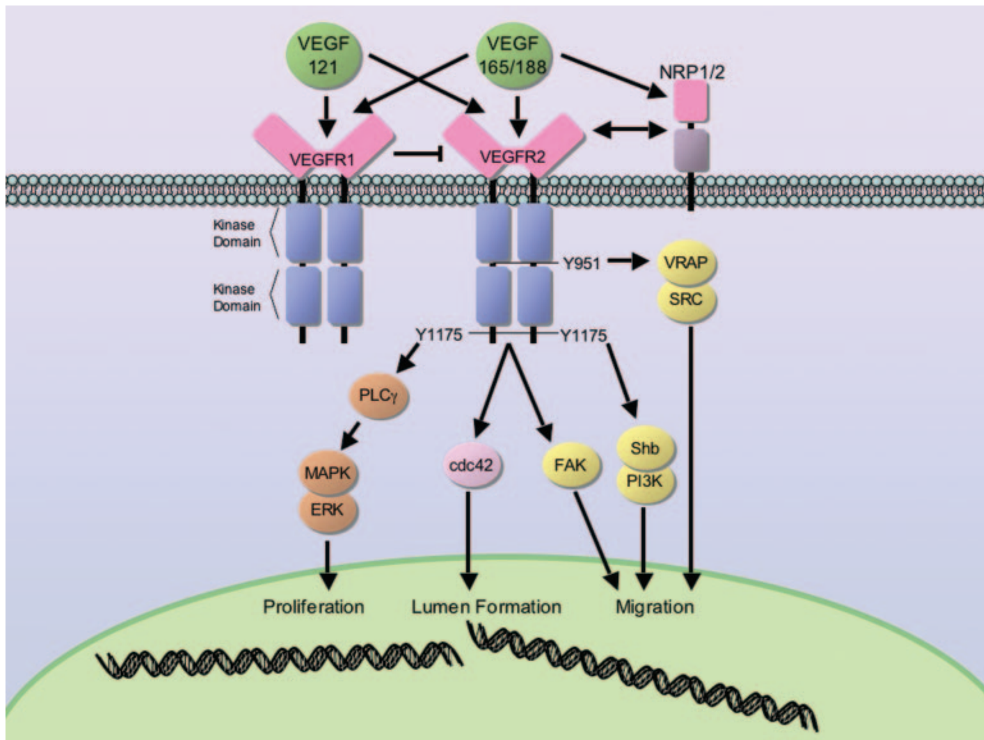

Figure 1. VEGF signaling in angiogenic ECs. VEGFR1 (Flt1) and VEGFR2 (Flk1/KDR) represent the 2 major VEGFRs on angiogenic ECs. VEGF121 binds to VEGFR1 and VEGFR2, whereas VEGF165 and VEGF188 also bind to the coreceptor neuropilin-1. Signaling downstream of VEGFR2 is complex, and only selected pathways are shown. VEGF drives EC migration, proliferation, and morphogenesis and gene expression. See the text for details.

Some insight can be gained into the complexities of VEGFR signaling during sprouting angiogenesis by the study of migrating and proliferating ECs in simple 2D culture. The VEGFRs are members of the receptor tyrosine kinase family and related to FGF receptor and PDGF receptor (PDGFR) (Figure 1). VEGFR2 binds VEGF-A, and the presence of Nrp1 or Nrp2 enhances VEGFR2 signaling in response to VEGF165 but not to VEGF121.[42] Enhanced proliferation downstream of VEGFR2 is dependent on binding of phospholipase Cγ to phosphorylated Tyr1175 (Tyr1173 in mice) and subsequent activation of the MAPK/ERK cascade.[43] Migration, on the other hand, seems to be mediated by several pathways. The adapter molecule Shb also binds to Tyr1175 and stimulates migration through phosphatidylinositol 3-kinase (PI3K).[44] VEGFR2-dependent migration is additionally stimulated through src, by VEGFR-associated protein (VRAP) binding to Tyr951 (Tyr949 in mice). Finally, VEGFR2 also interacts with focal adhesion kinase (FAK), which is essential for the turnover of focal adhesions.[45] VEGF has been reported to regulate actin remodeling through cdc42, which is known to have a critical role in lumen formation,[8] thus implicating VEGF signaling in this process. Whether these pathways are triggered simultaneously, sequentially, or independently in response to a specific state of the cell is not known. A comprehensive review of VEGFR signaling was recently published.[1]

In addition to its role in driving the basic processes of angiogenesis (EC migration, proliferation, tube formation, and vessel branching), VEGF is also implicated in arteriovenous specification. A detailed analysis of this process is beyond the scope of this review; however, the basic principals are that VEGF signaling through VEGFR2/Nrp1 induces notch and notch ligands such as delta4 (dll4), through a phospholipase Cγ–mitogen-activated protein kinase (MAPK) kinase/extracellular signal-regulated kinase (PLCγ–MEK/ERK) pathway, possibly via activation of the transcription factors Foxc1 and Foxc2.[46,47] Notch signaling then induces expression of the arterial marker ephrin B2 and suppresses expression of the venous marker EphB4.[48,49] In cells destined to form veins, COUP-TFII suppresses Nrp1 expression and reduces VEGFR2 signaling.[50] Phosphatidylinositol 3-kinase/Akt activation downstream of VEGFR2 suppresses the MEK/ERK pathway, thereby reducing expression of the arterial marker ephrin B2 and promoting a venous phenotype. Presumably, once arterial identity has been established, epigenetic changes at the ephrin B2 locus replace the need for further signaling through the MEK/ERK pathway.

Although the role of VEGFR2 in vascular development is clear, our understanding of the part played by VEGFR1 is still cloudy. It has tyrosine kinase activity and is phosphorylated in response to VEGF, although only at low levels. Mice lacking VEGFR1 die at embryonic day (E)8.5 to E9.0 as a result of EC overgrowth subsequent to hemangioblast overcommitment.[51–53] However, remarkably, these mice can be rescued by expression of a truncated VEGFR1 lacking the intracellular tyrosine kinase domain, suggesting that during development, the receptor acts as a sink for VEGF-A, regulating its availability for VEGFR2.[54] During pathological angiogenesis, VEGFR1 regulates EC migration but not proliferation.[55]

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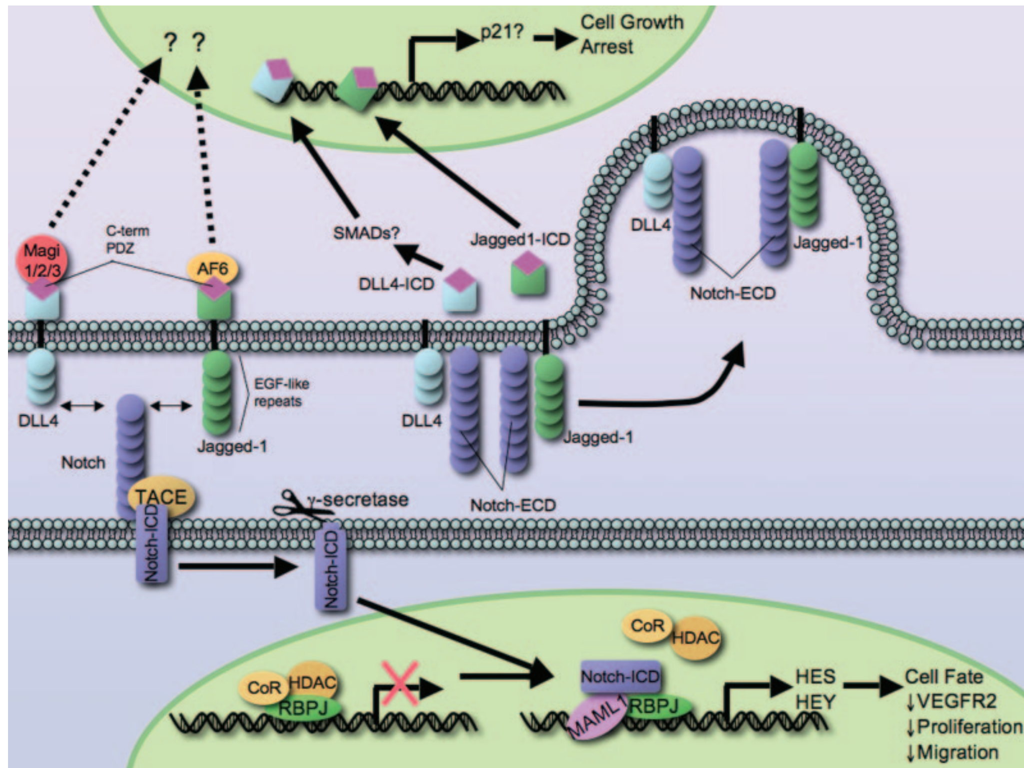

Figure 2. Notch–notch ligand signaling in angiogenic ECs. Interaction of notch with one of its ligands on an adjoining cell triggers the release of the NICD, which moves to the nucleus and drives transcription of target genes through the displacement of transcriptional repressors. Notch ligands potentiate notch signaling by internalizing the extracellular portion of notch, presumably making way for notch-processing enzymes to complete the cleavage that releases the NICD. Increasing evidence suggests that notch ligand intracellular domains may also "reverse" signal, either through interactions with scaffolding proteins such as AF6 and the MAGI proteins, all of which localize to adherens junctions (data not shown) or through direct effects on transcription. Notch signaling affects cell fate decisions, cell proliferation, and gene expression. See the text for details.

Finally, we should also note that VEGF was first identified as vascular permeability factor (VPF),[56] and this function may well have an important role in angiogenesis because leak of plasma proteins such as fibrinogen into the surrounding tissue can contribute to the formation of a provisional matrix capable of supporting angiogenic sprouting.

Role of Notch in Vascular Morphogenesis

Notch receptors are large transmembrane proteins that when activated by their ligands expressed on adjacent cells, regulate cell fate decisions in multiple lineages.[57–60] The notch signaling pathway is evolutionarily conserved and, in addition to mediating binary cell fate decisions, also controls establishment of tissue boundaries and cell proliferation.[57,58] There are 4 notch genes in mammals, notch1 to -4, and all are expressed as proteolytically cleaved, noncovalently associated heterodimers. There are 5 notch ligands in mammals: jagged-1 and jagged-2 and delta-like-1 (dll1), dll3, and dll4. When engaged by a ligand, the notch receptors undergo further proteolytic processing, which releases the notch intracellular domain (notch ICD or NICD). The final cleavage step is mediated by the γ-secretase complex.[57,60] NICD then translocates to the nucleus, where it complexes with the DNA-binding protein RBP-J and mediates the recruitment of histone acetylases and the transcriptional coactivator mastermind-like (MAML).[61] The best-characterized notch targets are members of the Hairy and enhancer-of-split (HES), and Hairy and enhancer-of-split-related (HEY, HESR, HRT, or CHF) gene families.[62–67] These basic helix–loop–helix (bHLH) proteins act largely as transcriptional repressors, either by direct binding to E- and N-boxes and recruitment of corepressors such as groucho (TLE in mammals) or by mechanisms that are independent of direct DNA binding.[63,68] A schematic of notch signaling is shown in Figure 2.

Notch4 and dll4 are largely endothelial-specific, and expression is seen in arteries and capillaries but not veins.[69–71] Notch 1 is expressed in many tissues, including the vasculature, where its expression is also largely restricted to arteries and capillaries.[71] An important role for notch in vascular development has emerged as each of the notch and notch ligand genes have been knocked out.[72] Notch1-deficient mice die at E10.5 because of a failure of cardiovascular development.[73] Specifically, the primary vascular plexi in the yolk sac and brain fail to remodel and large vessels are disorganized and smaller than wild type.[73] A vascular-specific knockout of notch1 has been made,[74] and this completely recapitulates the phenotype seen with global loss of notch1, indicating that notch signaling is specifically required in the ECs during vascular development. Similar conclusions of a cell autonomous requirement for EC notch1 in sprouting angiogenesis have also been reached in an in vitro system.[22] The loss of even a single allele of dll4 results in embryonic lethality at E9.5,[75–77] although there is variability in survival

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times in different mouse strain backgrounds.[75] Again, there is a failure to remodel the primary vascular plexus, a phenotype also seen in jagged1-deficient mice,[78] and RBP-J–deficient mice.[77] Overexpression of notch1 ICD or notch4 ICD blocks angiogenic sprouting,[67,79] as does expression of the notch target gene HESR1 (HEY1).[64,67] Consistent with these findings, knockout of the HEY genes also results in cardiovascular defects, with problems in vascular remodeling, arteriovenous specification, septation, and cushion formation[80,81]: a phenotype that closely resembles that of notch1 mutants.

Consistent with the restricted expression pattern of notch family members,[71,82] notch signaling has been shown to play a critical role in arteriovenous specification (see above), acting downstream of VEGF to regulate the expression of the arterial marker ephrin B2.[48] Both the notch1- and dll4-deficient mice show defects in arterial specification, as do mice lacking both HEY1 and HEY2.[80] An inducible system was used in mice to express activated notch4 in the vasculature, resulting in arteriovenous shunting and blood vessel enlargement, which led to lethality within a few weeks.[83] Remarkably, the defects were reversible when expression of the notch4 ICD was shut off.

Notch is clearly used reiteratively during development; it is required for arteriovenous specification and is necessary for remodeling of the early vascular plexus in multiple tissues, although the mechanism underlying the latter process has not been determined.[72] Recent work has also shown a role for notch signaling in the specification of tip cells. The first indication that notch signaling may be acting at this level came from in vitro studies in our laboratory.[22] We used a 3D fibrin gel model in which human ECs were induced to sprout in response to VEGF, basic FGF (bFGF), and undefined factors derived from myofibroblasts.[9] When notch signaling was blocked in this system, ectopic sprouting was observed with a consequent increase in tip cell number.[22] Notably, sprouts were seen to bifurcate at the tip, and 5-bromodeoxyuridine staining revealed that this was attributable to tip cell proliferation. Normally, division of tip cells is rare, proliferating ECs being restricted to the trunk of the vessel,[15,22] but in the absence of notch signaling, tip cell division was common. Conversely, when notch signaling was stimulated, branching and tip cell number were reduced. These data suggest that notch–notch ligand interaction is necessary for the maintenance of a single tip cell in this model. An additional phenotype was also apparent, namely an increase in vessel circumferential growth as a result of excess trunk cell proliferation, suggesting, again, that notch acts to suppress proliferation.[22] Given that notch and notch ligands are not only expressed in angiogenic capillary ECs but are also highly expressed in arteries, it is possible they play a similar role there in maintaining quiescence and suppressing branching.

Recently, these findings in vitro have been confirmed in several in vivo studies in retina,[16,19,23] in zebrafish,[10,18] and in transplantable mouse tumors.[20,21] For example, in dll4$^{+/-}$ mice, retinal vasculature is severely disrupted.[16,19,23] There is an increase in the number of tip cells and an increased expression of tip cell markers such as PDGF-B and Unc5b.[16,23] The increase in tip cells in these mice is at least partially attributable to an increase in proliferation of tip cells, as shown by 5-bromodeoxyuridine staining. When mice with floxed alleles of notch1 were crossed with VECad-CreER^{T2}/R26R mice, there were variable degrees of recombination, resulting in mice where only a subset of cells deleted notch1 (observed by LacZ staining). A disproportionately high percentage of these notch$^{-/-}$ cells were found at the tips of developing sprouts, providing further evidence that notch signaling suppresses the tip cell phenotype.[16] Development of arteries and veins in the retina appeared to proceed normally in the absence of dll4-notch signaling.[16] Similar findings were obtained using blocking antibodies to dll4,[19] a dll4-Fc fusion protein,[19] or a γ-secretase inhibitor,[16,23] again confirming the necessity of notch signaling in the ECs themselves. Consistent with all of these findings, activating notch signaling with a specific jagged-1 peptide decreased the number of tip cells and reduced branching.[16]

In developing zebrafish, dll4 transcripts were expressed in the dorsal aorta and ISV but not in the posterior cardinal vein.[18] Morpholino-mediated knockdown of dll4 in zebrafish resulted in enhanced filopodial extension and unchecked migration of the ISVs.[18] Instead of the normal single ISV connecting the dorsal aorta and the dorsal longitudinal anastomotic vessel (DLAV), the ISV branched several times to give an interconnected network of vessels. This increased branching could be blocked by inhibition of VEGFR signaling, suggesting that dll4 normally acts to regulate the EC response to VEGF. Similarly, loss of RBP-J in zebrafish produces excessive sprouting of segmental arteries, whereas activation of notch suppresses angiogenesis. Moreover, using mosaic analysis, it was found that cells with reduced notch signaling (RBP-J$^{-/-}$) preferentially localized to the tips of developing vessels, whereas cells with active notch signaling were specifically excluded from this location,[10] in agreement with the findings in retina.[16] Again, consistent with the findings in mice, there were no apparent defects in vessel specification.[18] Thus, in all of these studies (in mouse retinas, in zebrafish intersegmental arteries, and in an in vitro angiogenesis assay), the findings are congruent: notch normally acts in developing vessels to suppress filopodial extension, limit the number of tip cells, and limit proliferation in the trunk. Blocking notch signaling, by multiple approaches, leads to an excess of tip cells and branching, along with larger diameter vessels as a result of trunk cell proliferation.

Somewhat surprisingly, these findings are not easily reconciled mechanistically with the notch and notch ligand expression patterns that have been reported for retina and zebrafish.[10,15,16,18,84] Whereas dll4 expression is enriched in some tip cells in retina, others lack expression. In zebrafish, dll4 is not consistently enriched in tip cells at all.[18] Likewise, dll4 is expressed in some trunk cells but not others. Staining with an antibody specific for cleaved (activated) notch1 reveals sporadic and seemingly random expression of NICD throughout the developing retinal vasculature,[16] although both dll4 and activated notch do seem to be enriched in the vasculature of the outer third of the retinal disc.[16] A model incorporating transient expression of dll4 in tip cells seems to best fit the data and is described in detail below.

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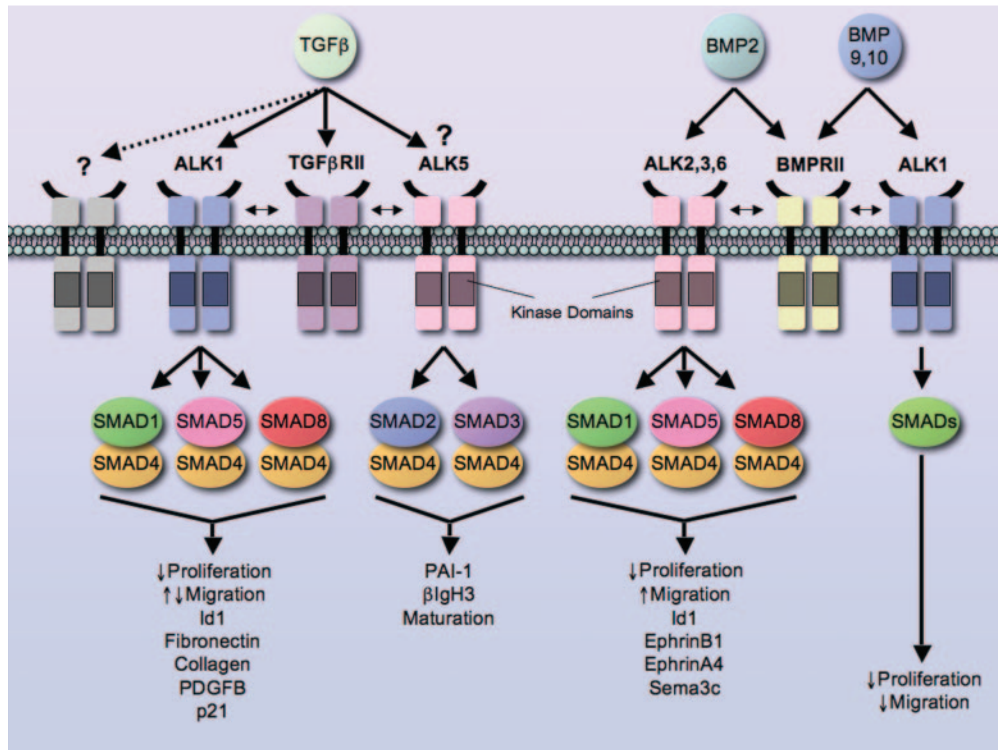

Figure 3. TGFβ/BMP signaling in angiogenic ECs. TGFβ is thought to signal through TGFβRII and Alk1 on ECs, which then activate smads. There are data suggesting that Alk5 is also involved; however, recent in vivo studies have shown that Alk5 is expressed only by cells in the vessel wall, likely smooth muscle cells, and not by the ECs lining the vessels. TGFβ generally suppresses proliferation and may suppress or increase migration depending on the presence of other factors, such as VEGF. Some BMPs, such as BMP2 and BMP4, are proangiogenic, increasing EC migration and inducing expression of guidance genes, whereas others suppress migration and proliferation (BMP9, BMP10). See the text for details.

It should also be noted that a number of reports have raised the possibility of reverse notch signaling, that is, notch-to-ligand.[85–88] Dll1, dll4, and jagged-1 all have a C-terminal PDZ-L domain that has been shown to interact with several intracellular adaptor and scaffold proteins, including MAGI-1, -2, and -3 and AF-6.[88] Dll1 and dll4 also bind to the tumor suppressor Dlg1, a member of the membrane-associated guanylate kinase (MAGUK) family of scaffolding proteins.[89] Interestingly, all of these proteins localize to sites of cell–cell contact, specifically adherens junctions. The potential significance of these findings is discussed in detail below. The dll1 intracellular domain induces cell growth arrest in ECs through the induction of p21,[87] and jagged ICD can activate a multimerized AP-1 reporter.[90] Provocative as these results are, what the physiological role of notch ligand signaling may be in angiogenic ECs is still to be determined.

Role of TGFβ in Vascular Morphogenesis

Perhaps more than most, the field of TGFβ and vascular morphogenesis has been riven with confusion. Only in the past few years has some kind of consensus been reached, and even that is looking less firm than it was. Much of the confusion stems from the breadth and diversity of the TGFβ superfamily of growth factors and morphogens and the equally diverse array of receptors and downstream signaling components.[91,92]

The TGFβ superfamily consists of close to 30 members, including TGFβs (TGFβ1, TGFβ2, and TGFβ3), bone morphogenetic proteins (BMPs), activins, and inhibins. The receptors fall into 2 major classes, both of which are serine–threonine kinases (Figure 3). type II receptors, of which there are 5 in human[93] including TGFβRII and BMP receptor (BMPR)II, undergo a conformational change on binding to their ligand, allowing them to phosphorylate and activate type I receptors. There are 7 known type I receptors, which once activated, phosphorylate various smads, which convey the signal from the membrane to the nucleus, where they activate transcription of target genes. Three classes of smads have been identified: receptor-activated smads (R-smads), including smad1, smad2, smad3, smad5, and smad8; the comediator smad smad4; and inhibitory smads, smad6, and smad7 (Figure 3). On activation, the R-smads bind to smad4 triggering translocation of the complex to the nucleus.[94]

The critical TGFβ receptors on ECs are the type II TGFβRII and the type I receptor Alk1, which activates smad1, smad5, and smad8. Alk5, a type I receptor that activates smad2 and smad3, may also be important on ECs. Alk5 is widely expressed in tissues, whereas Alk1 is more restricted to endothelium and especially to angiogenic ECs.[95] ECs also express a type III coreceptor called endoglin, which appears to potentiate TGFβ signaling.[96,97] Generation of mice lacking TGFβ or its receptors has revealed a critical role for this pathway in vascular development.[98] Deletion of TGFβ1 in mice results in 50% embryonic lethality at E9.5 to E10.5 because of defective yolk sac vasculogenesis.[99,100] Similarly, knockout of TGFβ receptor (R)II, Alk1, or Alk5 also leads to

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death at E10.5 resulting from vascular defects in the yolk sac and elsewhere.[101–103] Endoglin knockouts die a little later, at approximately E11.5, again with cardiovascular and angiogenesis defects.[104] Thus, most data point to a crucial role for TGFβ signaling at the vascular plexus-remodeling stage, at approximately E10.5. Importantly, in many cases in which TGFβ signaling has been disrupted in mice, there are also defects associated with vascular smooth muscle cells, which may account for some of the observed cardiovascular deficiencies.

To examine the direct angiogenic effects of TGFβ on ECs, many investigators have turned to in vitro assays. Most studies in 2D culture have determined that TGFβ1 is antiproliferative in the range of 10 pg/mL to 10 ng/mL when tested on ECs from a wide range of tissues.[105] The block of proliferation is at least partially accounted for by TGFβ induction of the cell cycle inhibitor p21, a response that can be overcome by c-myc.[106] Similarly, TGFβ in the same concentration range inhibits migration of ECs in a number of assays, including scrape-wounding of monolayers and Boyden chamber assays.[105] In 3D fibrin or collagen gel assays, TGFβ has no effect on sprouting when added alone but is additive with basic FGF or VEGF when added at low concentration (0.2 to 0.5 ng/mL). At high concentration (5 to 10 ng/mL), it is inhibitory.[107]

TGFβ regulates expression of a number of genes in ECs, particularly those involved in establishment of, and interaction with, the basement membrane (BM). For example, fibronectin and collagens I, IV, and V[105,108] are all induced by TGFβ, as are the integrins α5 and β1,[109] which together form an important receptor for fibronectin. TGFβ1 also induces PDGF-B, which is important for recruitment of pericytes to the maturing vessel and is highly expressed by tip cells.[15] The bHLH transcriptional regulator Id1 has been identified as a TGFβ/Alk target in ECs in a number of studies.[110–113] The Id proteins, which lack DNA-binding domains and inhibit other bHLH transcription factors by squelching, are required for EC proliferation and migration.[114]

To dissect the specific roles of the putative TGFβ receptors Alk1 and Alk5 in ECs, constitutively active (CA) forms have been generated. At least 4 reports have been published on the effects of these in different lines of ECs; however, they come to surprisingly different conclusions. Ota et al, using HUVECs, found that both CA-Alk1 and CA-Alk5 inhibited EC proliferation, and, similarly to TGFβ, CA-Alk5 blocked tube formation in collagen gels, whereas CA-Alk1 had no effect.[112] Goumans et al, on the other hand, used mouse embryonic endothelial cells (MEECs) and showed that CA-Alk1 increased migration 2.5-fold compared with control, whereas CA-Alk5 decreased it by 3.5-fold.[110] Lamouille et al used skin microvascular ECs, HUVECs, and a human microvascular line (HMECs) and found that CA-Alk1 blocked proliferation and migration,[115] in agreement with Ota et al[112] but in conflict with Goumans et al.[110] Finally, Mallet et al reported that both CA-Alk1 and CA-Alk5 reduced growth factor–induced sprouting from cultured embryoid bodies.[116] Two of these studies reported Id1 induction by CA-Alk1, one in which Alk1 activity appeared to be proangiogenic[110] and another in which it was antiangiogenic.[112] The discrepancies among these seemingly similar studies await resolution but probably relate to the origins of the cells used and their changing gene expression profiles over time in culture. Finally, a caveat that should be noted here is that many of the findings reported above are derived from overexpression of CA receptors in cultured cells. Lux et al studied gene expression in several lines of ECs downstream of low or high concentrations of TGFβ and CA-Alk1 and found a poor correlation.[111] For example, Id1 was induced in 3 lines of ECs by CA-Alk1 but was downregulated in HMECs by low concentrations of TGFβ and was unaffected by high concentrations.

The conclusion is that different cells may respond differently to TGFβ signaling and that this may or may not involve Alk1 and Alk5. It has been suggested that crosstalk between different TGFβ receptors can shift the balance of signaling from pro-to-antiproliferative and that this may depend on the local TGFβ concentration and the presence or absence of various BMPs.[110] For example, the inhibitory effect of Alk5 on EC proliferation and migration is counteracted by endoglin expression.[104] In addition, Alk1 inhibits Alk5 signaling in HepG2 cells by a mechanism involving phosphorylation of smad1 and smad5.[103]

A model has been proposed[96] in which low concentrations of TGFβ stimulate both receptors but Alk1 inhibits Alk5 signaling and induces expression of Id1 and endoglin, thereby potentiating TGFβ effects on proliferation and migration. At later times, perhaps during the maturation phase of angiogenesis, Alk5 signaling predominates, inducing growth arrest and extracellular matrix assembly.[96] However, disruption of the violet beauregarde (*Alk1*) gene in zebrafish results in increased numbers of ECs, suggesting that Alk1 in the fish normally acts to suppress EC proliferation,[117] as it may in cultured cells.[115,116] More recently, mice have been generated that express lacZ from the Alk5 locus. In contrast to Alk1, Alk5 was not seen in any endothelium but appeared to be localized to the vessel media, especially the smooth muscle cells.[118] In addition, whereas the vascular lumens in Alk1$^{-/-}$ mice are greatly dilated, lumen formation in Alk5$^{-/-}$ mice was normal. One interpretation of these data are that expression of Alk5 in vitro is not representative of expression in vivo and that a physiological role for Alk5 in endothelium may be limited.

Mutations in the TGFβ signaling pathway underlie the vascular disorders hereditary hemorrhagic telangiectasia-1 (HHT1) and HHT2 in human. In both cases, the condition presents as recurrent nosebleeds, multiple small vascular malformations (telangiectasias) in skin and various mucosa, and arteriovenous malformations in organs such as brain, lung, and liver. Lesions in brain and lung seem to predominate in HHT1, whereas HHT2 lesions are more common in liver. The reasons for these differences are not known but may relate to differential expression of the affected genes. HHT1 is attributable to mutations in endoglin, whereas HHT2 is associated with mutations in Alk1. Recently, vascular-specific targeting of TGFβRII, Alk1, and Alk5 has been reported.[119] Deletion of Alk1 completely recapitulated the vascular defects seen in HHT2, including vessel dilation, decreased wall thickness, and reduced and irregularly located

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vascular smooth muscle cells. Surprisingly, deletion of TGFβRII in ECs did not affect vascular morphogenesis. Given that TGFβRII is thought to be the major TGFβ type II receptor on ECs, the implication of these findings is that whereas TGFβ is required for vessel assembly and remodeling, it appears not to be required at the level of the ECs but may be important for pericytes or vascular smooth muscle cell function. Consistent with the reported nonendothelial expression of Alk5, deletion in ECs did not affect vascular morphogenesis.

Two recent reports have suggested that BMP9, and the closely related BMP10, are ligands for Alk1.[113,120] Several in vitro assays were used to demonstrate that BMP9/Alk1 signaling strongly inhibits both EC migration and proliferation. Importantly, these BMPs induced a set of genes that overlapped with those previously reported to be targets of CA-Alk1,[120] consistent with this receptor conveying antiproliferative and antimigratory signals to ECs. It should be noted, however, that BMP9 and BMP10 show quite restricted expression, with BMP9 being largely limited to liver, whereas BMP10 is expressed in developing and postnatal heart.

Germline mutations in BMPRII predispose to pulmonary arterial hypertension, characterized by a narrowing of the pulmonary arteries as a result of fibrosis and deregulated EC–smooth muscle cell interactions in the vessel wall.[121] Because a BMPRII knockout is embryonic lethal, its role in vascular homeostasis has been investigated by generation of hypomorphic epi-alleles using a short hairpin RNA approach.[122] These mice survive but show a progressive loss of vascular integrity and eventually die from massive intestinal hemorrhage. EC expression of PDGF-B is reduced in these mice, which likely contributes to the reduced recruitment of mural cells. Gene expression studies suggest that downregulated expression of Eph-Ephrins, jagged-2, and Sema3c may underlie these defects. In contrast to BMP9 and BMP10, BMP2 and BMP4 have positive effects on EC sprouting.[123,124] BMP2 is additive with VEGF in inducing vascularization in a sponge implant model in mice and induces phosphorylation of p38 MAPK and induction of Id1 in cultured ECs. In aggregate, these data suggest that BMP signaling through BMPRII is required for proper assembly and stabilization of the vasculature and that this may involve semaphorin–integrin crosstalk.

In summary, in the absence of TGFβ or BMP signaling in the vasculature, vessels are ectatic because of incomplete vessel wall maturation. EC–smooth muscle cell interaction is disrupted and vessels have a poorly formed basement membrane, both of which contribute to vessel rupture. Interestingly, the phenotype of mice lacking TGFβ signaling is quite similar to that seen in mice lacking fibronectin or the fibronectin receptor $\alpha5\beta1$ or in mice lacking PDGF-B or the PDGF receptor. All of these genes are TGFβ targets, suggesting, perhaps, a common mechanism underlying the vascular phenotype.[105] BMP receptors have been identified on ECs in a number of tissues in human and mouse, suggesting that BMPs may act in concert with TGFβ to regulate vessel formation in vivo.

Crosstalk Between the Notch, VEGF, and TGFβ Pathways

Historically, cell signaling pathways have been studied in isolation and treated as largely linear; however, the emerging field of systems biology emphasizes the growing appreciation of pathway crosstalk and the reality of signaling webs rather than pathways. Signals downstream of VEGF, notch, and TGFβ interact in a number of significant ways in the cardiovascular system, acting either in a synergistic or antagonistic manner.

VEGF has direct effects on notch signaling by inducing both receptor and ligand. This was first demonstrated in vitro, where notch1 and dll4 were both found to be upregulated by VEGF in human arterial ECs.[125] Signaling was mediated by both VEGFR1 and VEGFR2 and was dependent on phosphatidylinositol 3-kinase/Akt but not MAPK/ERK or src kinases.[125] More recently, VEGF induction of dll4 was demonstrated in the mouse retina.[19] Injection of VEGF164 into the vitreous increased expression of dll4 in retinas of p5 dll4$^{+/-}$ mice at 24 hours, whereas injection of the VEGF antagonist VEGF-Trap (a fusion protein of the VEGFR ligand binding domains with the Fc domain of human IgG1) reduced the expression of dll4. Dll4 signaling through notch establishes a negative feedback loop because notch signaling represses transcription of the VEGFR2 gene through upregulation of HESR1 (HEY1) (see below).[64,67,68,126] HESR1 binding to E-boxes in the VEGFR2 promoter is not required for repression, but, rather, interactions involving SP1 sites and the initiator element are critical.[68,63] Consistent with these findings, the level of expression of VEGFR2 in the retinas of dll4$^{+/-}$ mice is increased, and the zone of highest expression is no longer limited to cells at the leading edge of capillary growth.[23] TGFβ also directly downregulates VEGFR2 transcription via a GATA site in the proximal promoter.[127]

The expression pattern of dll4 in developing retina suggests that the gene may be regulated in an oscillatory manner such that an increase in expression corresponds with a cell taking on a tip cell phenotype and migrating toward the source of VEGF, whereas a subsequent fall in expression may correlate with, and be necessary for, subsequent steps in angiogenesis, including establishment of anastomoses, tube formation, and vessel maturation. A feedback loop comprising VEGF and notch signaling would greatly facilitate such a scheme and, indeed, the retinal model provides supporting evidence in that the dll4$^{+/-}$ phenotype of increased filopodia and branching can be mitigated somewhat by reducing VEGF levels with sFlt1 (soluble VEGFR1 extracellular domain) or by antibodies that block the VEGFR2 receptor,[23] consistent with at least a component of the dll4$^{+/-}$ phenotype being a consequence of increased VEGF signaling.[23] Despite the seeming simplicity of this model it is important to note that dll4 expression is also under the control of TGFβ.[128] Moreover, notch and TGFβ genetically interact at several additional levels (see below).

The same scheme of VEGF-induced notch and notch ligand expression has been shown to underlie arteriovenous specification in zebrafish. In VEGF morphants, the dorsal aorta loses arterial markers such as ephrin B2 and ectopically expresses the vein marker Flt4.[129] Importantly, arterial iden-

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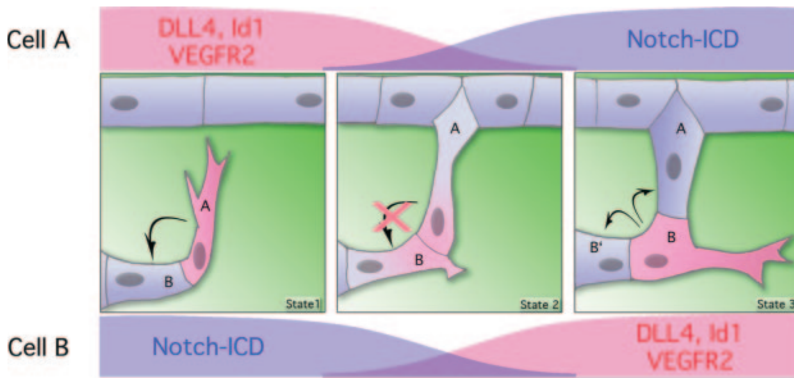

Figure 4. A 3-state model of tip cell formation incorporating VEGF, notch, and TGFβ signaling. In state 1, a tip cell (A) migrates in response to a VEGF gradient (green), trailed by a trunk cell (B). In state 2, the tip cell has anastomosed with another vessel and is becoming a trunk cell, as the former trunk cell is becoming a new tip cell. In state 3, the former tip cell is now a trunk cell (A), and the former trunk cell is now a tip cell (B). This system then oscillates under the control of transcriptional feedback loops (see Figure 5). Initially, the tip cell (A) has high expression of dll4 and VEGFR2 and likely also expresses Id1. It signals to the trunk cell (B) through dll4-notch interaction (arrow). The trunk cell (B), therefore, has activated NICD and low VEGFR2 and dll4. In state 2, both cells are in transition and notch signaling is at a minimum. Division occurs at this point to give cells B and B'. By the time state 3 is reached, the former trunk cell (B) now has elevated dll4 and VEGFR2 and decreased NICD and has become a tip cell. This new tip cell signals to trunk cells A and B' through dll4–notch. Thus, the former tip cell (A) has upregulated notch signaling, has downregulated dll4 and VEGFR2, and has become a trunk cell. TGFβ synergizes with VEGF and notch to drive migration, partly through induction of Id1, and to inhibit proliferation. See the text for details.

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tity can be rescued by activated notch in VEGF morphants but cannot be rescued by VEGF in notch mutants.[129] These epistasis experiments place notch downstream of VEGF in zebrafish arterial specification.

In the past several years multiple, complex interactions between notch and TGFβ signaling have emerged. Depending on the context, notch can either synergize with TGFβ/BMP signals to induce target genes or inhibit TGFβ/BMP signaling.[128,130–136] Generally, in the presence of other growth factors, TGFβ/BMP signaling is found to stimulate migration but block proliferation and notch either potentiates or antagonizes these responses. In cultured ECs, both notch1 ICD and notch4 ICD synergized with BMP6 to induce HEY1 expression, although BMP6 had no effect when added alone.[131] BMP6 binds to Alk2, -3, and -6 and activates receptor smad1, -5, and -8 and the coactivator smad4.[93] Consistent with this, NICD was found to interact with smad1 and smad5, and this was promoted by the coactivator p/CAF. Synergistic activation of the HEY1 promoter required the RBP-J site and GC-rich palindromic BMP response elements. Stimulation of

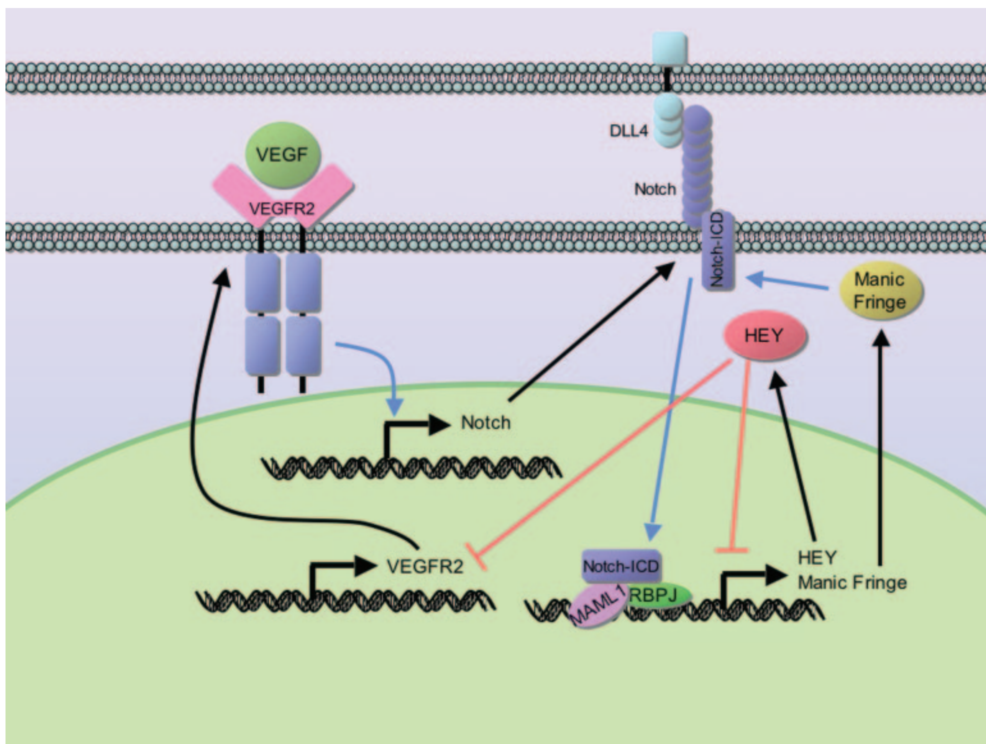

Figure 5. Notch signaling regulates coupled transcriptional feedback loops. Dll4–notch interaction induces upregulation of HEY and manic fringe through direct binding of NICD to the promoters. HEY then regulates its own promoter, as well as that of fringe and VEGFR2. Fringe acts on notch glycosylation to promote delta binding and signaling (jagged–notch interactions are reduced). VEGF binds to VEGFR2 and induces expression of notch (and dll4). TGFβ (data not shown) synergizes with VEGF to drive migration and also induces notch ligands. This model is based on the transcriptional oscillator that regulates somitogenesis in vertebrates and that is also based on notch–HES–fringe feedback. See the text for details.

ECs with BMP alone promoted expression of Id1 and cell migration. In the presence of notch signaling, Id1 expression was reduced and cell migration was inhibited. It was suggested that degradation of Id1 protein was directly promoted by HEY1, a phenomenon that was EC-specific.[131] Interestingly, the dominance of notch signaling over BMP signaling was dependent on cell–cell contact, suggesting a model whereby ECs not in contact with surrounding cells are stimulated by TGFβ to migrate until new cell–cell contact is established, at which point notch–notch ligand interaction induces HEY1 expression, Id degradation, and an arrest to further migration. In cultured ECs, therefore, BMP signaling promotes migration, and this is blocked by notch. However, given that ECs in vivo are rarely, if ever, separated from each other, this simple model may need to be modified (see below).

Of likely relevance to EC function are several studies on notch–TGFβ interaction in epithelial cells. Zavadil et al[136] found that TGFβ induced expression of the notch target gene HEY1 and the notch ligand jagged-1 in several epithelia, including mammary gland, kidney tubules, and epidermis. Interestingly, the induction of HEY1 was biphasic: the early phase was smad3-dependent, but jagged–notch–independent, whereas the latter phase was jagged–notch–dependent and still required smad3. These data imply that TGFβ-induced migration of epithelial cells depends not only on smad3 but also on jagged–notch interaction and the induction of HEY1.[136] In this case, in contrast to the findings in some ECs, notch signaling promotes the positive effect of TGFβ on migration. Niimi et al also studied epithelium and found that notch signaling is necessary for growth arrest by TGFβ.[128] Moreover, they found that fully one-third of TGFβ-induced genes required notch signaling for full expression. Again, TGFβ was shown to induce jagged-1, and knockdown of jagged-1 by siRNA led to a reduction in TGFβ-induced p21 and a rescue of TGFβ-inhibited proliferation.[128] The model that best fits these data has TGFβ inducing both c-myc, which stimulates cell cycle progression, and jagged-1, which through stimulation of notch and induction of p21 and p15, blocks cell cycle. Because induction of jagged-1 was rapid and transient, it is possible that a balance between TGFβ/notch-induced p21 and TGFβ/smad-induced c-myc may act as a switch to regulate cell proliferation. Others have found, however, that NICD relieves the TGFβ-induced block on cell proliferation,[133,134] possibly by sequestering the acetyltransferase p300 away from smad3.[133] To further complicate the picture, it has been reported that NICD forms a complex with smad1, p300, and p/CAF that synergistically induces HES5 and HEY1, both of which inhibit proliferation.[135]

It is clear that TGFβ signaling and its interactions with the notch pathway are complex and context-dependent. Switching between synergy and antagonism is likely to be subject to multiple variables, including which smads are activated, which notch is activated (notch4 differs from notch1 in lacking the transcriptional activation domain), the timing of pathway activation (simultaneous or sequential), the duration of signaling through each pathway, inputs from other pathways such as receptor tyrosine kinases and wnt and the local 3D environment. We have noted, for example, that although notch1 ICD blocks proliferation of ECs in 3D cultures, it stimulates proliferation in 2D monolayers (Sainson RCA, Hughes CCW, unpublished observations, 2005).

TGFβ has also been implicated in reverse signaling by notch ligands. In neural stem cells, the dll1 ICD was found to interact with smad2, -3, and -4, whereas no interaction with smad1 or smad5 was found.[86] The interaction between delta and smads enhanced TGFβ-stimulated transcription and promoted differentiation of P19 cells into neurons, as evidenced by blocking of this transition with a specific inhibitor of type I TGFβ receptors. The relevance of this finding to endothelial function is yet to be determined.

Regulation of Retinal Angiogenesis by Coupled Feedback Loops?

Although some findings on pathway interaction may turn out to be cell type–specific, a general synthesis of the present state of knowledge can be attempted (Figure 4). Here, we have incorporated as much of the data as we can to generate what we believe is a coherent model of VEGF-, notch-, and TGFβ-driven angiogenic sprouting. Where published in vitro data conflict, we have used those from 3D, rather than 2D, models because these seem to more closely mirror the in vivo situation, especially with regard to TGFβ signaling. Observations from mouse retina studies and live imaging in zebrafish suggest that during development, the tip cell–trunk cell duality is plastic and that tip cells can be "reabsorbed" back into the parent vessel, and, of course, it is from the trunk that new tip cells emerge.[137] In retina, it appears that there are, at most, 2 trunk cells between new tips, and, often, tip and trunk cells physically alternate along the leading edge of the plexus. To explain sprouting at the leading edge of the retina a 3-state model is proposed that incorporates a number of coupled feedback loops involving VEGF, notch, and TGFβ. It is quite possible that this scheme could be generalized to angiogenesis in other tissues.

In state 1, a distinct tip cell migrates toward a VEGF signal, trailed by a distinct trunk cell (Figure 4). In state 2, the tip cell has anastomosed with another vessel and is becoming a trunk cell, as the former trunk cell is becoming a new tip cell. In state 3, the former tip cell is now a distinct trunk cell, and the former trunk cell is now a distinct tip cell. This system then oscillates until a stop signal is received, likely the cessation of VEGF secretion.

The process initiates when tip cells, high in VEGFR2,[15] receive a VEGF signal that chemotactically drives migration but does not stimulate proliferation. TGFβ signaling synergizes with VEGF to drive migration, while also blocking proliferation. Dll4 expressed by the tip cell signals back to the trailing cell through notch. Notch induces HEY1, which represses VEGFR2 expression[64,67,126] and induces p21,[22] which suppresses progression through cell cycle. It should be noted, however, that in 2D cultures, notch has been shown to downregulate p21.[138] TGFβ signaling in the trunk cell may also contribute to regulation of p21.[115] Thus, at this point (state 1) the tip cell is migrating, and neither the tip cell nor the trunk cell is undergoing cell division. Once the tip cell makes contact with another trunk cell, migration ceases and

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anastomosis ensues. The cells are now entering state 2. At the same time, dll4 expression in the tip cell is downregulated, perhaps in response to signaling through receptor–ligand pairs at the site of anastomosis, which relieves notch signaling in the trailing cell. VEGF signaling in the tip/anastomosing cell now switches from driving migration to upregulating notch expression, while VEGFR2 expression in the trailing cell also rises, because of the loss of notch-mediated repression. The subsequent upregulation of VEGF signaling in the trunk cell, along with decreased p21 (attributable to decreased notch signaling), now initiates entry of the trunk cell into the cell cycle, potentially leading to cell division. Proliferation driven by VEGF often results in cleavage perpendicular to the long axis of the vessel, potentially orienting cells for a new round of sprouting.[41]

In state 3, under the influence of VEGF and TGFβ, dll4 expression increases in the trailing/trunk cell[125,128] (now becoming a new tip cell), and the dll4-notch interaction now reverses to promote a trunk cell phenotype in the former tip (now anastomosing) cell. Notch signaling in this cell now blocks the promigratory action of TGFβ. VEGF signaling in the formerly trailing/trunk cell now switches from promoting proliferation to promoting migration and the expression of dll4, and this cell now becomes a new tip cell. TGFβ also promotes migration of the tip cell directly through induction of Id1,[111] whereas BMP signaling induces guidance molecules such as ephrin B1, ephrin B2, EphA4, and Sema3c.[122] We speculate that whereas TGFβ (along with VEGF) is stimulating a tip cell to migrate, dll4 is maintained in the area of tip cell–trunk cell contact by interaction with MAGI proteins in adherens junctions.[89,139–141] At this location, dll4 signals through notch to the trailing/trunk cell to maintain its phenotype, as described above. Once anastomosis begins, dll4 is released from the adherens junction and can then block promigratory TGFβ signaling.[89] Loss of dll4 from the area of cell–cell contact relieves notch signaling in the trailing/trunk cell, initiating its transition to a new tip cell (see above). In support of this hypothesis, we have found that decreasing dll4 expression in cultured ECs with antisense increases their migration, whereas blocking notch signaling with a γ-secretase inhibitor does not, consistent with the phenotype being attributable to loss of dll4 (and the potential for reverse signaling) rather than the loss of dll4-induced notch signaling (Sainson RCA, Hughes CCW, unpublished observations, 2005). Dll4 in the new tip cell then signals back to the new trunk cell (formerly tip cell) through notch to downregulate VEGFR2 expression, and this could be potentiated by TGFβ directly, or through independent induction of HEY1. The system thus oscillates, with notch signaling switching back and forth between adjacent cells as they alternately become trunk and tip cells. The activity of notch then regulates VEGF signaling and both cooperate with TGFβ/BMP.

How this oscillation may be regulated is an interesting question. Extensive work in mouse, chick, and zebrafish[142,143] has identified an oscillatory mechanism that regulates somitogenesis. A transcriptional oscillator in the presomitic mesoderm regulates the expression of a number of genes involved in formation of the somites, such that 1 new somite is formed for each cycle of the clock.[144,145] Most of the genes so far identified are part of the notch signaling pathway, including delta, mindbomb, and lunatic fringe.[142–144] Mathematical modeling has shown that relatively simple feedback loops can generate oscillatory behavior in this system and that a critical parameter is a short half-life for the relevant proteins.[144] How might this work in angiogenesis? Dll4 signaling through notch induces expression of HEY genes and fringe genes (we have found regulation of manic fringe in angiogenic ECs; unpublished observations, 2006). HEY, in turn, represses its own transcription[66] and the transcription of fringe (Figure 5). In addition, fringe alters the glycosylation of notch promoting its binding to delta at the expense of jagged.[146] Under the right conditions, these coupled negative feedback loops will result in oscillating activity of the notch pathway in ECs. It is unlikely that the oscillation runs independent of outside influences, and so we suggest that this mechanism may operate in conjunction with both the regulation of delta expression and its subcellular localization, as well as with inputs from other pathways.

Many other questions remain, the most important of which is how the switch between migration and proliferation in response to VEGF is regulated. A thorough analysis of point mutations in the intracellular domain of VEGFR2, in the context of EC migration and proliferation, may shed some light on this question. In particular, the role of pathway-specific phosphatases may be worth investigating. This switch may also involve synergistic and/or antagonistic interactions with TGFβ/BMP signaling. The interactions between notch ICD and smads, and between notch ligands and MAGI proteins, is only partially understood and not at all at the temporal level, which is likely to be key. The role of endoglin, and especially Alk5, in ECs is still undetermined as are the relative roles of TGFβ and the positively and negatively-acting BMPs. The model we have proposed does not directly account for how the first tip cell to emerge from a formerly quiescent vessel is determined, only how new tip cells are formed in an ongoing angiogenic environment. It is likely that notch signaling is involved, and the possibility exists that quiescent vessels contain cells that are predetermined to become tip cells once an angiogenic stimulus is received. Finally, the existence of a transcriptional oscillatory mechanism in ECs is still speculative, although all of the components required in somitogenesis are also expressed in angiogenic ECs. Misexpression in angiogenic ECs of some of these components, mutated to alter the half-life of the protein, may provide a means to test this hypothesis directly.

In summary, VEGF, notch, and TGFβ act in concert to pattern the vasculature. Hopefully, this review has made it clear that a complex phenomenon such as vascular morphogenesis will only be understood when it is studied at a temporal and systems level.

Acknowledgments

We thank members of our laboratory for helpful discussions and apologize to those whose work we have been unable to cite because of space limitations.

Sources of Funding

The angiogenesis work in our laboratory is supported by NIH R01 grants HL086959 and HL60067.

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Disclosures

None.

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Circ Res. 2008;102:637-652
doi: 10.1161/CIRCRESAHA.107.167171
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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