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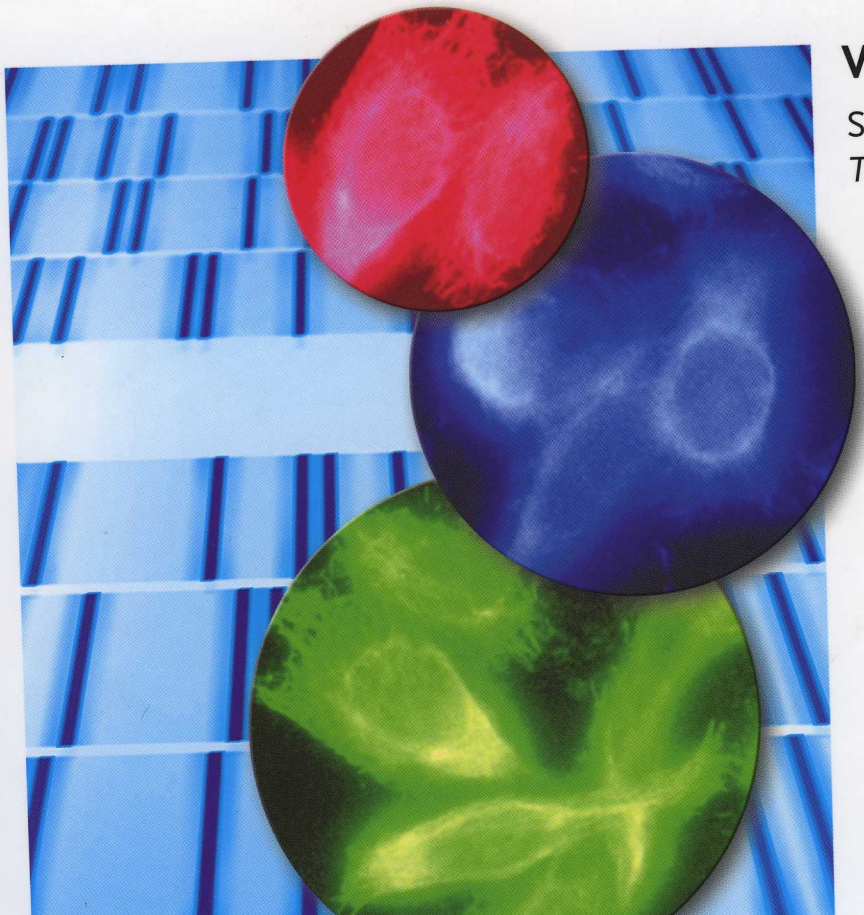

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Vascular Development and Angiogenesis

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Keywords

Angiogenesis
The process by which new blood vessels are originated through the sprouting of endothelial cells from preexistent vessels.

Vasculogenesis
Differentiation of endothelial and precursor cells from the mesoderm and their coalescence into tubes to form a primary vascular plexus.

The development of the vascular system is initiated by the local differentiation of mesenchymal cells into hemangioblasts. These are pluripotent progenitors for both endothelial and hemtopoetic cells. Subsequently, endothelial cells form vascular channels that coalesce and circulation begins thereafter. Recruitment of smooth muscle cells and pericytes contributes to the remodeling stage characterized by the emergence of a hierarchical tree of vessels. Concomitantly, a second array of vessels emerges from the cardinal vein, the lymphatic vasculature. The use of molecular biology and genetics has allowed us to identify the key signaling pathways involved in the specification, differentiation and homeostasis of both the vascular and lymphatic systems. Here we have discussed the details associated with the formation of the vasculature in concert with the key signaling pathways responsible for these events.

1 General Overview

The vascular system develops shortly after gastrulation. The formation of the embryonic vasculature is initiated by the appearance of blood islands from progenitor cells (hemangioblasts) in the visceral yolk sac. Within the blood islands, hemangioblasts differentiate into either hematopioetic or endothelial cells, with the former located within the channel space and the endothelial cells lying on the edges. This first phase of blood vessel formation is known as *vasculogenesis*. A second phase then begins, which is referred to as *angiogenesis*, involving active sprouting of vessels from preexisting blood vessels. Interconnection of these primitive vessels results in the formation of a primary vascular plexus, which undergoes a complex process of remodeling. Eventually, the fetal vasculature emerges from angiogenic growth, selective fusion, and regression of primitive vessels. Figure 1 illustrates the major processes in blood vessel formation and highlights the key signaling pathways required for each step.

The morphogenesis of the heart is concomitant with the differentiation of blood islands. Cardiac progenitor cells migrate to the ventral midline, forming a linear heart tube. As the inflow and outflow tracks are formed, the heart is joined to the yolk sac and flow begins. All subsequent vascular development occurs in the presence of blood flow.

The circulatory system is the first functioning organ system to develop in vertebrate embryos and it is essential for viability and survival. This dependency has been advantageous to investigators. The unsuspected contribution of several regulatory molecules has been revealed by hemorrhage and embryonic lethality. During the last two decades, the use of homologous recombination to ablate gene function has provided major breakthroughs in our understanding of vascular development. Key signaling pathways to vascular morphogenesis include vascular endothelial growth factor (VEGF), Notch, angiopoietins, ephrins, transforming growth factor (TGF)-β, and platelet-derived growth factor (PDGF).

The identification of genes involved in embryonic vascular morphogenesis is important to gain a concrete understanding of how vessels are formed. Many of the events that take place during development are recapitulated in situations of neoangiogenesis in the adult. Thus,

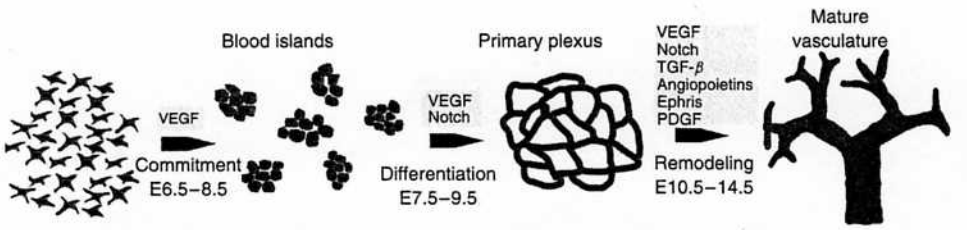

Fig. 1 Development of blood vessels: initially, undifferentiated mesenchymal cells under the instructive signals of VEGF become committed to hematopoietic and endothelial cell fate originating small groups of cells, known as *blood islands*. These clumps of cells gain lumen, coalesce, and form a primary plexus. Subsequently, a series of remodeling events takes place with selective fusion and regression, as well as active expansion by sprouting of new vessels. The end result is a hierarchic mature vascular plexus.

this information is central to the generation of novel and more effective therapies for the manipulation of vascular growth during pathological conditions in the adult.

2
A Stem Cell for the Vascular System: The Hemangioblast

The notion of a common progenitor for both endothelial and hematopoietic cells was first suggested in the early part of the twentieth century by Florence Sabin, based on her studies of vessel formation in the chick blastodisc. Those initial observations were morphological and based on the proximity between endothelial cells and hematopoietic cells in the embryos. Today, we know that endothelial and hematopoietic progenitors share expression of many genes, including VEGFR1, VEGFR2, Tie1 and Tie2, Tal1/SCL, and Runx1. We also know that genetic ablation of VEGFR1 and R2 affects both hematopoietic and endothelial development. Although these findings did not provide a concrete lineage link, they were consistent with at least an interdependency of both cell types.

Subsequently, the utilization of "ES cell *in vitro* differentiation assays" offered an opportunity to further explore the lineage association between endothelial and hematopoietic cells. Using a subpopulation of embryonic stem (ES) cells that expressed VEGFR2, Choi and colleagues demonstrated that under appropriate culture conditions those cells could give rise to both blast-colony forming cells (BL-CFC) and endothelial cells. Subsequent analysis demonstrated that not all VEGFR2 cells could generate BL-CFC, but cells coexpressing both VEGFR2 with Tal1/SCL are enriched for BL-CFC. Tal1/SCL is a transcription factor required for the initiation of primitive and definitive hematopoietic development. Overexpression of Tal1/SCL in Flk1-expressing ES cells significantly increased the number of BL-CFCs in culture. Consistent with those observations, ES cells lacking Tal1/SCL do not differentiate into endothelial cells or hematopoietic cells. However, some degree of endothelial cell development has been noted in Tal1-null mice.

Additional evidence for the existence of a common link between endothelial and hematopoietic cells comes from the finding that during development, certain endothelial cells give rise to hematopoietic progenitors. Functional evaluations *in vivo* have demonstrated that definitive hematopoietic stem cells (HSC) can arise from yolk sac, and in the intraembryonic aorta-gonad-mesonephros (AGM) region. A large morphological body has also noted budding of hematopoietic progenitors from the ventral region of the dorsal aorta, umbilical, and vitelline arteries in chicken, mouse, and human embryos. This potential of endothelial cells to give rise to hematopoietic progenitors is temporally restricted to early developmental stages. Currently, there is no evidence that mature endothelial cells can give rise to hematopoietic progenitors.

Primordia of blood vessels form by the interconnection of hemangioblast clumps that coalesce into blood lakes, and subsequently give rise to a primitive network of endothelial tubes. The process, known as *vasculogenesis*, marks the initiation of vascular development. While, initially confined to an extraembryonic location (yolk sac), shortly thereafter (8–12 h) vasculogenesis is also noted within the embryo proper.

3
Development of the Vascular System

3.1
Early Vascular Morphogenesis

The primitive vascular plexus is composed of differentiated endothelial cells that initiate their function as barrier between the bloodstream and tissues. The main characteristic of this plexus is its uniformity. Channels of the vascular plexus are of the same width, and are separated by similar distances, with no evidence of vascular hierarchy. The vascular plexus is transient and must be altered to accommodate the physical forces associated with the pressures of blood flow. Failure to differentiate this primitive vascular plexus results in embryonic death, generally by E9.5 to E12.5, a feature that has enabled the identification of several genes critical to vascular remodeling.

The vascular plexus expands by vascular sprouting (angiogenesis). During angiogenesis, endothelial cells migrate and proliferate in response to various stimuli, assembling into tubules containing cell–cell tight junctions characteristic of the endothelium. Recently, studies in the developing retina have indicated that the column of cells forming a sprout is heterogeneous. The leading cell, referred to as the *tip cell* within a vascular sprout, does not proliferate in response to growth factors. The tip cell responds to VEGF signaling by sending cellular processes (filopodia) and sensing its environment. The presumption is that, much like the neurons, these cells act to provide directionality to the sprout and the location of the future vascular bed. Recruitment and differentiation of supporting cells associated with mature vessels, such as smooth muscle cells, fibroblasts, and pericytes is also initiated at this time, but is not as prevalent in this stage. Recruitment events are highly regulated and require the coordinated action of a large number of molecules. However, the main group of signaling pathways that is essential to the early morphogenetic events include: VEGF and Notch. Genetic inactivation of molecules within these two signaling pathways results in lethality at E10.5. In addition, haploinsufficiency has been noted in VEGF and delta4 (ligand for Notch) embryos, suggesting that dosage of these ligands is critical to vascular morphogenesis.

3.2
Vascular Patterning and Remodeling

As it develops, the vascular system forms stereotypical blueprints reproducible within the same species and in a few cases even conserved across ontogeny. Patterning results from the combined effect provided by directionality of sprouting, selective vascular fusion, and apoptosis. Thus, some vessels are eliminated, while others increase in size providing the typical treelike structure of blood vessels. Although in both events, patterning and remodeling occur concurrently, for didactic they will be addressed sequentially.

3.2.1 Vascular Patterning

The remarkable conservation of vascular patterning suggests that specific genetic programs coordinate its formation. Interestingly, there is a growing body of experimental evidence to indicate that many of the signaling pathways involved in neural pathfinding also guide endothelial cells during development. The interconnection between neural and vascular patterning has been demonstrated by the finding that

sensory nerves instruct arterial differentiation and blood vessel patterning in the skin. There is an expanding list of neural guidance gene families that regulate migration and proliferation of endothelial cells, suggesting that developmental similarities coupled with physical associations between the vascular and nervous system rely on common molecular mechanisms.

Recently, the sprouting and selective fusion of growing capillaries has been documented by recording zebra fish embryos labeled with fli-EGFP, a construct that enables visualization of endothelial cells. This method has shown that tip cells in sprouts exhibit numerous active filopodia, which extend and retract in an intermittent fashion. Their directionality is guided by the combined set of attractive and repulsive cues, which, in turn, determine the selection of the branching site, the direction, and the fusion. Elucidation of the signaling pathways involved in this process is still in its infancy; however, a subset of these has been identified. Clearly, VEGF plays an important role in the guidance of sprouts, but more specifically the VEGF isoforms that bind to matrix proteins (VEGF188) are responsible for stimulation of sprouts and guidance cues.

Neuropilin, a receptor initially identified for its ability to promote axonal guidance, was later found to bind specific splice isoforms of VEGF. Genetic experiments later confirmed that ablation of neuropilin 1 resulted in abnormal vascular development. In particular, these mice exhibit poor branching in the subventricular zone of the brain indicating that neuropilin 1 is necessary for tip cell guidance, at least in the brain.

Another important group of signaling molecules in patterning are the plexins. In particular, loss of plexin D1 in both zebra fish and in mouse result in abnormalities in pathfinding. Plexin D1 null mice showed abundant branching in the intersomitic vessels with concomitant loss of the normal vascular pattern. The results indicate that disruption of plexin D1 signaling removes the repulsion signals that are necessary for correct guidance during vascular sprouting events. The ligand for plexin D1, Semaphorin 3E has also been inactivated in mouse and reveals a very similar phenotype.

3.2.2 Vascular Remodeling

Remodeling is best understood in the retina. The relatively planar distribution of the capillaries combined with postnatal development make this an excellent system to study vascular morphogenesis. In this system, it is easy to note how specific vessels fuse to form larger vascular channels and others regress giving the vasculature its typical hierarchical structure.

Several pathways have been implicated in vascular remodeling. Essentially, an interruption in the evolution of the primary plexus into the hierarchical vascular tree is considered as a remodeling defect. Signaling molecules, which contribute to the recruitment of mural cells, such as Tie2 and Angiopoietin-1 result in remodeling defects. Also, pathways that affect the lumen and the stability of vessels have also been implicated in remodeling, such as Notch 1. These signaling pathways and their specific contributions will be described later in this chapter.

The contribution of extracellular matrix molecules appears to be extremely important in vascular stabilization and, consequently it can play a role in remodeling events. There is experimental evidence, for example, that type XVIII collagen, a component of basement membranes, is

required for the regression of hyaloid vessels in the developing eye. The hyaloid vessels are formed to provide nutrients to the lens capsule. After this structure differentiates, these vessels regress as part of the normal development of the eye. Absence of type XVIII collagen prevents this regression. The specific mechanisms that drive the regression of these vessels by type XVIII collagen are yet to be determined. It is likely that the signals from matrix molecules are conveyed through integrins. Several integrin proteins have been inactivated and some have revealed important roles in vascular stability, including α v, $\alpha 5$, and $\beta 3$ among others. Thus, it appears that extracellular matrix proteins, through integrins, are important contributors to vascular remodeling and stability.

3.3 Arteries and Veins

A functional vascular circuit requires the separation of arterial and venous networks that only interconnect within the capillary beds of distal target organs. Whereas the endothelial tubes that makeup primitive vascular plexus are morphologically indistinguishable, mature arteries and veins differ in their arrangement of extracellular matrix, smooth muscle cells, and other supporting cells that provide specialized mechanical and physiological properties. Thus, it is conceivable that the formation of arterial and venous networks might differ from one another. Indeed, it appears that during differentiation, endothelial cells become committed to either arterial or venous identities. However, their specific timing of differentiation and segregation during vascular morphogenesis is poorly understood.

Acquisition of arterial or venous features was previously attributed to local environmental factors. Specifically, hemodynamic forces such as blood pressure and blood flow imprinted arterial or venous character to vessels, depending upon the pressure load imposed. During development, however, this was not to be the case. Genetic predetermination drives fate of arterial and venular endothelial cells before any physical regulatory event takes place. Evidence of a genetic program that directs the development of arterial–venous identity comes largely from the characterization of gene-targeted mice and mutational analysis in zebra fish. The first compelling data arose from the observation that the transmembrane ligand, ephrin B2, and its cognate tyrosine kinase receptor, EphB4 are differentially expressed within the arterial and venous endothelium, respectively, of embryonic and extraembryonic vessels prior to the onset of circulation. Genetic inactivation of either ephrin B2 or EphB4 disrupts angiogenic remodeling of arteries and veins that is likely due to inappropriate interactions between arterial and venous angiogenic sprouts. As a result, animals die at midgestation from vascular anomalies and lack of remodeling.

More recently, the Notch signaling pathway has been implicated as an important mediator of arterial differentiation in zebra fish. In fact, inactivation of Notch in zebra fish blocks arterial differentiation, as determined by the expression of arterial-specific markers. Supporting these findings, expression of Notch ligands and receptors in mice is restricted to the arterial vasculature. Similarly, expression of neuropilin 1 and 2 is also segregated in arteries and veins, respectively. Combined, the findings support a critical role for genetic programs regulating arterial and venous fates. However, these genetic programs do not explain how arterial and venous endothelial cells are guided to common

distal targets along parallel but distinct paths.

Failure to establish or maintain proper arterial–venous boundaries in mutants may partially relate to a disruption of normal sprouting mechanisms during angiogenesis. Normally, arteries and veins sprout from the primitive vasculogenic vessels to form parallel but distinct vascular networks that only interconnect at the capillary beds of the target organs. Patients afflicted by hereditary hemorrhagic telangiectasia (HHT) suffer from multiple arteriovenous malformations, direct, and abnormal connections between arterial and venous vascular beds. As will be discussed in more detail, HHT is caused by haploinsufficiency in either activin receptor-like kinase 1 (ALK-1) or endoglin, both are members of the TGF-β superfamily of receptor and ligands. Mice lacking Akt1 or endoglin lose the morphological, molecular, and functional distinctions between arteries and veins. These observations led to the hypothesis that genes responsible for guiding endothelial tubes to their appropriate destinations might be differentially expressed in mutant mice lacking Akt or endoglin. Differential evaluation of these mice resulted in the identification of Robo-4, an endothelial-specific member of the Robo family of proteins. Combined, it appears that establishment of arterial and venous identity has to occur in parallel to patterning and selective fusion of new sprouts.

Finally, it is important to stress that arterial and venous molecular signatures, although established early during development, may not be sufficient to maintain the arterial–venous identity. It has been experimentally demonstrated that after transplanting the arterial endothelium into veins and vice versa, the transplanted endothelium adopts the specific molecular programs of the host vessel, suggesting that local cues modulate vessel specificity. The specific identity of these cues is unknown; however, it is also likely that mechanical forces, including flow and pressure might play important roles.

4 Lymphangiogenesis

In addition to blood vessels, the circulatory system also includes a group of lymphatic vessels. Unlike the circulatory loop formed by the heart and blood vessels, the lymphatic vasculature consists of blind-ended capillaries that permeate most tissues to drain interstitial fluid and proteins, and return them to the blood circulation by large lymphatic conduits (thoracic duct) that drain into the left subclavian vein. Lymphatics are also responsible for fat uptake from the gut and play an essential role in the immune system by directing leukocytes and antigen presenting cells to the lymph nodes. The blood and lymphatic systems complement each other to maintain tissue homeostasis; an interdependence that is also reflected in their coordinated development during embryogenesis.

As first described by Florence Sabin in 1901, the lymphatic vessels arise in midgestation (E10.5 in mouse) after the development of the blood vascular network from progenitor cells in the cardinal vein. Subsequent to the formation of the lymph sacs, progressive expansion of lymphatic vessels occurs by active sprouting, in a manner similar to blood vessel sprouting, but under regulatory control of a different cast of molecules. Prox-1, a homeobox gene related to the Drosophila Prospero, is one of such molecules. Mice lacking this transcription factor fail to develop lymphatics and die

at midgestation from generalized edema. It appears that Prox-1 is essential for the commitment and differentiation of endothelial cells from the cardinal vein to the lymphatic lineage. Figure 2 illustrates the process of lymphangiogenesis from the budding of endothelial cells from the cardinal vein to the organization of lymphatic capillaries. In the absence of Prox expression, cells that bud from the cardinal vein continue to display a cell-surface marker profile of the blood vascular system suggesting that expression of Prox-1 is sufficient for the specification of lymphatic endothelial cells. In fact, overexpression of Prox-1 is sufficient to reprogram cultured blood vascular cells into lymphatic endothelial cells.

Maturation of the lymphatic vascular system, as well as homeostatic function of lymphatics vessels requires expression of VEGF-C and VEGFR3. Activation of VEGFR3 by either VEGF-C or D leads to enhanced proliferation, migration, and survival of cultured human adult lymphatic endothelial cells. In addition, activation of VEGFR3 by overexpression of VEGF-C in adult transgenic mice results in lymphangiogenesis, and blocking of VEGFR3 signaling *in vivo* by expression of a soluble form of the receptor leads to lymphedema in adult. Finally, the association of missense mutation of the human VEGFR3 gene in patients with chronic lymphedema (Milroy's disease) supports a requirement for VEGFR3 signaling in the adult. It should be stressed that expression of VEGFR3 is initially not restricted to lymphatic vessels. In fact, VEGFR3 is highly expressed in vascular endothelium during development and mice die from disruptions in the morphogenesis of the vascular system. After E13.5 in the mouse, VEGFR3 is downregulated in blood vessels and becomes highly expressed in lymphatic endothelium.

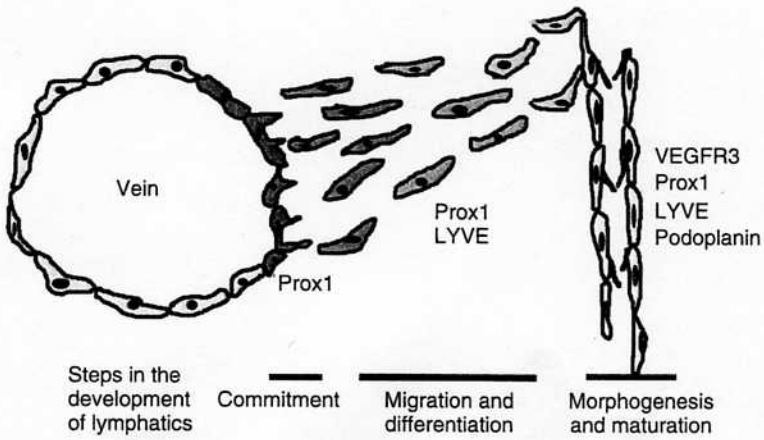

Fig. 2 Lymphangiogenesis: the initiation of lymphatic vessels occurs by the specification of subdomains within veins. These showed commitment to the lymphatic fate by expressing Prox1, a transcription factor that regulates expression of certain lymphatic genes. Subsequently these cells depart from the parental veins and migrate to organize a parallel vascular system that only drains its contents into major veins, but it is otherwise completely independent from blood vessels. Markers for lymphatic endothelial cells include: VEGFR3, Prox1, LYVE, and podoplanin.

VEGFR2 is expressed in lymphatic endothelium, as well as in blood vascular endothelium. In lymphatic vessels, VEGF-C is able to activate both VEGFR2 and VEGFR3. However, mice deficient for VEGF-C display a defect in the formation of the initial lymphatic vessels sprouts from veins, but specification of the lymphatic lineage is not impaired. Evaluation of heterozygous mice for VEGF-C revealed that dosage of this ligand is critical for normal lymphatic function in the adult. In fact, VEGF-C +/− mice display lymphedema in the lower limbs as well as chylous ascites from impaired lymphatic function in the gut.

The angiopoietin-Tie2 signaling pathway is also important in lymphangiogenesis. The contribution of Ang-Tie, however, appears to be more important for the function rather than to the development of lymphatics. Mice lacking Ang2 develop chylous ascites, an accumulation of interstitial fluid in the gut that is associated with a loss of gut lymphatic function. This requirement for Ang2 in the lymphatic system can be substituted by Ang1, as demonstrated by gene replacement. In addition, a subset of embryos deficient for Tie1 displays severe tissue edema that is independent of placental or cardiac dysfunction.

Abnormal development of peripheral lymphatics results in severe edema and impairment of lymphatic development in mouse models can be lethal. Equally importantly, lymphatic vessels are created by tumors and these vessels are used by tumors to metastasize from their site of origin. Growth of lymphatics in some tumors even appears to be rate limiting for metastasis. Thus, identification of molecular pathways regulating lymphatic growth may provide new strategies to treat vascular and neoplastic diseases.

5
Key Signaling Pathways in Vascular Morphogenesis

The molecular nature of the signaling pathways involved in blood vessel development has become an intense area of investigation, and great strides have been made toward elucidating the functions of the required genes. The mouse has proven to be a robust model system for studying vascular morphogenesis, not only because its developmental processes are similar to humans but also because of the wealth of available methods to manipulate the genome and create mutant animals. Many mutant mouse lines with a multiplicity of early and late vascular defects are currently available and allow for detailed analysis of the role of single genes or combination of genes during development, homeostatic functions, and under pathological conditions.

There are four most extensively studied ligand/receptor families that are essential for vascular morphogenesis: VEGF-VEGFRc; Notch-DSL; angiopoietin(Ang)-Tie2, and ephrin B2-ephB4. Their critical roles during development have been established by diverse ranges of approaches including the use of genetically ablated mouse lines for each gene. In addition, the TGF-β and PDGF pathways are also essential and will be discussed. Figure 1 provides an overall assessment of the impact of each of these pathways during vascular maturation. While we will constrain our comments to these major signaling pathways, it is important to stress that a large number of extracellular matrix proteins and their receptors, adhesion molecules, and other growth factors contribute extensively to the overall process of vascular maturation.

5.1
The VEGF Signaling Pathway

Initially identified for its ability to mediate vascular permeability, VEGF was later found to be a mitogenic agent for endothelial cells. Today, VEGF is recognized as an essential cytokine for the development and homeostasis of the cardiovascular system. Inactivation of a single VEGF allele results in embryonic lethality at midgestation due to severe cardiovascular defects. Interestingly, increase of VEGF in the heart, also leads to embryonic lethality. In the adult, overexpression of VEGF by basal epidermal cells (expressing keratin-14 promoter) results in increased microvascular density, leukocyte adhesion, and hypersensitivity to inflammation. Together these results indicate that unlike most genes, alterations in VEGF levels can result in significant pathological outcomes.

Although the major focus of interest has been on VEGF-A (or simply VEGF), this molecule is only one member of a family of proteins that also comprises VEGF-B, VEGF-C, VEGF-D, VEGF-E, and PlGF (placenta growth factor). These proteins interact with three major tyrosine kinases: VEGFR1, VEGFR2, and VEGFR3 and two nonreceptor tyrosine kinases; neuropilin 1 and neuropilin 2, which also bind to other ligands. Figure 3 provides a schematic view of these receptors and their respective specificity for each ligand. While this chapter will focus mostly on VEGF-A, referred herein as VEGF, much research has been done in the biological functions of the other family members.

The biologic activities of VEGF are mediated principally by two related

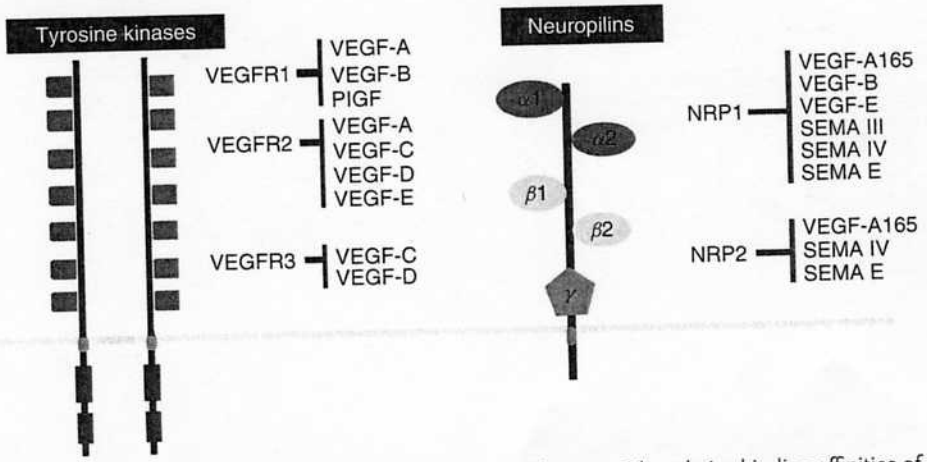

Fig. 3 VEGF receptors and ligands: VEGF signaling is essential for blood vessel development and homeostasis. This pathway includes a series of tyrosine kinases: VEGFR1, VEGFR2, and VEGFR3 that belong to the class III receptor tyrosine kinases of the platelet-derived growth factor receptor superfamily. All three receptors are characterized by the presence of seven immunoglobulin homology domains (in the extracellular region) and two intracellular kinase domains. The relative binding affinities of each receptor for the ligands is indicated. Neuropilins and accessory receptors to the VEGF pathway. The structure of neuropilin (NRP) 1 and 2 comprise two α, two β, and one γ subdomains, in addition to a short intracellular tail that does not show significant homology to any known proteins. The interaction of NRP 1 and 2 with their respective ligands is shown on the right.

cell-surface receptors, Flt1/VEGFR1 and KDR/Flk-1/VEGFR2 with extracellular ligand-binding domains and intracellular protein-tyrosine kinase domains. It has been determined that VEGFR1 is the high affinity receptor, while VEGFR2 is the low affinity receptor. In addition, some VEGF isoforms can bind to neuropilin receptors; these receptors will be discussed separately. VEGFR2 is the main signaling receptor for VEGF. Activation of this receptor has been shown to promote differentiation of progenitors, migration, mitogenesis, survival, nitric oxide release, and vascular permeability. In contrast, the biological role of VEGFR1 remains enigmatic. Treatment of VEGFR1-expressing endothelial cells with VEGF typically does not stimulate migration or DNA synthesis. Other reports using chimeric receptors or neutralizing anti-VEGFR1 antibodies to distinguish VEGFR1 and VEGFR2-mediated pathways suggest that VEGFR1 may indeed transmit signals that down-modulate endothelial cell responses to VEGF via VEGFR2. VEGFR1 appears to suppress both VEGF-stimulated chemotaxis and proliferation in endothelial cells.

Other evidence indicates that VEGFR1 performs important functions during vascular development. These might be mediated by VEGF, but could also be directed through activation by placenta-derived growth factor, another ligand for VEGFR1. Furthermore, in the adult, VEGFR1 appears to regulate monocyte function.

In situ hybridization reveals that patterns of VEGFR1 expression partially parallel those of VEGFR2 and VEGF in the developing mouse embryo. Moreover, homozygous knockout of VEGFR1 in mice results in embryonic lethality between days E9.5 and E11.5 characterized by a superabundance of endothelial cells and their hemangioblast precursors and the failure to form vascular channels. It was postulated that VEGFR1, as a buffer for embryonic VEGF, serves to keep endothelial cell expansion within limits appropriate for efficient vascular channel assembly. The VEGFR1-null phenotype is distinct from that seen in VEGFR2 homozygous knockout mice, which died *in utero* between days E8.5 and E9.5 with marked deficits in hematopoietic and endothelial cell numbers. Intriguingly, transgenic mice homozygous for tyrosine kinase–null VEGFR1 containing intact extracellular and transmembrane domains developed a normal, functional vasculature, but showed defects in monocyte chemotaxis. These findings reinforce the concept that, in endothelial cells, VEGFR1 may serve a modulatory function by virtue of its ligand-binding activity and potential to interfere with VEGFR2 or neuropilin 1, in addition to a signaling function.

While in neonatal mice expression of VEGF is required for survival, the function of VEGF signaling in adult vessels is less clear. There is evidence that activation of this signaling pathway is required for the maintenance of fenestrations in certain vascular beds. Blockade of VEGF signaling in adult mice has resulted in reduced capillary density and proteinuria.

5.1.1 Soluble VEGFR1, an Endogenous VEGF Antagonist

In addition to the major VEGFR1 mRNA species of 6.5 to 8.5 kb (presumed to encode the full-length receptor protein), Northern blotting typically detects other VEGFR1 hybridizing mRNA, originally detected in human placenta and endothelial cells, which arises by alternative RNA splicing. This mRNA encodes a protein, termed *soluble* or *secreted* VEGFR1 (or

sFlt1), in which a unique intron 13-encoded C-terminal peptide replaces the membrane-anchoring and tyrosine kinase segments, resulting in release of VEGF-binding receptor fragment from cells. Mouse VEGFR1 gene structure reveals that sFlt-1 mRNA is generated by the failure to excise intron 13 and the use of a presumed intronic polyadenylation signal. Recombinant sFlt1 binds VEGF with high affinity (Kd 20 pM) and acts as a potent inhibitor of VEGF biological activities *in vitro*. sFlt1 binds heparin, which may favor its retention close to sites of release in tissues containing heparan sulfate proteoglycans. Additional soluble forms of VEGFR1 have been described although their origin and properties remain unclear.

sFlt1 and chimeric analogs have been used experimentally as potential therapeutic agents and to test the role of VEGF in a variety of physiologic and pathological processes *in vivo*. For example, sFlt1 has been investigated as an experimental therapy for neoplasms that constitutively produce VEGF. Increased expression of sFlt1, either via transfected or infected tumor cells or administration of adenoviral vectors can dramatically inhibit vascularization and/or growth of tumor xenografts in mice. Similarly, virus-directed expression of sFlt1 reduced subretinal neovascularization in rats and ischemia-induced retinal neovascularization in mice. In a small-scale study, sFlt1 protein in the systemic circulation was marginally increased in patients with proliferative retinopathy compared to healthy controls.

Although it is clear that sFlt1, when overexpressed, can effectively inhibit biological actions of VEGF *in vivo*, little is known about the physiological function(s) of sFlt1 or the mechanisms controlling its biosynthesis. Since sFlt1 expression can occur only where the parental VEGFR1 pre-mRNA is produced, the fundamental level of control is transcriptional, governed by the endothelial-selective promoter elements for VEGFR1. In cell cultures or tissue explants, expression of mRNAs for VEGFR1, but not for VEGFR2, can be induced by hypoxia. This pattern of VEGFR1 inducibility can be recapitulated in animals exposed to systemic hypoxia.

Neuropilins and semaphorins. Neuropilins are transmembrane receptors best understood for their interaction with semaphorins. However, recently it has become clear that in addition to semaphorins, neuropilins also bind to VEGF isoforms 164 and 188. Eight classes of semaphorins have been identified and most of these appear to be specific to the neural system. They provide negative neuronal guidance cues: repel axons and collapse growth cones. Class-3 semaphorins, however, bind to neuropilin receptors also expressed by endothelial cells, in addition to neurons. There are six members of class-3 semaphorins (sema A–F) and they exhibit some relative degree of specificity. Thus, sema-3A activates neuropilin 1 and sema 3F activates neuropilin 2.

Clear demonstration of the impact of semaphorins in the cardiovascular system has been obtained from genetic knockout studies. Sema-3A–null mouse in 129/SV background die from cardiac failure approximately 3 days after birth. In the CD-1 background, they also exhibit a number of vascular defects in the head and abnormalities in trunk vessels. At E9.5, cranial blood vessels of the mutant embryos fail to remodel and the formation of the anterior cardinal vein is disrupted. Neuropilin 1–null mice also showed vascular remodeling defects with onset of lethality at E12.5 to E13.5. They also display agenesis of some branchial arch-derived vessels and

transposition of aortic arches. Overexpression of neuropilin 1 in mice also results in vascular defects. These include: vascular dilation, excess of vascular growth, and cardiac malformation.

Sema-3C–null mice die just after birth due to aortic arch malformations and septation defects in the heart. Interestingly, these were not noted in neuropilin 2–null mice. Instead, neuropilin 2 deficiency showed reduction of small lymphatic vessels and capillaries.

Interestingly, removal of both neuropilins showed a more severe vascular phenotype than either receptor alone. Embryonic lethality occurs at E8.5 with defects in yolk sac and embryonic vasculature. Overall, there seems to be a decreased number of endothelial cells in a manner similar to the one observed in VEGFR2–null mouse. Together these findings indicate that activation of neuropilins by semaphorins is an essential component of vascular morphogenesis.

5.2 The Notch Signaling Pathway

The Notch signaling pathway impacts a large variety of cell types including hematopoietic, neuronal, muscle, epithelial, and more recently, endothelial. It has been shown to participate in cell fate decisions either by initiating differentiation processes or by maintaining the undifferentiated state of cells. The contribution of Notch to the vasculature was initially recognized by the association of ligands and receptors with hereditary vascular anomalies. CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), is a disease of adult onset manifested by strokes, migraines, and progressive dementia. CADASIL has been linked to mutations in notch 3 resulting in a progressive degeneration of the smooth muscle layer surrounding cerebral and skin arterioles. Alagille syndrome has been attributed to mutations in jagged 1. Patients suffering from this syndrome exhibit abnormally formed blood vessels, arterial stenosis, and heart disease, in addition to hepatic lesions and skeletal defects.

In mammals, there are four distinct Notch receptors (notch 1–4) and five ligands (jagged 1–2 and δ1, 3, and 4). Unlike the VEGF signaling pathway, Notch receptors and ligands are transmembrane, thus interaction requires cell–cell contacts. Most frequently, associations occur between cells (homotypic or heterotypic) resulting in trans-signaling events. However, activation of receptors in cis can also occur. There does not appear to be ligand specificity between the ligands and receptors; however, recent experimental evidence suggests that not all receptor/ligand pair results in signaling. Activation of Notch requires a series of proteolytic events that are trigged by binding to receptors. The enzymes implicated in processing include members of the ADAM family (ADAM10/Kuzbanian and gamma secretase). Interestingly, genetic inactivation of both these enzymes has resulted in embryonic lethality with vascular anomalies similar to those found in Notch mutants. Once released, the intracellular domain of Notch (ICD) translocates to the nucleus where it binds to the transcription factor RBPJ-k (also known as *CBF-1/RJBk* in mammals, and Su(H) in Drosophila). The complex then activates a cohort of specific downstream effectors. The best characterized direct targets of Notch activation include the Hairy genes in Drosophila and the related bHLH transcription factor genes, Hes/Hey in vertebrates.

Targeted inactivation of notch 1 has confirmed its relevance to the development of the cardiovascular system. Embryos homozygous for a null allele of notch 1 die by day 9.5 with clear defects in somitogenesis and severe cardiovascular anomalies. Targeted mutation of another Notch receptor, notch 4, showed no defects in homozygous mutant mice, but compound homozygous for notch 1 and 4 had a more severe vascular defect than embryos homozygous for the notch 1 receptor alone. Interestingly, gain of function of notch 4 in the endothelium has an abnormal vascular development and early lethality. Like VEGF, it seems that vascular morphogenesis is extremely susceptible to levels of Notch signaling.

Several Notch ligands have been inactivated by homologous recombination. Embryos lacking a functional jagged-1 gene die at embryonic day 10.5, with vascular defects including lack of vascular remodeling and absent vitelline vessels. More recently, inactivation in delta4 results in haploinsufficiency with lethality at E 9.5. Mice die from lack of remodeling of the primary vascular plexus, a finding that was evident in both the yolk sac and in the embryo proper.

While all Notch receptors and ligand mutants do not link this pathway in hemangioblast or endothelial cell specification, a possible contribution in Notch signaling in cell fate choice might occur in the lymphatic system. Activated Notch appears to directly transactivate VEGFR3, and thus, it might contribute to the specification of lymphatic endothelial cells from veins.

5.3 Angiopoietins – Tie Signaling Axis

Initially isolated through a secretion-trap expression strategy aimed at identifying the ligand for Tie2, Angiopoietin-1 belongs to the family of ligands currently comprising four members (Ang 1–4). From these, Ang 1 and 2 are more understood with respect to their roles in developmental and pathological angiogenesis. Although these two ligands bind to the receptor tyrosine kinase Tie2 with similar affinities, Ang2 can inhibit Ang1 under some specific physiological contexts. Some studies *in vitro*, however, have shown that Ang2 can induce Tie2 phosphorylation in endothelial cells depending on dose, duration of exposure, and whether the source is autocrine or paracrine. Interestingly, Ang2 is expressed at angiogenic and vascular remodeling sites and it contributes to the detachment of smooth muscle cells. This enables the exit, migration, and proliferation of endothelial cells during the process of angiogenesis. These findings explain why Ang2 can either participate in the regression of the formation of new blood vessels, since destabilization of vessels is also a requirement for angiogenesis.

Inactivation of Tie2 by homologous recombination results in embryonic lethality by midgestation (E12.5) from cardiovascular abnormalities. Mice exhibit lack of remodeling of the primary plexus and sprouting deficiency. Genetic ablation of Ang-1 results in a similar phenotype, indicating that at least during development, these two molecules are codependent. Gain of function of Ang1 results in increased vascularization and abnormal permeability.

Although ligands for the related kinase, Tie1, are yet to be identified, inactivation of this receptor is also embryonically lethal (from E13.5 to neonates). Deficiency in Tie1 results in edema and microvascular fragility with hemorrhage. Together the data indicates that Tie1 is not required for the early differentiation of endothelial cells

and, unlike Tie2, is not necessary for initial patterning.

In adult settings, it is clear that Ang1 is a natural antipermeability factor protecting against excessive plasma leakage and counteracting the pathological permeability induced by VEGF.

5.4 Ephrins and Eph Receptors

The family of Eph receptors includes a large group of receptor tyrosine kinases (14 in total) that regulate an impressive array of biological effects, including: embryonic patterning, neuronal targeting, epithelial differentiation, and vascular development, among others.

Activation of Eph receptors by their ephrin ligands requires cell–cell contact, as ephrins are also membrane bound. Signaling between ligands and receptors is bidirectional and dependent on the dimerization state of the ligand. The effects of ephrins have been well studied in neurons. In this system, ephrins provide guidance by repulsion of contact of the growing axon. There have been eight ephrin ligands identified thus far; however, only a subset of these appear to impact the cardiovascular system.

The first indication that Eph signaling was involved in vascular morphogenesis came from genetic inactivation analysis. In fact, removal of either ephrin B2 or EphB4 results in early embryonic lethality (E10.5) with alterations in vascular remodeling and lack of arterial and venular identities. Interestingly, expression analysis of LacZ reporter mice under the regulatory control of ephrin B2 and EphB4 revealed complementary expression patterns, with ephrin B2 in arteries and EphB4 in veins. Expression of ephrin B2 and EphB2 is also found in mesenchymal cells, suggesting a possible role for this signaling system in the recruitment of mural cells during remodeling events.

The signaling consequences initiated by Eph-ephrins have been also explored *in vitro*. Thus, signaling of EphA2 by ephrin A1 in aortic endothelial cells attenuates proliferative signals mediated by VEGF, through what appears to be the Erk pathway. In addition, this system regulates cell–cell recognition, as was anticipated by the defects noted in the null mouse.

5.5 TGF-β Signaling

TGF-β is a member of a large superfamily that includes: bone morphogenetic proteins, activins, inhibits, and Mullerian inhibitory substance all relevant to developmental processes. Three members of the TGF-β family (TGF-β1–3) have been identified all with partially overlapping expression, but distinct functions. The growth factors are secreted as latent forms and its activation is dependent on either proteolytic processing or binding to thrombospondin-1. Signal transduction by TGF-β requires a series of serine/threonine receptors, accessory receptors, Smad proteins, and Smad transcription factors that convey these signals to specific genes.

Because of its early expression and broad distribution, it was extremely surprising to find that targeted disruption of the TGF-β1 gene does not necessarily lead to embryonic lethality or congenital anomalies and mice tend to die 3 weeks after birth from immunological deficiencies. Initially, the potential issue of redundancy was a plausible explanation. Subsequently, it was shown that TGF-β could be transferred from mother to fetuses by the placenta

and to pups through the milk. This transfer is likely to account for the lack of a more severe phenotype, as the transfer would rescue the lack of endogenous production of the growth factor. While the maternal source of TGF-β could rescue some animals, still only about 50% of homozygous mice were recovered. The lethality was associated with defects in both hematopoiesis and vasculogenesis resulting in lethality around E9.5 to E11.5.

Genetic inactivation studies have clearly shown that TGF-β signaling is important for differentiation of endothelial cells. However, a further understanding of its contribution has been difficult to ascertain, particularly since the effects of TGF-β appear to be multiple and altered by other factors. In two-dimensional cultures, TGF-β has been shown to inhibit endothelial cell proliferation. Interestingly, in three-dimensional cultures, TGF-β does not affect proliferation, but appears to modulate differentiation. More recently, these opposing results have been conciliated by a more concrete understanding of TGF-β signaling pathways.

TGF-β signaling is mediated by its type I and II serine/threonine kinase receptors. There are two type I receptors: ALK-1 and 5 and one type II receptor, also known as TGF-β receptor II (TGFβRII). By activation of Smad1/5, it has been shown that ALK1 is responsible for the stimulation of endothelial cell proliferation and migration; while ALK5 through activation of Smad2/3 results in inhibition of cell proliferation and migration. Consequently, the net expression of these two receptors and their respective target Smads dictates the end result of TGF-β response in endothelial cells.

Mutations in ALK1 or on its accessory receptor, endoglin, have been linked to HHT. Consistent with these findings, ALK1-null mice die at E10.5 to E11.5 with defects in smooth muscle cell development and angiogenesis. ALK1 knockout animals also display vascular branching defects, dilations, and abnormal vascular connections (arterial–venular fusions). Mice lacking either TGF-βRII or ALK5 die at E10.5 due to defects in vascular development clearly seen in the yolk sac. Mice lacking endoglin also display embryonic lethality, with defects in vascular remodeling and abnormalities in arterial–venous boundaries. The contribution of this signaling pathway to vascular development has also been reinforced by the experimental inactivation of several Smad proteins in mice.

5.6 Platelet-derived Growth Factor

The PDGF family of dimeric growth factors shares a significant degree of sequence similarity to VEGF, yet its expression patterns and functional properties are clearly distinct. PDGFs and their tyrosine kinase receptors are expressed and impact a large number of tissues including fibroblasts, smooth muscle cells, neurons, and endothelium. This expression pattern explains why deregulation of this pathway has been associated with a myriad of human diseases, including atherosclerosis, fibrosis, and cancers.

The classical PDGFs include homo- and heterodimeric associations of the A and B chains, forming PDGF AA, BB, and AB respectively. The A and B polypeptides share similar protein domains, and are secreted as fully active factors. Like VEGF, these classical PDGFs also contain "retention matrix" motifs that allow them to interact with extracellular matrix proteins and regulate their biological availability to specific sites. Signaling occurs by

three receptors resulting from the association of two transmembrane polypeptides: PDFGR-α and β that also function as homo and heterodimers.

The contribution of PDGFs to the vasculature has been revealed by gene inactivation in mice. Phenotypic analysis of null mice implicated PDGF-B in the maturation stages of vascular development. PDGF-BB is expressed by endothelial cells and its receptor, PDGF-β, is located in vascular smooth muscle cells and pericytes. Absence of PDGF-B results in reduced pericyte association with capillaries, vascular fragility, and hemorrhage with subsequent lethality. Both PDGF-B and PDGFR-β-null mice had decreased numbers and proliferation of smooth muscle cells/pericytes progenitors. This was more noticeable in the brain, heart, and brown adipose tissue.

Recently, two additional growth factors have been identified in this family: PDGF-C and D. In contrast to A and B, the novel PDGFs are secreted as zymogens and require activation by proteolytic cleavage of their N-terminal CUB domains. Following proteolytic processing, PDGF-C binds as a homodimer to PDGFR-α, whereas active PDGF-D interacts with PDGFR-β. Both are also able to activate PDGFR-α and β heterodimers in cells that expressed these two chains. Genetic inactivation of PDGF-C results in defects in secondary palate formation and the dermis. The contribution, if any, of PDGF-C and D in the vascular system is yet to be elucidated.

6
Multiple Forms of Vascular Expansion

Angiogenesis is also a vital and dynamic process in adult vertebrate organisms. Vascular expansion is essential for normal tissue growth, wound healing, endometrial cycling, and pregnancy. Moreover, angiogenesis can be a beneficial adaptation to myocardial ischemia and peripheral vascular insufficiency.

Postnatal neovascularization has been thought to occur by a sprouting mechanism, that is, angiogenesis. While this process has been well studied and characterized during development, additional modes of vascular expansion have been recently proposed. In particular, the contribution of circulating endothelial cell progenitors (ECPs) has been clearly documented in a wide array of models and the existence of "resident-cell progenitors" that contribute to the growth of vessels upon injury has also been recognized. In addition, it appears that a mechanism of intraluminal bridging of vessels, known as *intussusception*, has also been noted as a mode for vascular expansion. These processes are discussed in greater length in the following sections.

6.1
Circulating Endothelial Cell Progenitors

The growth of blood vessels in adult animals has been traditionally considered to occur only as the result of angiogenesis, sprouting from preexisting vessels, rather than *de novo* endothelial recruitment from progenitor cells. Recent studies, however, have documented the existence of bone marrow–derived endothelial cells that participate in adult vascular growth, particularly in the context of rapidly generated tumor vasculature. Characterization of these circulating endothelial precursors has demonstrated that they are likely hematopoietic in origin and are recruited by VEGF signals to home in sites of vascular growth.

Similar to embryonic progenitors, adult bone marrow–derived endothelial precursors, including Sca1, appear to share antigenic determinants with hematopoietic progenitors. They also respond to similar factors, prominent among them is VEGF, which mobilizes Sca1+ cells, and stimulates their proliferation and migration, and probably their differentiation. ECPs have been detected in the peripheral blood and have the capacity to differentiate *in vivo* and *in vitro*. Their incorporation into sites of neovascularization has also been documented. Specifically, bone marrow–derived endothelial cell precursors (BMD-ECP) have been shown to participate in neoangiogenesis after postmyocardial ischemia, limb ischemia, wound healing, atherosclerosis, endothelialization of vascular grafts, retinal neovascularization, and cardiac impaired neovascularization during aging. Furthermore, the growth of at least certain tumors appears to be dependent on the recruitment of ECPs from bone marrow. Transplantation of wild-type marrow into *Id3/4* knockout mouse was reported to rescue the otherwise impaired tumor neovascularization of *Id3/4* deficient animals. At least in this model system, tumor vascularization was dependent on bone marrow–derived endothelial cells.

Contributions of bone marrow cells to the vascular smooth muscle cell compartment have been examined as well. Smooth muscle cells from arterioles in the infarcted heart were found to have bone marrow origin. Furthermore, smooth muscle–like cells can be derived from cultures flk-positive ES cells. These studies indicate that bone marrow cells can contribute to smooth muscle cell formation in pathological conditions, but do not address their potential role in normal vascular formation.

While investigators have agreed on their presence, the relative contribution of vascular progenitors to sites of neovascularization in the adult has been a point of heated debate, mostly because the assessments are not equivalent. Engraftment of ECPs, either from blood circulation or adult tissues have been reported to range from 3.5 to 10%. The information contrasts other studies that indicate progenitor contribution of up to 95%. Again, the source of bone marrow (or purified subpopulation) and the experimental models have been variable, therefore, this question remains to be answered.

If, indeed, the contribution of bone marrow–derived progenitors to the growth of the tumor vasculature is significant, manipulation of the ECP population might result in suppression of angiogenesis and tumor expansion. Consequently, several groups have now initiated significant efforts to ascertain the potential value of ECPs for therapeutic intervention. However, progress in this arena has been hampered by lack of a solid means to identify these cells.

The cell-surface makeup and full characterization of ECPs is still unclear and the subject of active investigation. Experiments using CD34+ sorted precursors have shown that ECPs display this marker, both while in the bone marrow and also in the circulation. In contrast, other studies have reported the presence of tissue-resident CD34 negative cells that can contribute to endothelial cells. These latter studies were performed with local progenitors, so it is possible that there are indeed multiple precursors, some bone marrow–derived; and some resident. More consistently, CD34+, AC133 (CD133 or prominin A in mouse) and VEGFR2 are the markers most agreed upon in the literature. Why has it been difficult to

fully characterize this population of bone marrow–derived cells? Models that can identify these cells are currently missing, and the isolation of ECPs using CD34 is challenging, with low yield. Other investigators have used a combination of markers, yet the proof of principle for their identity relies on vascular integration. This adds another layer of difficulty, as once they become incorporated, the differentiation pathways are initiated and the makeup of the precursor population is lost. Thus, the transient nature of the phenotype has hindered progress in this area. Clearly, genetic markers that could trace this population of cells can add a strong advantage to this research.

6.2 Vascular Intussusception

The term *intussusception* is used to describe the process of intravascular growth by the formation of transendothelial cell bridges. These bridges are subsequently reinforced by tissue columns that grow into the lumen of these vessels. Subsequently the bridges gain mural cells, that is, pericytes and/or smooth muscle components, and undergo remodeling. Through this process a large vessel can branch, or be subdivided into smaller daughter vessels. Little information is available in the literature about this process. At this point, it is difficult to ascertain the frequency of intussusception *in vivo* and its relevance to vascular expansion.

7 Mechanical Forces and Angiogenesis

Mechanical factors, such as shear stress, wall tension, and stretch, have long been implicated in vascular growth. Ligation of vessels and subsequent interruption in flow results in severe disruption in normal vascular patterning and morphogenesis. However, caveats in the interpretation of these experiments include the possible contribution of oxygenation.

Technological advances have allowed the application of laminar, pulsatile, and circular shear stress on cultured endothelial cells to study the effect of these forces on gene expression. There is increasing experimental evidence that shear stress, and other consequences of vascular activity, modulate gene expression and that different types of stress activate distinct gene programs. Stretch of cardiomyocytes activates several second messenger pathways including Ras/MAP kinase, tyrosine kinases, and protein kinase C. Stretch has been shown to activate all three MAP kinase family members. Furthermore, it has been shown that stretching of skeletal muscle results in transcriptional activation of VEGF.

In vivo evidence also indicates a role for stretching in angiogenesis. Stretching the left ventricle for 30 min via an intraventricular balloon resulted in nearly a sixfold increase in VEGF message level. Stretching of endothelial cells also precipitates changes that activate and facilitate angiogenesis. They include rearrangement of the cytoskeleton and the release of plasminogen activators and metalloproteinases, which disrupt the basement membrane thus enabling cell migration, proliferation, and activation of ion channels with consequent mobilization of calcium. These factors modulate the relative generation of cellular force by the cytoskeleton and integrins, which combined with the relative rigidity offered by the matrix results in either thinner or wider capillary tubes *in vitro*. Clearly, a concrete understanding of how mechanical

forces are interpreted by cells during angiogenesis is precarious; however, this is an active area of investigation that is likely to reveal important functions of hemodynamics forces in the vasculature.

8
Angiogenesis Inhibitors

The concept that tumor progression could be regulated by pharmacological and/or genetic suppression of blood vessel growth has engendered a long-standing interest in the identification of molecules or synthetic compounds that block angiogenesis. Among recognized angiogenesis inhibitors are: platelet factor 4 (PF4), thrombospondin-1 (TSP1) and 2 (TSP2), angiostatin, endostatin, pigment epithelial-derived factor, and more recently proteolytic fragments of type IV collagen. Interference in the signaling pathways of specific stimulators, such as VEGF/VPF (vascular permeability factor) has proven effective in blocking blood vessel growth. In addition, blockage of integrin binding, in particular, $\alpha_v ß_3$, has been shown to suppress angiogenesis in several tumor types.

Antiangiogenesis has been appealing for cancer therapy for three main reasons: (1) it is likely that most tumors are dependent on angiogenesis, thereby providing a common target in the treatment of widely heterogeneous disease; (2) endothelial cells are considered to be less likely to develop adaptations to bypass drug effects (i.e. drug-resistant phenotype, as seen in some tumors); (3) it is anticipated that tumor vessels are proliferative providing a differential target than the quiescent vessels present in normal tissues.

Results from angiogenesis research during the last decade have questioned some of those earlier assumptions. Clinical trials designed to test antiangiogenic therapy have met with variable and rather disappointing success. In particular, not all tumor vessels appear equally susceptible to a single modality of antiangiogenic therapy. The reasons for this outcome are likely multiple. For example, the participation of tumor cells to the vascular wall ("vascular mimicry") has been demonstrated in uveal carcinoma, and more recently in melanoma. How responsive are these cells to antiangiogenic therapy? The molecular nature of tumor microvessels appears to be more variable than anticipated and differences in the tumor microenvironment will likely influence therapeutic outcome. It has been well acknowledged that the "context" poses a direct (and reciprocal) effect in the tumor cells. Additional reasons for the "disconnect" between bench and clinical results have been attributed to poor design of trials and efficacy of some of the tested drugs. More recently, the evaluation of "combination therapy" (antiangiogenic and "metronomic" chemotherapy) has received experimental attention with a higher degree of success than either therapy alone. Regardless, the relative success of therapeutic approaches using VEGF has engendered hope for this modality of therapy both in cancer (to block vessels) and in cardiac ischemia (to enhance angiogenesis).

VEGF has shown experimental success in animal models to treat vascular insufficiency in the heart and in peripheral blood vessels. Accordingly, clinical studies have been undertaken in which VEGF has been administered as recombinant protein or expressed from plasmids or viral vectors for stimulatory reasons. The situation is particularly complex in diabetic patients, where the potential benefits of

proangiogenic therapy for peripheral vascular disease must be weighed against the risks of exacerbating the progression of "diabetic retinopahy," a condition characterized by excessive vascular proliferation. Inhibition of VEGF signaling has been used for suppression of vascular growth during cancer progression. In addition, the role of VEGF in other processes, such as atherosclerosis, arthritis, and retinopathies has engendered a lot of interest in attempting to block this signaling pathway in pathologies other than tumors. The efficacy of these treatments is currently under evaluation. It is clear that understanding the control of cellular events associated with VEGF-dependent angiogenesis will facilitate the rational design of therapeutic interventions.

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