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Development of the Endothelium

A. M. Suburo[1] · P. A. D'Amore[2] (✉)

[1]Facultad de Ciencias Biomédicas, Universidad Austral, B1629AHJ Buenos Aires, Argentina

[2]Schepens Eye Research Institute and Harvard Medical School, Boston MA, 02114, USA
pdamore@vision.eri.harvard.edu

Abstract Our understanding of the regulation of vascular development has exploded over the past decade. Prior to this time, our knowledge of vascular development was primarily based on classic descriptive studies. The identification of stem cells, lineage markers, specific growth factors and their receptors, and signalling pathways has facilitated a rapid expansion in information regarding details of the mechanisms that govern development of the vascular system.

Keywords Embryo · VEGF · Haemangioblasts · Endothelial determination and differentiation · Mural cells

1 Introduction

Endothelial cells (EC) derive from early precursors that proliferate and then coalesce to form complex vascular networks. During this developmental process, EC precursors receive appropriate developmental signals, inducing expression of specific genes and stimulating proliferation and migration. At the same time, EC are able to direct differentiation of neighbouring tissues, including cells that will form periendothelial vascular structures and the parenchyma served by the developing vessels. The result is a quiescent tissue, finely tuned to functional demands of nearby tissues. This review will describe fundamental steps of endothelial developmental processes as a pathway to the phenotypic diversity that is seen throughout the vascular system. In addition, we will review the anomalies of endothelial development and the possibility of reactivation of developmental processes under situations of stress and disease.

Differentiation of EC precursors is followed by formation of primitive endothelial tubes, and development and maturation of a vascular network. These processes involve changes in shape and adhesivity of EC and their precursors, sprouting and splitting of primitive vascular tubules, and remodelling of existing vessels plus their investment with mural cells-vascular smooth muscle cells (SMC) and pericytes.

Co-ordinated operation of numerous receptor-mediated signalling pathways and the activation of specific transcription factors are required for EC differentiation. Expression of receptors for vascular endothelial growth factor (VEGF)-A, which has been implicated in virtually all aspects of cardiovascular system formation, including heart development, haematopoiesis, vasculogenesis, angiogenesis and endothelial survival (Zachary 2003), is considered a hallmark of endothelial development. However, VEGF-A signals must be co-ordinated with many other intra- and extracellular messengers that contribute to the development of structurally and functionally mature blood vessels.

2
Early Endothelial Precursors

Vasculogenesis is the differentiation and coalescence of mesodermal precursor cells to form vessels, whereas angiogenesis involves the migration and division of EC from pre-existing vessels to form new vasculature. The existence of the haemangioblast, a common progenitor for endothelial and haematopoietic lineages, was first postulated at the beginning of the last century, and it was considered that separation of both lineages occurred in early stages of yolk sac development. Contemporary findings, however, indicate a more complicated differentiation pathway (summarised in Fig. 1).

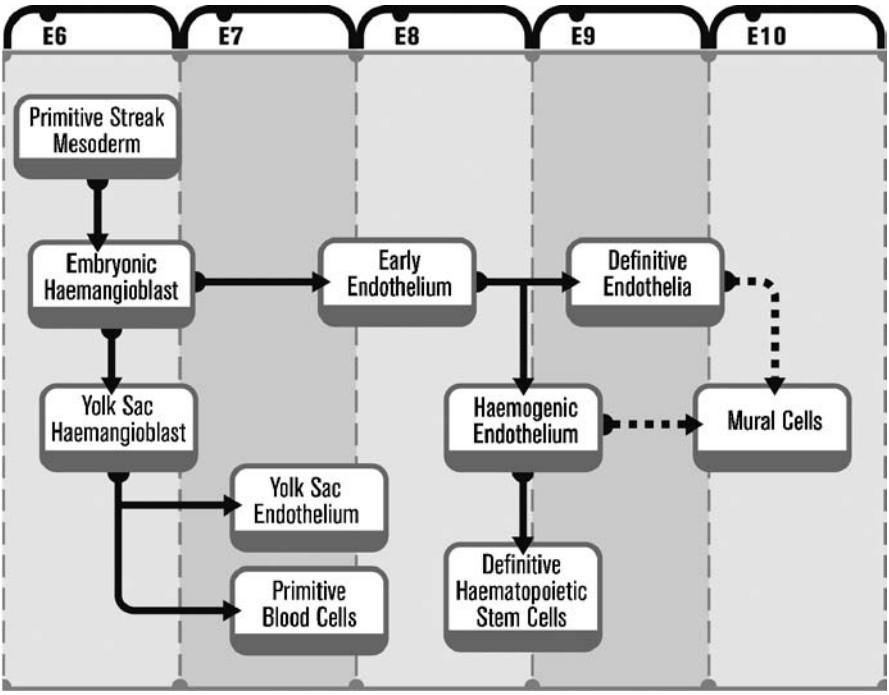

Fig. 1 Timetable of endothelial differentiation. In the mouse embryo, major steps of endothelial differentiation take place between embryonic day E6 and E10. Haemangioblasts differentiate within the mesoderm of the primitive streak and migrate to the yolk sac where they form blood islands that give rise to endothelium and primitive blood cells. Blood islands fuse to form the extraembryonic vessels. Within the embryo, endothelial precursors, presumably derived from similar haemangioblasts, differentiate to the endothelium of large intraembryonic vessels. Through angiogenesis, this early endothelium is the origin of the rest of the vasculature. Certain regions of the early endothelium are specialised into the haemogenic endothelium, which is the source of definitive haematopoietic cells. Some evidence suggests that endothelium and haematopoietic cells may be able to differentiate into mural cells

2.1
Haemangioblasts in the Yolk Sac

Haemangioblasts have recently been defined as a subpopulation of mesoderm cells that originate in the posterior region of the primitive streak. They co-express brachyury (also known as T) and VEGF-A receptor 2 (VEGFR-2; Flk1 in mouse and KDR in human) genes, and are first detected at the mid-streak stage of gastrulation (Huber et al. 2004). Thus, the earliest stages of haemangioblast differentiation probably occur before their migration to the extraembryonic mesoderm of the presumptive yolk sac (Fig. 2). Haemangioblasts aggregate in presumptive blood islands (also known as mesodermal cell masses or angioblastic cords) that appear in the extraembryonic mesoderm between mouse embryonic day (E)7 and E7.5. Cells at the outer aspect

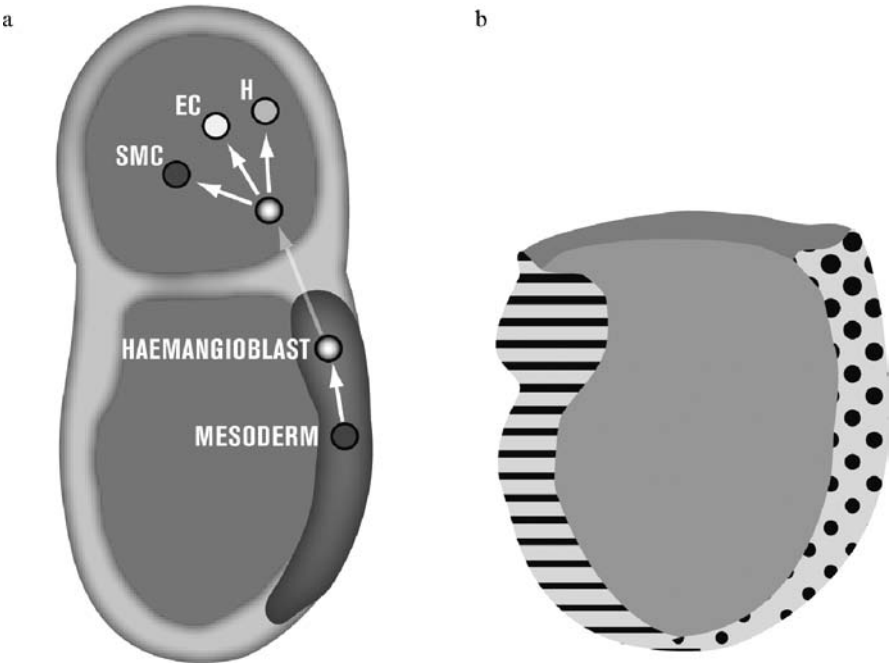

Fig. 2 **a** Schematic representation of a 7.0-day mouse embryo illustrating haemangioblast development and migration to the yolk sac. The haemangioblast is a Bry^+ and VEGFR-2^+ cell derived from mesodermal Bry^+ cells located in the region of the primitive streak (*black*). Haemangioblasts migrate onto the yolk sac where they differentiate into haematopoietic cells (H), EC and SMC. Adapted from Huber et al. (2004). **b** Representation of the spatial distribution of VEGF-A and VEGFR-2 transcripts in an E7.75 embryo transversely sectioned through the amnion. VEGF-A is present throughout the whole embryo, but is at higher levels in the cephalic region (*striped region*) where the neural plate is developing. Conversely, VEGFR-2 is also widely distributed but predominates caudally (*dots*) where EC precursors arise in the region of the primitive streak. (Adapted from Hiratsuka et al. 2005)

of the blood islands assume a spindle shape as they differentiate into EC, whereas inner cells progressively lose their intercellular attachments as they differentiate into primitive blood cells. Shortly thereafter, blood islands fuse to form the first endothelial tubes. A three-dimensional network, the primary vascular plexus, takes shape and then undergoes reorganisation, sprouting and remodelling to form the large vitelline vessels. Remodelling is accompanied by the recruitment and differentiation of vascular SMC (Drake and Fleming 2000).

At the three-somite stage, vascular development has spread throughout the yolk sac, but primitive red blood cells remain restricted to the blood islands of the proximal yolk sac, suggesting that there are haemangiogenic and angiogenic regions within the yolk sac (McGrath et al. 2003). On the other hand, the presence of cells giving rise to both endothelial and haematopoietic lineages in the allantois, placenta and somitic tissue (Alvarez-Silva et al. 2003; Finkelstein and Poole 2003), indicates that haemangioblasts could be far more extensively dispersed than previously thought.

2.2 Development of Primitive Intraembryonic Vessels

Vasculogenesis and angiogenesis are regulated by the capacity of EC and their precursors to adhere to each other and form new tubes. These cells can undergo dramatic changes in their shape, and their plasma membranes can engage in extensive protrusive activity with directionally oriented processes recognising and contacting neighbouring EC precursors to form cord-like cellular assemblies. At the same time, EC flatten and assume the spindle shape characteristic of differentiated EC. Tensional forces contribute to the creation of a single cell-layered vascular lumen. Continued vascular fusion can combine neighbouring small-calibre vessels into larger ones. The earliest intraembryonic endothelial populations appear in regions fated to give rise to the heart before vasculogenesis. The quantity of these cells increases dramatically before the aortic primordia first become discernible. Intraembryonic vasculogenesis is initiated in the cranial region of E7.3 embryos. Bilateral aortic primordia become discernible by E7.8 and their fusion is completed by E8.3. The lateral vascular networks are formed between E8.2 and E8.5. These early vascular channels develop before links with the vitelline vessels are established (Drake and Fleming 2000).

2.3 The Haemogenic Endothelium

Groups of 25–100 rounded cells, possessing the same ultrastructural features of primitive haematopoietic cells of the yolk sac blood islands (Tavian et al. 1996; Godin and Cumano 2002), are attached to the ventral luminal wall of

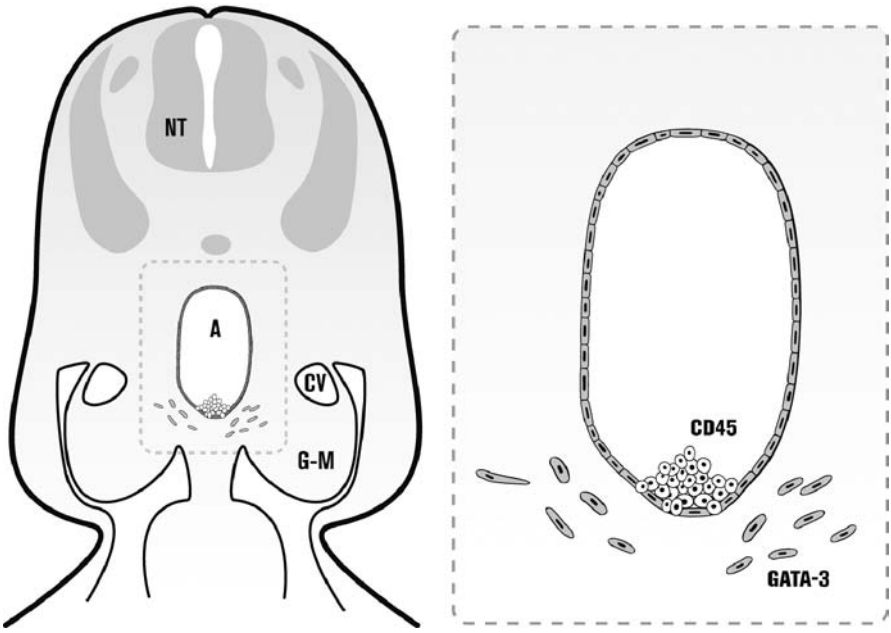

Fig. 3 Schematic representation of the embryo at the level of the truncal aorta-gonad-mesonephros (*AGM*). The area of haemogenic activity, including the aorta and subaortic patches, is outlined. *NT* is the neural tube and *CV* is the cardinal vein. The enlargement of the aortic region illustrates the intra-aortic clusters, which are restricted to the ventral part (floor) of the vessel and exhibit CD45. The subaortic patches are found bilaterally. (Based on Tavian et al. 1996)

the main arteries, aorta, omphalomesenteric and umbilical arteries (Fig. 3). These cells, which exhibit haematopoietic markers, are only observed during a brief stage in gestation (E9-11.5 in mice and ED30-40 in humans). This time period coincides with the one in which multipotent definitive haematopoietic stem cells can be isolated from the aorta-gonad-mesonephros (AGM) region, defined as the region of the murine embryonic splanchnopleuric mesoderm bounded by the dorsal aorta, gonadal ridge and pro/mesonephros. No intra-aortic clusters are visible outside the AGM in the post-umbilical caudal region of the embryo. Cytological features of the aortic floor, such as the presence of "bottled-shaped" cells and the absence of a basal membrane, suggest that cell migration can occur across this endothelium (Godin and Cumano 2002). A special group of mesenchymal cells, the subaortic patches, are located below the haematopoietic clusters, but their relationship with differentiation of the intra-aortic clusters has still to be clarified (Fraser et al. 2003).

3 Molecular Differentiation of EC

3.1 The Yolk Sac and Extraembryonic Vasculogenesis

Early haemangioblasts ($Bry^+/VEGFR\text{-}2^+$) apparently arise in the primitive streak region; however, the yolk sac probably provides them with a suitable environment inducing divergence of primitive EC and primitive blood cells. The yolk sac is composed of two cell layers, an extraembryonic mesodermal layer and a visceral endoderm layer. Members of the GATA family of transcription factors are important for mesodermal development. In mouse embryos, the loss of *GATA-1* leads to a qualitative defect in primitive erythroid cell differentiation, whereas the loss of *GATA-2* has a modest quantitative effect at the yolk sac (Fujiwara et al. 2004). In later stages, definitive haematopoietic stem cells are highly dependent on *GATA-2*, which is expressed in the aortic endothelium and neighbouring mesenchymal cells (Ling et al. 2004).

The haematopoietically expressed homeobox (Hex) gene is transiently expressed in the nascent blood islands of the visceral yolk sac and later in embryonic angioblasts and endocardium. Hex is required for the transition from the definitive haemangioblast to a definitive haematopoietic stem cell, and to a somewhat lesser extent, EC, since $Hex^{-/-}$ embryos can form some vessels before they die at day 12 (Guo et al. 2003). Other transcription factors, encoded by the stem cell leukaemia (SCL, also known as TAL-1) and LMO-2 genes, are essential for the development of both primitive erythropoiesis and definitive haematopoiesis. SCL is expressed in the presumptive yolk sac region in the mid/late streak stage of mouse embryos, coincident with VEGFR-2, and continues to be expressed in haemangioblasts, definitive haematopoietic stem cells, some haematopoietic lineages and, at lower levels, in EC precursors and some EC. Expression of SCL follows expression of VEGFR-2, and is not detected in $VEGFR\text{-}2^{-/-}$ embryos (Ema et al. 2003). $SCL^{-/-}$ mouse embryos contain no primitive or definitive haematopoietic cells in the yolk sac and die around E10.5 because of defective embryonic haematopoiesis. Although these embryos generate EC, suggesting that this transcription factor is only required for blood cell commitment, they also show defective remodelling of primary vascular networks (Gottgens et al. 2002).

Signalling from the endoderm is a critical early determinant of haematopoietic and vascular development. Indian hedgehog (Ihh) but not Sonic hedgehog (Shh) is expressed in the visceral endoderm of gastrulating mouse embryos and mature yolk sacs. Ihh alone is sufficient to activate embryonic haematopoiesis and vasculogenesis in epiblasts in the absence of visceral endoderm (Dyer et al. 2001), and $Ihh^{-/-}$ yolk sacs can form blood vessels, but they are fewer in number and smaller, perhaps owing to their inability to undergo vascular remodelling (Byrd et al. 2002).

VEGF-A signalling is pivotal for vascular differentiation because its inhibition prevents vascular development from its beginning and consistently inhibits tumour vascularisation. The VEGF ligand family includes VEGF-A, VEGF-B, placenta growth factor (PlGF), VEGF-C and VEGF-D. VEGF-A interacts with three tyrosine kinase receptors, VEGFR-1 (Flt1), VEGFR-2 and VEGFR-3 (Flt4). VEGF-A function is required for development of the yolk sac mesenchyme and recruitment of haematopoietic precursors to the yolk sac, expansion of the primitive erythroid compartment, survival of primitive erythrocytes, and angiogenic sprouting of blood vessels, but not for EC specification (Duan et al. 2003; Martin et al. 2004). The extraembryonic visceral endoderm and the yolk sac mesodermal sheet are the first tissues to express VEGF-A, and expression in the visceral endoderm seems to be necessary and sufficient for normal development of the yolk sac vasculature (Damert et al. 2002). In blood islands, outer EC are VEGFR-2^+, whereas "core" cells, representing the primitive haematopoietic lineage, are VEGFR-2^- (Drake and Fleming 2000) and $CD41^+$ (Ferkowicz et al. 2003). Embryos lacking VEGF-A or VEGFR-2 genes have few or no blood vessels (Shalaby et al. 1997). *VEGFR-*$2^{-/-}$ mice do not develop yolk sac blood islands or blood vessels, and die between E8.5 and E9.5, whereas *VEGFR-*$1^{-/-}$ die due to an overgrowth of vascular EC and disorganisation of blood vessels.

Transforming growth factor-β1 (TGF-β1)/bone morphogenetic protein (BMP) families of factors and their receptors are required for extraembryonic vasculogenesis. BMP4 is secreted by extraembryonic mesoderm at the posterior end of the primitive streak and, in *BMP4*-null mice that survive beyond gastrulation, both haematopoiesis and vasculogenesis are greatly reduced. BMP4 acts through activation of the Smad/5 downstream signalling molecules, and mice deficient in Smad1 or Smad5 also display defects in haematopoietic and vascular development (Tremblay et al. 2001). Deficiency of retinoic acid synthesis also generates embryos with multiple anomalies, including missing organised extraembryonic vessels in the yolk sac. Lack of retinoic acid leads to suppression of TGF-β1 and fibronectin production in EC and downregulation of VEGF-A, Ihh and fibroblast growth factor (FGF)-2 in visceral endoderm; these changes are correlated with enhanced EC growth, decreased visceral endoderm survival and lack of capillary plexus remodelling (Bohnsack et al. 2004).

3.2 Endothelial Differentiation in Embryoid Bodies

Under certain in vitro conditions, embryonic stem (ES) cells differentiate into embryoid bodies (EB) that contain precursors for multiple lineages. Differentiation of haematopoietic and endothelial lineages in this model parallels that of the normal mouse (Feraud et al. 2003). Thus, Bry^+ mesodermal progenitors can originate blast colony-forming cells (BL-CFCs) expressing VEGFR-2 and will grow blast colonies in response to VEGF-A (Faloon et al. 2000). Since

blast colonies contain both haematopoietic and EC precursors, BL-CFCs are postulated to represent the haemangioblast (Chung et al. 2002). In serum-free conditions, ES cells develop only to the mesodermal stage. BMP4 is required for the transition of ES cells to mesoderm, from mesoderm to VEGFR-2^+ cells and from VEGFR-2^+ to SCL^+ cells. VEGF-A then acts through VEGFR-2 to expand SCL^+ cells. TGF-β1 and activin A further modulate the expansion of haematopoietic and EC lineages (Park et al. 2004). In addition, BMP-binding endothelial cell precursor-derived regulator (BMPER) is specifically expressed in VEGFR-2^+ cells and directly interacts with BMP2, BMP4 and BMP6, and antagonises Smad5 activation, possibly modulating local BMP activity during EC differentiation (Moser et al. 2003).

BL-CFCs have provided a suitable model system to analyse the divergence of haematopoietic and EC lineages in vitro. Initially, a subset of VEGFR-2^+/GATA-1^+ mesodermal cells, representing the primitive erythroid lineage, loses the capacity to give rise to EC (Fujimoto et al. 2001). The remaining VEGFR-2^+/GATA-1^- cells express vascular endothelium (VE)-cadherin, the major component of endothelial adherens junctions. A subset of VE-$cadherin^+$ cells, giving rise to definitive haematopoietic progenitors and to EC, probably represents the "haemogenic" EC (Fujimoto et al. 2001). Primitive endothelial-like cells derived from human ES cells also express platelet endothelial cell adhesion molecule-1 (PECAM-1; CD31), but not CD45, and give rise to endothelial and haematopoietic lineages (Wang et al. 2004a). Wild-type EB give rise to BL-CFCs differentiating into endothelial and haematopoietic cells, but $SCL^{-/-}$ EB can only differentiate into EC (Faloon et al. 2000).

VEGF-A regulates cellular properties required for migration, including invasive activity, motility and adhesion/de-adhesion to matrix substrates. In cystic EB, VEGF-A expression is both temporally and spatially correlated with development of a vascular network. By contrast, EB derived from *VEGF-A*-null ES cells contain PECAM-1-positive EC that do not form tubes. Addition of VEGF-A partially rescues the formation of vascular networks in the VEGF-A-null EB, whereas addition of FGF-2 results in increased EC proliferation but does not rescue vascular morphogenesis (Ng et al. 2004).

3.3 Intraembryonic Differentiation of EC

Using mice embryos (E7.25-E7.75) in which the lacZ gene is driven under the control of the endogenous VEGFR-2 promoter, EC precursors can be traced as they migrate from the caudal to the cephalic region, where they are incorporated to the developing heart and aorta. EC precursors derived from wild-type or *VEGFR-*$2^{+/-}$ mice rapidly move in a cephalic direction, whereas cells derived from *VEGFR-*$2^{+/-}$ mice carrying a truncated VEGFR-1 migrate very little. Direction of migration is correlated with sites of VEGF-A synthesis, which is much higher in the cephalic than in the caudal region. VEGFR-1

and VEGFR-2 are mainly expressed caudally (Fig. 2b), where both receptors localise to the same cells. In vitro migration of embryo-derived VEGFR-2^+ cells is stimulated both by VEGF-A and PlGF, a specific ligand for VEGFR-1 (Hiratsuka et al. 2005).

PECAM-1 is expressed by early endothelial precursors, first within the yolk sac and then in aortic primordia at E7.8, whereas CD34, VE-cadherin, and Tie2 appear the next day. PECAM-1 expression is initially associated with the entire cell surface, but later becomes localised to sites of cell-cell contact (Drake and Fleming 2000). VE-cadherin promotes cell adhesion and is required for the assembly of the yolk sac primary plexus and remodelling of embryonic blood vessels (Bazzoni and Dejana 2004).

Cell clusters associated with the endothelial floor of the 5-week human embryonic aorta express, among other molecules, the transcription factors SCL, GATA-2, GATA-3 and Runx1 (Godin and Cumano 2002). The haemogenic endothelium expresses GATA-2, c-KIT, tenascin C, VWF, VEGFR-2, PECAM-1, CD34, endomucin, VEGFR-1, VEGFR-2, Flt3L, SCL, Tie2, VE-cadherin and VEGF-A (Godin and Cumano 2002). Embryonic cells selected by surface expression of CD34 or CD31 yield myelo-lymphoid cells in culture, thus supporting the haemogenic nature of intra-aortic clusters (Oberlin et al. 2002). A transient population of cells expressing both CD45 and VE-cadherin probably represents an intermediate stage between EC and blood cells (Fraser et al. 2003). VEGFR-2^+/CD34^- cells persist in the para-aortic splanchnopleura or subaortic patches until the disappearance of aorta-associated haematopoietic cell clusters, and it is speculated that these cells represent the intraembryonic haemangioblastic precursor of haematopoietic and endothelial lineages (Cortes et al. 1999).

The transcription factor Runx1 (also known as AML1 or CBFA2), a frequent target of chromosome translocations in acute myeloid leukaemia, is first detected in mesenchymal cells of the yolk sac at E7.5. Clusters of Runx1^+ cells, also expressing the pan-leucocyte marker CD45, can be detected inside the aorta, vitelline and umbilical arteries (Fraser et al. 2003). Although Runx1-null embryos show no dramatic defects in primitive erythropoiesis, they fail to generate definitive haematopoietic lineage cells. Main EC and haematopoietic differentiation markers are summarised in Fig. 4.

3.4 VEGF-A Transcription and Signalling in Differentiation of EC

Molecular responses to oxygen gradients contribute to the differentiation and maintenance of the cardiovascular system. Hypoxia-sensitive genes include erythropoietin, transferrin and its receptor, VEGF-A and its receptors, platelet-derived growth factor (PDGF)-B, FGF-2, and multiple genes encoding glycolytic enzymes (Ramirez-Bergeron et al. 2004). Hypoxia-inducible factor (HIF), consisting of HIF-1α (or HIF-2α) and aryl hydrocarbon receptor nuclear

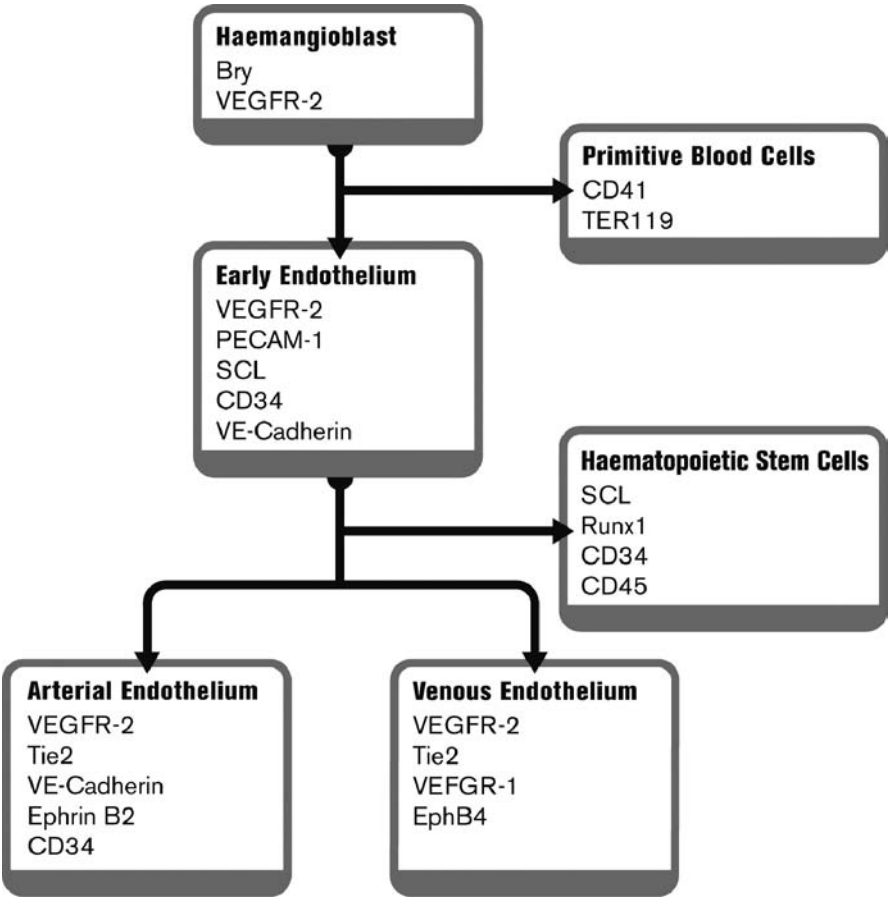

Fig. 4 Gene markers at different stages of endothelial and haematopoietic differentiation. Development of these lineages requires the concerted action of many genes, but those included in the chart have been shown to perform essential differentiation steps. Data were collected from several references included in the text

translocator (ARNT, also known as HIF-1β) subunits, activates multiple genes in response to oxygen deprivation. VEGF-A expression can be activated by HIF-1α or HIF-2α, but only the latter can activate expression of VEGFR-2 (Elvert et al. 2003). In differentiating ES cells, hypoxia accelerates the expression of Bry, BMP4 and VEGFR-2, and proliferation of BL-CFCs (Ramirez-Bergeron et al. 2004).

Other effectors, however, must be involved during early embryogenesis, since oxygen is distributed by diffusion and its levels seem to be almost the same throughout the embryo (Hiratsuka et al. 2005). Many transcriptional regulators have been associated with VEGF-A expression under pathological conditions, but few of them have been studied during embryonic development.

Ets transcription factors could be involved in the control of VEGF-A and other genes involved in angiogenesis, such as VEGFR-1, VEGFR-2, Tie1 and Tie2. Ets-1 is highly expressed in the lateral mesoderm when VEGFR-2 starts to be expressed in EC precursors, and HIF-2α co-operates with Ets-1 in activating transcription of this receptor (Elvert et al. 2003). ErbB2, one of the receptors for the family of epidermal growth factor (EGF) ligands, has also been implicated as a positive modulator of VEGF-A expression (Loureiro et al. 2005).

Most biologically relevant VEGF-A signalling in EC is mediated via VEGFR-2. Major pathways include survival signalling through phosphoinositide (PI)-3-kinase-dependent activation of the anti-apoptotic kinase Akt/protein kinase B (Zachary 2003). VEGFR-1 has a tenfold higher affinity for VEGF-A than VEGFR-2 but with a much weaker tyrosine kinase activity. VEGFR-1 is expressed as a full-length molecule in blood vessels and capillaries of developing organs, closely resembling the pattern of VEGFR-2 distribution, and as a soluble form that consists of the extracellular domain. Since VEGFR-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice (Hiratsuka et al. 2005), it has been suggested that VEGFR-1 may function as a "decoy" receptor to negatively regulate VEGFR-2-mediated actions. Such a role is supported by increased VEGFR-2 tyrosine phosphorylation in differentiated ES cell cultures lacking VEGFR-1 (Roberts et al. 2004).

4
Development of Mural Cells

Pericytes are the mural cells of capillaries and post-capillary venules, whereas SMC are associated with arteries, arterioles and veins. Mural cells contribute to the developing vascular wall through cell proliferation and production of extracellular matrix components such as collagen, elastin and proteoglycans. Most mural cells are of mesodermal origin, but unlike other tissues, a discrete population of mural cell precursors cannot be distinguished in the developing organism. SMC in the proximal aorta, aortic arch and pulmonary trunk are derived from neural crest, whereas SMC in the coronary arteries are derived from epicardium, and those in the descending aorta originate from mesoderm and possibly from transdifferentiated endothelium (Mann et al. 2004). Various clonal lines of multipotent, self-renewing cells called mesoangioblasts have been isolated from embryonic dorsal aorta (Minasi et al. 2002).

In vitro experiments suggest that EC or EC precursors may give rise to mural cells. Thus, VEGFR-2^+ cells derived from ES cells can differentiate into both endothelial and mural cells and can form capillary-like structures in vitro. The same cells can also incorporate into blood vessels as either EC or pericytes when injected into chick embryos (Yamashita et al. 2000). SMC are also produced from ES-derived BL-CFCs, and VEGFR-2^+ cells retain the capacity to form this phenotype after the time of haematopoietic cell formation (Ema et al. 2003).

The absence of mural cells during vascular development results in endothelial hyperplasia, abnormal EC shape, alteration of junctional proteins, increased capillary diameter vessel dilation and microaneurysms, abnormal vascular remodelling and increase of permeability. Affected embryos frequently die from embryonic or perinatal haemorrhage (Hellstrom et al. 2001; Uemura et al. 2002).

4.1 Regulation of Pericyte/SMC Phenotype

Understanding phenotypic regulation of SMC during development is particularly important, since changes of SMC associated with diseased vascular tissue partially recapitulate normal fetal and neonatal development. Different molecular transitions occur during SMC differentiation, leading to the development of the cytoskeleton, acquisition of contractile function and differentiation of arterial and venous SMC. Transcripts for α-smooth muscle actin (α-SMA) and SMα22, a calponin-related protein, are expressed in the developing dorsal aorta at E9.5, in the umbilical vessels and other cephalic vessels at E10.5, and in most vessels at E14.5. These genes, however, are also expressed in the early tubular heart, myotome and skeletal muscles. A more specific marker, smooth muscle-myosin heavy chain (SM-MHC), does not appear in the aorta until E10.5 (Li et al. 1996). In the retina, mural cell precursors express NG2 proteoglycan (or its human homologue, high molecular weight-melanoma associated antigen) and α-SMA, whereas mature pericytes express NG2 and desmin. Calponin and caldesmon, required for the contractile response, are markers of highly differentiated SMC (Hughes and Chan-Ling 2004). Diversity of gene products generated by alternative splicing can be enormous and is especially relevant for development of different muscle phenotypes, e.g. the expression of different smoothelin isoforms in vascular and visceral SMC (Rensen et al. 2002). Tissue-specific alternative splicing characterises the differentiated vascular SMC phenotype and is rapidly lost during vascular disease.

Little is known about the maturation of vascular SMC, but Notch3 (see Sect. 6.4) and angiotensin receptor 2 (AT2) may be involved. In fetal blood vessels, the AT2 receptor is expressed at late gestation but decreases to very low levels in the adult. Levels of the regulatory proteins calponin and caldesmon are below normal in the aorta of *AT2*$^{-/-}$ mice. Since AT2 is re-expressed in vascular injury, it may have a role in late vascular remodelling; however, this remains controversial (Perlegas et al. 2005).

Most SMC genes are under the control of the serum response factor (SRF) that binds to a *cis* element known as a CArG box. The SM-MHC gene includes three positive-acting CArG elements that are selectively required for the different SMC phenotypes. Mutation of an intronic CArG results in an arterial phenotype, with complete silencing of SM-MHC expression in the aorta, common carotid arteries and the main trunks of subclavian arteries (Manabe and

Owens 2001). Three CArG sites also present in the SMα22 promoter region appear to be involved in vascular SMC differentiation (Ding et al. 2004). Myocardin and related molecules MRTF-A and MRTF-B are SRF co-activators that are expressed in a subset of vascular and visceral SMC, usually preceding expression of SMC-specific genes. Interfering with myocardin expression results in embryonic death at E11.5 from a lack of vascular SMC. It has been proposed that the reversible association of myocardin with SRF could be the basis of the switch between muscle-specific and growth-regulated genes during embryological and pathological SMC differentiation (Wang and Olson 2004).

4.2 Differentiation of Pericytes and SMC

Mural cells are expanded and recruited to angiogenic sprouts by proliferation and migration (Beck and D'Amore 1997). Association of mural cells with newly formed blood vessels appears to regulate EC proliferation, survival, migration, differentiation and stability (Antonelli-Orlidge et al. 1989; Hirschi et al. 1999). Differentiation of mesenchymal cell precursors (10T1/2 cells) into pericytes is not only accompanied by the expression of α-SMA and NG2, but also by the induction of VEGF-A (Hirschi et al. 1998; Darland and D'Amore 2001a). Vascular development is conveniently studied in the retinas of mice, which are vascularised postnatally. In this model, a subset of pericytes was shown to express VEGF-A, further supporting the observation that contact-induced pericyte differentiation leads to a localised source of VEGF-A (Darland et al. 2003) and other growth factors (see Sects. 4.2.3 and 4.2.4). Pericytes as a source of a local survival factor may explain the regression of pericyte-deficient vessels, and the prevention of regression by the administration of VEGF-A. Conversely, pericytes suppress EC proliferation and migration in vitro (Orlidge and D'Amore 1987; Sato and Rifkin 1989), possibly explaining lesions observed in diabetic retinopathy (Hammes et al. 2002) and various mouse mutants (Hellstrom et al. 2001), where the loss of pericytes precedes retinal EC proliferation. These interactions between EC and mural cells are critical to mural cell differentiation and vessel remodelling, and reflect the collective activity of several signalling molecules, including those described in the following sections.

4.2.1 S1P Phosphate and S1P Receptors

Sphingosine-1 (S1P) is a lipid mediator derived from sphingomyelin that can signal through S1P receptors (S1P1-S1P5), a family of G protein-coupled receptors also known as endothelial differentiation genes (EDG). These receptors and sphingosine kinase are expressed in pre-vascularised embryonic tissues

and during vasculogenesis and angiogenesis (Allende et al. 2003). Exogenous S1P or sphingosine, but not VEGF-A or FGF-2, can replace the requirement for serum in promoting vasculogenesis in cultured allantois explants. In the absence of S1P, failure of the cells to move, coupled with the continued proliferation due to the mitogenic effects of VEGF-A, results in small vascular networks with abnormally high cell numbers (Argraves et al. 2004).

The receptor S1P1 is highly expressed in EC and developing SMC, whereas S1P2, is strongly expressed in adult SMC (Lockman et al. 2004). Mice lacking S1P1 die around E12.5-E14.5 from severe haemorrhage, and exhibit incomplete SMC ensheathment of dorsal aorta and large arteries. Endothelial-specific deletion of S1P1 leads to a similar phenotype, whereas deletion targeted to vascular SMC produces viable animals (Allende et al. 2003). Other receptors are probably involved, since S1P stimulates expression of multiple SMC differentiation markers in primary SMC cultures and in 10T1/2 cells, through the activation of an SRF co-factor (Lockman et al. 2004).

4.2.2 Wnts

Wnts are secreted glycoproteins that are likely to play an important role in normal and pathologic angiogenesis and in neointimal hyperplasia (Goodwin and D'Amore 2002). Three major Wnt signalling pathways have been identified: the canonical or β-catenin-dependent cascade, the Wnt/Ca^{++} pathway and the planar cell polarity (PCP) pathway that co-ordinates polarisation of cells within the plane of epithelial sheets (Huelsken and Behrens 2002).

EC and SMC in culture express components of the canonical pathway, including the Frizzled (Fzd) receptors Fzd-1, Fzd-2 and Fzd-3. The mouse gene Fzd5 is strongly expressed in the yolk sac after E9.5, and the placental blood vessels as late as E10.5. Fzd5 ligands, Wnt5a and Wnt10b, are also expressed in the early yolk sac. Homozygous Fzd5 knock-out mice are lethal, owing to defects in the yolk sac vasculogenesis. Wnt2 is also a Fzd5 ligand, and Wnt2-deficient embryos show placental defects suggesting its importance for vascular growth during later stages of development (Ishikawa et al. 2001).

Engagement of Fzd receptors results in recruitment of dishevelled (Dvl), which inhibits β-catenin phosphorylation. About 50% of Dvl2-deficient mice die perinatally due to severe cardiovascular outflow tract defects that have been related to alterations of neural crest (Hamblet et al. 2002). Dvl2, which mediates both the canonical and PCP pathways, has recently been detected in the cytoplasm of cultured EC (Wechezak and Coan 2005). Secreted Fzd-related proteins (FRP) compete with Fzd receptors for Wnt binding. The secreted Frizzled FrzA (or sFRP-1) promotes EC migration and organization into capillary-like structures (Ezan et al. 2004), probably explaining the reduction in size of experimental infarct in mice overexpressing this protein (Barandon et al. 2003). In vitro experiments suggest that Wnt-1 is also co-localised with β-catenin

in adherens junctions, probably accounting for the enhanced adhesiveness of transfected EC (Wechezak and Coan 2003).

4.2.3 Platelet-Derived Growth Factors Family

The PDGF family of growth factors is composed of four different polypeptide chains: PDGF-A, PDGF-B, PDGF-C and PDGF-D, which form five dimeric ligands. PDGF-B is secreted by vascular endothelium, PDGF-C by vascular SMC and PDGF-D by adventitial fibroblasts, whereas the receptor PDGFR-β is present in vascular mural cells (Hoch and Soriano 2003). Endothelial expression of PDGF-B occurs during vascular development and is downregulated in quiescent EC. Thus, as development progresses, PDGF-B expression becomes restricted to short capillary segments probably representing angiogenic sprouts. PDGFR-β is expressed by developing pericytes and SMC of arteries/arterioles (Hellstrom et al. 2001).

The ability of EC from different sources to recruit presumptive mural cell precursors is blocked by a neutralising antibody to PDGF-B (Hirschi et al. 1998), indicating that this ligand is a chemotactic, and perhaps survival, signal for PDGFR-β-expressing pericyte/SMC progenitors. Mice lacking PDGF-B or PDGFR-β die perinatally with extensive haemorrhaging, as a result of absence of microvascular pericytes and subsequent microaneurysm formation and capillary rupture (Hoch and Soriano 2003).

Deletion of the extracellular retention motif of PDGF-B by gene targeting in mice results in defective pericyte investment in the microvasculature and delayed formation of the renal glomerulus mesangium. In these mutants, pericytes appear partially detached and with processes directed away from the vessels, suggesting that extracellular retention of PDGF-B may act to restrict pericyte migration to the abluminal surface of microvessels (Lindblom et al. 2003).

4.2.4 Angiopoietins and the Tie Receptors

The two endothelial-specific receptors, Tie1 and Tie2 (tyrosine kinase receptors with immunoglobulin and EGF homology domains), are expressed in the vascular system from the earliest embryonic stages and remain endothelial-specific throughout adult life (Thurston 2003). Angiopoietins (Ang-1 to -4) are the ligands for the Tie2 receptor, but the identity of the Tie1 ligand(s) remains unknown. Ang-1 is expressed by perivascular cells during development and in adult tissues. Ang-1 and -4 stimulate Tie2, whereas Ang-2 and -3 block Ang-1-induced tyrosine phosphorylation of Tie2.

4.2.4.1 Angiopoietin-1 and Tie2

Ang-1 consists of four alternatively spliced isoforms. The 1.5-kb isoform is the activating ligand of Tie-2, whereas the smaller isoforms probably represent dominant-negative regulatory molecules. Both *Ang-1* and *Tie2* knock-out mice exhibit reduced embryonic pericyte/SMC formation and die with cardiac failure and haemorrhage. Initial phases of blood vessel formation occur normally, but there is no remodelling, and vascular networks exhibit no hierarchical organisation (Thurston 2003). Intravitreal Ang-1 injections to newborn mice slightly accelerate the rate of vascular development and partially restore defects induced in neonatal retinal vasculature by depletion of mural cells (Uemura et al. 2002).

Endothelial loss of Tie2 expression correlates with EC apoptosis in haemorrhagic regions of the embryo (Jones et al. 2001), probably reflecting the inactivation of the Akt survival pathway. Akt effects are mediated through members of the FOXO subclass of forkhead transcription factors. Deletion of FOXO1 (but not that of FOXO3a or 4) causes embryonic death on E10.5 because of incomplete vascular development (Hosaka et al. 2004). Since FOXO1 regulates EC apoptosis as well as many genes associated with vascular destabilisation and remodelling (including Ang-2), Ang-1 blockade of the FOXO1 cascade promotes vessel stability (Daly et al. 2004).

Some familial forms of venous malformations, characterised by the formation of low-resistance vessels with insufficient SMC investment, have been associated with point mutations in the kinase domain of Tie2. The means by which Tie2 mutation leads to these abnormal vessels is unclear (Morris et al. 2005).

4.2.4.2 Angiopoietin-2

Ang-2, produced by EC and stored in Weibel-Palade granules, binds Tie2 but does not transduce a signal (Fiedler et al. 2004). Ang-2 controls EC quiescence and responsiveness, probably by inhibition of Ang-1-mediated Tie2 activation. Ang-2 is not essential for embryonic vascular development, but it is required for subsequent postnatal vascular remodelling. Newborn pups lacking Ang-2 have the beginnings of a normal eye vasculature, with well-formed hyaloid vessels. However, the hyaloid vasculature does not regress and the peripheral retina remains avascular; this defect is not rescued by expression of Ang-1 (Gale et al. 2002). Ang-2-null mice also exhibit defects in their lymphatic vasculature, which can be rescued by Ang-1. Mice overexpressing Ang-2 display vascular anomalies similar to mice lacking Ang-1 (Thurston 2003). Availability of VEGF-A appears to switch Ang-2 functions from anti- to pro-angiogenic. In the pupillary membrane, Ang-2, in the presence of VEGF-A, promotes a rapid

increase in capillary diameter, remodelling of the basal lamina and sprouting of new blood vessels. By contrast, Ang-2, in the absence of VEGF-A, promotes EC death and vessel regression (Lobov et al. 2002).

4.2.4.3 Tie1

Mice deficient in Tie1 die between E13.5 and E18.5, depending on the genetic background. These embryos show signs of oedema, local haemorrhage and microvessel rupture, but the major blood vessels appear intact (Thurston 2003). Tie1 and Tie2 are also expressed in haematopoietic cells and they are specifically required during postnatal bone marrow haematopoiesis (Puri and Bernstein 2003).

4.2.5 Transforming Growth Factor-β1

Signalling by TGF-β1 family members occurs through a receptor complex formed by two type I (also termed activin-receptor-like kinases, ALKs) and two type II transmembrane serine/threonine kinases. In most cells, TGF-β1 signals via a type II receptor and ALK5 to induce Smad2 and Smad3 phosphorylation, whereas in EC, TGF-β1 also activates an ALK1-promoting Smad1/5 phosphorylation. Smad3 can be proangiogenic through stimulation of VEGF-A expression, whereas Smad2 can be antiangiogenic via thrombospondin-1 (TSP-1) expression (Nakagawa et al. 2004). Thus, EC regulation of the various TGF-β1 intracellular cascades remains to be elucidated. Effects of members of the TGF-β1 superfamily are mediated through a consensus TGF-β1-controlling element (TCE), which is common to regulatory regions of SMC-marker genes. TCE-binding factors act as potent repressors of SMC differentiation marker genes (Ding et al. 2004).

Mice lacking TGF-β1 show defects in the yolk sac vasculature, including decreased vessel wall integrity, reduced contact between EC and mesenchymal cells, and incomplete maturation of SMC. The yolk sac vessels are large and leaky with abnormal endothelial adhesion. Mice lacking the TGF-β1 type II receptor exhibit a similar vascular phenotype, with additional abnormalities in other organ systems (Oshima et al. 1996). Conversely, diverse cell types, including 10T1/2, a line of multipotent mesenchymal cells, murine ES cells and rat neural crest stem cells, differentiate into SMC upon TGF-β1 treatment (Mann et al. 2004). TGF-β1 is also involved in the inhibition of EC growth induced by pericytes and SMC (Antonelli-Orlidge et al. 1989) and cord formation in EC and 10T1/2 co-cultures (Darland and D'Amore 2001b). EC, SMC and 10T1/2 secrete latent TGF-β1 that is locally activated upon contact between the EC and either SMC or 10T1/2 cells (Antonelli-Orlidge et al. 1989; Hirschi et al. 1998). 10T1/2 cells engineered to form defective gap junctions cannot

activate endogenous TGF-β1 but can respond to exogenous TGF-β1 (Hirschi et al. 2003). Other members of the TGF-β1 family might also be involved in the control of the SMC phenotype; however, their role during embryonic vascular development has yet to be studied.

4.2.6 Interactions Between Signalling Cascades

Complex interactions exist between PDGF-B, Ang-1 and TGF-β1 (Fig. 5). In mural cell precursors, PDGF-B upregulates Ang-1 and TGF-β1 expression, via the PI3-kinase and PKC pathways for Ang-1 and the MAPK/ERK pathway for TGF-β1. In addition, TGF-β1 partially inhibits endogenous Ang-1 expression and completely blocks expression induced by PDGF-B. In EC, either Ang-1 or TGF-β1 alone marginally downregulates PDGF-B expression, but a combination of these factors produces a much stronger downregulation (Nishishita and Lin 2004).

S1P and PDGF-B seem to co-ordinate EC-mural cell interactions required for development and stability of the vessel wall. In vitro, S1P potently stimulates PDGF-A and -B chain messenger RNA (mRNA) and protein expression in vascular SMC (Usui et al. 2004). On the other hand, PDGF-B acts on SMC to stimulate S1P release, resulting in stimulation of cell migration via activation of muscular S1P receptors in an autocrine/paracrine fashion (Hobson et al. 2001). More recent evidence suggests that PDGFR-β integrates a pre-formed complex with the S1P1 receptor that, upon PDGF stimulation, is internalised through endocytic vesicles and activates a MAPK cascade (Waters et al. 2005).

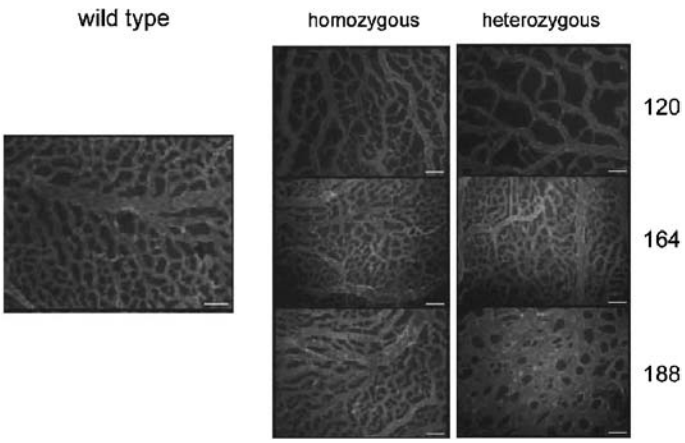

Fig. 5 Yolk sac vasculature of E10.5 mice that express single VEGF-A isoforms. Shown are yolk sacs isolated from embryos of wild-type mice that express all three VEGF-A isoforms and mice that express VEGF120 alone, VEGF164 alone or VEGF188 alone. Yolk sacs were stained with anti-PECAM antisera to visualise the vasculature

Pericyte growth and differentiation are differentially regulated by antagonistic signalling cascades involving FGF-2 and TGF-β1. FGF-2 markedly stimulates pericyte growth, whereas its removal and/or the addition of TGF-β1 causes the withdrawal of pericytes from the growth cycle and the induction of a contractile phenotype (Papetti et al. 2003).

5
Endothelium Morphogenesis

5.1
Angiogenic Sprouting

Angiogenic sprouting involves specialised endothelial tip cells that respond to chemoattractant and repellent guidance cues. Tip cells display long filopodia that sense extracellular VEGF-A gradients through VEGFR-2. Whereas tip cells do not proliferate, activation of VEGFR-2 is interpreted differently by sprout stalk cells, which are induced to proliferate (Gerhardt et al. 2003).

Different VEGF-A protein isoforms, VEGF120, VEGF164 and VEGF188, have a different affinity for heparan sulphate proteoglycans (HSPG) and heparin (Ng et al. 2001). This is the basis for the selective spatial distribution of VEGF-A, a primary mechanism controlling directed EC migration and the vas-

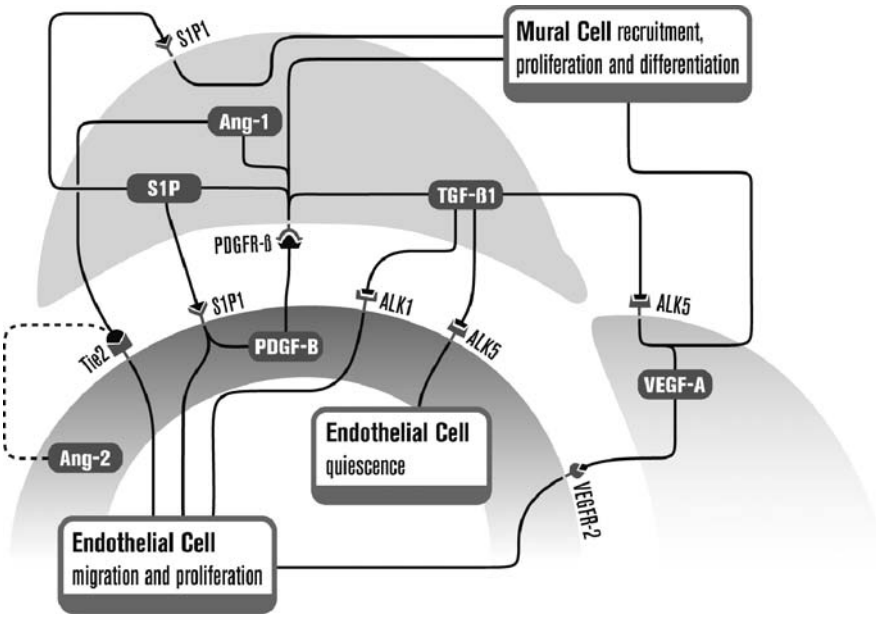

Fig. 6 Factors involved in assembly and remodelling of the vessel wall

cular pattern (Fig. 6). HSPG-binding properties have also been demonstrated for a wide range of growth factors, including members of the FGF, TGF-β1, EGF, insulin-like growth factor (IGF), PDGF-B, Wnt families and many other chemokines and cytokines (Iozzo and San Antonio 2001).

5.2 Attraction and Repulsion of Angiogenic Sprouts

5.2.1 Patterning of the Embryonic Midline

Vessel formation takes place throughout the embryonic disc, with the exception of the midline region surrounding the notochord, where no vessels grow during the early stages of development. This vascular exclusion zone is not determined by a lack of endothelial growth factors, but by notochordal production of the BMP antagonists Chordin and Noggin, which provide strong inhibitory cues (Reese et al. 2004). The neural tube, a localised source of VEGF-A, plays a role in patterning the midline vasculature, since it recruits somite precursors that develop into the perineural vascular plexus surrounding the developing brain and spinal cord. Sprouts from this plexus do not invade the neural tissue until later in development, suggesting that negative or repulsive cues also originate from the neural tube (Hogan et al. 2004).

5.2.2 Semaphorins

Neuropilin 1 (NRP-1) and NRP-2 are related transmembrane receptors that respond to two different extracellular ligands, class 3 semaphorins (SEMA3) and VEGF164, which are competitive inhibitors of one another in binding and in EC motility assays. Transgenic mice lacking both NRP-1 and NRP-2 die in utero at E8.5 with avascular yolk sacs. *NRP-1*-null mice die between E11 and E14 with cardiovascular and neuronal defects, whereas many *NRP-2*-deficient mice survive to adulthood but show lymphatic and neurologic defects. Cardiovascular defects in *NRP-1*-null mice include transposition of great vessels and persistent aorticopulmonary truncus (Takashima et al. 2002). *NRP-1*-deficient mice exhibit a defect in tip cell guidance that leads to paucity of sprouting, which in the presence of EC proliferation results in development of aneurysmatic malformations (Gerhardt et al. 2004). A knock-in mouse expressing the variant NRP-1^{Sema-}, unreactive to semaphorin but retaining VEGF-A 165 responses, survives until birth and has normal cardiac outflow tracts, indicating that semaphorin-NRP-1 signalling is not critical for embryonic viability (Gu et al. 2003).

Semaphorins induce the association of NRPs with transmembrane proteins of the plexin family such as plexinD1, which is expressed by most embryonic

and adult vascular EC. PlexinD1-null embryos show severe defects of the cardiac outflow tract and a deficiency of differentiated SMC in the developing 4th and 6th aortic arch arteries (Gitler et al. 2004). SEMA3E can bind directly to plexinD1 without intervention of a neuropilin. This property is not shared by any of the other known SEMA3. In E10.5-E11.5 mouse embryos, SEMA3E expression is localised to the somites, where it acts as a repulsive cue for plexinD1-expressing EC of adjacent intersomitic vessels (Gu et al. 2005). SEMA3A signalling inhibits integrin-mediated adhesion to the ECM, and no vascular remodelling is found in *SEMA3A*$^{-/-}$ embryos (Serini et al. 2003).

5.2.3 Netrins and Their Receptors

Netrins are guidance molecules related to laminin. Two families of netrin receptors are known, the deleted in colorectal cancer (DCC18) and UNC-5 families. DCC18 receptors mediate attraction, while UNC-5 mediates repulsion (Mehlen and Mazelin 2003). Receptor UNC-5B, selectively localised to arterial EC and endothelial tips, controls filopodial activity. UNC-5B mutant embryos develop a normal vascular plexus, but remodelling produces 40% more branching points than in wild-type embryos. Mutants die around E12.5 with heart failure probably resulting from increased peripheral resistance. Increased branching is associated with a larger number of tip filopodial extensions, and reflects the lack of UNC-5B negative regulation by netrin-1 stimulation. Intravitreal injection of netrin-1 during retinal angiogenesis leads to a marked decrease in filopodial extension (Lu et al. 2004).

5.2.4 Calcineurin/NFAT

Calcineurin, a protein phosphatase that is downstream of VEGFR-2, activates the nuclear factor of activated T cells (NFATc1-c4). This pathway leads to the transcriptional activation of various proangiogenic genes and can be counterbalanced by upregulation of the Down syndrome critical region 1 (DSCR-1) gene, a calcineurin inhibitor with antiangiogenic properties (Yao and Duh 2004). Signals transduced by Ca^{2+}, calcineurin, and NFATc3/c4 promote the proper anatomical patterning of the developing vascular system, as shown by disorganised vascular growth in mice doubly mutant for the NFATc3 and c4 genes. In these mutants, intersomitic vessels ignore somitic or neural boundaries, suggesting that NFAT signalling normally prevents abnormal growth of vessels into these tissues (Graef et al. 2001). EC show a low degree of NFATc4 expression, but perivascular mesenchyme typically expresses high levels of NFATc4, reflecting its importance for recruitment of pericytes and SMC. Calcineurin and NFATc1 direct neural crest stem cells to a SMC fate, whereas

DSCR-1 decreases SMC differentiation. DSCR-1 and NFATc1 are upregulated in response to TGF-β1, and expression of either calcineurin or NFATc1 mimics the effects of TGF-β1 on neural crest stem cells, suggesting that TGF-β1-dependent differentiation of SMC is mediated by calcineurin signalling (Mann et al. 2004).

6
Development of Arteries and Veins

Developmental remodelling includes structural and functional differentiation of arteries and veins, and establishment of an organ-specific microvascular network. Circulatory dynamics were thought to play a major role in establishing these differences; however, it has been demonstrated recently that the identities of arterial and venous endothelium are defined early in development, even before the start of circulation (Wang et al. 1998). Ephrins and their receptors, Eph, seem to be the earliest markers of arteriovenous differences, except for the recent description of the apelin (APJ) receptor as an even earlier marker for developing retinal veins (Saint-Geniez et al. 2003).

6.1
Ephrins and Eph Receptors

Eph, receptor tyrosine kinases that are typically activated by ligands anchored to the membrane of adjacent cells, regulate cellular adhesion, migration or chemorepulsion, and tissue/cell boundary formation. Reverse signalling, downstream of membrane-anchored ephrin ligands, can also occur. In all vertebrates, ephrin-B2 is expressed in arterial EC, while its receptor, EphB4, is expressed predominantly in venous EC. Ephrin-B2 also appears in perivascular mesenchyme and developing mural cells (Wang et al. 1998). Ephrin-B1 is co-expressed with ephrin-B2 in EC, whereas EphB3 and ephrin-B3 are co-expressed with EphB4 in venous EC. In the adult vasculature, expression of ephrin-B2 and EphB4 extends into the smallest-diameter capillaries, suggesting that they can also have arterial and venous identity (Shin et al. 2001). Eph-ephrin signalling is the basis for endothelial propulsive and repulsive activities that mediate EC guidance signals during angiogenesis, as well as the positional control of EphB receptor- and ephrin-B ligand-expressing cells towards each other. Forward EphB4 signals may direct EC in a repulsive manner avoiding areas where ephrin-B2 is expressed, whereas promotion of EC migration may occur if ephrin-B2-expressing EC are activated by EphB4. These propulsive and repulsive activities may also segregate EC from each other to limit cellular intermingling and control arterio-venous positioning of cells (Fuller et al. 2003).

Ephrin-B2 and EphB4 are also involved in mural cell development. Stromal cells expressing ephrin-B2 support the proliferation of ephrin-B2$^+$ EC, suppress

the proliferation of ephrin-B2 EC, promote vascular network formation and induce the recruitment and proliferation of α-SMA$^+$ cells. Conversely, stromal cells expressing EphB4 inhibit vascular network formation, ephrin-B2$^+$ EC proliferation and α-SMA cell recruitment and proliferation (Zhang et al. 2001).

Targeted disruption of either ephrin-B2 or EphB4 results in embryonic lethality at E11 and E9.5-10, respectively, due to defects in angiogenic remodelling of arteries and veins, and alterations of myocardial trabeculation. Early vasculogenesis is also abnormal, since EphB4-deficient EB display delayed expression of VEGFR-2 (Wang et al. 2004b). The initial commitment of ephrin-B2$^+$ or EphB4$^+$ EC could be the trigger for determining the arterial or venous fate of developing vessels. However, determination of arterial or venous fates probably requires the action of other upstream signals (see Sects. 6.2 and 6.5).

6.2 Hedgehog in Arteriogenesis

Hh proteins act as morphogens in many tissues during embryonic development. Signalling requires the interaction of Hh protein with its receptor, Patched-1 (Ptc1), leading to activation of a transcription factor, Gli, that induces expression of downstream target genes including Ptc and Gli themselves. Zebrafish embryos lacking Shh activity fail to express ephrin-B2a within their blood vessels, and a similar failure occurs in embryos lacking VEGF-A or Notch. In these embryos, ectopically expressed Shh induces ectopic formation of ephrin-B2-expressing vessels (Lawson and Weinstein 2002). A determinant role for Hh proteins in arteriogenesis of higher vertebrates has not been as clearly demonstrated as in Zebrafish. However, in the murine corneal angiogenesis assay, Shh produces large, branching vessels, whereas VEGF-A results in capillaries of lesser lumenal calibre. Moreover, Shh is involved in arteriogenesis during revascularisation of adult ischaemic tissues (Pola et al. 2003).

6.3 VEGF-A in Arteriogenesis

The association of peripheral nerves and expression of arterial markers during development has led to the suggestion that neurally derived VEGF-A directs arteriogenesis. Nerves express VEGF-A at a higher level than surrounding mesenchymal tissue. Moreover, expression of ephrin-B2 can be induced in embryonic EC by incubation with VEGF-A or co-culture with neurons or Schwann cells. In these experiments, only 50% of EC cultures express the arterial ephrin, suggesting that VEGF-A could represent a permissive inducing signal rather than an instructive determinant of arterial identity (Mukouyama et al. 2002). Since major receptors for VEGF-A are expressed on all EC, the arteriogenic effect of this factor has been ascribed to the co-receptor NRP-1 that

is preferentially expressed on arteries, whereas NRP-2 tends to be expressed in veins/lymphatic vessels (Yuan et al. 2002).

Defective vascular development in mice expressing single VEGF-A isoforms illustrates the complexity of VEGF-A signalling in arterial specification. In the early developing retina, prior to mural cell differentiation, the arterial marker ephrin-B2 is detected in about 50% of the retinal vessels, and NRP-1 shows a similar distribution, being localised in retinal arterioles with very low expression in retinal venules. Arteries and veins develop normally in $VEGF^{164/164}$ mice, but severe arterial defects accompanied by relatively normal veins and capillaries appear in $VEGF^{188/188}$ mice. $VEGF^{120/120}$ mice show severe retinal vascular defects, but 50% of early retinal vessels express ephrin-B2, suggesting unimpaired arterial specification. After remodelling, however, arterial development appears to lag behind venous development, suggesting that expression of NRP-1 is not the only mechanism driving the arterial specificity of the VEGF-A-response (Stalmans et al. 2002).

6.4 Notch Pathways

Notch receptor-ligand interaction results in proteolytic cleavage of the Notch receptor, producing a C-terminal intracellular fragment (NotchIC) that translocates to the nucleus. NotchIC binds to a transcriptional repressor, derepressing or co-activating the expression of various lineage-specific genes. Since the Notch cascade has a role in determining cell identities, it is probably involved in the events distinguishing EC from mural cells, artery from vein, pulmonary from systemic vessels, and large vessels from capillaries (Iso et al. 2003).

Several Notch pathway ligands and receptors are selectively localised in EC and their supporting cells. $Notch1^{-/-}$ and $Notch1^{+/-}/Notch4^{-/-}$ embryos arrest early in development with severe defects in the yolk sac and embryonic vessels. The primary vascular plexus develops normally, but both small capillaries and large vitelline collecting vessels fail to form, and embryonic large blood vessels are severely malformed (Krebs et al. 2000). Constitutive activation of Notch4 causes defects in vascular remodelling, whereas mice deficient in Jagged1, one of the Notch ligands, die from haemorrhage early during embryogenesis (Uyttendaele et al. 2001; Leong et al. 2002). Notch4 activation in EC promotes mesenchymal transformation, evidenced by downregulation of EC-specific proteins such as VE-cadherin, and upregulation of mesenchymal proteins, such as α-SMA, fibronectin and PDGFR-β (Noseda et al. 2004).

The Notch ligands, Jagged1, Jagged 2 and Dll4, as well as the receptors Notch1, Notch3 and Notch4, are selectively expressed in arteries. Notch1 and Notch4 are expressed in EC, whereas Notch3 is localised specifically to SMC (Villa et al. 2001). Heterozygous deletion of Dll4 results in absence of well-defined arterial vessels, including the internal carotid artery, although a rela-

tively normal venous plexus is present. SMC coverage of large arterial vessels is often lacking or markedly deficient (Gale et al. 2004). In *Dll4*$^{-/-}$ mice embryos, EC do not express the arterial markers ephrin-B2, connexin37 and connexin40 (Duarte et al. 2004).

Effectors of the Notch cascade are also involved in arterial differentiation. Loss of RBP-J (mammalian suppressor of hairless), one of the primary transcriptional mediators, results in the production of arteriovenous malformations (AVM), including fusion of the dorsal aorta with the common cardinal vein (Krebs et al. 2004). *Hey1* and *Hey2*, two other targets of Notch signalling, are preferentially expressed in embryonic arteries. *Hey1*$^{-/-}$/*Hey2*$^{-/-}$ mice display a phenotype resembling that produced by Notch1 deficiency, including defects in yolk sac vascular remodelling and lack of the arterial markers CD44, neuropilin1 and ephrin-B2 (Fischer et al. 2004). In Zebrafish, Notch-induced arterial differentiation is downstream of VEGF-A signalling (Lawson and Weinstein 2002). This is likely to be the case in mammals, since in vitro VEGF-A stimulation upregulates Notch1 and Dll4 transcription (Liu et al. 2003).

In humans, mutations of the ligand Jagged 1 are associated with Alagille syndrome, a developmental disorder that includes vascular defects (Gridley 2003). Cerebral cavernous malformation (CCM), a vascular malformation characterised by thin-walled vascular cavities that haemorrhage, has been linked to loss-of-function mutations in a locus termed CCM1. *CCM1*$^{-/-}$ mouse embryos exhibit progressive dilatation of cephalic vessels, with marked enlargement of the aorta and branchial arch arteries, downregulation of Dll4 and Notch4, and lack of ephrin-B2 expression and SMC recruitment in arteries. Consistent with the murine data, Notch4 is not detected in human cavernous lesions, and is markedly reduced in brain arteries adjacent to the vascular malformations (Whitehead et al. 2004).

Missense mutations in Notch3 have been implicated in a neurovascular disorder known as cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), an arteriopathy that involves regression of arterial vascular SMC. In mice, the absence of Notch3 function is compatible with normal angiogenesis and remodelling, but arterial SMC is severely affected and resembles venous SMC, both by its orientation and by the lack of smoothelin (Domenga et al. 2004).

6.5 TGF-β1 Receptors

Hereditary haemorrhagic telangiectasia (HHT) is a vascular dysplasia characterised by localised vascular malformations. Mutations in endoglinCD34(ENG, CD105) have been linked to HHT type 1, whereas mutations in the gene coding for ALK1 are associated with HHT type 2. ALK1, a receptor for TGF-B1 and activins, is predominantly expressed in arterial capillary EC. In ALK1-null mice, there is downregulation of ephrin-B2, loss of arterial-specific haematopoiesis,

defects in development of mural cells, and arteriovenous malformations between major arteries and veins (Seki et al. 2003).

ENG is a component of the TGF-β1 receptor complex that is uniformly expressed in all vessels, including liver sinusoids (Jonker and Arthur 2002). The most recent evidence indicates that ENG stimulates TGF-β1/ALK1-induced Smad1/5 responses and indirectly inhibits the TGF-β1/ALK5 signalling pathway, thereby promoting endothelial activation (Lebrin et al. 2004). The loss of ALK1 or ENG does not disrupt de novo assembly of large vessels, but impairs the ability to maintain the arterial and venous beds as distinct circuits during remodelling (Sorensen et al. 2003). CD34 is a cell-surface glycoprotein that is expressed on the surface of haematopoietic, as well as EC, but is normally expressed at a much higher level on arterial endothelium. In early $ALK1^{-/-}$ and $ENG^{-/-}$ embryos, CD34 is strongly expressed in venous vessels, suggesting a progressive conversion of venous endothelium to arterial haemogenic endothelium. The appearance of venous endothelial haematopoiesis could reflect an intrinsic defect in definitive haematopoietic stem cells, which also express ENG (Chen et al. 2002).

7 Concluding Remarks

The identification of a large number of growth factors and their signalling pathways, in conjunction with observations of mice in which these molecules have been genetically deleted, has provided an enormous body of information regarding their roles in vascular development. These data have made it clear that the formation of the vasculature is a highly complex process that involves a large number of growth factors and cell-cell interactions. Although use of knock-out mice has indicated a role for many factors, the precise role that each molecule plays is not known. In particular, the contextual role of such factors has not been elucidated concerning how the actions of a specific factor are modified by the environment and/or by the presence of other factors. Further, the tissue specificity of the various developmental pathways has not been systematically studied. Thus, though there has been a virtual explosion of knowledge regarding the development of the vascular system, many important questions remain to be answered.

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