**Endothelial and smooth muscle cells interact with each other to form new blood vessels. In this review, the cellular and molecular mechanisms underlying the formation of endothelium-lined channels (angiogenesis) and their maturation via recruitment of smooth muscle cells (arteriogenesis) during physiological and pathological conditions are summarized, alongside with possible therapeutic applications.**

# Mechanisms of angiogenesis and arteriogenesis

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Normal tissue function depends on adequate supply of oxygen through blood vessels. Understanding how blood vessels form has become a principal, yet challenging, objective of the last decade. Unraveling these mechanisms would offer therapeutic options to ameliorate or perhaps even cure disorders that are now leading causes of mortality. In this millenium, we will be required to answer such questions as: Will it be possible to treat ischemic heart disease by stimulating myocardial angiogenesis, and will it be feasible to cure cancer or inflammatory disorders by suppressing excessive vessel growth? Unfortunately, research on angiogenesis has for too long remained descriptive, mainly because the molecular 'players' were not identified. The recent discovery of candidates able to stimulate or inhibit endothelial cells has stirred a growing interest in using these molecules for therapeutic applications. This overview provides an update on the present understanding of the basic molecular mechanisms of how endothelial and smooth muscle cells interact with each other to form blood vessels, as a basis for design of future (anti)-angiogenic treatments.

### Development of an endothelium-lined vasculature

Blood vessels in the embryo form through vasculogenesis; that is, through *in situ* differentiation of undifferentiated precursor cells (angioblasts) to endothelial cells that assemble into a vascular labyrinth[1] (Fig. 1). Historically, the term angiogenesis was first used to describe the growth of endothelial sprouts from preexisting postcapillary venules (Fig. 1). More recently, this term has been used to generally denote the growth and remodeling process of the primitive network into a complex network. This involves the enlargement of venules, which sprout or become divided by pillars of periendothelial cells (intussusception) or by transendothelial cell bridges, which then split into individual capillaries (Fig. 1). New vessels in the adult arise mainly through angiogenesis, although vasculogenesis also may occur (Fig. 2). Because vasculogenesis only leads to an immature, poorly functional vasculature, angiogenesis is a therapeutic goal. As the cellular and molecular mechanisms of angiogenesis differ in various tissues (vessels in psoriatic skin enlarge, but they sprout in ischemic retina), the therapeutic stimulation or inhibition of angiogenesis should be adjusted to the target tissue.

### Smooth muscle–endothelial cell interactions

Although endothelial cells have attracted most attention, they alone can initiate, but not complete, angiogenesis; periendothelial cells are essential for vascular maturation (Fig. 3). During 'vasular myogenesis', mural cells stabilize nascent vessels by inhibiting endothelial proliferation and migration, and by stimulating production of extracellular matrix (Fig. 1). They thereby provide hemostatic control and protect new endothelium-lined vessels against rupture or regression. Indeed, vessels regress more easily as long as they are not covered by smooth muscle cells[2]; the loss of pericytes around retinal vessels in diabetic patients causes aneurysmal dilatation, bleeding and blindness.

During the subsequent arteriogenesis, vessels become covered by a muscular coat, thereby endowing blood vessels with viscoelastic and vasomotor properties, necessary to accommodate the changing needs in tissue perfusion (Fig. 1). Periendothelial cells also assist endothelial cells in acquiring specialized functions in different vascular beds[3]. Arteriogenesis is recapitulated during the pathological enlargement of preexisting collateral vessels (Fig. 2). Therefore, strategies to promote sustainable and functional new blood vessels should not be restricted to the induction of capillary angiogenesis, but should include the stimulation of arteriogenesis. Likewise, the therapeutic regression of 'muscularized' vessels may require strategies other than the inhibition of endothelium-lined vessels.

### Vasculogenesis: the formation of a primitive network

Endothelial and hematopoietic cells share a common progenitor (the hemangioblast). In the yolk sac, hemangioblasts form aggregates in which the inner cells develop into hematopoietic precursors and the outer population into endothelial cells (Fig. 1). Angioblasts may migrate extensively before *in situ* differentiation and plexus formation. Vascular endothelial growth factor (VEGF), VEGF receptor (VEGFR) 2 and basic fibroblast growth factor (bFGF) influence angioblast differentiation[4–7], whereas VEGFR1 suppresses hemangioblast commitment[8]. The molecular mechanisms of how transforming growth factor (TGF)-β1 and TGF-β receptor 2 affect vasculogenesis remain mostly undetermined[9]. Molecules mediating interactions between endothelial cells and matrix macromolecules, fibronectin or matrix receptors ($\alpha_5$ integrin), also affect vasculogenesis. The $\alpha_v\beta_3$ integrin mediates vasculogenesis in avian but not in murine embryo[10].

Little is known about the mechanisms governing endothelial cell fate: *Ets-1*, *Hex*, *Vezf1*, *Hox* and *GATA* family members, basic helix–loop–helix factors and their inhibitors of differentiation may be involved[11]. Such molecules may be of therapeutic value, as they could determine the 'decision' of endothelial cells to become angiogenic during pathological conditions (called 'angiogenic switch')[12]. The fate of endothelial cells to become integrated into arteries or veins is mediated by the bHLH transcription factor gridlock at the angioblast stage, and, subsequently, by members of the ephrin family, signals that are also involved in guidance of axons and repulsion of neurons[14]. It was once believed that endothelial precursors only exist during embryonic life. However, endothelial precursor cells have been identified in bone marrow and in peripheral blood in adults. VEGF, granulocyte–monocyte colony-stimulating factor, bFGF and insulin-like growth factor (IGF)-1 stimulate their differentiation and mobilization[15,16]. Such precursors colonize angiogenic sites and vascular prostheses in the adult and may hold promise for future therapy (Fig. 2).

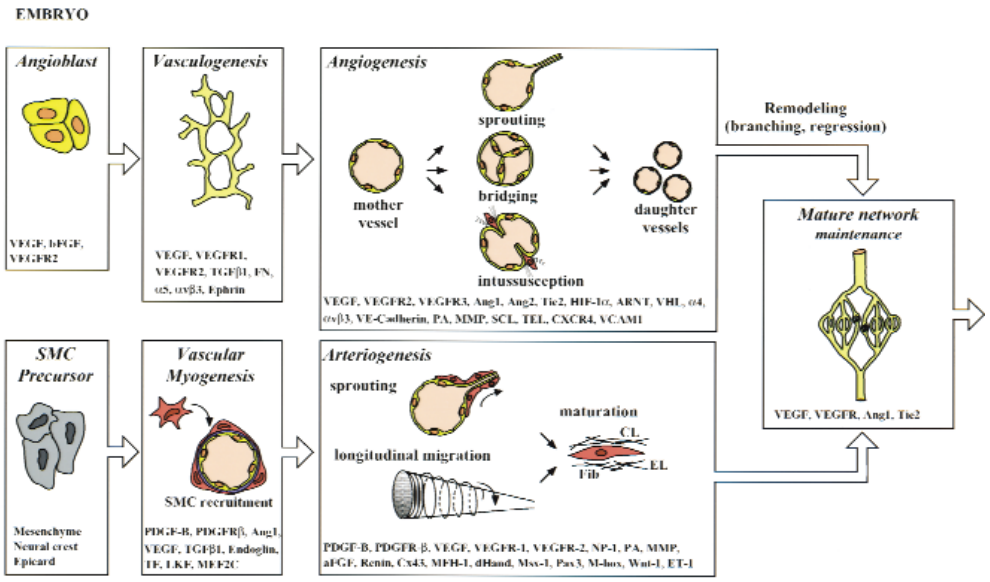


**Fig. 1** Endothelial precursors (angioblasts) in the embryo assemble in a primitive network (vasculogenesis), that expands and remodels (angiogenesis). Smooth muscle cells cover endothelial cells during vascular myogenesis, and stabilize vessels during arteriogenesis. CL: collagen; EL: elastin; Fib: fibrillin (Fib).

## Angiogenesis: sprouting and remodeling

Angiogenic sprouting is one, but not the only, mechanism of blood vessel formation in the adult; however, it has been studied most extensively. The molecular basis of angiogenesis in the embryo seems to differ from that of pathological angiogenesis in the adult (Figs. 1 and 2). Several steps have been determined (Fig. 3).

*Vasodilation, endothelial permeability and periendothelial support*: Angiogenesis initiates with vasodilation, a process involving nitric oxide (Fig. 1). Vascular permeability increases in response to VEGF, thereby allowing extravasation of plasma proteins that lay down a provisional scaffold for migrating endothelial cells. This increase in permeability is mediated by the formation of fenestrations, vesiculo–vacuolar organelles and the redistribution of platelet endothelial cell adhesion molecule (PECAM)-1 and vascular endothelial (VE)–cadherin, and involves Src kinases[17]. Although permeability is good for angiogenesis, excessive vascular leakage can be bad and lead to circulatory collapse, intracranial hypertension, formation of adhesion, metastasis, premenstrual discomfort or blindness. Angiopoietin (Ang) 1, a ligand of the endothelial Tie2 receptor, is a natural inhibitor of vascular permeability, tightening preexisting vessels. When acutely administered to adult vessels, Ang1 protects against plasma leakage without profoundly affecting vascular morphology.[18]

For endothelial cells to emigrate from their resident site, they need to loosen interendothelial cell contacts and to relieve periendothelial cell support; that is, mature vessels need to become destabilized (Fig. 3). Ang2, an inhibitor of Tie2 signaling, may be involved in detaching smooth muscle cells and loosening the matrix[14,19]. Proteinases of the plasminogen activator, matrix metalloproteinase (MMP), chymase or heparanase families influence angiogenesis by degrading matrix molecules and by activating or liberating growth factors (bFGF, VEGF and IGF-1), sequestered within the extracellular matrix[20]. Urokinase-type plasminogen activator (u-PA) is essential for revascularization of myocardial infarcts[21], whereas antagonists of the u-PA receptor inhibit tumor angiogenesis. MMP-3, MMP-7 and MMP-9 affect angiogenesis in neonatal bones[22] and tumors[23], whereas tissue inhibitors of MMPs 1 or 3) or PEX, the naturally occurring fragment of MMP-2, by preventing binding of MMP-2 to $\alpha_v\beta_3$, inhibit tumor angiogenesis[24]. Paradoxically, tissue inhibitor of MMPs 1 and plasminogen activator-inhibitor 1 are risk factors for a poor prognosis in cancer patients and promote tumor angiogenesis in mice by preventing excessive proteolysis (Bajou *et al*, manuscript submitted)[23]. In support of this, endostatin inhibits tumor angiogenesis by increasing plasmin generation (A. Reijerkerk *et al.*, manuscript submitted).

*Endothelial cell proliferation and migration*: Once the path has been cleared, proliferating endothelial cells migrate to distant sites. VEGF (ref. 4), placental growth factor (PLGF), VEGF-B, VEGF-C, VEGF-D and their receptors VEGFR2, VEGFR3 (ref. 25) and neuropilin-1 (a co-receptor of VEGFR2; ref. 26) have specific functions: VEGF and its receptor VEGFR2 affect embryonic, neonatal and pathological angiogenesis and are therapeutic targets, although much remains to be learned about the involvement of the distinct VEGF isoforms or of the heterodimers of VEGF family members[4–6,27]. $VEGF_{120}$ alone initiates but does not complete angiogenesis[28]. VEGFR3 is involved in embryonic angiogenesis[29] and is expressed in pathological angiogenesis, whereas VEGF-C (a ligand of VEGFR3) is angiogenic in adult pathology[30]. The angiogenic or lymphangiogenic activity of VEGF-C depends on its processing. Truncation of VEGFR1 at the tyrosine kinase domain does not impair embryonic angiogenesis, but the involvement of VEGFR-1 signaling during pathological angiogenesis remains undetermined[31]. Indeed, the loss of PLGF specifically impairs pathological but not physiological angiogenesis, by increasing the responsiveness of VEGFR2 to VEGF through increased VEGFR2 tyrosine phophorylation (P.C., manuscript submitted). Loss of VEGF-B affects coronary function after coronary occlusion. Ang1 phosphorylates tyrosine in Tie2 and is chemotactic for endothelial cells, induces sprouting and potentiates VEGF, but fails to induce endothelial proliferation[14,32]. In contrast to VEGF, Ang1 itself does not initiate endothelial network organization, but stabilizes networks initiated by VEGF, presumably by stimulating the interaction between endothelial and periendothelial cells. This indicates that Ang1 may act at later stages than VEGF (Fig. 3)[14,32]. Ang2, at least in the presence of VEGF, is also angiogenic. However, more recent data indicate that overexpression of Ang1 in tumors suppresses their growth, but whether this means that Ang1 acts physiologically to promote angiogenesis by inducing vessel maturation and stabilization, but that this function may inhibit growth of immature and stable tumor vessels, remains to be determined (P. Maisonpierre, personal communication). Low

levels of phosphorylated Tie2 have been detected in the adult quiescent vasculature, indicating involvement of Tie2 in vascular maintenance.

Members of the fibroblast growth factor and platelet-derived growth factor (PDGF) family are redundant during normal development[33,34], but they affect angiogenesis when administered, probably by recruiting mesenchymal or inflammatory cells. TGF-β1 and tumor necrosis factor (TNF)-α can either stimulate or inhibit endothelial growth, and may be involved in tumor dormancy[35]. Molecules involved in cell–cell or cell–matrix interactions, such as the $\alpha_v\beta_3$ integrin, which localizes MMP-2 at the endothelial cell surface, mediate endothelial spreading, explaining why $\alpha_v\beta_3$ antagonists inhibit angiogenesis[36]. PECAM-1 and EphrinB2 (G. Yancopoulos, personal communication) may also be involved in pathological angiogenesis. Nitric oxide, a downstream effector of VEGF, TGFβ-1 and other angiogenic factors, is not essential for embryonic vascular development, but affects pathological angiogenesis and improves the reendothelialization of denuded vessels[37]. A growing list of molecules is being discovered that are angiogenic after exogenous administration, but whose endogenous angiogenic function remains undetermined: erythropoietin, IGF-1, neuropeptide-Y, leptin, Thy-1, epidermal growth factor, tissue factor (initiator of blood coagulation), hepatocyte growth factor, interleukins hormones and chemokines.

Angiogenic sprouting is controlled by a balance of activators and inhibitors. Angiogenesis inhibitors suppressing the proliferation or migration of endothelial cells include angiostatin (an internal fragment of plasminogen)[38], endostatin (a fragment of collagen XVIII; ref 39), antithrombin III, interferon-β, leukemia inhibitory factor and platelet factor 4. Naturally occurring angiogenesis inhibitors may be involved in tumor dormancy and are being tested for anti-cancer treatment.

*Lumen formation*: Endothelial cells often assemble as solid cords that subsequently acquire a lumen. Intercalation or thinning of endothelial cells and fusion of preexisting vessels allow vessels to increase their diameter and length. In contrast to normal vessels, tumor vessels are often abnormally enlarged, but blood flow in tumor vessels is often chaotic, slow and not efficient in meeting metabolic demands[40]. $VEGF_{189}$ decreases luminal diameter, whereas $VEGF_{121}$, $VEGF_{165}$ and their receptors increase lumen formation, in addition to increasing vessel length. In certain tissues (such as psoriatic skin), VEGF mainly exerts a morphogenetic activity by enlarging existing vessels. Ang1 in combination with VEGF also increases luminal diameter[32]. Other molecules affecting lumen formation are integrins ($\alpha_v\beta_3$ or $\alpha_5$) and the myocyte enhancer binding factor 2C (MEF2C) transcription factor. Excessive proteolysis may lead to cystic assembly of endothelial cells and prevent tube formation. Thrombospondin (TSP)-1 is an endogenous inhibitor of lumen formation.

*Endothelial survival*: Once assembled in new vessels, endothelial cells become quiescent and survive for years. The importance of endothelial survival is demonstrated by findings that reduced survival causes vascular regression in the embryo[41]. Endothelial apoptosis is a natural mechanism of vessel regression in the retina and ovary after birth and a frequent effect of (therapeutic) inhibitors of angiogenesis. Endothelial apoptosis is induced through deprivation of nutrients or survival signals when the lumen is obstructed by spasms, thrombi or the shedding of dead endothelial cells, or when a change in the angiogenic gene profile occurs[27,28,42]. For example, exposure of premature babies to hyperoxia reduces VEGF levels and causes vessel regression in the retina[43]. The survival function of VEGF depends on an interaction between VEGFR2, β-catenin and vascular endothelial (VE)–cadherin[41]. Ang1 also promotes, whereas Ang2 suppresses, endothelial survival, at least in the absence of angiogenic stimuli (Fig. 3), and has been suggested to contribute to the regression of 'co-opted' tumor vessels[14,32,44] or of hyaloid vessels (G. Yancopoulos, personal communication). Disruption of the interaction with matrix macromolecules, using $\alpha_v\beta_3$ antagonists or the desintegrin accutin, also results in endothelial apoptosis, but, as $\alpha_v\beta_3$ is only expressed in proliferative cells, pre-existing quiescent blood vessels remain unaffected[36]. Different vascular beds may have specific survival mechanisms, such as brain-derived neurotrophic factor for coronary endothelial cells (B. Hempstead, personal communication). Hemodynamic forces are essential for vascular maintenance, as physiological shear stress reduces endothelial turnover and abrogates TNF-α mediated endothelial apoptosis. Endothelial apoptosis can be also induced by nitric oxide, reactive oxygen species, angiostatin, TSP-1, the metallospondin METH-1, interferon-γ, tissue factor pathway inhibitor and vascular endothelial growth inhibitor (VEGI).

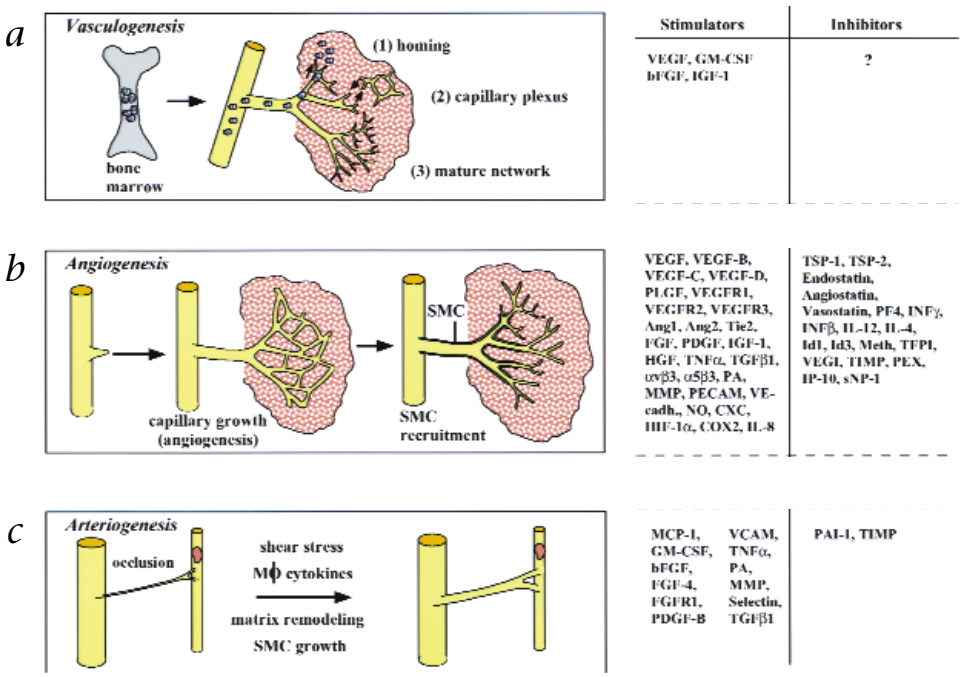


**Fig. 2** Pathological vascular growth in the adult may occur via vasculogenesis (angioblast mobilization), angiogenesis (sprouting) or arteriogenesis (collateral growth).

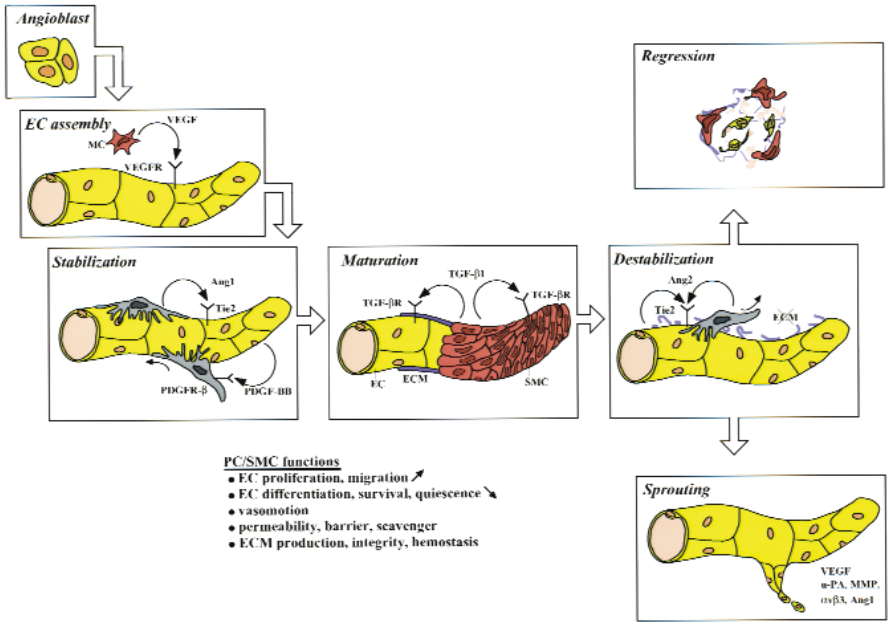


**Fig. 3** VEGF initiates assembly of endothelial cells (EC), PDGF-BB recruits pericytes (PC) and smooth muscle cells (SMC), whereas angiopoietin-1 (Ang1) and TGF-b1 stabilize the nascent vessel. Angiopoietin-2 (Ang2) destabilizes the vessel, resulting in angiogenesis in the presence of angiogenic stimuli, or in vessel regression in the absence of endothelial survival factors.

Several endothelial survival factors (VEGF, Ang1 and $\alpha_v\beta_3$) suppress p53, p21, p16, p27 and *Bax*, whereas they variably activate the survival PI3-kinase/Akt, p42/44 mitogen-activated protein kinase, Bcl-2, A1 and survivin pathways. The mechanism of action remains unknown for many other angiogenesis inhibitors, including prothrombin kringle-1 and kringle-2, TSP-2, PECAM-1 antagonists, interleukins 4 and 12, interferon-α, cyclooxygenase-2 (Cox2)-inhibitors, 1,25-dihydroxyvitamin-$D_3$ and the N-terminal fragment of prolactin. The transcription factor *Braf* may be involved in endothelial survival.

*Endothelial differentiation*: To accommodate local physiological requirements, endothelial cells acquire specialized characteristics that are determined in part by the host tissue[45]. For example, an interaction of astroglial cells expressing glial fibrillary acidic protein, pericytes and normal angiotensinogen levels is essential for development of the blood–brain barrier[34]. In contrast, endothelial cells in endocrine glands, involved in the exchange of particles, become discontinuous and fenestrated; this is possibly mediated by interactions between VEGF and the extracellular matrix. Endothelial cells in tumors are abnormal in many ways: They are multilayered, protrude extensions bridging and splitting vessels, contain intercellular and transcellular holes, show relatively uncontrolled permeability and undergo constant remodeling. The recent and controversial finding that tumor vessels are 'mosaic' and are lined by both endothelial cells and malignant 'vasculogenic' tumor cells (or 'vasculogenic mimicry') may have considerable consequences for anti-angiogenesis tumor therapy (ref. 46 and R. Jain, personal communication). Epitopes specific for tumor endothelial cells are attractive targets for the 'homing' of pro-apoptotic or thrombotic molecules in anti-cancer therapy[47]. Microenvironmental factors also determine the endothelial barrier in tumors, as endothelial cells contain more fenestrae when tumors are grown under the skin than in the brain.

*Remodeling*: So far, very little is known about the spatial cues guiding endothelial cells into correct patterns and three-dimensional networks, a goal for therapeutic angiogenesis. Maturation of the endothelial network involves remodeling and 'pruning' capillary-like vessels with uniform size, and irregular organization into a structured network of branching vessels (Fig. 1). Intussusception, resulting in replacement of vessels by matrix, underlies 'pruning' and branching. Gene inactivation studies indicate a morphogenetic function for the distinct VEGF isoforms and VEGFR3 (refs. 5, 6, 28, 29), the endothelial 'orphan' receptor Tie1 (ref. 48), the T-cell-leukemia protein stem cell leukemia factor/tal-1, *TEL* (a member of the Ets family of transcription factors), the GTP-binding protein $G\alpha_{13}$, Jagged, chemokine receptor 4, vascular cell adhesion molecule 1, $\alpha_4$ integrin and fibronectin. Recent studies in zebrafish have identified the candidate gene responsible for *gridlock*, a vascular patterning defect resembling coarctation of the aorta[13]

## Vascular myogenesis

*Smooth muscle cell fate*: Smooth muscle cells have a complex origin depending on their location (Fig. 1). A puzzling question, therefore, is whether distinct growth factors/receptors mediate their fate in different vascular beds. This would have substantial therapeutic consequences, as stimulating collateral vessels in the heart or limb would then require the use of different therapeutic factors. This idea is supported by recent findings that *Bves* is a specific marker for coronary vascular smooth muscle cells, whereas brain-derived neurotrophic factor is a specific survival factor for coronary endothelial cells. Smooth muscle cells can transdifferentiate from endothelial cells, differentiate from mesenchymal cells *in situ* in response to as-yet-unidentified endothelial-derived stimuli, or from bone marrow precursors or macrophages. Smooth muscle cells of the coronary veins are derived from atrial myocardium, whereas mural cells of the coronary arteries are recruited from the epicardial layer[49]. The large thoracic blood vessels, which are often affected by congenital malformations, contain smooth muscle cells, derived from cardiac neural crest cells[50]. Smooth muscle fate involves transcriptional control by the serum response factor, *Prx-1* and *Prx-2*, *CRP2/SmLIM*, capsulin, and members of the *Hox*, *MEF2* and *GATA* family.

*Smooth muscle cell recruitment and growth*: PDGF-BB is a chemoattractant for smooth muscle cells[34] (Fig. 1). VEGF also promotes mural cell accumulation, presumably through the release of PDGF-BB or binding to VEGF receptors[2,28]. Ang1 and Tie2 affect growth and maintenance of blood vessels by stabilizing the interaction of mural cells with nascent endothelial channels, and by inducing branching and remodeling[14,19,33,51]

(Fig. 3). Hereditary dysfunction of Tie2 in humans induces vascular malformations, characterized by vessels with fewer smooth muscle cells. TGF-β1, TGF-βR2, endoglin (an endothelial TGF-β binding protein) and Smad5 (a downstream TGF-β signal) are involved in vessel maturation in a pleiotropic manner: they inhibit endothelial proliferation and migration, induce smooth muscle differentiation and stimulate extracellular matrix production, thereby 'solidifying' the endothelial–mural cell interactions[9,52] (Fig. 3). Patients lacking endoglin suffer hereditary hemorrhagic telangiectasia type 1. N-cadherin seems to 'glue' endothelial and mural cells in close apposition. Endothelin-1, produced by endothelial cells of thoracic blood vessels, is chemotactic for neural crest cells, transforming into smooth muscle cells[53]. Tissue factor of coagulation promotes pericyte recruitment, possibly through the generation of thrombin and/or a fibrin-rich scaffold. Other candidates are heparin-binding epidermal-growth-factor-like factor and the transcription factors *LKLF*, *COUP-TFII* and *MEF2C* (ref. 54). Ang2 and EphrinB2 are also expressed by vascular smooth muscle cells in particular tissues, but their involvement remains undefined (G. Yancopoulos, personal communication).

### Arteriogenesis

*Smooth muscle cell migration and growth*: Once mural cells have been recruited, they further 'muscularize' the nascent vasculature by sprouting or by migrating alongside preexisting vessels, using these as guiding cues (Fig. 1, longitudinal migration), such as in the retina or in the heart where smooth muscle coverage proceeds in a epicardial-to-endocardial direction. In mesenchyme-rich tissues, such as in the lung, *in situ* differentiation of mesenchymal cells contributes to muscularization. Presumably, signals similar to those mediating smooth muscle cell recruitment and growth during initial vascular myogenesis are involved in arteriogenesis. Fibroblast growth factors may be involved in branching of coronary arteries, whereas the renin-angiotensin system may be involved in initiation, branching, and elongation of the renal arterial tree[55].

A pathological type of arteriogenesis is the 20-fold enlargement of preexisting collateral arterioles after occlusion of a supply artery[56] (Fig. 2). As a result of the increased collateral flow, endothelial cells express monokines (monocyte chemotactic protein 1) and monocyte adhesion molecules (such as intracellular adhesion molecule 1). The recruited monocytes infiltrate the vessel wall and destroy the media, using proteinases and death factors (TNF-α) (ref. 56). Activated endothelial cells then upregulate bFGF, PDGF-B and TGF-β1, thereby inducing the regrowth of smooth muscle cells and vessel enlargement. Arteriogenesis is impaired in *op/op* mice lacking macrophage colony-stimulating factor. A deficiency in PLGF prevents collateral growth by impairing monocyte recruitment, extravasation of fibronectin and smooth muscle cell growth (P.C., manuscript submitted).

*Smooth muscle cell differentiation*: Mural cells acquire specialized characteristics, including contractile components (Fig. 1). Loss of the intermediate filament desmin results in smooth muscle hypoplasia and degeneration, whereas a deficiency in MEF2C results in impaired smooth muscle differentiation[54]. Interstitial matrix components provide the developing arteries viscoelastic properties (elastin and fibrillin-2) and structural strength (collagen and fibrillin-1). A deficiency in these components, as in gene-inactivated mice or in humans with hereditary Marfan syndrome or atherosclerotic media destruction, results in weakening and aneurysmal dilatation of the arteries[57]. However, elastin also (negatively) regulates smooth muscle cell growth, as mice or humans lacking elastin die of obstructive intimal hyperplasia[58]. During pathological conditions (such as restenosis or atherosclerosis), smooth muscle cells often 'de-differentiate' to an embryonic phenotype, reverting from their 'contractile' to a 'synthetic' phenotype.

*Remodeling*: The large thoracic vessels undergo considerable remodeling during development. Genetic analysis has shown that loss of MFH-1, dHand or Msx1, Pax-3, Prx1, retinoid acid receptors, the neurofibromatosis type-1 gene product, Wnt-1, connexin 43 or endothelin-1 (ref. 53) induce aortic arch malformations. Prostaglandins mediate closure of the neonatal ductus arteriosus[59]. Signals involved in neuronal patterning also seem to be involved in vascular patterning. In the avian heart, there is a close spatial juxtaposition between coronary arteries and Purkinje cells of the myocardial conduction system. Endothelin-1, locally generated in the coronary artery, is an instructive cue for the differentiation of cardiomyocytes into Purkinje cells[60]. Loss of semaphorin-3C (J. Epstein, personal communciation) or of neuropilin-1, a receptor for neurorepulsive semaphorins, induces abnormal patterning of the large thoracic vessels[61]. Arterial rarefication also occurs during pulmonary or systemic hypertension. An imbalance between endothelin-1 and nitric oxide initially induces vasospasms, but when sustained, this progresses to irreversible vascular loss. Loss of PLGF or u-PA protects against pulmonary vascular remodeling (P.C., manuscript submitted).

### Modulation of vascular growth

*Involvement of hypoxia and nutrients*: Hypoxia-inducible transcription factors (HIF-1β, HIF-1α and HIF-2α) trigger a coordinated response of angiogenesis and arteriogenesis by inducing expression of VEGF, VEGFR1, VEGFR2, neuropilin-1, Ang2, nitric oxide synthase, TGFβ-1, PDGF-BB, endothelin-1, interleukin-8, IGF-II, Tie1, cyclooxygenase-2 and so on[62]. The von Hippel Lindau tumor suppressor gene product suppresses expression of hypoxia-inducible target genes during normoxia. Gene inactivation studies have shown that angiogenesis, not vasculogenesis, is regulated by hypoxia[62,63]. Tumors lacking HIF-1β or HIF-1α fail to develop vascularization and lack hypoxic induction of VEGF expression[64], whereas stabilization of HIF-1α by peptide regulator 39 induces angiogenesis in the myocardium[65]. Hypoxia-inducible factors and hypoxia-response elements are now being tested for angiogenic (gene) therapy of tissue ischemia. Metabolic stimuli, including hypoglycemia and low pH, also stimulate vessel growth, but their mechanisms remain to be determined.

*Involvement of mechanical factors:* Vasculogenesis occurs mostly independently, whereas angiogenesis coincides with the onset of and is influenced considerably by flow. As a result of the higher blood pressure in the capillaries proximal to the aorta, coronary arteries become covered by smooth muscle cells at earlier times than do veins[66]. Remodeling of the developing thoracic arteries or of collateral vessels after arterial occlusion also depends on flow[56]. Gene inactivation studies have shown that shear-stress-induced vascular remodeling is affected by nitric oxide and P-selectin, that the response of resistance arteries to flow is determined by vimentin, and that vascular tone is affected by bFGF (ref. 33). Mechanical forces modulate vascular function through shear-stress-responsive gene transcription.

### Lymphangiogenesis

The molecular mechanisms governing lymphangiogenesis remain mostly undefined because of the lack of specific molecular markers. Lymphatic vessels sprout from VEGFR3-expressing veins at a time when fluid extravasated by the increased blood pressure needs to be reabsorbed. VEGF-C is lymphangiogenic, as demonstrated by the lymphatic hyperplasia and sprouting that occurs after administration or transgenic overexpression of this molecule[67]. A similar although less-potent function has been recently identified for VEGF-D (K. Alitalo, personal communication). LYVE-1 (lymphatic vessel endothelial hyaluronan receptor) and VEGFR3 mark lymphatic vessels in the embryo and the adult[25], and genetic mutations of VEGFR3 have been identified in patients with herediatry lymphoedema (K. Alitalo, personal communication). *Prox-1* transcriptionally regulates lymphatic sprouting and budding[68]. Gene targeting studies also indicate involvement of Ang2 in lymphatic development (G. Yancopoulos, personal communication).

### Physiologic or pathologic angiogenesis: different or alike?

Growth of new blood vessel in the adult occurs through vasculogenesis (mobilization of bone marrow-derived endothelial stem cells), angiogenesis or arteriogenesis[56] (Fig. 2). Several mechanisms mediating pathological blood vessel growth resemble those during embryogenesis. However, evidence is emerging that distinct mechanisms may govern adult blood vessel formation, although some of these apparent differences may reflect our incomplete understanding (Figs. 1 and 2). The requirement for different signals is not unexpected, given that endothelial cells are loosely connected and actively growing in the embryo, whereas they are quiescent and encapsulated by a thick mural coat in the adult. Examples of molecules not or minimally involved in embryonic vascular development but substantially affecting pathological angiogenesis include Cox2, PLGF (P.C., manuscript submitted), $\alpha_v\beta_3$ (ref. 36), proteinases[21], plasminogen activator inhibitor 1 (ref. 23), nitric oxide[37] and TSP-2. Many stimulators and inhibitors affect adult blood vessel formation, although we do not understand their functions before birth, if indeed they have any at all. Another difference between physiological or pathological angiogenesis, is that the latter is often induced by (some degree of) inflammation. Monocytes/macrophages, platelets, mast cells and other leukocytes are 'chemoattracted' to sites of inflammation or wound healing, in part by angiogenic factors such as VEGF. These blood-borne cells produce angiogenic and arteriogenic factors (VEGF, bFGF, TGFβ-1, interleukin-8, PDGF, IGF-1, monocyte chemotactic protein 1, TNF-α and proteinases) that, in turn, attract endothelial and smooth muscle cells, fibroblasts, leukocytes or platelets[20,56,69].

### Therapeutic consequences, questions and perspectives

The recent insights in the molecular basis of angiogenesis have resulted in treatment paradigms to promote or inhibit angiogenesis. Although these approaches are in their infancy, they are promising. However, because of the rapid evolution and enthusiasm of the field, angiogenic molecules are often being tested in clinical trials, without a complete understanding of their mechanism of action. In addition, many questions remain unanswered. For example, is administration of a single angiogenic molecule sufficient? $VEGF_{120}$ alone initiates but does not complete angiogenesis and arteriogenesis in transgenic mice[28], and mice expressing $VEGF_{164}$ alone are more normal than $VEGF_{120}$ mice; transgenic overexpression of VEGF and Ang1 induces more-numerous and more-stable, yet more-irregular, vessels[14,32]. If one is not sufficient, how should angiogenic molecules be administered in combination: simultaneously or sequentially? Will it be feasible to administer a potent molecule like VEGF in quantitities sufficient for therapy without causing toxicity (hypotension, edema), and, conversely, how do we explain the beneficial effect of administration of minimal VEGF? Are the other VEGF homologs (PLGF, VEGF-B or VEGF-C) attractive, perhaps safer, angiogenic targets? Are Ang1 and Ang2 molecules that stimulate, stabilize or suppress vessels, or does their pro- or anti-angiogenic activity depend on the microenvironment, tissue and context? Are hypoxia-inducible factors safe 'master switches' to use for therapeutic angiogenesis, given their possible involvement in cell death[64]? How much do bone marrow-derived endothelial precursors contribute to pathological angiogenesis, or is local proliferation of angiogenic endothelial cells *in situ* more important? Which (anti)-angiogenic gene therapy methods and routes should be used to avoid infiltration of pro-angiogenic inflammatory cells? Do we deliver angiogenic drugs to the non-ischemic or peri-ischemic myocardium? How long should angiogenic factors be administered: will therapeutic angiogenesis lead to a sustainable and functional vasculature or will vessels regress upon ending therapy? Should treatment not be targeted to arteriogenesis instead of angiogenesis, and will therapeutic angiogenesis/arteriogenesis only succeed in ischemic regions? Will we be able to extrapolate to patients the success of angiogenesis inhibitors (endostatin, angiostatin, vasostatin, thrombospondin, troponin, MMP-inhibitors and so on) in murine models of (ectopic) tumor implantation? Are proteinase inhibitors a good choice to suppress angiogenesis, given the poor prognostic value of plasminogen activator inhibitor 1 in cancer patients[23], or instead should we use molecules (like endostatin) that increase plasmin proteolysis? How much do we need to understand of the mechanism of action of (anti)-angiogenic candidates and/or become aware of their side effects before initiating clinical trials: will therapeutic angiogenesis promote growth of dormant tumors or atherosclerotic plaques; will inhibition of tumor angiogenesis be feasible when tumor vessels are mosaically lined by tumor cells and tumors easily find escape routes to switch on alternative angiogenic programs? Considering the link between angiogenesis and neurogenesis[14], and the recent finding that VEGF improves ischemic neuropathy[70], will long term (anti)-angiogenic treatment cause undesired neuronal effects? Finally, should angiogenic treatment be tailored to individual patients and require genetic prescreening? Indeed, recent genetic studies in mice indicate that loss of u-PA prevents therapeutic myocardial angiogenesis with VEGF (ref. 21). Furthermore, angiogenesis differs more than 10-fold in mice of various genetic backgrounds, and some strains respond by angiogenic sprouting, wereas others by morphogenetic remodeling[71]. Clearly, more work is needed in the future, but—at least—the outlook has become a promising one.

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