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Review

Molecular mechanisms of blood vessel growth

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1. From avascular to vascular

From the earliest stages, the embryo develops in the absence of vascularization, receiving its nutrition by diffusion [1]. In an ordered and sequential manner, however, the embryo rapidly transforms into a highly vascular organism, survival being dependent on a functional, complex network of capillary plexuses and blood vessels. 'Vasculogenesis' refers to the initial events in vascular growth in which endothelial cell precursors (angioblasts) migrate to discrete locations, differentiate in situ and assemble into solid endothelial cords, later forming a plexus with endocardial tubes (Fig. 1). The subsequent growth, expansion and remodeling of these primitive vessels into a mature vascular network is referred to as 'angiogenesis' (Fig. 1). This process is characterized by a combination of sprouting of new vessels from the sides and ends of pre-existing ones, or by longitudinal division of existing vessels with periendothelial cells (intussusception), either of which may then split and branch into precapillary arterioles and capillaries. Depending on the ultimate fate with respect to the type of vessel (artery, vein, capillary) and vascular bed, activated endothelial cells that are migrating and proliferating to form new vessels, forming anastomotic connections with each other, become variably surrounded by layers of periendothelial cells — pericytes for small vessels and smooth muscle cells for large vessels ('vascular myogenesis') (Fig. 1) [2]. During this dynamic period, extracellular matrix produced by mural cells, serves to stabilize the network. Finally, further functional modifications of larger arteries occur during 'arteriogenesis' as a thick muscular coat is added, concomitant with acquisition of viscoelastic and vasomotor properties (Fig. 1).

It is clear that for vasculogenesis and angiogenesis to effectively proceed during physiological and pathological conditions, it is essential that a complex array of angiogenic and anti-angiogenic factors, interacting with multiple cells and tissues, be tightly regulated. Although endothelial cells have attracted the most attention, they alone cannot complete the process of vessel growth and development, as peri-endothelial cells and matrix components play essential roles. Innovative gene technologies and advances in animal modeling have enabled research scientists to make major advances in understanding the mechanisms of blood vessel growth. The ultimate therapeutic goals, which are to mitigate against angiogenesis during pathological processes such as inflammation and tumorigenesis, and to enhance angiogenesis to prevent and/or treat ischemic events, have become realizable at the clinical level, and thus caught the imagination and attention of all those involved in health care and research [3,4]. In the past 15–20 years, major breakthroughs have resulted in the emergence of useful paradigms to help explain vasculogenesis and angiogenesis. This review provides an update on some of the basic molecular mechanisms governing how endothelial cells, smooth muscle cells, matrix molecules, and several critical receptors and their ligands, interact with each other to form blood vessels. As will become evident, the rapid growth in our understanding has provided scientists with new and exciting potential options to address the therapeutic goals. However, there are still many aspects of vasculogenesis and angiogenesis that remain challenges to our knowledge. These are being met with remarkable rapidity.

2. Vasculogenesis

Clear evidence of blood vessel development first appears

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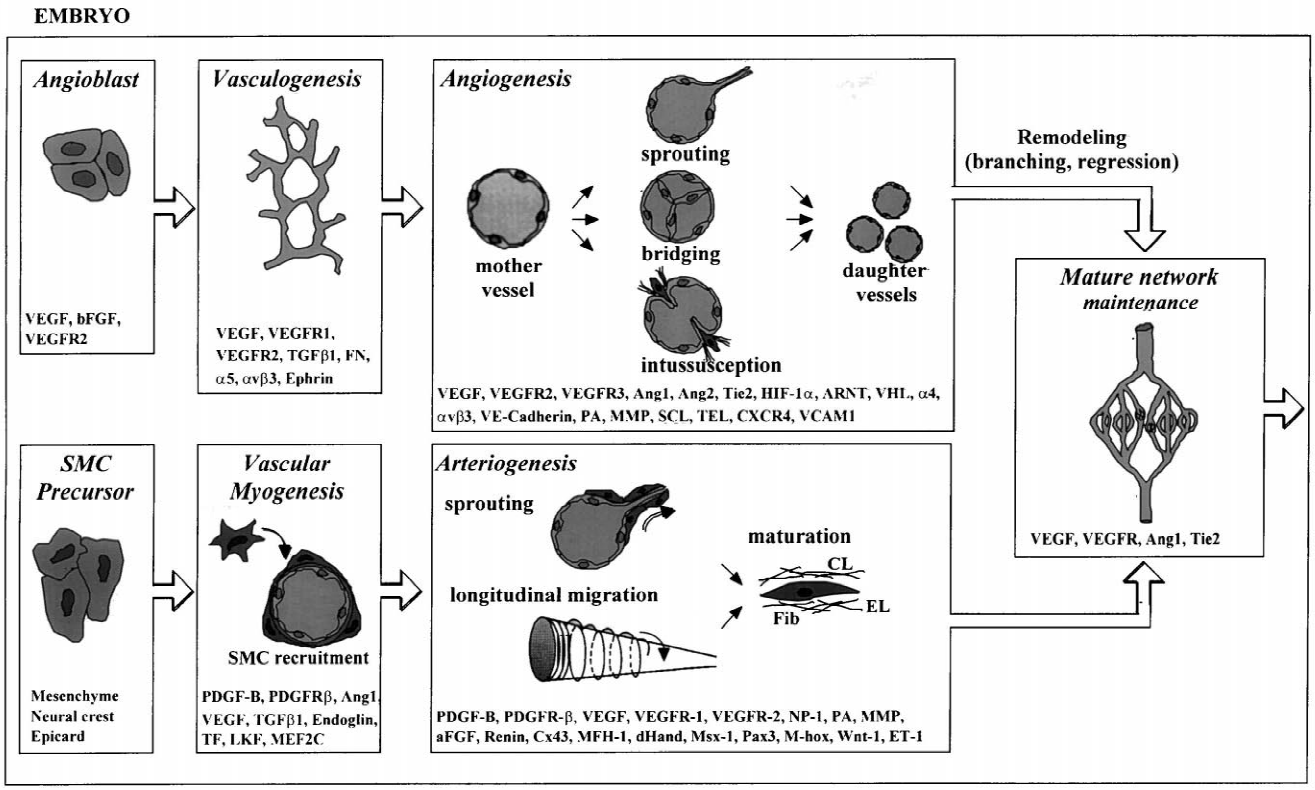

Fig. 1. Endothelial precursors (angioblasts) in the embryo assemble in a primitive network (vasculogenesis), that expands and remodels (angiogenesis). Smooth muscle cells cover endothelial cells during vascular myogenesis, and stabilize vessels during arteriogenesis. CL, collagen; EL, elastin; Fib, fibrillin. With permission from Ref. [4].

outside of the embryo proper, on the yolk sac, as focal aggregations of mesenchymal cells, known as blood islands, form within the mesoderm adjacent to the extraembryonic endoderm. While the earliest studies suggested that the embryo proper becomes vascularized from branching and growth of extraembryonic blood vessels which in turn are derived from fusion and channeling of blood islands, intraembryonic origins of a vascular network were recognized as early as 1900 when it was determined that cells from isolated embryos were able to give rise to blood vessels. It was at this time that the term angioblast, referring to the cells from which all endothelial cells arise, was described. It was also during this early period that the presence of the hemangioblast — a common precursor for both endothelial cell and hematopoietic cell — was first postulated (Fig. 2). Notably, it was almost 100 years later that the presence of the hemangioblast was confirmed [5]. Differentiation of pluripotent embryonic precursor cells into hemangioblastic cells is induced to at least some extent by fibroblast growth factor (FGF) via protein kinase C signaling [6]. Hemangioblasts undergo their first critical steps of differentiation within the blood islands. Cells at the perimeter of the blood islands give rise to precursors for endothelial cells, while those at the center constitute hematopoietic precursors. The molecular signals determining the fate of the hemangioblast are not fully elucidated. However, several genes have been identified that may play a role in this early event [7]. These include *Ets-1* [8], *Hex* [9], *Vezf* [10], *Hox* [11,12], members of the *GATA*-family, basic helix-loop-helix (bHLH) factors [13,14], and the *Id*-proteins [15]. The earliest markers common to endothelial and hematopoietic precursors so far identified are CD31, CD34 and the receptor tyrosine kinase type-2 of vascular endothelial cell growth factor (VEGFR-2 or KDR/Flk1) [16]. Inactivation of the *VEGFR-2* gene in mice results in embryonic lethality, with lack of development of both hematopoietic and endothelial cell lineages, supporting the critical importance of this receptor at that developmental stage [17], although not defining the steps regulating differentiation into endothelial versus hematopoietic cell.

As the yolk sac vasculature begins to form ~7.5 days post coitum (dpc) in the mouse, angioblasts that have migrated to the paraxial mesoderm assemble into aggregates, proliferate and subsequently differentiate to form a plexus with endocardial tubes, leading to formation of the dorsal aortae, cardinal veins and the embryonic stems of yolk sac arteries and veins (Fig. 3). Vasculogenesis was

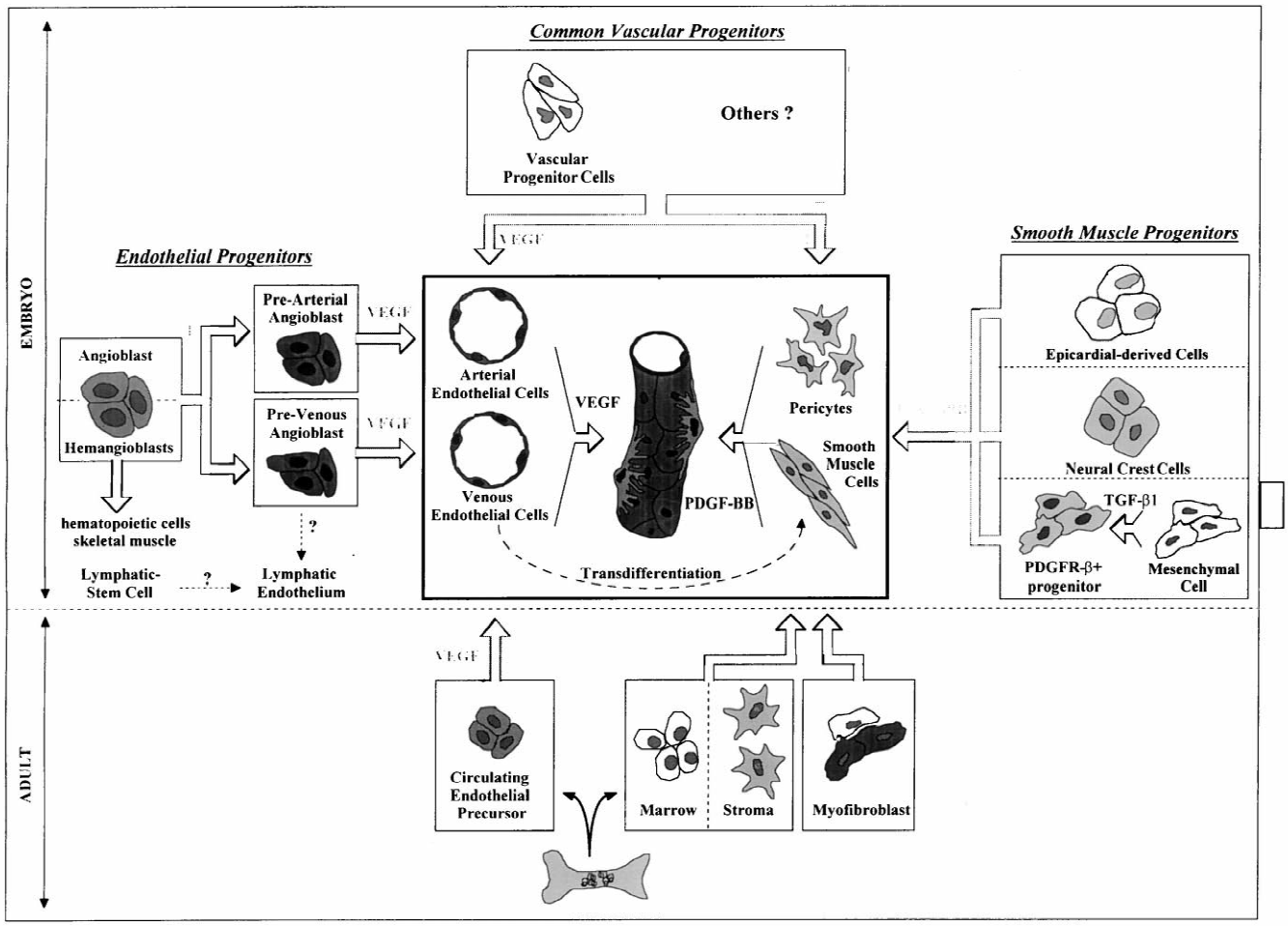

Fig. 2. A common origin for the two types of blood-vessel cell. Endothelial and smooth muscle cells arise from separate types of precursor. Endothelial cells arise from precursors called angioblasts or hemangioblasts in the embryo, or from circulating endothelial progenitors in the adult. Angioblasts give rise to arterial and venous lineages. Smooth muscle cells and pericytes, by contrast, can form from a variety of progenitors. These include mesenchymal cells, neural crest cells, and progenitors in the epicardium in the embryo. Progenitors in the bone marrow and its stroma, and mesenchymal myofibroblasts also give rise to smooth muscle cells. A new common vascular progenitor cell that gives rise to both types of blood-vessel cell has been recently identified. Vascular endothelial growth factor (VEGF) promotes the development of endothelial cells from this precursor. TGF-β1 has been involved in differentiation of mesenchymal cells to progenitors, that express the receptor for PDGF-BB. The latter stimulates their development into smooth muscle cells and pericytes and is responsible for their recruitment around nascent vessels. Adapted from Ref. [114].

originally believed to be restricted to embryonic development. However, it has been established that angioblasts not only migrate intraembryonically, but may circulate postnatally, and may be recruited for in situ vessel growth [18–20] (Fig. 4). Unlike shed cells, circulating CD34/VEGFR-2/AC133-positive marrow-derived angioblasts in the adult have a high proliferation rate [21]. How angioblasts 'know' where and when to initiate vasculogenesis is largely a mystery, although a variety of growth factors and receptors, including vascular endothelial growth factor (VEGF), granulocyte monocyte-colony stimulating factor (GM-CSF) and other cytokines have been implicated, as they have been shown to be able to recruit bone-marrow derived angioblasts to sites of neovascularization postnatally [18,22,23]. Angioblast differentiation may be promoted by VEGF, VEGFR2 and basic fibroblast growth factor (bFGF) [24–27], while VEGF receptor-1 (VEGFR-1; Flt1) has been determined to suppress hemangioblast commitment [28]. The findings that endothelial cells still develop in mice engineered to lack VEGF but do not develop any longer in the absence of VEGFR-2, suggests that there are additional molecules, binding to VEGFR-2, that determine endothelial cell fate. Although vasculogenesis is closely linked to hematopoiesis, precursors of definitive hematopoietic cells arise only from the dorsal aorta, and subsequently populate the liver, spleen and bone marrow. A basic understanding of the molecular mechanisms that separate hematopoiesis from vasculogenesis will

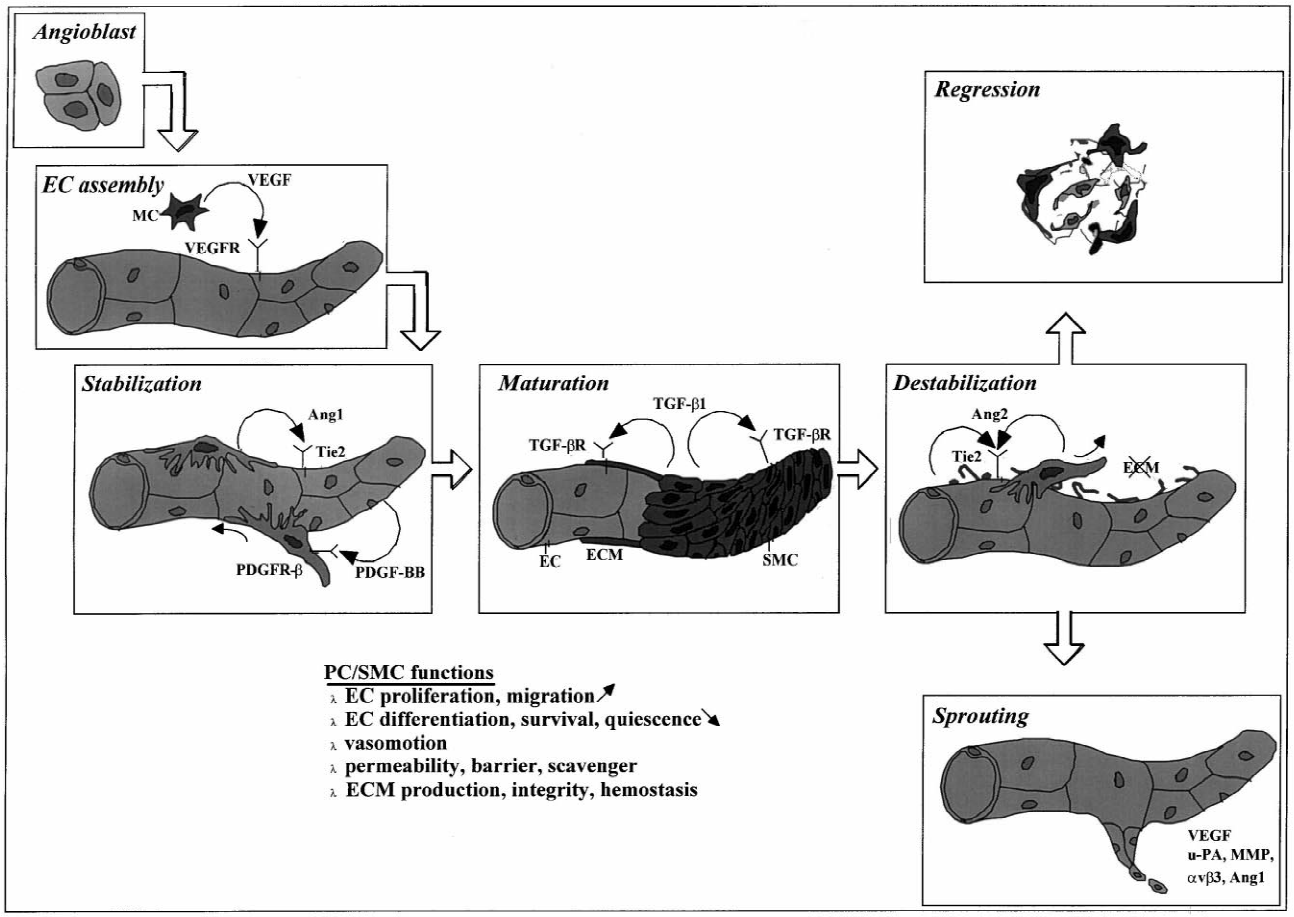

Fig. 3. Pathological vascular growth in the adult may occur via vasculogenesis (angioblast mobilization), angiogenesis (sprouting) or arteriogenesis (collateral growth). With permission from Ref. [4].

not only impact on angiogenic therapies, but also on treatment of a variety of hematologic disorders.

3. Angiogenesis

The molecular basis of angiogenesis is most easily characterized by viewing the process as a step-wise progression (Figs. 3 and 4). The initial vasodilation of existing vessels is accompanied by increases in permeability and degradation of surrounding matrix, which allows activated and proliferating endothelial cells to migrate and form lumens. The endothelial cells of these 'sprouting' new vessels differentiate to accommodate local requirements, supported by a surrounding network of similarly differentiated peri-endothelial cells and matrix, all of which mature through remodelling into a complex vascular network, ultimately lined by endothelial cells that have acquired critical survival factors, optimized to function under a variety of conditions. Our understanding of the cellular and molecular mechanisms in angiogenesis, in cancer and other diseases is still limited. Capillary growth in tumors appears to be particularly complex, possibly because the malignant cells derail the process via chaotic expression of angiogenic factors. As each of these steps is better elucidated, greater potential for therapeutic intervention will be forthcoming.

3.1. Existing vessels dilate, vascular permeability increases and extracellular matrix is degraded

Largely in response to nitric oxide (NO), vasodilation is one of the earliest steps in angiogenesis. VEGF, transcriptionally upregulated in part by NO [29], mediates an increase in vascular permeability, accomplished through redistribution of intercellular adhesion molecules, including platelet endothelial cell adhesion molecule (PECAM)-1 and vascular endothelial (VE)-cadherin, and alterations in cell membrane structure via induction of a series of kinases [30,31]. Extravasation of plasma proteins follows, similarly induced by VEGF, these being required for creating a temporary support structure, as activated endothelial cells subsequently migrate. Not surprising, excessive vascular permeability could result in pathological outcomes, such as intracranial hypertension or circulatory collapse. Conse-

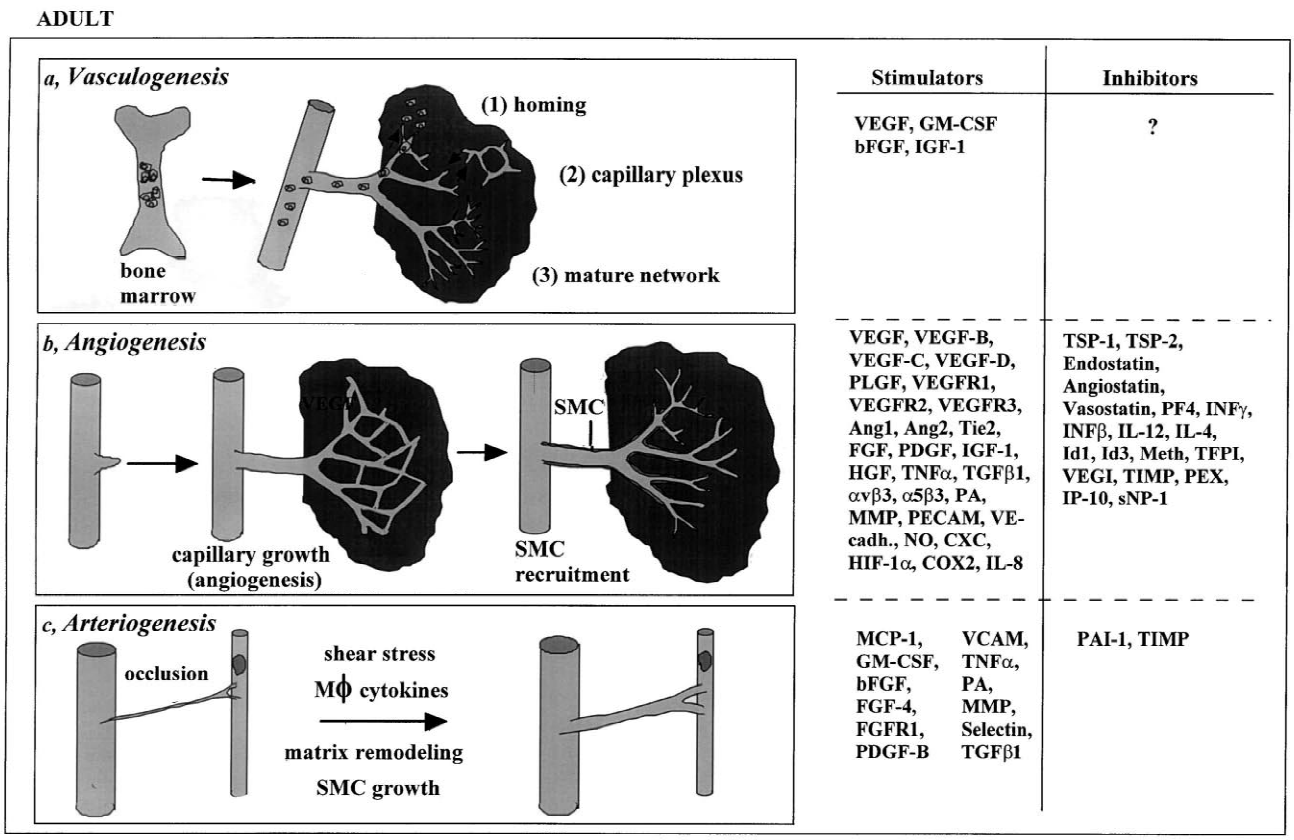

Fig. 4. VEGF initiates assembly of endothelial cells (EC), PDGF-BB recruits pericytes (PC) and smooth muscle cells (SMC), whereas angiopoietin-1 (Ang1) and TGF-β1 stabilize the nascent vessel. Angiopoietin-2 (Ang2) destabilizes the vessel, resulting in angiogenesis in the presence of angiogenic stimuli, or in vessel regression in the absence of endothelial survival factors. With permission from Ref. [4].

quently, permeability changes must be tightly regulated. Angiopoietin-1 (Ang1), a ligand for the endothelial receptor Tie2, is a natural anti-permeability factor, that provides protection and a balance against excessive plasma leakage [32].

Endothelial sprouting is further enhanced by another member of the angiopoietin family, Ang2, an inhibitor of Tie2 signalling and a natural antagonist of Ang1. Ang2, appearing at angiogenic and vascular remodelling sites, is involved in detaching smooth muscle cells and loosening underlying matrix, thereby allowing endothelial cells to migrate as inter-endothelial cell contacts are relieved [31,33]. Degradation of extracellular matrix involves an array of proteinases which not only provides 'room' for the migrating endothelial cells, but also results in the liberation of growth factors, including bFGF, VEGF and insulin-like growth factor-1 (IGF-1), which otherwise remain sequestered within the matrix. Over 20 matrix metalloproteinases (MMPs) have been described and implicated in angiogenesis, tumorigenesis and cell proliferation (reviewed in Ref. [34]). In addition, they can expose cryptic adhesion sites, hidden in non-proteolyzed matrix components. As the name implies, MMPs play a central role in degrading extracellular membranes and basement membrane structures, allowing endothelial migration to occur. Natural inhibitors of MMPs include circulating protease inhibitors, such as tissue-localized inhibitors of metalloproteinases (TIMPs) [35]. It is at least partly through the secretion of MMP-2, MMP-3 and MMP-9, and suppression of TIMP-2 that Ang1 induces sprouting [36]. Similarly, MMPs 3, 7 and 9 have been shown to induce angiogenesis in neonatal bones and tumors [37]. MMPs do not uniformly enhance angiogenesis, however. Temporal and spatial factors likely dictate their function. For example, MMPs 1 and 3 may also inhibit tumor angiogenesis by interfering with binding of MMP-2 to integrins [38], while MMPs 7 and 9 generate angiostatin from circulating plasminogen, thereby inhibiting endothelial cell proliferation [39]. Interactions with other proteins also alter their roles in angiogenesis. Thrombospondin-1 (TSP-1) is believed to be anti-angiogenic by preventing activation of MMP-2 and MMP-9 [40]. Optimal angiogenic function of MMP-9 may require cell-surface localization with the hyaluronan receptor CD44 and TGF-beta [41]. Other proteinases have also been implicated in matrix degradation enabling endothelial cell migration. Notably, urokinase-type plasminogen activator (u-PA) is essential for revascularization of myocardial infarcts [42,43], while antagonists of u-PA or its interaction with the u-PA have in vivo anti-angiogenic therapeutic potential [43,44]. A fine-tuned balance between proteinases

and their inhibitors is, however, essential as excessive plasmin proteolysis prevents pathological angiogenesis in cancer and inflammation [45]. This may also explain why the u-PA inhibitor PAI-1 is a poor prognostic factor for several cancers.

3.2. Endothelial cells proliferate and migrate

As the physical barriers are dissolved, proliferating endothelial cells are free to migrate to distant sites. At this step, an interplay between the various forms of VEGF, angiopoietins, FGFs and their receptors are responsible for mediating embryonic, neonatal and pathological angiogenesis, although additional factors have also been implicated in the process. There is likely considerable redundancy — however, several distinct functions for many of the components have been delineated. Although VEGF has profound effects throughout angiogenesis [46,47], homologues of VEGF are more restricted. For example, placental growth factor (PlGF) [48] is redundant for embryonic vascular development, but an essential amplifier of VEGF-driven angiogenesis during pathological conditions (unpublished findings). VEGF-B has been implicated in extracellular matrix degradation via activation of plasminogen, and is believed to regulate coronary artery function [49,50]. VEGF-C has been demonstrated to stimulate angiogenesis in the adult, although its endogenous role in pathology remains undefined [47]. Its receptor, VEGFR-3, is highly expressed during embryonic development, and is required for vascular remodeling and angiogenesis [47,51]. Ang1, via phosphorylation of Tie2, is chemotactic for endothelial cells, induces sprouting, and stimulates the interaction between endothelial and peri-endothelial cells [31,52,53]. Ang2, in concert with VEGF is also angiogenic, although in the absence of VEGF, Ang2 may actually induce vessel regression [33].

Several additional factors have been implicated. Fibroblast growth factors stimulate endothelial cell growth and recruit mesenchymal and/or inflammatory cells, producing a myriad of angiogenic factors [54]. Platelet-derived growth factor (PDGF) is angiogenic for microvascular sprouting endothelial cells and recruits pericytes and smooth muscle cells around nascent vessel sprouts [55,56]. From gene inactivation studies in mice, endothelial nitric oxide synthase (eNOS) was determined to have in vivo angiogenic properties, inducing endothelial growth following denudation injury or hind limb ischemia [57]. Additional studies have demonstrated that VEGF stimulates eNOS and release of NO. Members of the TGFβ superfamily, such as activin A and tumor necrosis factor (TNF)-α, are multifunctional cytokines that have been variably reported to both stimulate and inhibit endothelial growth in different models [58]. TGF-β1 suppresses tumor angiogenesis [59], while TNF-α was recently reported to modulate VEGF induced endothelial proliferation via interference with VEGFR-2 phosphorylation [60]. More recently, several chemokines, including monocyte chemotactic protein (MCP)-1, have been demonstrated to induce endothelial growth [61]. As the endothelial cells proliferate and migrate, directed in part by signaling through integrins $\alpha_V\beta_3$ and $\alpha_5\beta_1$ [62], PECAM-1 [63], and Eph/ephrin receptor-ligand pairs [64–66], they contact other endothelial cells. Endothelial cell junctions are established with gap proteins such as VE-cadherin and members of the connexin family [67,68]. Integrins may localize MMPs to the endothelial cell surface and extracellular matrix, thereby explaining why antagonists of integrins might be therapeutically effective anti-angiogenic agents [69].

Numerous additional molecules with angiogenic or anti-angiogenic effects following administration in various animal models, are currently being evaluated (Table 1). Those that are angiogenic are, among others, erythropoietin, insulin, IGF-1, neuropeptides, leptin, epidermal growth factor, hepatocyte growth factor, interleukins, monocyte activating peptides, etc. [4,70]. Similarly, natural inhibitors of endothelial proliferation are being uncovered (Table 1). For instance, chondromodulin-1 is expressed in the avascular zone of cartilage in developing bone, withdrawal of which results in capillary in-growth [71]. Some of the endogenous angiogenesis inhibitors that are being evaluated for clinical use include angiostatin [72], endostatin [73], anti-thrombin III, interferons, platelet factor-4, and others [4,70]. The specific endogenous role of each of these molecules during embryonic, post-natal and adult vessel growth, remains largely unknown.

3.3. Endothelial cells assemble, form cords and acquire lumens

As endothelial cells migrate into the extracellular matrix, they assemble into solid cords, and subsequently acquire a lumen. This is accomplished by intercalation and thinning of the endothelial cells along with fusion of existing vessels to create longer and greater diameter vessels. Tumor vessels are structurally and functionally abnormal. They are highly disorganized: vessels are tortuous and dilated, with uneven diameter. They have excessive branching and shunts. This may be due to an imbalance of angiogenic regulators, such as VEGF and angiopoietins, or alternatively, due to different molecular signals. Consequently, the blood flow in tumors is chaotic, changing from one location to the next and from one time to the next [74]. Lumen diameter is tightly regulated by several factors. While $VEGF_{121}$ and $VEGF_{165}$ and their receptors increase lumen formation, $VEGF_{189}$ decreases lumen diameter. In combination with VEGF, Ang1 augments lumen diameter [52]. Multiple integrins ($\alpha_5\beta_1$ and $\alpha_v\beta_3$) are also involved in lumen formation and cyclic RGD compounds may abrogate their function, another potential for therapeutic intervention [75]. Finally, there are also several endogenous inhibitors of lumen formation,

Table 1
Angiogenesis activators and inhibitors[a]

Activators	Function	Inhibitors	Function
VEGF, VEGF-C, PlGF and homologues[b]	Stimulate angiogenesis, permeability; VEGF-C: stimulates lymphangiogenesis; PlGF: role in pathologic angiogenesis	VEGFR-1, soluble VEGFR-1 and neuropilin-1 (NP-1)	Sink for VEGF, VEGF-B, PlGF (VEGFR-1) and for $VEGF_{165}$ (NP-1)
VEGF receptors (VEGFR)	VEGFR-2: angiogenic signaling receptor; VEGFR-3: (lymph)angiogenic signaling receptor; neuropilin-1 (NP-1): binds specifically $VEGF_{165}$; coreceptor of VEGFR-2	Angiopoietin-2	Antagonist of Ang1: induces vessel regression in the absence of angiogenic signals
Angiopoietin-1 (Ang1) and Tie2-receptor[b]	Ang1: stabilizes vessels by tightening endothelial-smooth muscle interaction; inhibits permeability; Ang2: destabilizes vessels before sprouting	Thrombospondin-1 (TSP-1)	Extracellular matrix protein; Type I repeats inhibit endothelial migration, growth, adhesion, survival; related TSP-2 also inhibits angiogenesis
PDGF-BB and receptors	Recruit smooth muscle cells	Meth-1, Meth-2	Inhibitors containing metalloprotease, thrombospondin and disintegrin domains
TGF-β1[c], endoglin, TGF-β receptors	Stabilize vessels by stimulating extracellular matrix production	Angiostatin and related plasminogen kringles	Proteolytic fragments of plasminogen; inhibit endothelial migration and survival
FGF, HGF, MCP-1	Stimulate angiogenesis (FGF, HGF) and arteriogenesis (FGF, MCP-1)	Endostatin	Fragment of type XVIII collagen; inhibits endothelial survival and migration
Integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$	Receptors for matrix macromolecules and proteinases (MMP2)	Vasostatin, calreticulin	Calreticulin and N-terminal fragment (vasostatin) inhibit endothelial growth
VE-cadherin, PECAM (CD31)	Endothelial junctional molecules; essential for endothelial survival effect; antibodies block tumor angiogenesis	Platelet factor-4	Heparin-binding CXC chemokine inhibits binding of bFGF and VEGF
Ephrins	Regulate arterial/venous specification	Tissue-inhibitors of MMP (TIMPs), MMP-inhibitors, PEX	Suppress pathologic angiogenesis; PEX: proteolytic fragment of MMP2, blocks binding of MMP2 to $\alpha_v\beta_3$
Plasminogen activators, matrix metalloproteinases	Proteinases involved in cellular migration and matrix remodeling; liberate bFGF and VEGF from the matrix; activate TGF-β1; generate angiostatin	Tissue-inhibitors of MMP (TIMPs), MMP-inhibitors	Suppress pathological angiogenesis
Plasminogen activator inhibitor-1	Stabilizes nascent vessels by preventing matrix dissolution; poor cancer prognosis	Interferon (IFN) α, β, γ; IP-10, IL-4, IL-12, IL-18	Cytokines and chemokines, inhibiting endothelial migration; IFNα downregulates bFGF
Nitric oxide synthase, cyclooxygenase-2	Nitric oxide and prostaglandins stimulate angiogenesis and vasodilation; Cox2 inhibitors suppress tumor angiogenesis	Prothrombin kringle-2, anti-thrombin III fragment	Fragments of the hemostatic factors suppress endothelial growth
Other activators	AC133 (orphan receptor involved in angioblast differentiation); chemokines[c] (pleiotropic role in angiogenesis); inhibitors of differentiation (Id1/Id3; helix-loop-helix transcriptional repressors)	Other inhibitors	16 kDa-prolactin (inhibits bFGF/VEGF); canstatin (fragment of the α_2-chain of collagen IV); maspin (serpin); troponin-I (inhibits actomyosin ATPase); VEGI (member of TNF family); restin (NC10 domain of collagen XV); fragment of SPARC (inhibits endothelial binding and activity of VEGF); osteopontin fragment (contains RGD sequence)

[a] Selected list. See text for abbreviations.
[b] Suppresses angiogenesis in some contexts.
[c] Also present in or affecting non-endothelial cells.

including thrombospondin-1 (TSP-1), and a recently identified novel gene, tubedown (tbdn)-1, that has homology to acetyltransferases [76]. Ultimate selection of factors responsible for specific regulation of tumor angiogenesis is a major focus of current research.

3.4. Long-term survival of vascular endothelium

Once new vessels have assembled, the endothelial cells become remarkably resistant to exogenous factors, and are quiescent, with survival measured in years. Diminished endothelial survival — or endothelial apoptosis — is characterized by vascular regression in the embryo [77], which in the retina or ovary after birth, is a natural and physiologic process. The list of factors identified that regulate endothelial apoptosis is extensive [78,79], and these vary considerably according to the developmental time point, the specific site, function and type of vessel, in addition to surrounding physiological and/or pathological stimuli. The molecular mechanisms by which a 'quiescent', confluent endothelium is able to maintain its physiological function in various vascular beds for long periods of time, is unclear. However, some insights have been gained from in vivo and in vitro studies. Deprivation of nutrients following obstruction of vessels by spasm or thrombi, results in release of pro-apoptotic signals and

effective suppression of anti-apoptotic stimuli, leading to endothelial programmed cell death and vessel regression. In premature babies, exposure to hyperoxia reduces VEGF levels and causes retinal vessel regression [80,81]. The role of VEGF as a survival factor depends on its interaction with VEGFR-2, PI3-kinase, β-catenin and VE-cadherin [77]. Lack of the cytoplasmic domain of VE-cadherin results in endothelial apoptosis via interruption of VEGF signalling, leading to diminished activation of the protein kinase Akt and lack of upregulation of the anti-apoptotic *bcl*-2 gene [77]. While the endothelial survival function of VEGF is evident in the embryo, adult quiescent vessels appear to be less sensitive to VEGF deprival.

Other factors known to play a role in endothelial survival include the angiopoietins via their cognate receptors, Tie1 and Tie2 — Ang 1 promotes, while Ang2 suppresses survival, particularly with respect to tumor vessel growth [31,52,82]. Ang1 has further been determined to exert survival properties by enhancing expression of the anti-apoptotic gene, *survivin*, via activation of Akt through Tie2 signalling [83]. Maintenance of vascular integrity in different vascular beds also requires hemodynamic shear forces, which not only provide appropriate metabolic requirements to target tissues, but regulate and reduce endothelial turnover and abrogate endothelial apoptosis caused by TNF-α [84]. Molecular mechanisms implicated in mediating cell cycle arrest and survival of vascular endothelial cells include several factors involved in regulation of cell-cycle and apoptosis, such as p53, p21, p16, p27, Bax and p42/44 mitogen activated protein kinase [85]. Many other angiogenesis inhibitors provide survival advantages to endothelium, however the mechanisms of action are similarly unknown. Examples include prothrombin kringles-1 and -2, thrombospondin-2, antagonists of PECAM-1, interleukins 4 and 12, and cyclo-oxygenase-2 (Cox) inhibitors.

3.5. Vascular endothelium differentiates to meet local needs

With maturation of the vascular network, local physiological requirements must be met. To this end, endothelial cells acquire highly specialized characteristics to provide the functional needs within specific tissues and organs. For example, development of the blood–brain barrier requires interactions between astroglial cells that express glial fibrillary acidic protein, pericytes and adequate angiotensinogen levels [55]. The tight junctional complex between endothelial cells consists of numerous integral membrane and cytosolic proteins from, for example, the families of cadherins, occludins, claudins, and membrane-associated guanylate kinase homologous proteins [86,87]. The plasma membranes of those endothelial cells reaching confluence necessarily undergo major structural and functional modifications, crucial for the regulation of vascular permeability. Other proteins, such as 7H6, cingulin and JAM participate in monoctye transmigration and regulation of permeability [87,88]. In contrast, endothelial cells in endocrine glands lack the tight junctions of the blood–brain barrier. Rather, the endothelium is discontinuous and fenestrated, allowing high-volume molecular and ion transport. Overall, the factors that regulate acquisition of specific endothelial properties are largely unknown. However, it appears that the host environment, in concert with VEGF, plays a major role [89,90]. Endothelial differentiation in tumors is abnormal and results in vessels with multi-layered endothelium, irregularly sized lumens, and non-uniform junctional complexes that tend to be highly permeable [70]. Recent findings suggest that tumor vessels may be lined not only by endothelial cells, but by tumor cells themselves which have attained ‘vasculogenic’ properties or a mosaic of cancer and endothelial cells [91]. For example, 15% of vessels in xenografted and spontaneous human colon carcinomas are mosaic in nature [92]. So-called ‘vasculogenic mimicry’, which implies de novo generation of vasculature without participation of endothelial cells and independent of angiogenesis, would have a major impact on therapy designed to interfere with new vessel growth associated with tumorigenesis [93].

3.6. Remodeling of vessels yields complex, functional networks

The three-dimensional organization of the vascular network has fascinated many scientists for a long time [54]. One of the first was Aristotle, who wrote: ‘‘the system of blood vessels in the body may be compared to those of water-courses which are constructed in gardens: they start from one source, or spring, and branch off into numerous channels, and then into still more, and so on progressively, so as to carry a supply to every part of the garden’’. Several mechanisms have been identified that could result in the branching of vessels. First, new vessel branches can sprout towards a cluster of cells in the surrounding mesenchyme, that produce the angiogenic stimulus. Second, vessels can split into individual daughter vessels by the formation of transendothelial cell bridges. Third, vessels can branch via intussusception, based upon insertion of interstitial tissue columns into the lumen of pre-existing vessels [94,95]. The subsequent growth of these columns and their stabilization — in part via ingrowth of periendothelial cells — results in partitioning of the vessel lumen and remodeling of the vascular network.

A number of angiogenic signals have been identified to affect one or more of these branching processes, although the distinction between these processes is often blurred [96]. For the sake of clarity and brevity, some typical examples are illustrated. VEGF contributes to the complexity of the vascular network by stimulating vascular splitting and sprouting [97]. Embryos lacking a single *VEGF* allele have fewer vascular sprouts [25,26]. In addition,

mice that only express the short $VEGF_{120}$ isoform die of ischemic heart disease and suffer a markedly reduced coronary perfusion reserve [98]. This resulted from a significantly impaired branching of myocardial vessels. Expression of PDGF-BB, which is known to affect airway branching, was reduced in these hearts, but it is also possible that VEGF also acts as a 'branching factor' by directly affecting endothelial and/or smooth muscle cells. Ang1 and its receptor Tie2 induce angiogenic sprouts, but may also affect microvascular intussusceptive growth [31]. Indeed, gene inactivation of Ang1 and Tie2 resulted in dilated capillary-like vessels of uniform size that fail to remodel in a mature, complex network of large vessels ramifying into smaller branches. Renin is a 'branching factor' for renal arteries [99], while acidic FGF is a 'branching factor' for myocardial arteries [100]. Such a role of FGF-1 in the vasculature is consistent with the role of the FGF-related signals in airway branching. *Notch* signaling has been implicated in the singling-out process of budding airway cells. Indeed, the FGF-like *branchless* upregulates expression of *Delta*, a ligand for *Notch*, at the tip of the tracheal branches. Activated *Notch* then restricts the *branchless*-signal to the tip of the branches by downregulation of *branchless* in neighboring cells via lateral inhibition. A challenge for the future will be to unravel whether FGF-1 also induces a similar finely tuned cascade of cellular and molecular events in the heart as *branchless* and FGF-10 accomplish in the lung. For instance, it remains to be determined whether *Notch*, *Delta*, and *Jagged* [101]— molecules of which we only now start to reveal their role in angiogenesis [102,103] — are also involved in singling out sprouting endothelial cells via lateral inhibition in response to FGF-1.

Several gene targeting studies implicate other factors in vessel patterning and remodeling, including VEGFR-3, the endothelial receptor Tie1, T-cell leukemia protein stem cell leukemia factor/tal-1, members of the Ets family of transcription factors, some GTP-binding factors, VCAM-1, α_4 integrin and fibronectin which all have distinct roles in the patterning of the vasculature during development (reviewed in Ref. [4]). In several diseases, vessels do not grow, but primarily remodel. For instance, skin vessels enlarge in psoriasis or vascular malformations, preexisting collaterals in the heart or peripheral limbs expand more than 20-fold after arterial occlusion, and pulmonary vessels become invested by smooth muscle cells during pulmonary hypertension (see below). For effective, targeted angiogenic therapy, insights into the complex means by which correct patterning can be effected in different tissues, will be invaluable.

3.7. Vein or artery?

Interestingly, the molecular signals determining which endothelial and peri-endothelial cells will belong to an artery, which to a vein, and which to a capillary, are largely unknown. Basic helix-loop-helix (bHLH) transcription factors often determine cell fate and differentiation, including that of endothelial cells [7]. Recently, the bHLH-transcription factor gridlock was identified to determine arterial specification [104]. A common angioblast may give rise to pre-venous and pre-arterial angioblasts — gridlock would favor differentiation of pre-arterial at the expense of pre-venous angioblasts (Fig. 2). *Notch* signaling is associated with cell fate determination via lateral specification and inductive signaling between distinct cell types. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephaly (CADASIL) is a late onset vascular disease, caused by a mutation in the *Notch3* gene. Recent evidence indicates that *Delta4*, a Notch ligand, is expressed in arterial endothelium [105] and that defective *Notch* signaling caused vascular remodeling defects [102,103]. The hairy-related bHLH factor HeyL, an effector of Notch, is expressed in smooth muscle of all arteries, overlapping with that of Notch3, mutations of which underlie the CADASIL vascular disorder [106].

There is mounting evidence to indicate that members of the large ephrin family may play a role. Ephrins are ligands for their corresponding Eph receptors, the latter forming a family of at least 14 receptor tyrosine kinases [31,66]. Ephrins must be membrane bound to activate their receptors. Ephrin A1 (or B61) and ephrin A2 are angiogenic, with specificity based on vascular bed [107]. Ephrins B1 and B2 induce sprouting. Ephrin B2 is restricted to arteries, whereas its receptor, EphB4 is found in veins — not arteries [108]. Inactivation of the *ephrin B2* gene in mice results in normal vasculogenesis but abnormal angiogenesis, the latter with disrupted remodeling of both arteries and veins into large and small branches, and diminished vessel maturation with decreased association of peri-endothelium [109,110]. Complex ligand–receptor bidirectional signalling interactions via ephrins and ephrin receptors have been described, with responses dependent on a variety of factors, including phosphorylation, multimerization, and the presence of adaptor proteins, such as Grb2, Grb10 and Nck. A novel cytoplasmic tyrosine kinase gene, bone marrow tyrosine kinase (Bmx), was identified in arterial endothelium [111]. Future studies will be required to unravel the differentiation characteristics of veins, arteries and capillaries [112].

3.8. Critical role of peri-endothelial cells and surrounding matrix

Although the endothelium has received the most attention in angiogenesis research, the surrounding peri-endothelial cell layers and extracellular matrix are critical for ongoing structural and functional support of the vascular network. Vascular smooth muscle cells stabilize nascent vessels by inhibiting endothelial proliferation and migration. Indeed, vessels regress more easily when not covered by smooth muscle cells in case angiogenic stimuli become

limiting [56,113]. Furthermore, peri-endothelial cells are metabolically active, and express a variety of vasoactive peptides, growth factors and cytokines that impact on the overall function of the vasculature. The extracellular matrix is also critical for normal vessel growth and maintenance, by providing not only a 'solid' scaffold through which new vessels may migrate, but to store and mobilize necessary growth factors, to mediate appropriate intercellular signals. Overall, an understanding of the means by which peri-endothelial cells are recruited to and encase the endothelial tubes, differentiate to serve a local function, and finally interface with the extracellular matrix and endothelium, is essential to ultimately designing angiogenic therapies.

The origin of smooth muscle cells is a subject of considerable research, with still many unanswered questions [114] (Fig. 2). Smooth muscle cells may differentiate from endothelial cells, from mesenchymal cells, or from bone marrow precursors or macrophages. Coronary vein smooth muscle cells are derived from the atrial myocardium, while those of the coronary arteries come from the epicardial layer [115]. Cardiac neural crest cells are the source of smooth muscle cells of the large thoracic blood vessels, a not infrequent site of congenital malformations [116]. Recently, a novel common vascular progenitor has been identified that gives rise to endothelial cells upon exposure to VEGF and to smooth muscle cells when treated with PDGF-BB [117]. Similarly to vascular smooth muscle cells, pericytes covering arterioles, venules and capillaries serve multiple functions, modulating blood flow and vascular permeability, regulating growth of blood vessels, and providing signals to endothelium and matrix via secreted and cellular molecules [118].

Recruitment of peri-endothelial cells is mediated by an array of local factors (Fig. 3). PDGF-BB is chemoattractant for smooth muscle cells [55], while VEGF, possibly via release of PDGF or binding to VEGF receptors, also contributes. Interactions between the endothelial cells of nascent vessels and the peri-endothelial cells are stabilized by Ang1 and Tie2, the latter of which also induces branching and remodeling [31,53,55]. There is strong clinical evidence of the importance of these receptor-ligand systems in vessel maturation. Dysfunction of Tie2 results in diminished smooth muscle cells and vascular malformations in humans. Members of the TGF-β family, including TGF-β1, TGF-β receptor-2, endoglin and Smad5, also work in concert to induce vessel maturation by stimulating smooth muscle cell differentiation and extracellular matrix deposition, while inhibiting endothelial proliferation and migration. Patients who lack endoglin have a prominent vascular bleeding disorder — hereditary hemorrhagic telangiectasia — characterized by vascular malformations [119]. Mice lacking endoglin die in utero of vascular abnormalities due to poor vascular smooth muscle cell development and arrested endothelial remodeling [120]. There are several other molecules that reportedly play a role in peri-endothelial cell recruitment and growth. For example, endothelin-1 is chemotactic for neural crest cells and transforms these into smooth muscle cells in the thoracic vasculature. Tissue factor promotes pericyte recruitment, possibly through initiation of coagulation and generation of thrombin. Ephrin-Eph ligand–receptor interactions may also transfer signals between endothelium and mesenchymal cells [31]. The basic helix-loop-helix transcription factor, dHAND, is required for normal reciprocal interaction between vascular mesenchymal cells and endothelial cells, as well as smooth muscle cell differentiation. Lack of dHAND in mice results in severe vascular abnormalities during development, with consequent lethality [121]. Finally, adhesion molecules such as N-cadherin, further provide more 'permanent' connections between endothelium and mesenchymal cells.

The extracellular matrix is critical for angiogenesis, and should be viewed as a dynamic player in the process. In addition to providing a site for storage of growth factors and pro-enzymes, such as the MMPs, for release and activation, its components serve as binding sites and targets for endothelial and mesenchymal cell-derived integrins and growth factors. For example, integrin $\alpha_v\beta_3$ that mediates attachment to collagen, is essential for vessel survival and maturation of blood vessels during angiogenesis, and inhibition of this interaction result in apoptosis of endothelium, vessel regression, and interference of the angiogenic effects of VEGF and TGFβ [122,123]. Other matrix components include fibronectin, laminin, vitronectin, osteopontin, fibrin, hyaluronic acid, and thrombospondin, each of which variably interacts with integrins and other growth factors, facilitating endothelial and peri-endothelial cell migration, tube formation and vascular network maturation.

3.9. Arteriogenesis

Once the peri-endothelial cells are recruited, they continue to migrate along sprouting vessels or pre-existing vasculature (Fig. 1). PDGF-BB plays an essential role in this process — at least in organs where peri-endothelial cells are not recruited from the local mesenchyme [56]. Although the cues regulating the spatial organization are not yet delineated, there are definite patterns of peri-endothelial cell migration as exemplified in the heart, where smooth muscle cell coverage proceeds in an epicardial to endocardial direction. Depending on the vessel type and site, additional smooth muscle cell layers are added, as the cells proliferate, differentiate and thereby acquire specialized functions, including contractility. In this respect, a number of receptors are expressed that respond to hormones, neuromodulators, and other effector molecules that are necessary to maintain appropriate tone and function under different physiological and pathological conditions. Interstitial matrix components, such as elastin and fibrillin-2, provide arteries with viscoelastic properties,

while collagen and fibrillin-1 add structural strength. Several defects in arteriogenesis have been described. Inactivation of the gene encoding the transcription factor MEF2C results in an embryonic lethal phenotype in which the severe vascular disorder is characterized by lack of differentiation of vascular smooth muscle cells [124]. Deficiency of fibrillin-1 in mice recapitulates the vascular defects of Marfan syndrome in humans, characterized by weakening and aneurysmal dilatation of the arteries [125]. Elastin both regulates smooth muscle proliferation and stabilizes arterial structure. Elastin deficiency leads to obstructive intimal hyperplasia with features similar to those found in atherosclerosis [126].

Inflammatory cells have been implicated in the growth of pre-existing collateral arterioles after occlusion of a supply artery in the myocardium and peripheral limbs [127,128] (Fig. 4). This process has been termed 'adaptive arteriogenesis' to distinguish the distinct cellular and molecular mechanisms from those in true angiogenesis (capillary growth). As a result of the increased collateral flow, endothelial cells express chemokines (MCP-1) and adhesion molecules (ICAM-1). The recruited monocytes infiltrate and proteolytically remodel the vessel wall [127]. Activated endothelial cells then upregulate bFGF, PDGF-B and TGF-β1, which stimulate smooth muscle cell growth and vessel enlargement. It is possible that flow provides the necessary survival signals for maintenance of collaterals. Adaptive arteriogenesis finally results in functional and structurally normal arteries, which ameliorate the detrimental effects of vessel obstruction [128]. These vessels may be superior to newly formed capillaries (formed by angiogenesis), because they are able to sustain proper circulation and to adapt to changes in physiological demands of blood supply. Therefore, we should critically consider whether therapeutic stimulation of new blood vessels in ischemic tissues should be aimed at improving angiogenesis or, perhaps preferably, arteriogenesis.

3.10. Oxygen levels regulate vessel growth and remodeling

The role of oxygen tension in regulating VEGF levels is critical to its function as an angiogenic factor. Accumulation of VEGF mRNA is induced by exposure to low oxygen levels both in vitro and in vivo [129]. Not surprisingly, many other genes directly or indirectly involved in angiogenesis, are also upregulated in response to hypoxia. These include, among others, the VEGF receptors VEGF-R1, VEGF-R2, neuropilin-1, neuropilin-2, Ang2, nitric oxide synthase, TGFβ1, PDGF-BB, endothelin-1 and IL-8 [130]. A transcriptional complex, composed of hypoxia inducible factors (HIF) — HIF-1α, HIF-1β (aryl hydrocarbon receptor nuclear translocator; ARNT) and HIF-2α — serve to augment expression of several of the genes involved in angiogenesis and cell survival. Some of those, including VEGF, erythropoietin and VEGFR-1 contain a consensus hypoxia responsive element (HRE) in the 5′ promoter region of the target genes with which HIF associates [131]. HIF-1α is itself induced by hypoxia, while the subunit HIF-1β is constitutively expressed. During normoxia, the product of the gene responsible for von Hippel Lindau disease, VHL, targets HIF-1α for rapid degradation [132]. During hypoxia, however, HIF-1α is stabilized [132]. Inactivation of the *HIF-1α* or *HIF-1β* genes in mice supports a role for hypoxia in regulating angiogenesis rather than vasculogenesis [133,134]. Of potential therapeutic impact is the finding that blood vessel formation is reduced in tumors that lack either HIF-1α or HIF-1β [135–137]. Further delineating the molecular regulation of VEGF and other angiogenic molecules via hypoxia inducible factor, hypoxia responsive elements, and in turn, the means by which these are functionally stabilized or degraded by, for example, VHL, will lead hopefully to more effective angiogenic and anti-angiogenic treatment approaches to disease.

3.11. Shear stress and vascular remodeling

Although oxygen tensions in different vascular beds have profound effects on vascular development and growth, other factors have also been implicated in regulating both VEGF expression and angiogenesis. There is considerable evidence to show that blood flow shear stresses and blood pressure affect vascular remodeling and the development of collateral circulation both under physiological and pathological conditions [138]. As with hypoxia, vasculogenesis appears to proceed unaffected by these factors. During development, smooth muscle cell covering of coronary vessels is spatio-temporally related to the generation of adequate capillary pressure proximal to the aorta, a process that may lead to the differentiation of arteries from veins. Shear stress has profound effects on the functional expression of many endothelial and smooth muscle cell proteins, including transcription factors such as c-Fos and Egr-1, enzymes such as angiotensin converting enzyme and nitric oxide synthase, growth factors including PDGF-A and B, and TGF-β, and finally several signaling molecules, integrins and adhesion molecules — an array of complex interactions that result in vascular remodelling under a variety of conditions. Further insights into the specific roles of many of these molecules have been provided by gene-targeting studies in mice [139,140].

4. Summary

The basic molecular mechanisms governing how endothelial cells, peri-endothelial cells and matrix molecules interact with each other and with numerous growth factors and receptors, to form blood vessels have been presented. The many insights gained from this basic knowledge are being extended to further understand pathological angio-

genesis associated with disorders such as arterial stenosis, myocardial ischemia, atherosclerosis, allograft transplant stenosis, wound healing and tissue repair. As a result, novel angiogenic and anti-angiogenic molecules are rapidly entering the clinic, with the promise of relief from a host of medical disorders.

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