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ENDOTHELIAL SIGNAL INTEGRATION IN VASCULAR ASSEMBLY

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■ **Abstract** Regulated assembly of a highly specialized interconnecting network of vascular endothelial and supportive cells is fundamental to embryonic development and organogenesis, as well as to postnatal tissue repair in metazoans. This review advances an "endotheliocentric" model that defines tasks required of endothelial cells and describes molecular controls that regulate steps in activation, assembly, and maturation of new vessels. In addition to the classical assembly mechanisms—angiogenesis and vasculogenesis—endothelial cells are also recruited into vascular structures from the circulatory system in adult animals and from resident mesenchymally derived progenitors during organogenesis of kidney and other organs. Paracrine signaling cascades regulated by hypoxia initiate a sequentially coordinated series of endothelial responses, including matrix degradation, migration, proliferation, and morphogenetic remodeling. Surface receptors on committed endothelial lineage progenitors transduce cues from extracellular-matrix–associated proteins and cell-cell contact to direct migration, matrix attachment, proliferation, targeting and cell-cell assembly, and vessel maturation. Through their capacity to spatially segregate and temporally integrate a diverse range of extracellular signals, endothelial cells determine their migratory paths, cellular partners, and life-or-death responses to local cues.

INTRODUCTORY COMMENTS

Toward an Integrated Model for Vascular Assembly

Endothelial cells are the central cellular organizational unit of vascular structures. Their lineage commitment, expansion, organization, and assembly into ordered and tissue-specific interconnecting vascular structures are required for organogenesis and successful embryonic development. In mature subjects, expansion, contraction, and remodeling of microvascular structures underlie wound healing, reproductive tissue cycles, tumorigenesis, and a number of other pathological conditions involving inflammation (1). Endothelial cells are integrators, trans-

ducers, and effectors of local environmental signals (2). Their tightly balanced proliferation, migration, and morphogenic responses to angiogenic or angiostatic stimuli are context appropriate in two critical features: (*a*) they maintain integrity of the vascular barrier function, and (*b*) they conform with fidelity (under physiological situations) to the architectural cues of adjacent nonvascular tissue structures to integrate critical functions of such tissues as the mammalian kidney.

This review considers spatial and temporal problems faced by endothelial cells as they assemble and remodel vascular structures, from an "endotheliocentric" vantage. Maintenance of vascular integrity requires that endothelial cells spatially and temporally segregate responses to local cues in the context of cell-cell and cell-matrix attachments. Indeed, endothelial shape and tractional forces that influence it are critical determinants of gene expression, signaling, and apoptosis (3). Although useful in integrating recently obtained information, the model is, at some level, a conceptual artifice that underplays many crucial features of vascular development and neovascularization, summarized in recent reviews (4–6). The timing and morphological features of vascularization failure in mouse embyros that are null for molecules regulating vascular development have provided some insight into the necessity for specific receptors, their ligands, matrix-interactive proteinases, and cell-cell–targeting machinery. The pattern of vascularization failure in homozygous animals that are null for intermediaries is evolving as a gold standard to define molecular features, yet considerable overlap in the morphological characteristics exists, and specifics of organogenesis and vascular bed–specific neovascularization may not be uncovered until conditional gene deletion strategies are expanded. In this review, we highlight recent advances that provide insight into emerging pictures of integrated response.

PROCESSES OF ENDOTHELIAL INCORPORATION

Endothelial Progenitor Spatio-Temporal Tasks

Schematically represented in Figure 1 is the process of vasculogenic assembly, in which individual endothelial progenitor cells display markers of lineage commitment and assemble vessels de novo. This contrasts with so-called angiogenic assembly (Figure 2), in which new vessels arise from existing vessels through endothelial branching, sprouting, migration, proliferation, and anastomotic interconnection with endothelial cells residing in existing vessels (4). Compelling evidence defines a common role for vascular endothelial growth factor (VEGF) to support both processes through its actions on endothelial cells and progenitors (7, 8), and recent definition of hypoxia-sensitive transcriptional mechanisms that regulate VEGF production in tissue sites underserved by vascular supply are emerging (9) (see below). Yet requirements of integrated endothelial-cell function in the neovascularization process extend far beyond roles for VEGF and its recep-

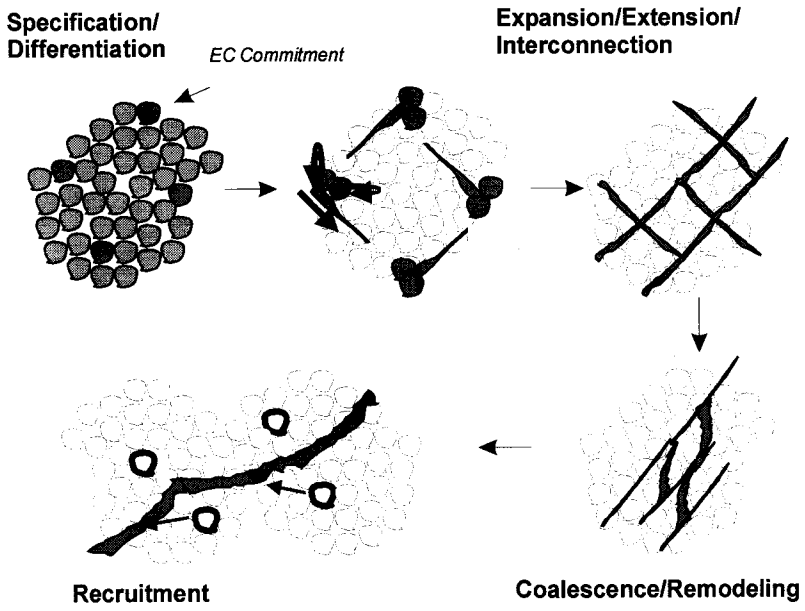

Figure 1 Vasculogenesis. Early flk-1 (+) angioblasts in the paraxial mesoderm are specified, expand in clusters, and extend long projecting processes that interconnect to form a network or primary vascular plexus. Through coalescence of this network, a linear structure evolves that undergoes remodeling to form a single central lumen within the developing aorta. Supportive smooth muscle cells are recruited and coordinately participate in vessel maturation and morphogenesis. A similar process evolves through the primary plexus stage in the extraembryonic circulation, where hemangioblasts migrate and expand in clusters (blood islands) that contain central hematopoietic progenitors and peripheral angioblasts.

tors, obligating additional cellular processes, including cell-cell discrimination, recruitment, and remodeling.

A developmental progression of endothelial events was chronicled in the 1930s (10). It is now possible to frame endothelial cellular events within the context of molecular mediators that are likely to contribute. During angiogenesis, endothelial cells in existing vessels are initially activated by a net imbalance favoring angiogenic over angiostatic factors (2). Activated endothelial cells break down and penetrate existing subendothelial basement membrane through actions of proteinases, such as matrix metaloproteinase (MMP)-9 (11). Long, filopodial-like cellular processes migrate, tracking along fibrillar extracellular-matrix components, through migratory responses that are mediated by α_v and α_5 integrins (12). These endothelial processes contact and discriminate among inappropriate cell partners, such as fibroblasts and inflammatory cells, to approach an existing vessel. Through currently unknown mechanisms, a collaborator endothelial cell is acti-

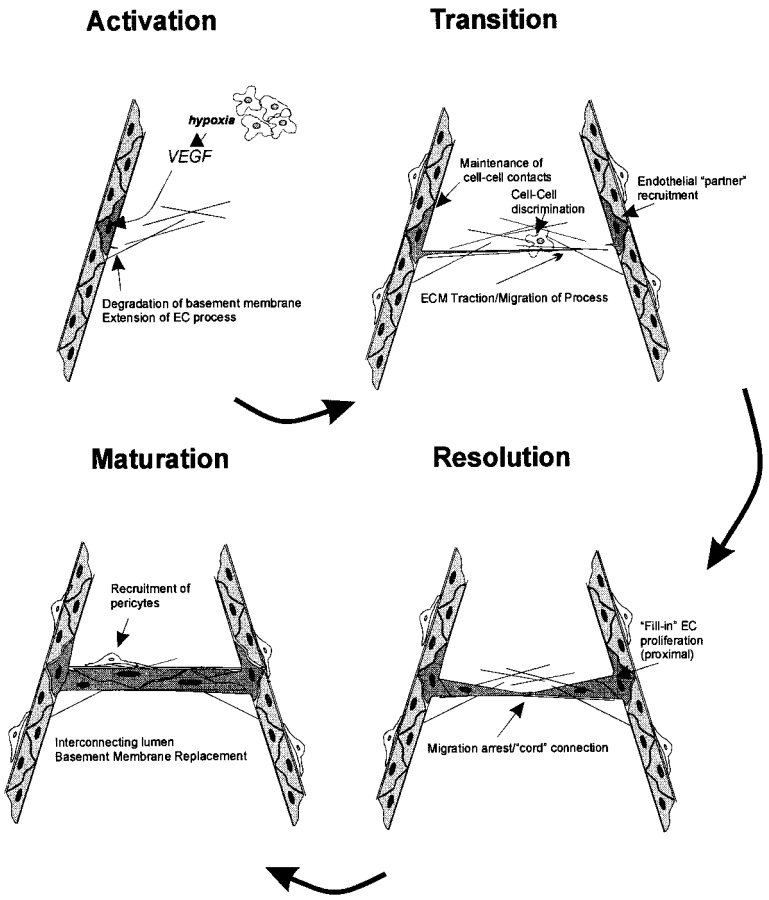

Figure 2 Endotheliocentric stages in angiogenesis. Expansion of new vessels from existing vascular networks proceeds in response to tissue hypoxia, a primary stimulus for VEGF production and release. A receptive endothelial cell responds (Activation) by degrading subjacent basement membrane, extending an elongating cellular process by traction upon fibrillar connective tissue elements, while maintaining integrity of the existing vessel on the trailing end. The extending process discriminates inappropriate partner cells, approaches an existing vessel, and through unknown means (likely release of chemokine or other soluble factor), signals a collaborating partner endothelial cell to penetrate basement membrane, and extend a cell process (Transition). A stable interconnecting cord-like scaffold is formed (Resolution), about which proliferation, migration, and morphogenesis ensue to create an interconnecting lumen. Pericytes are recruited and basement membrane elaborated (Maturation). Adapted, with modern interpretation, from Clark & Clark (10).

vated to project a reciprocating process through its basement membrane. Cell-cell recognition machinery, likely involving Eph/ephrin juxtacrine signaling (13), initiates molecular coupling events that proceed through establishment of VE-cadherin–containing junctional complexes, connexin-integrated gap junctions, and focal contacts (14). Morphogenetic events then establish a lumen interconnecting with existing vessels, basement membrane is reestablished, and pericytes are recruited as critical elements of vessel maturation and maintenance (6). Coordinated recruitment of pericytes and smooth muscle cells provides not only structural support but also paracrine signals implicated in endothelial maturation and vessel integrity such as angiopoetins (5) and transforming growth factor (TGF) β (15).

These spatial problems require integration of signals linked with and regulated by endothelial cell-cell and cell-matrix interactions. Each interval step of the process of vascular assembly appears critical to the next, based on embryonic vascularization defects in animals homozygous for targeted gene deletions. Distinctions in the timing and morphology of defective vascularization in gene knockout mice illustrate the sequential features of the process during early development, at a time when much of the vascularization process is temporally compressed and synchronized (Table 1).

An additional mechanism for incorporation of endothelial cells into new vessels has recently been described (Figure 3). Endothelial progenitor cells (EPCs) are also recruited to sites of neovascularization in mature mammals from a cir-

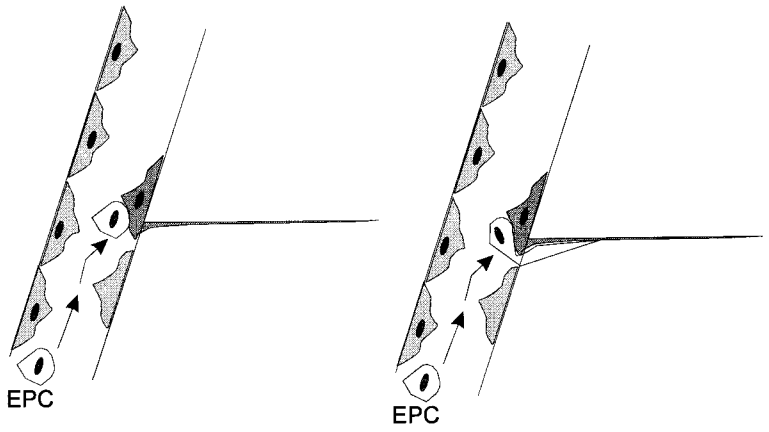

Figure 3 Circulating endothelial progenitor cell (EPC) incorporation: targeting to sites of neovascularization. With the demonstration that marrow-derived EPCs are incorporated into new vessels, questions arise about the mechanisms responsible for their targeting. If activation (Figure 2) at local sites recruits substantial numbers of EPCs from the circulation, their recruitment may depend upon their cell-cell interactions at the lumen interface with activated endothelial cells.

TABLE 1 Molecular identity of genes necessary for embryonic vascularization

Molecular class	Embryonic stage lethality	(Vascular phenotype extraembryonic/intraembryonic)
Transcription factors		
HIF1α or ARNT (HIF1β)	$\leq E_{10.5}$	Defective yolk sac vascularization with intact EC differentiation, fusion but maturation failure (38, 103, 104)
MEF2C	$\leq E_{9.5}$	Normal EC differentiation, with failure to organize a primitive vascular network. Failure of SMC differentiation defective yolk sac vessels with failure of anterior cardinal vein and dorsal aortae formation (106)
LKLF	$E_{12.5\text{-}14.5}$	Normal vasculogenesis and angiogenesis, failure of vessel wall stablization (107)
TEL	$E_{10.5\text{-}11.5}$	Normal EC differentiation with defective yolk sac angiogenesis and normal hematopoiesis (36)
Ets2	$< E_{12.5}$	Defective trophoblast migration/differentiation with persistent ECM and defective MMP-9, 3, and 13 production (35)
Transcription factor interactor		
PVHL	$E_{10.5\text{-}12.5}$	Extraembryonic vasculogenesis failure after $E_{9.5}$ (105)
Receptor tyrosine kinases		
VEGFR1 (flk1)	$E_{8.5\text{-}9.5}$	Endothelial and hemangioblast migration and differentiation failure (29, 41)
VEGFR2 (flt1)	$E_{8.5\text{-}9.5}$	Endothelial cell differentiation intact. Vascular channels disorganized, overpopulated with angioblasts (42)
VEGFR3	$E_{9.5}$	Vasculogenesis/angiogenesis intact; large vessel disorganization with lumen defects (44)
Tie1	$E_{13.5\text{-}14.5}$	Defective vascular integrity/endothelial survival in angiogenesis, edema, and hemorrhage (57, 108)
Tie2	$E_{10.5}$	Defects in organization, remodeling, sprouting; heart trabeculations (56)
EphB2/EphB3	$E_{10.5}$	Defects in sprouting, vessel remodeling, and organization (63)
PDGFβR	P_1	Failure of mesangial recruitment, glomerular development (24, 109)

Other receptors		
Endoglin (HHT1)	$\leq E_{11,5}$	Normal vasculogenesis; defects in SMC recruitment/endothelial remodeling (110)
Other membrane proteins		
VE Cadherin$^{-/-}$	$E_{10.5}$	Defective anterior large vessels and failure to establish yolk sac vascular plexus (111)
Integrin $\alpha_v{}^{-/-}$	$E_{10.5} \rightarrow P_1$	Vasculogenesis and early angiogenesis intact. Placental (labyrinthine) defects. Intracerebral, intestinal hemorrhage (112)
Ligands		
VEGF$^{+/-}$	$E_{11.5}$	Rudimentary dorsal aorta, reduced ventricular mass (39, 40)
VEGF$^{-/-}$	$E_{10.5}$	No dorsal aorta; defective hematopoiesis (39, 40)
Ephrin-B2$^{-/-}$	$E_{11.5}$	Failure of extraembryonic vessel fusion/remodeling (64)
Angiopoietin 1$^{-/-}$	$E_{10.5}$	Defective organization, remodeling (113)
Angiopoietin 2$^{-/-}$	$E_{12.5} \rightarrow P_1$	Defects in vessel integrity, hemorrhage (5)
JAG1$^{dDSL/dDSL}$	$E_{10.5}$	Normal vasculogenesis, with dysmorphic, small vessels; failure to remodel primary plexus in yolk sac and embryo (114)
TGFβ 1$^{-/-}$	$E_{10.5}$	Failure to remodel the primary vascular plexus of yolk sac and cranial vessels (47)
PDGF BB$^{-/-}$	P_1	Failure of glomerular development (25)
Protease/coagulation factor		
[Tissue Factor (TF)$^{-/-}$]	$E_{8.5}$	Extraembryonic vascular failure with failure of SMC/pericyte recruitment (115)

culating, marrow-derived population of progenitor cells (16, 17). This population of circulating EPCs is mobilized by regional ischemia or administration of either GM-CSF or VEGF, and increased numbers of marrow-derived EPCs are incorporated into neovascularization sites after VEGF administration (18, 19). At present, incorporation of these circulating EPCs into sites of neovascularization appears to obligate some yet undefined targeting machinery to recruit circulating cell participation. Such a function may be served by Eph/ephrin or other juxtacrine-targeting interactions, as defined below. Finally, vascular remodeling and formation of networks may also involve formation of pillars within existing vascular lumen space, to create bissected "hallways" and new capillary networks (20, 21).

During organogenesis, mesenchymally derived EPCs within fields of differentiating mesenchyme contribute to vascularization of such organs as mammalian kidney (22, 23). These cells assimilate into new vessels through coordinated recruitment to vascularization sites, such as the developing glomerulus, through a process that shares features with both vasculogenesis and angiogenesis (Figure 5, see color insert). In addition to endothelial-endothelial assembly, endothelial cells actively participate in recruitment of supportive pericytes and equivalent mesangial cells through expression of growth factors such as platelet-derived growth factor (PDGF) BB. Developmental vascularization fails, as demonstrated by glomerulogenesis defects and cerebral circulation defects, in mice null for PDGF B (an endothelial product) or PDGF β receptors (expressed on pericytes) (24–26).

Embyronic Origins and Commitment of Endothelial Cells

Vascular development in mouse embryos initiates around embryonic day 7 (E7.5). At that time, intraembryonic angioblasts, the earliest EPCs, arise as individual cells from paraxial- and lateral-plate mesoderm under the influence of inducing factors (8, 27, 28). Those that contribute to yolk sac vasculature migrate through the primitive streak into the extraembryonic tissues and assemble first into mesodermal-cell aggregates that contain both endothelial and hematopoietic precursors (4). Common lineage is defined by shared expression of CD34, CD31, and Flk-1, a VEGF receptor that is required for development of both lineages during mouse development (29). During maturation of these blood islands these lineages segregate, with endothelial precursors lining spaces containing the hematopoietic progenitors. In contrast, angioblast precursors of intraembryonic vessels arise in paraxial mesoderm as individual cells expressing Flk-1 and SCL/TAL-1, an HLH transcription factor (30). There they proliferate locally, extending sprouts that interconnect into a loose meshwork and undergo both cranio-caudal and dorsoventral progression into a primary vascular plexus of cells expressing PECAM, CD34, and the angiopoeitin receptor, Tie-2, in that progression. This network subsequently fuses and remodels through morphogenesis into the earliest intraembryonic vessels (Figure 1) (30).

Specification of Endothelial Lineages Although Flk-1 and SCL/TAL1 are among the earliest markers for cells with endothelial potential, the signals and transcriptional controls regulating specialization and specification as distinct from hematopoietic lineages are not yet clear. In vitro differentiation of embryonic stem cells suggests that Flk-1^+ cell populations destined for endothelial differentiation subsequently express in sequence VE-cadherin, PECAM, and CD34 and that the earliest marker for hematopoietic cells not expressed on the common endothelial lineage precursor is α_4 integrin (31, 32).

Among other early molecular controls regulating specification of angioblasts, several transcription factors have been evaluated in developmental systems. Ets-1 and Ets-2 are expressed in early vascular sites, and putative Ets-1–interacting *cis* elements have been identified in genes expressed in endothelium, including MMP-1, MMP-3, MMP-9, and u-PA (33). Ets-1 expression is induced in angiogenic endothelial cells adjacent to imposed wounds in vivo and in vitro (34). Homozygous null mutations in Ets-2 are embryonic lethal, as a consequence of impaired trophoblast development in ectoplacental cone formation, with suppressed expression of MMP-9 (gel B) (35).

The Ets-related helix-loop-helix factor TEL is implicated in a number of leukemias through genetic rearrangements that create fusion proteins with PDGF receptor (PDGFR), Abl, AML-1, and others. Yet, TEL-null embryos die between E10.5 and E11.5 with failure of yolk sac angiogenesis, apparently the consequence of failure to maintain and mature the yolk sac vessels, whereas intraembryonic vasculature appears normal (36). Surprisingly, hematopoietic lineages derived from explanted yolk sacs are unaffected. Prominent mesenchymal-cell apoptosis suggests TEL plays a critical role in endothelial survival.

Regulation of Endothelial Activation

Hypoxia and Molecular Controls for VEGF Expression The hypoxia-inducible factor (HIF)-basic helix-loop-helix-PAS family of transcription factors has recently surfaced as a control system that regulates VEGF expression. The product of a tumor suppressor gene responsible for von Hippel Lindau disease, *VHL*, is implicated in this hypoxia-sensitive regulation (9). The *VHL* protein product, pVHL, associates with HIF-1α and HIF-2α and appears to target them for ubiquitination and rapid degradation under normoxic conditions (37). In hypoxic cells, HIF-1α is stabilized by an undefined oxygen-sensitive sensor mechanism, permitting it to form an active complex with HIF-1β [aryl hydrocarbon receptor nuclear translocator (ARNT)] that induces VEGF transcription. In VHL-deficient cells, VEGF production is constitutively elevated as a consequence of HIF-α subunit stabilization, even under normoxic conditions. $ARNT^{-/-}$ embryonic stem cells fail to induce VEGF expression in response to hypoxia, and null embryos die before E10.5 with failure to develop yolk sac vessels, similar to the defects in $VEGF^{-/-}$ embryos (38). Thus ARNT deficiency reduces VEGF levels sufficiently to impose vascular consequences. The molecular identity of the oxygen

sensor and how it may be modified in settings where neovascularization is impaired are critical issues yet to be defined.

VEGF and Its Receptors Under influence of VEGF supplied by adjacent cells, the Flk-1 (VEGFR2)-positive angioblast or hemangioblast population expands during development, extending sprouts that initiate formation of the primary vascular plexus (8). It now appears that VEGF administration and VEGF produced in response to ischemic injury can also induce release of marrow-derived endothelial progenitor cells (EPCs) that may be recruited to neovascular sites in mature animals (Figure 3) (19). Tight regulation of VEGF availability appears to determine vascular progenitor survival, proliferation, and migration. The critical nature of this signal is highlighted by effects of deficiency of a single *VEGF-A* allele to cause developmental failure in both embryonic and extraembryonic circulation (39, 40). Flk-1 (VEGR2) expression and activation are critical for early vasculogenesis. Flk-1 null mice die between E8.5 and E9.5, with defects in blood island formation and lack of organized blood vessels in either the yolk sac or embryo proper (41).

Although a second VEGF receptor, VEGFR1 (Flt-1), is also required for early embryonic vascular development, null animals do develop blood islands, but they include abnormally mixed angioblasts, which suggests overexuberant proliferation (42). Flt-1 may play a role in sequestering and damping VEGF responses through Flk-1, because it has higher affinity and its ectodomain is sufficient to mediate normal vascular development (43). Although VEGFR-3 has high affinity for the VEGF-C isoform implicated in lymphangiogenesis, mice null for functional VEGFR-3 show failure of embryonic vascularization as well (44).

VEGF has also been shown to be functionally linked to eNOS activity. VEGF, but not fibroblast growth factor (FGF), stimulates accumulation of eNOS- and nitric oxide–dependent in vitro assembly of endothelial capillary-like structures (45). Consistent with a downstream role for nitric oxide in VEGF action, dietary supplementation with L-arginine promotes angiogenesis in a hind-limb ischemia model, and ischemia-induced angiogenesis is impaired in $eNOS^{-/-}$ mice (46).

Transforming Growth Factor β Signaling Strong genetic evidence supports the function of TGFβ family proteins and their receptors in vascularization and vessel integrity. TGFβ1-null mice have a vascular embryonic lethal phenotype, depending on genetic background (47). Moreover, distinct subsets of families with hereditary hemorrhagic telangiectasia (HHT) have mutations in genes encoding TGFβ receptors or homologous proteins. Familial mutations in endoglin, a type-III TGFβ receptor homolog, or activin-like kinase (ALK)-1, a type-I TGFβ receptor, are implicated in the angiodysplastic lesions in these patients (48, 49). A single mutant allele of either gene is sufficient to evoke the angiodysplasia typical of this disease.

TGFβ ligands are bound initially by type-II receptors, which recruit and phosphorylate type-I receptors, such as ALK-1, that signal specific downstream

responses (50). Endoglin does not bind ligand independently, but does associate with type-II receptors to which TGFβ1 and TGFβ3 have bound, to form serine/threonine kinase signaling complexes (51). Endoglin also associates with type-I receptors for bone morphogenetic protein (BMP)-7 and activin-A, which suggests it functions as an accessory protein of multiple receptor complexes within this TGFβ superfamily (51). Among the seven type-I TGFβ receptors, involvement of ALK-1 in HHT and ALK-5 as an important intermediary of TGFβ1 signaling appears most important in mediating endothelial responses to TGFβ, yet required to assemble the cardiac valves (52). Confirmation of a vascular role for endoglin was recently provided by the phenotype of null mice, with failure of extraembryonic endothelial development into syncytiotrophoblasts and placental failure (53).

Although endothelial responses to TGFβ and related ligands are critically defined by their expression of specific receptors, many regulatory aspects of TGFβ expression and processing are interactive with endothelial proteases, integrins (54), and extracellular matrix proteins such as thrombospodin-1 (55). Synthesized as a single propeptide chain from which an N-terminal latency-associated peptide (LAP) is cleaved, TGFβ1 is inactive in this small latent complex. LAP-binding proteins are disulfide linked and appear to target TGFβ1 to potential sites of action. Protease release from this complex, through plasmin or other proteases that cleave LAP in conjunction with its interaction with mannose-6-phosphate/insulin-like growth factor (IGF)-II receptors, has, until recently, appeared to be the likely physiological mechanism of activation.

New evidence defines the capacity for thrombospondin-1 (TSP-1) to bind LAP and change the conformation of associated TGFβ1 to promote its activation (55). Moreover, TSP-1–null mice have a phenotype strikingly similar to that of TGFβ1-null animals. Interactions between $\alpha_v\beta_6$ integrin and LAP have also been shown to activate TGFβ in specific cell presentation contexts (54). It appears likely that TGFβ plays an angiomodulatory role at several steps during angiogenesis, with the most notable net effect exerted on the maturation phase required to stabilize vascular structures.

Angiopoietins and Tie-2 Receptor A third receptor-ligand system is critically important in embryonic vascular development. Initially identified as orphan receptor tyrosine kinases restricted to endothelial expression, Tie-1 and Tie-2 functions were evaluated by gene deletion and dominant negative transgenic experiments (5). Mouse embryos null for a functional angiopoietin receptor, Tie-2, and its structural homolog, Tie-1, display vascular lethal outcomes. Tie-2–null or –dominant-negative animals die before E10.5 with malformation of vascular networks (56). In contrast, the majority of Tie-1–null mice survive to die immediately after birth from respiratory failure and edema attributed to lack of vessel integrity (57).

Among at least four different angiopoietins identified to date, Ang-1 is an activator of the Tie-2 kinase, whereas Ang-2 binds without activating (58). Knockout embryos lacking angiopoietin 1 expression display a picture quite simi-

lar to the Tie-2–null embryos, with failure of normal endothelial cell adherence and interaction with subjacent supporting cells and extracellular matrix (59). Similarly, endocardial cell attachment and subjacent myocardial trabeculations are disordered in both Tie-2– and Ang-1–null embryos. Transgenic mice overexpressing Ang-2 during embryogenesis display vascular lethal phenotypes similar to those of either Tie-2– or Ang-1–null mice (58). Based on endothelium-restricted expression of Tie-2 and the dominant smooth muscle cell expression of angiopoietins, it appears that recruitment of smooth muscle cells or pericytes into proximity with endothelial cells of newly formed vessels is required for Tie-2 activation. Local overexpression of Ang-2, as a Tie-2 receptor antagonist, appears to disrupt developmental vessel maturation as effectively as Tie-2 deficiency. This argues for a delicately balanced role for Tie-2 signaling in the maturation phase (Figures 1 and 2) and suggests that important biological functions attend both receptor activation and subsequent antagonism. Temporally staged expression of Ang-1, then Ang-2, during the progression of ovarian follicle vascularization and regression provides support for this sequential process (58).

Regulation of Endothelial Targeting

In both vasculogenic (Figure 1) and angiogenic (Figure 2) neovascularization, a critical task required of migrating or extending endothelial cells is the recognition and recruitment of appropriate partners for anastomosis and interendothelial self-assembly.

Eph/Ephrin Interactions The Eph/ephrin receptor/counter-receptor system has been identified as an important mediator of early developmental patterning (60) and neural targeting (61, 62). This system participates importantly in vascular development (5, 63, 64). Gradients of membrane-bound ephrins appear to explicitly direct the targeting of axons through spatially defined migratory fields. These Eph/ephrin receptors are candidates to signal interendothelial cell-cell recognition.

Function in the vasculature was first recognized when the tumor necrosis factor (TNF)α-inducible ephrin-A1 (B61) was shown to mediate corneal angiogenesis responses through EphA2 (65). More recently, homozygous deletion of ephrin-B2 was shown to cause failure of extraembryonic vascularization at a stage when vascular plexus fusion is normally seen between an arterial limb plexus expressing ephrin-B2 and a venous limb plexus expressing its receptor, EphB4 (64). This was a striking observation because it demonstrated endothelial "chimerism" in Eph/ephrin expression that defined anatomical and biochemical distinctions in commitment to venous or arterial function before competency of vascular flow. Subsequent experiments have expanded evidence of endothelial heterogeneity. Mice null for both EphB2 and EphB3 also display variable penetrance of embryonic vascularization defects, manifest at the same developmental stage (>E9.5). Yet the expression pattern shows endothelial bed–selective differences that are

not limited to the arterovenous border (63). Cultured endothelial cells derived from distinct vascular beds also display differential attachment and self-assembly responses to specific ephrins A or ephrins B (66).

One model for how endothelial cell-cell contact could participate in cell-cell fusion functions to provide cell recognition addresses has been advanced by function linkage between EphB1 activation and $\alpha_v\beta_3$ integrin. Shown in Figure 4 (see color insert), EphB1 functions as a molecular switch, not only distinguishing whether it is engaging ephrin counter-receptor, but also reading the oligomerized form of ephrin-B1 to relay different signals that control integrin-mediated cell attachment and migration (13). At a biochemical level, the composition of EphB1 signaling complexes is also critically regulated by the state of ephrin oligomerization (67). Thus EphB1 receptors are poised to discriminate spatial signals on cell surfaces to regulate movement and attachment.

As outlined above, juxtacrine cell-cell discrimination is a critical task facing endothelial cells during neovascularization, whether vasculogenic (Figure 1), angiogenic (Figure 2), or through recruitment of EPCs from a circulating pool (Figure 3). The Eph/ephrin system also meets another expectation imposed on cell-cell recognition, that of reciprocity. Reciprocal signaling has been demonstrated, transduced through ephrin-B counter-receptors upon engagement of the EphB2 ectodomain (68, 69). Thus, this system provides an ideal early recognition "molecular sensor" capable of "reading" counter-receptor density like an address to direct cell-cell assembly of appropriate collaborative cell partners through correct targeting (70).

Extracellular Matrix and Matrix-Associated Matricellulins in Angiogenesis

An exceptionally strong body of evidence implicates $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins in neovascularization responses to defined stimuli, such as VEGF and FGF, as well as in tumor-responsive neovascularization (12, 71). It appears that integrins not only provide structural links to extracellular matrix for attachment and motility, they also bind metalloproteinases or inactive fragments to regulate endothelial invasiveness (72).

Recent findings further highlight the intimate interaction between matrix-associated proteins that have been described as matricellulins, SPARC and thrombospondin-1 (TSP-1), and specific angiomodulatory growth factors. For example, SPARC and peptides derived from selected domains inhibit VEGF stimulation by direct binding to VEGF and by reducing the association of VEGF with endothelial cell surface receptors (73). This provides a mechanism for matrix sequestration and inactivation of secreted VEGF. In addition, SPARC is a counter-adhesive protein that reduces endothelial spreading and acts to dissolve focal adhesions between endothelial cells and extracellular matrix (74).

As noted above, TSP-1 is an important regulator of TGFβ activity. It controls conversion of the latent TGFβ complex to active forms, by binding through a defined peptide loop, KRFK, to the LAP component (55). It apparently sequesters LAP and dissociates it from TGFβ in an activation step. This biochemical mech-

anism has been confirmed by the striking phenotype similarity of TSP-1–null mice to those with inactivated TGFβ1. It is noteworthy that TSP-1 also has intrinsic antiangiogenic activity, mediated through its binding to CD36 through a different domain (75). Thus, these matricellulins display independent functions resident within modular domains, including those that sequester and alter activity of matrix-associated growth factors such as VEGF and TGFβ.

VASCULAR DEVELOPMENT OF THE MAMMALIAN KIDNEY

Although the kidneys are among the most richly vascularized organs in mammals, mechanisms regulating the development of the renal vascular system are only now beginning to be understood. The permanent, metanephric kidney originates at ~E10 in mice, ~E11 in rats, and ~5 weeks gestation in humans, when the ureteric bud projects dorsolaterally from the nephric duct into a group of metanephric blastemal mesenchymal cells (Figure 5, *upper left panel*; see color insert) (76). Reciprocal inductive signals emitted by cells of the ureteric bud and metanephric mesenchyme, respectively, lead to repeated branching of the bud (which ultimately forms the collecting system of the kidney) and aggregation of mesenchymal cells at each branch tip.

Each of these mesenchymal aggregates subsequently converts into a cluster (vesicle) of epithelial cells that ultimately differentiate into the glomerular and tubular epithelial cells of individual nephrons (76–78). Early in nephron development, a vascular cleft forms near the base of each vesicle to produce a comma-shaped nephric figure (Figure 5, *upper right panel*). Vascular elements assemble within this cleft, which give rise to the glomerular capillary tufts and mesangial cells (76, 79). Concurrent with these events, the epithelial cells above the vascular cleft ultimately produce the proximal convoluted tubule, Henle's loop, and distal tubular segments of the nephron, which connects to the branching collecting system.

The glomerular and peritubular capillaries form rapidly. The period from initial nephron induction to glomerular filtration and tubular reabsorption is only a few days in the mouse. The first nephrons and glomeruli induced to form in the mouse (at ~E11) occupy the juxtamedullary region of the fully developed kidney cortex, whereas the last nephrons that form (~postnatal day 7) are found in the outer cortex immediately beneath the capsule. This unique centrifugal pattern for nephrogenesis makes the kidney particularly attractive for studying a number of spatio-temporal developmental events, including formation of the vascular system.

Along with VEGF, all of its receptor tyrosine kinases are expressed in the embryonic kidney, as are many of the other growth factor receptor and signaling systems important for vascular assembly and referred to earlier. Because mice

with targeted null mutations for VEGF, Flk-1, and Flt-1 die before the kidney develops, the exact roles for these signaling molecules in renal vascular development specifically are not fully understood. A cascade of overlapping events appears to govern the orderly formation and stabilization of glomerular and peritubular capillaries, and VEGF and its receptors are clearly among dominant regulators of this process.

VEGF is expressed in glomerular visceral epithelial cells (developing podocytes), which are located beneath the vascular cleft of comma-shaped nephric figures, and it continues to be expressed by podocytes of later-stage glomeruli and into adulthood (80–83). Likewise, the VEGF receptors Flk-1/KDR and Flt-1 are found in glomerular and other kidney endothelial cells in both fetal and adult humans (80, 81). By using in situ hybridization (84), *lacZ* reporter gene expression (85), and protein immunolocalization in the embryonic mouse (23), Flk-1 has been observed in metanephric angioblasts, developing microvessels, and glomerular endothelium of immature kidneys.

The expression of VEGF by podocytes and of the VEGF receptor Flk-1 by adjacent endothelial cells clearly implicates this ligand-receptor system in juxtacrine regulation of glomerular vascularization. As a test for this, injection of anti-VEGF antibodies into newborn mouse kidney cortex results in the formation of avascular glomeruli [resembling those that develop under normoxic conditions in organ culture (see below)], providing further evidence that VEGF is crucial for glomerular endothelialization (86). The sustained expression of both VEGF and Flk-1 in fully mature glomeruli of adult kidneys is unusual, however, because fully developed glomeruli are remarkably stable vascular structures. The data therefore suggest that both VEGF and Flk-1 are needed for maintenance of the extensively fenestrated phenotype of the highly differentiated glomerular endothelium (87, 88).

As explained above, the roles for TGFβ1 and its receptors in blood vessel development have been difficult to unravel, and it now appears that TGFβ1 may exert its angiogenic effects in vivo indirectly by stimulating VEGF production (89, 90). When neutralizing anti-TGFβ1 antibodies are infused into newborn rat kidneys, early glomeruli lack endothelial cells, a consequence similar to that seen after injection of anti-VEGF, except that the endothelium in more mature glomeruli is also affected as it fails to flatten and form fenestrae (91). Overall, however, kidney VEGF levels are unchanged after infusion of anti-TGFβ1 antibodies, so exactly how TGFβ1 mediates glomerular vascularization remains undefined.

Several morphological investigations reviewed in detail previously (76, 92) considered the two likely origins of endothelial cells in the embryonic kidney: (*a*) in situ differentiation of mesenchymal endothelial precursors (angioblasts) into vascular endothelial cells (vasculogenesis) or (*b*) ingress of angiogenic sprouts from preformed vessels outside the metanephros (angiogenesis). Evidence in support of this second possibility came from observations that, despite the organotypic tubulogenesis and glomerulogenesis that occurs when fetal rodent kidneys are maintained under standard organ culture conditions, the glomeruli that form

in vitro are avascular (93, 94). Additionally, when fetal mouse kidneys are grafted onto avian chorioallantoic membranes, glomeruli within grafts contain endothelial cells of host (avian) lineages (95).

More recently, however, new evidence indicates that kidney microvessels may instead originate from intrinsic kidney angioblasts. For example, when fetal kidneys are cultured under hypoxic conditions, there is an upregulation of VEGF, and under these conditions, renal microvessels do assemble in vitro (96). As referred to above, this finding is consistent with activation of VEGF transcription by ARNT/HIF-1α heterodimers, which are stabilized specifically in hypoxia. Additionally, this points to an ability by the kidney to form vessels from its own internal resources and fits with immunolocalization and reporter gene expression data showing that dispersed mesenchymal cells in the metanephric cortex that express Flk-1 are candidate angioblasts (23, 85). On the other hand, when embryonic kidneys are cultured under routine normoxic conditions, there is a marked downregulation of Flk-1 expression in vitro (85). When these cultured kidneys are then grafted into anterior eye chambers, however, Flk-1 expression resumes, and endothelial cells, which are derived exclusively from the engrafted kidney, constitute an extensive microvasculature that forms in oculo (85). In aggregate, these data demonstrate that (*a*) both VEGF and Flk-1 expression are necessary for renal microvessel formation and (*b*) the kidney is capable of establishing its own microvascular network independent of external vessels, presumably through activation of resident angioblasts.

Whereas an abundant amount of data shows that complementary expression of VEGF and its receptors is likely to be a major controlling element for initial glomerular endothelial-cell development, most experimental evidence in cultured endothelial cells indicates that this signaling system results mainly in increased mitotic and cell motility behavior. Although enhanced mitosis and motility would be crucially important for seeding and maintenance of a renal angioblast stem cell population in vivo, different activities need to be invoked for the actual targeting of differentiating endothelial cells into glomeruli and the capillary nets that surround renal tubules. As suggested previously, the Eph/ephrin families of cell surface receptor-ligand pairs, which are capable of inducing endothelial network formation in vitro, are probably more important than VEGF and its receptors for endothelial-cell targeting and aggregation in vivo. When the distributions of EphB1 and ephrin-B1 were evaluated in the embryonic kidney, both members of this receptor-ligand pair were identified in metanephric angioblasts and on endothelial cells of developing and maturing glomeruli (66). These patterns were in fact indistinguishable from those seen for cells bearing Flk-1 (66) and Tie-1 (97). Although it is too soon to know for certain whether all of these membrane proteins colocalize exactly to the same cells, the possibility seems highly likely. Taken together with the evidence reviewed earlier on the respective roles for VEGF and its receptors and the Eph/ephrin families, these data from the developing kidney suggest that VEGF/Flk-1 mediates renal angioblast activation and, at least for glomerular endothelial cells, may also be necessary for maintenance of the dif-

ferentiated state. The expression of Eph/ephrin by these same cells may direct partnering between activated angioblasts and the subsequent formation of spatially restricted vascular networks.

Once the basic vascular framework is established, additional signaling systems are required for modulating endothelial-cell mitotic activity and stabilizing the network. Among the more promising candidates for this role is ECRPTP/DEP-1, a type-III receptor protein tyrosine phosphatase. Cells expressing ECRTP/DEP-1 have the same distribution pattern in developing kidney as those expressing the endothelial lineage restricted protein, vascular endothelial-cadherin, and this receptor phosphatase accumulates at points of inter-endothelial contact in vessels and in cultured endothelial cells (98). Although not implicated directly in endothelial differentiation, PDGF B functions as an attractant signal to recruit pericytes and other myofibroblasts to developing vessels. In the immature kidney, PDGF B is expressed by epithelial cells of early nephrons, whereas PDGFRβ is found on interstitial cells and undifferentiated mesenchyme (99). As glomeruli develop, both PDGF B and PDGFRβ are concentrated on mesangial cells, which suggests a paracrine and then autocrine signaling system for mesangial cell recruitment and maintenance.

In mice with targeted mutations of either PDGF B (25) or PDGFRβ (24), glomerular mesangial cells are absent, and the glomeruli that form are characterized by a large, irregular capillary loop. Although PDFGFA and PDGFRα are coordinately expressed in collecting-duct epithelium and vascular smooth muscle, respectively, indicating an involvement in recruitment of renal arterial adventitial cells (99–101), no renal arteriolar defects are apparent in null mutants (24, 102).

CONCLUSION

With further molecular definition of the endotheliocentric responses that direct proliferation, migration, cell-cell discrimination, and assembly of vascular structures, we anticipate further understanding of the molecular code read by endothelial cells as they assemble and remodel the interconnecting vascular network that is so integral to tissue structure and function.

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