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Endothelial cells and VEGF in vascular development

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The intricate patterning processes that establish the complex vascular system during development depend on a combination of intrinsic pre-patterning and extrinsic responses to environmental parameters. Mutational studies in mice and fish have shown that the vascular system is highly sensitive to genetic disruption and have identified potential targets for therapeutic interventions. New insights into non-vascular roles of vascular endothelial growth factor and the requirement for endothelial cells in adult organs and stem-cell niches highlight possible side effects of anti-angiogenic therapy and the need for new targets.

The development of the vascular system is one of the earliest events in organogenesis. The early blood vessels of the embryo and yolk sac in mammals develop by aggregation of *de-novo*-forming angioblasts into a primitive vascular plexus (vasculogenesis), which then undergoes a complex remodelling process, in which growth, migration, sprouting and pruning lead to the development of a functional circulatory system (angiogenesis; Fig. 1). Many of the events that occur during the normal progression of vascular development in the embryo are recapitulated during situations of neoangiogenesis in the adult[1]. Most notably, many tumours promote their own growth and dispersion to form metastases by recruiting host blood vessels to grow into the vicinity of the tumour (so-called tumour angiogenesis). In addition, neovascularization induced after tissue damage is a key component of the repair and healing process.

In recent years, there have been major breakthroughs in our understanding of the genetic control of the normal processes of vascular development and remodelling, especially from the characterization of vascular-mutant phenotypes in mice[2]. Embryonic phenotypes that fail to develop different phases of the normal vasculature have been reported for the vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR) and angiopoietin/Tie families of vascular-specific signalling molecules. Similar results were reported for certain members of more widely used signalling pathways including platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), ephrin, Notch and pathways thought to be primarily involved in axon guidance (such as semaphorins, netrins and Robo). Excitingly, many of these signalling pathways are reactivated in situations of neoangiogenesis[3]. This finding opens up a new era of rational therapeutics for prevention of tumour angiogenesis or promotion of new blood-vessel growth.

In this review, we focus on recent studies in fish, chicks and mice that have dissected early patterning of the vascular endothelial system, with particular reference to the role of endothelial cells and VEGF. Later events and signalling pathways that are equally important in vascular development, such as PDGF, TGF-β and angiopoietin signalling in pericyte and smooth-muscle-cell recruitment and vascular remodelling, have been reviewed elsewhere[4–6]. The process of lymphangiogenesis is reviewed in this issue by Alitalo, Tammela and Petrova (p. 946). Furthermore, a comprehensive list of genes that are essential for vascular development has been compiled and is given in Supplementary Table 1.

Endothelial cells might probably have more interesting roles than just acting as channels for blood circulation. They can promote stem-cell development and organ formation by acting as sources of

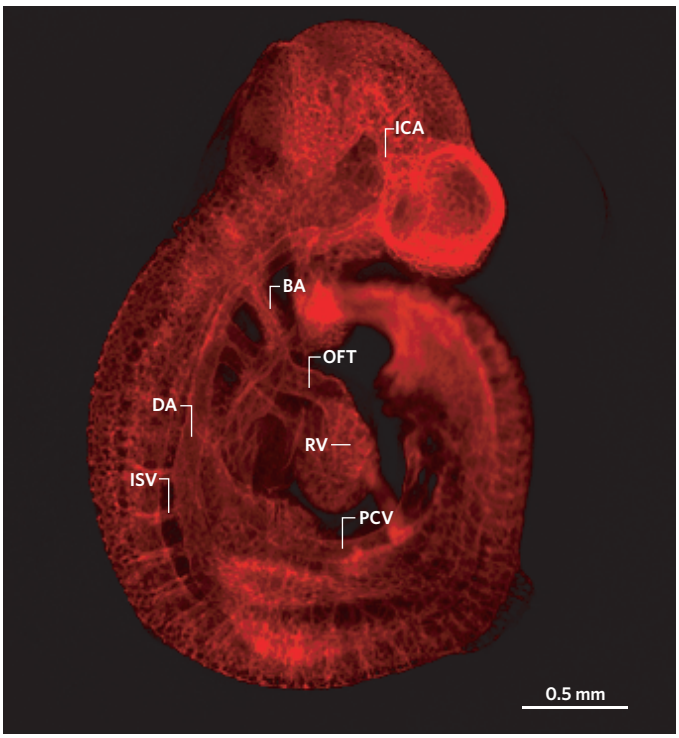

Figure 1 | Murine embryonic vasculature. Projection from optical projection tomography[100] of a normal mouse embryo at embryonic day (E) 9.5 showing developing vasculature labelled with CD31 (PECAM) immunofluorescence staining (K.C. and J.R., unpublished data). BA, branchial arteries; DA, dorsal aorta; ICA, intercarotid artery; ISV, intersomitic vessels; OFT, outflow tract; PCV, posterior cardinal vein; RV, right ventricle. (Image courtesy of L. Davidson, Mouse Imaging Centre, Hospital for Sick Children, Toronto, Canada.)

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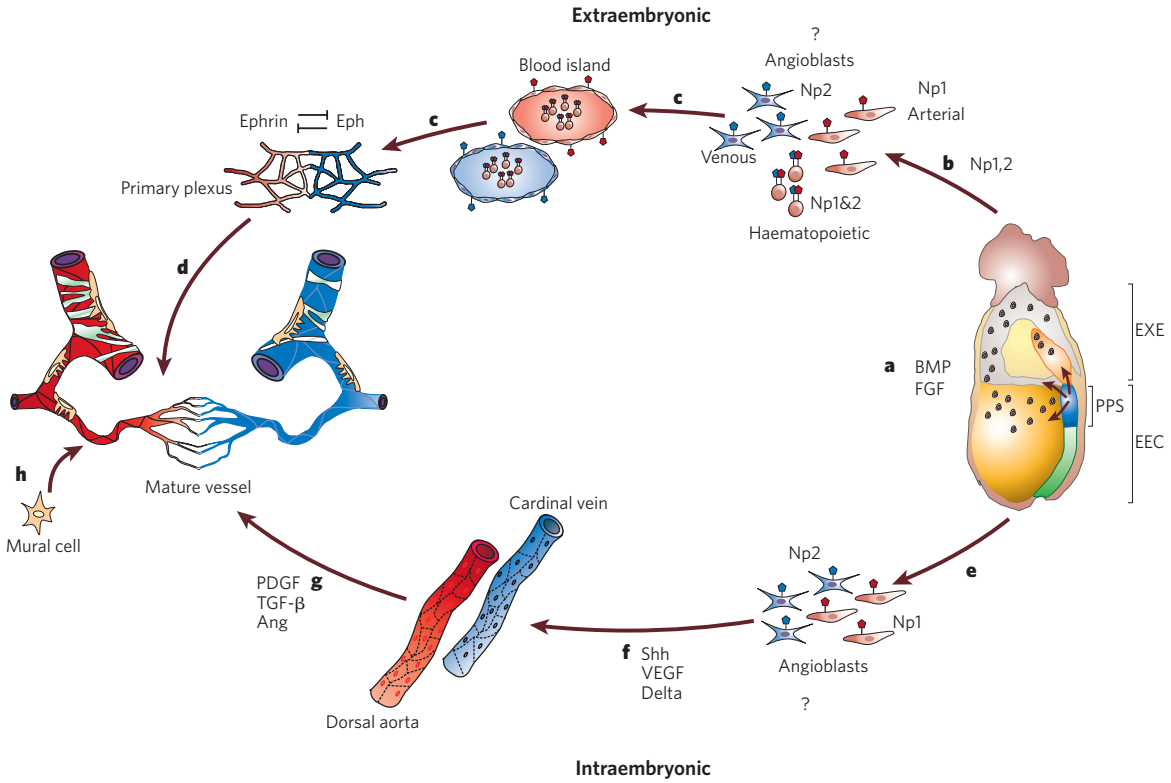

Figure 2 | Formation of a functional circulation from endothelial progenitors. **a**, Vascular progenitors appear in response to basic fibroblast growth factor (bFGF) and bone morphogenetic protein 4 (BMP4) in the posterior primitive streak (PPS) as vascular endothelial growth receptor-2 (VEGFR-2)/Flk1-positive mesodermal cells. **b**, Flk1-positive cells in the primitive streak give rise to both blood and endothelium (haemangioblasts), but are restricted to haematopoietic or angiogenic fate after emigrating into extra-embryonic sites (extra-embryonic ectoderm (EXE), yolk sac and allantois) and intra-embryonic sites (embryonic ectoderm (EEC)). **c**, In the yolk sac, these progenitors aggregate into endothelial-lined blood islands that then fuse to generate a primary capillary plexus (see main text). **d**, The primary capillary plexus undergoes remodelling along with intra-embryonic vessels to form a mature circulation (**g**). **e**, Intra-embryonic angioblasts migrate along distinct pathways before (**f**) aggregating directly into the dorsal aorta or cardinal vein, without a plexus intermediate. **g**, The primary vessels (capillary plexus, dorsal aorta and cardinal vein) then remodel, together with the extra-embryonic plexus, to form a mature vasculature, which along with VEGF and Notch involves the angiopoietins and Tie receptors[4]. **h**, Mural cells (pericytes and smooth-muscle cells) proliferate and differentiate in response to transforming growth factor-β (TGF-β) signalling, and are recruited to vessels by platelet-derived growth factor (PDGF) secreted by endothelial cells[5,6]. Ang, angiopoietin; Eph, Eph receptor family; Shh, sonic hedgehog; Np, neuropilin.

paracrine signals to surrounding cells, both in development and in ongoing endothelial niches in the adult. In addition, the signals that are primarily used to promote endothelial development might also act directly on other cell types, reinforcing the complex interplay between the vasculature and surrounding tissues. The second part of this review critically evaluates the evidence for the role of VEGF signalling in non-vascular tissues, as well as the role of the vasculature in inducing organogenesis and differentiation. Both of these processes have serious implications for the specificity of vascular therapies.

The haemangioblast/angioblast connection

In the mouse, the earliest marker of angioblast precursors is Flk1 (VEGFR-2), which is the major receptor for VEGF-A. Flk1 marks a subset of Brachyury-positive cells in the primitive streak, which then migrate into the extra-embryonic yolk sac to form a disperse vascular plexus, part of which contains clusters known as the blood islands (Fig. 2). The outer cells of the blood islands are endothelial, whereas the inner cells give rise to haematopoietic progenitors. In the embryo itself, angioblast precursors, marked by Flk1 expression, are dispersed throughout the head mesenchyme and other areas. The dorsal aorta and cardinal veins develop directly from aggregating angioblasts, whereas, in other areas, local vascular plexuses develop and slowly remodel and refine into the major vessels and capillary beds of the embryo. Flk1 is not only a marker for the earliest progenitors of the vascular and haematopoietic system, active VEGF-A signalling is also required for normal development of both systems. Embryos lacking *Flk1* die at around 9 days of development and show no development of blood vessels or haematopoietic cells[7,8]. Mutation of the ligand, VEGF-A, also leads to early lethality. Interestingly, VEGF-A is haploinsufficient — heterozygotes die early in gestation with severe reduction in the size and calibre of their developing blood vessels[9,10].

The loss of both vascular and haematopoietic cells in *Flk1* mutants is one of many pieces of information linking endothelial and haematopoietic development through a possible common progenitor, the haemangioblast (reviewed in ref. 11). Notably, Keller, Choi and colleagues showed that Flk1-positive cells sorted from differentiating embryonic stem (ES) cells can give rise to single-cell-derived blast colonies — so-called BL-CFCs — that can produce both endothelial and haematopoietic cells, thereby providing formal proof of the haemangioblast[12,13]. In the embryo itself, not all endothelial progenitors have such bipotential fate. In the chick, only a subset of endothelial progenitors have haematopoietic potential. In the mouse, there are clear Flk1-positive progenitors in regions of the head mesenchyme and in non-blood-island regions of the yolk sac that do not have haematogenic potential[11]. However, Flk1-positive cells with bipotential activity can be isolated from the primitive streak in greater numbers than from the early yolk sac[14]. This raises the possibility that all early Flk1-positive cells have the potential to become either haematopoietic or endothelial cells.

Isolation of single Flk1-positive cells that can give rise to both endothelial and haematopoietic cells *in vitro* is clear proof of a com-

mon progenitor for the two lineages. However, it does not exclude the possibility that the isolated precursor is a more primitive multipotent precursor, the potency of which has not been revealed by the assays used. During embryonic stem (ES) cell differentiation, isolated Flk1-positive progenitors can produce smooth-muscle-type cells[15,16] or cardiomyocytes *in vitro*[17] under appropriate culture conditions. These results suggest a broader potential for Flk1-positive progenitors than simply haematopoiesis and vasculogenesis, and imply that the Flk1-positive cells isolated from the primitive streak and from ES cells *in vitro* represent a multipotent mesodermal progenitor that later specializes into different mesodermal lineages. Whether endothelial progenitors in later embryonic or adult development have multipotent capacity is not known; however, this could help to explain recent evidence linking *Flk1* expression with multipotent stem-cell populations, such as MAP-C cells isolated from adult bone marrow[18] and multipotent mesodermal (mesoangioblast) cell lines derived from developing dorsal aorta[19].

Other studies have challenged the concept of a bipotential haemangioblast. Cell-lineage tracing of cells from the primitive streak to the yolk sac failed to reveal a common haematopoietic and endothelial progenitor[20], and other studies have suggested that haematopoietic and endothelial progenitors can be distinguished by their differential expression of CD41 as soon as they exit from the primitive streak[21]. What might seem contradictory results could really reflect issues of timing of the progression of lineage commitment from the primitive streak. Recent advances in imaging technologies[22] might finally allow us to follow the fate of Flk1 progenitors in the living embryo and resolve these controversies.

From endothelial progenitors to a functioning circulation

Once the vascular progenitors have been specified, they begin to form a disperse vascular plexus, which is then gradually reorganized into a functional circulation. As vessels begin to be remodelled, they undergo localized proliferation and regression, as well as programmed branching and migration into different regions of the body. They need to be specified into different calibres and types of vessel, including division into arteries, veins and lymphatics, with further subdivision into large vessels, venules, arterioles, capillaries and so on. In addition, they need to recruit supporting cells, smooth-muscle cells and pericytes, to ensure the stability of the vessels formed (Fig. 2). Although we do not fully understand the intricacies of these processes, it is clear that the final outcome is determined by a combination of hard-wired genetic programming and extrinsic influences, such as hypoxia[23] and haemodynamic flow[24].

One of the first events that takes place during maturation of the circulatory system is the specification of arteries versus veins, and this serves as a good example of the interplay of intrinsic and extrinsic factors. Until fairly recently, it was assumed that specification of arterial versus venous fate was a late event in development and was instigated by haemodynamic-flow differences in the two types of vessel. This view changed when specific markers of venous versus arterial fate, such as ephrinB2 for arteries and its receptor EphB4 or veins[25–27], were detected on subsets of developing blood vessels before the onset of circulation. In the chick, the early extra-embryonic blood islands contain a random mixture of subpopulations of cells expressing neuropilin 1 (NP1), which is the VEGF co-receptor later restricted to arteries, and NP2, which is the vein-specific receptor. By the 13-somite stage, the expression of the two genes is segregated to the future arterial and venous parts of the plexus, despite the absence of blood flow[28]. This study raises the possibility that arterial versus venous fate is established in early progenitors, which then segregate; a similar conclusion was suggested by cell-lineage tracing in zebrafish[29].

Although the pendulum has swung towards intrinsic specification of arterial versus venous fate, there is still considerable plasticity in the early vasculature. Grafting experiments in the chick have shown that, up to embryonic day 7, ectopic grafts of individual arteries or veins can lead to respecification of cell fate in early stages, but plasticity is gradually lost later in development[30]. Elegant time-lapse cinematography in the chick and zebrafish has shown that the establishment of the final pattern of circulation between artery and vein involves selective disconnection and reconnection of small vessels, and concomitant switches in markers of arterial versus venous fate[24,31]. Manipulating flow physically (chick) or genetically (fish) showed that it could change gene expression and cell fate. Therefore, the overall vascular architecture is probably refined by the haemodynamics of circulatory-flow patterns on top of an underlying genetic programming of arterial versus venous fate[24,29].

The Notch signalling pathway has been implicated as a prime player initially, from studies in zebrafish, in establishing arterial versus venous fate. Injection of a dominant-negative form of Suppressor of Hairless (the common downstream effector of Notch signalling) resulted in decreased expression of arterial markers, such as ephrinB2, and ectopic expression of venous markers in the dorsal aorta[29,32]. Conversely, expression of an activated Notch construct induced ectopic arterial markers in the posterior cardinal vein[32]. This suggests that during normal development, activation of the Notch signalling pathway in developing artery cells is required to suppress the venous fate and allow arterial differentiation. The hairy-related basic helix–loop–helix transcriptional repressors of the Hes/Hey family are direct downstream targets of Notch signalling in many situations in which Notch determines cell-fate choice. The zebrafish gridlock gene[33] encodes a member of this family of proteins. Gridlock is expressed specifically in the aorta, not the developing veins, which is consistent with its being a Notch target *in vivo*[33]. Graded reduction in gridlock expression leads to progressive loss of the artery and expansion of the adjacent vein, in a similar manner to the effect seen by blocking Notch signalling with a dominant-negative Su(H) construct[29].

Four different Notch receptors (Notch 1–4) and five ligands (delta-like (Dll)1, Dll3, Dll4, Jagged-1 (Jag1) and Jag2) have been identified in mammals[34]. Genetic analysis in mice and humans has revealed various types of vascular defect associated with Notch-pathway mutants (Supplementary Table 1), although defective primary separation of arterial versus venous fate seems to be a consistent feature of these complex phenotypes. However, mutations in Dll4, which is a Notch ligand specifically expressed in developing arterial but not venous endothelium, lead to defective development of the dorsal aorta and cardinal veins, with development of arterio-venous shunts[35,36]. This is associated with downregulation of arterial markers and upregulation of venous markers in the dorsal aorta. Dll4 seems to act primarily in an autocrine manner on the arterial endothelium, through the receptors Notch1 and Notch4. Double mutants of Notch1 and Notch4 have a similar phenotype to loss of Dll4, and endothelial-specific knockout of RBP, the Su(H) orthologue, also leads to complex vascular defects, including loss of arterial specification[37]. Double mutation of Hes1 and Hey1, mammalian orthologues of gridlock, also produces loss of arterial markers and vascular shunts[38]. Thus, activation of Notch signalling and its downstream-response genes seems to be a conserved requirement for specification of arterial-cell fate in vertebrates, with venous fate being the 'default' state. Recent evidence has shown that the orphan nuclear receptor COUP-TFII is expressed specifically in venous endothelium and that mutation leads to activation of arterial markers in veins[39]. Expression of COUP-TFII ectopically in arteries suppresses the expression of NP1 and other arterial markers. Together, these results suggest that COUP-TFII is a part of an active pathway promoting venous fate and suppressing Notch signalling.

Patterning the developing vasculature along the body axis

Although levels of Notch signalling might be crucial to establishing arterial versus venous fate, there must be other mechanisms that establish the positions in which the various primary blood vessels develop. The formation of the dorsal aorta and cardinal veins in chicks, and presumably in mice, occurs by local aggregation of angioblast progenitors in the midline and recruitment by inward migration of haematogenic progenitors to the ventral side of the dorsal aorta (Fig. 3). In zebrafish,

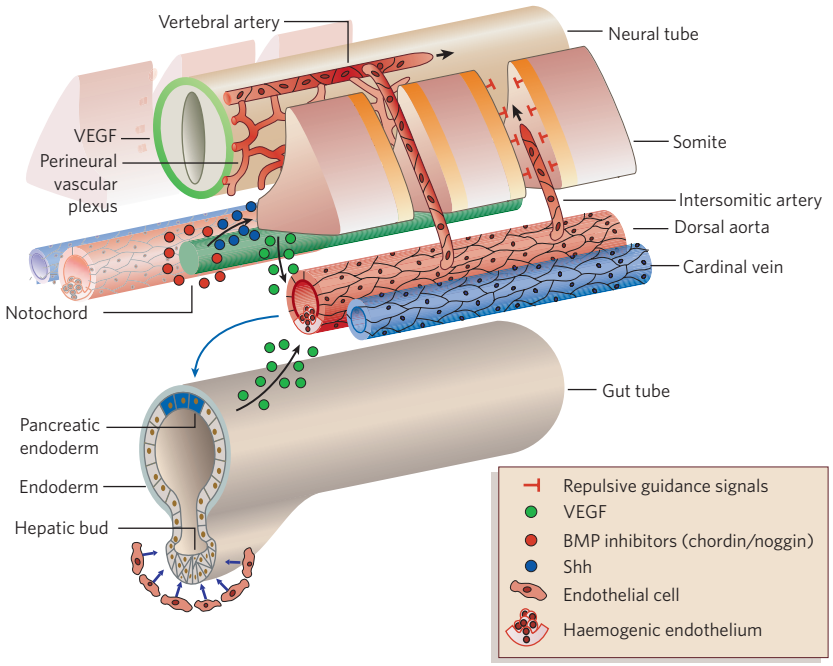

Figure 3 | Vascular development along the mammalian body axis. Formation of the dorsal aorta and cardinal veins occurs by *de novo* aggregation of angioblasts along the anterior/posterior body axis in response to VEGF from the endoderm. Somites, in response to hedgehog from the notochord, might also produce VEGF required for development of these axial vessels. Bone morphogenetic protein (BMP) inhibitors, noggin and chordin, produced by the notochord establish an avascular midline zone. Intersomitic vessels sprout from the dorsal aorta and cardinal vein (not shown) in a VEGF-dependent manner, and are then guided between the somites by repulsive cues, such as ephrinB2, semaphorin3A (sema3A) and sema3E in the somite boundary. Intersomitic arteries bifurcate once they reach the medial edge of the somite, fusing with adjacent intersomitic arteries to form the vertebral artery. The perineural vascular plexus surrounds the neural tube and is formed by angioblasts migrating from the somites in response to VEGF from the neural tube (green band). Once specified by VEGF from the endoderm, the dorsal aorta signals back to the endoderm to induce pancreatic bud formation by means of an unknown signal (blue arrow). Endothelial cells surrounding the prospective hepatic endoderm also provide an unknown signal (blue arrows) to induce hepatic bud development.

the single dorsal aorta might be induced by signals from the notochord, as *floating-head* and *no-tail* mutants lacking a notochord also lack a dorsal aorta. Ectopic transplantation of notochord cells allows assembly of aortic primordia[40,41]. In *Xenopus*, the hypochord, which is a structure ventral to and induced by the notochord, is required for dorsal aorta formation[42,43]. There is no hypochord in mice or chicks, but the definitive endoderm ventral to the aorta might take over the role of the hypochord in inducing dorsal aorta formation in these organisms. The common signal in all cases again seems to be VEGF-A, which acts in this context as a graded attractant to angioblasts to promote aorta formation. The hypochord in frogs, and definitive endoderm in chicks and mice, are potent sources of VEGF[42,44], whereas VEGF produced by the somites in response to sonic hedgehog (Shh) from the notochord promotes recruitment of cells to form the midline vessels in zebrafish[45]. Hedgehog signalling also seems to be important in dorsal aorta formation in chicks and mice, as mutants for the hedgehog signalling component *smoothened*, or use of *smoothened* inhibitors, cause defective dorsal aorta formation[46]. The nature of the hedgehog signal is less clear, as loss of Shh does not lead to loss of the dorsal aorta in mice, suggesting the involvement of other hedgehog ligands. The role of the notochord in dorsal aorta formation in chicks and mice is also not clear. *Brachyury* mutants lack the posterior notochord, but still form a posterior dorsal aorta[47]. However, this does not rule out the role of the notochord in the anterior region, where the *smoothened* phenotype is most severe[46].

The notochord does have at least one key role in patterning the axial vasculature in chicks and mice by secreting bone morphogenetic protein (BMP) inhibitors, noggin and chordin, which define an avascular region around the developing notochord[48,49]. Later, secretion of VEGF from the neural tube is involved in recruiting somite-derived angioblasts to form the perineural vascular plexus, which will encase the neural tube at midgestation[50]. From there, angiogenic sprouting within the neural tube will occur and is dependent upon Shh signals from the ventral neural tube and neighbouring tissues[51].

The formation of the intersomitic arteries from the dorsal aorta is a highly stereotyped process that begins at an early somite stage and spreads caudally as somites develop. The intersomitic vessels sprout dorsally from the dorsal aorta in the region between somites (Fig. 3). Once they reach the medial surface they are deflected along the surface of the somite, where they fuse with adjacent sprouts and form the longitudinal vertebral arteries aligning with the neural tube. Beginning at the eight-somite stage, presumptive cardinal vein angioblasts send processes to join the vertebral artery and become the intersomitic veins dorsal to the intersomitic arteries[52]. What controls the position and timing of sprouting and migration of the intersomitic vessels? Again, VEGF has a role. Mutation of Flt1 (VEGFR-1), which is normally considered a negative regulator of VEGF signalling, reduced sprouting in ES cells *in vitro*, and intersomitic sprouts *in vivo* suggest that precise levels of bioactive VEGF-A and perhaps spatial localization of the VEGF signal are likely to determine proper localized intersomitic sprout formation[53]. Notch signalling might also be important in determining the basic patterning and number of branch points, as well as in artery–vein specification. Several *in vitro* studies show a role for active Notch signalling in restricting branching morphogenesis and *in vivo* mutation studies support this. Loss of Dll4 (ref. 35) or Notch1 (ref. 54) leads to excessive and misdirected intersomitic branching, while activation of Notch signalling in the endothelium suppresses branching of vessels.

Once formed, intersomitic sprouts need to be properly guided in their migration between adjacent somites. This process, like many other examples of vessel formation and migration, has several mechanistic and genetic similarities to axon guidance. The roles of axon-guidance cues in blood-vessel development have recently been reviewed elsewhere[55].

Clearly, even the earliest phases of vessel patterning involve a complex hierarchy of signals from VEGF through Notch to specific vessel-guidance mechanisms using molecular strategies first implemented in the nervous system. These pathways also seem to be reactivated in the adult in situations of neoangiogenesis, and therefore become interesting new targets for pro-angiogenic or antiangiogenic therapies (see review in this issue by Ferrara and Kerbel, p. 967). The formation of the developing vasculature depends on tight regulation of developmental cues with some signalling pathways, such as VEGF and Dll4, showing haploinsufficient defects. If this sensitivity is manifested in situations of adult neoangiogenesis, then there is real hope for effective therapeutic interventions.

Endothelial cells and vascular signalling in organogenesis

The crucial pro-angiogenic properties of VEGF, as described above, have made it a prime candidate for therapeutic intervention involving

angiogenesis. The intimate association of endothelial cells with their cognate organs throughout life, and the potentially pleiotropic nature of VEGF signalling, might, however, lead to significant off-target effects from these interventions. In shaping the vascular system, organs signal to the vessels that service them, influencing the endothelial cells to adopt functional specialties, such as the blood–brain barrier and fenestrated endothelium in the kidney glomeruli[56]. However, there is increasing evidence that endothelial cells, in turn, provide instructive morphogenetic cues to promote organ formation and patterning both in development and in the adult.

Endothelial cells, and liver and pancreas induction

The liver first appears as a multilayered epithelium in the ventral foregut endoderm. Endothelial cells surround this presumptive liver bud, then invade it and aggregate into sinusoids as hepatoblasts begin to migrate from the endoderm into the underlying septum transversum[57] (Fig. 3). A role for endothelial cells in liver development was suggested by studies of *Flk1*-mutant mice, which fail to form mature endothelial cells[7]. In these mice, thickening of the foregut endoderm and expression of liver-specific genes occurred normally. However, there was no migration or proliferation of the hepatoblasts into the surrounding septum transversum to form the liver bud[57]. Explant cultures demonstrated that the effect of endothelial cells on liver development was independent of circulation[57]. Endothelial cells activated by VEGF also support hepatocyte proliferation and survival in adult animals[58]. In response to VEGF, liver sinusoidal endothelial cells (LSEC) released hepatic mitogens, hepatic growth factor (HGF) and interleukin-6 (IL-6), in a VEGFR-1-dependent manner, promoting hepatic growth and protecting hepatocytes from toxic insult *in vivo*. Therefore, endothelial cells provide essential cues to the developing liver and can be stimulated to provide both nutritional and trophic support to a damaged adult liver.

Endothelial cells seem to have a similar role in promoting early pancreatic development. The developing pancreas forms in close association with the dorsal aorta and vitelline veins (Fig. 3)[59]. Pancreatic islet endocrine cells (such as insulin-producing cells) also associate closely with endothelial cells, secreting hormones directly into the blood. Reciprocal signalling seems to occur between endothelial and endocrine cells in order to establish a functional pancreas. Explant studies showed that the dorsal aorta was necessary and sufficient for insulin expression in endoderm tissue, and ectopic endothelial cells could induce insulin expression in non-pancreatic endoderm[59]. Endocrine cells then signal back to endothelial cells through VEGF to induce capillary invasion of the islets and fenestration of endothelial cells occupying the islets[60]. VEGF was not required for endocrine pancreas development itself[60]. Analysis of *Flk1*-mutant mice suggested that endothelial cells were not required for initial specification of the pancreas from foregut endoderm but were required for the subsequent emergence of the pancreatic bud and expression of Ptf1a, which is a transcription factor necessary for pancreatic development, as well as endocrine genes[61].

Studies in zebrafish have not supported this early role for endothelial cells in liver and pancreas development, as liver and pancreatic budding occurs normally in zebrafish mutants lacking endothelial cells[62,63]. This might reflect evolutionary differences in the source of morphogenetic signals to compensate for differences in development and function of these organs between fish and mammals[64]. The signals provided by the endothelium to promote liver and pancreatic bud morphogenesis are currently unknown. Determining the nature of these signals, their conservation between fish and mammals, and whether they are expressed by all endothelial cells or only those in close juxtaposition to the liver and pancreas will provide interesting areas for future research.

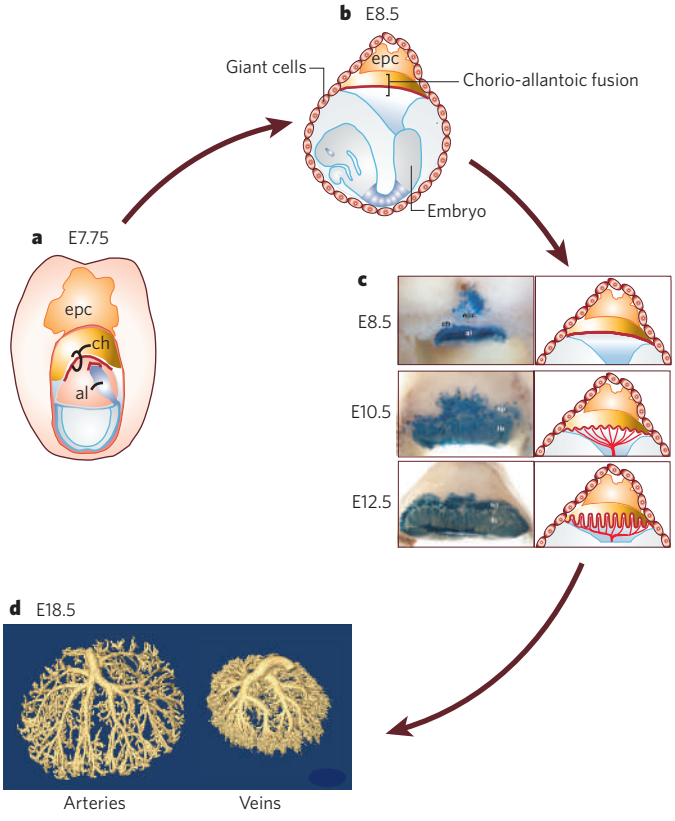

Figure 4 | Formation of the fetal vasculature in the chorio-allantoic placenta. **a**, Fetal components of the chorio-allantoic placenta as shown at embryonic day (E) 7.75 are the chorionic membrane (ch) and allantois (al). Future fusion areas are highlighted in red. **b**, At the four-to-six-somite stage, the allantois fuses to the chorion, forming the chorio-allantoic placenta. **c**, Shortly after fusion, the chorion branches, forming the labyrinth layer that is invaded by fetal vasculature from the allantois. The panels on the left show whole-mount staining for Flt1, revealing a subset of ectoplacental cone cells, spongiotrophoblasts, and trophoblasts and endothelial cells in the labyrinth layer. (Image reproduced with permission from ref. 73.) Vessels invading the labyrinth layer undergo extensive remodelling to form a complex vascular structure of fetal arteries and veins. **d**, Surface renderings derived from reconstructed micro-computed tomography showing placental vasculature at E18.5, displaying the intricate nature of the fetal vasculature of the placenta. Contrast agent was injected separately into the placental arteries or veins via the umbilical vessel vasculature. (Image courtesy of M. Y. Rennie, S. L. Adamson and J. G. Sled of the Mouse Imaging Centre, Hospital for Sick Children, Toronto, Canada, and Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada.)

Endothelial cells, VEGF and the kidney glomerulus

Numerous studies in mice have shown that signalling between endothelial cells and podocytes is essential for proper development and maintenance of the filtration function of the kidney glomerulus[65,66]. Podocytes, which are the specialized cells that make up the support structures of the functional glomerulus, normally express VEGF at high levels. Homozygous deletion of VEGF specifically in podocytes prevented recruitment and maturation of endothelial cells in the glomerulus and led to abnormal podocyte and mesangial-cell maturation and perinatal lethality[65]. Mesangial-cell contribution to glomeruli was also abnormal in mice specifically lacking PDGFB in endothelial cells[67]. Together, these results suggest that endothelial cells, which are recruited, matured and maintained by VEGF from podocytes, promote further maturation of podocytes and mesangial cells, and formation of a functional glomerulus. Endothelial-cell maintenance through regulated VEGF levels is crucial for continued glomerular function in adults. Heterozygous loss of VEGF in podocytes resulted in the disappearance of endothelial fenestrations in adult mice, followed by loss of podocyte foot processes. Clinical symptoms of hypertension and proteinuria were followed by end-stage kidney failure[65]. Administration of VEGF-neutralizing agents results

in glomerular dysfunction, suggesting that the glomerulus is highly dependent on endothelial function controlled by precise VEGF levels throughout life[68].

Endothelial cells and placental development

The mammalian placenta is a complex organ, the main functions of which depend on the establishment of a structure in which oxygen, waste products, nutrients and growth factors can be interchanged between the maternal and fetal environments. Establishment of the vascular-interchange bed on the fetal side involves fusion of the allantois to the chorion and branching invasion of the fetal capillaries into the chorionic trophoblast (Fig. 4). On the maternal side, the developing spiral arteries entering the placenta undergo a process of invasion by fetal trophoblasts, which eventually replace the endothelial cells, ensuring that maternal blood directly bathes fetal trophoblasts and thereby enhancing exchange.

Despite some detailed differences in architecture, the overall gross anatomical features and molecular pathways underlying placental development are similar in humans and mice[69]. Mouse mutants in many pathways affecting vascular development cause placental problems[69] and the same pathways are probably relevant to human placental development. However, on the whole, the pathways involved in placental vascular development are similar to those involved in the establishment of other vascular beds. A few special features do arise, which might be related to the rapid and extensive neovascularization and remodelling that the placenta needs. First, the placenta secretes a number of specific signalling molecules that seem to be involved in promoting vascular development, beyond the standard angiogenic factors. For example, the placental lactogen-related hormones, proliferin and proliferin-related protein, exert angiogenic and anti-angiogenic actions on the placental vasculature[70]. In addition, one member of the VEGF family, placental-like growth factor (PlGF), is strongly expressed in the placenta and is thought to play its normal physiological role there. Although mice lacking PlGF show no apparent physiological defects in normal development[71], they do show defects in situations of induced angiogenesis, such as tissue ischaemia. Binding of PlGF to Flt1 enhances VEGF signalling through Flk1 (ref. 72), suggesting a mechanism by which this molecule might have a modulatory role in the developing placenta.

Interestingly, in both mice and humans, Flt1 (the VEGFR that preferentially binds PlGF) is expressed not only in the fetal vasculature but also on subsets of trophoblast cells within the placenta, suggesting that PlGF also has a direct role in promoting placental development. However, chimaeric mouse placentas, in which trophoblast cells lack Flt1 but fetal vascular expression is intact, are morphologically normal and support fetal growth to term[73]. Therefore, trophoblast-specific expression of Flt1 is dispensable for establishment of the maternal–fetal interface and the formation of placental vasculature. This leaves open the possibility that the placental expression of PlGF and Flt1 could still have an important modulatory role in the placenta itself or, by virtue of the production of the soluble form of Flt1, sFlt1, on the maternal system.

Pre-eclampsia is a relatively common disorder affecting about 2.5–5% of pregnancies (reviewed in ref. 74). It appears in two stages: the preclinical phase is characterized by failure of cytotrophoblast invasion of maternal spiral arterioles, leading to a hypoxic placenta and, hence, upregulation of the production of hypoxic-responsive placental factors secreted by the trophoblast, including components of the VEGF/PlGF pathway. In turn, this local defect leads to the second phase of pre-eclampsia whereby systemic responses in the mother result in hypertension, proteinuria, blood clotting and various other internal organ dysfunctions predominantly due to endothelial dysfunction. Pre-eclampsia can be life threatening to both mother and baby and can be cured only by delivery of the placenta. Recent evidence has suggested that increased circulating levels of sFlt1, along with reduced levels of VEGF and placental growth factor (PIGF) in maternal serum 5 weeks before clinical onset of pre-eclampsia, can be predictive of disease[75]. Importantly, delivery of sFlt1 directly to pregnant rats recapitulated many hallmarks of preeclampsia, including hypertension, proteinuria and kidney endotheliosis[68]. Therefore, blocking sFlt1 is an attractive avenue to manage pre-eclampsia. The finding that Flt1 is not required for placental development itself in mice is encouraging, as it suggests that there might not be unwanted placental side effects of any such therapy.

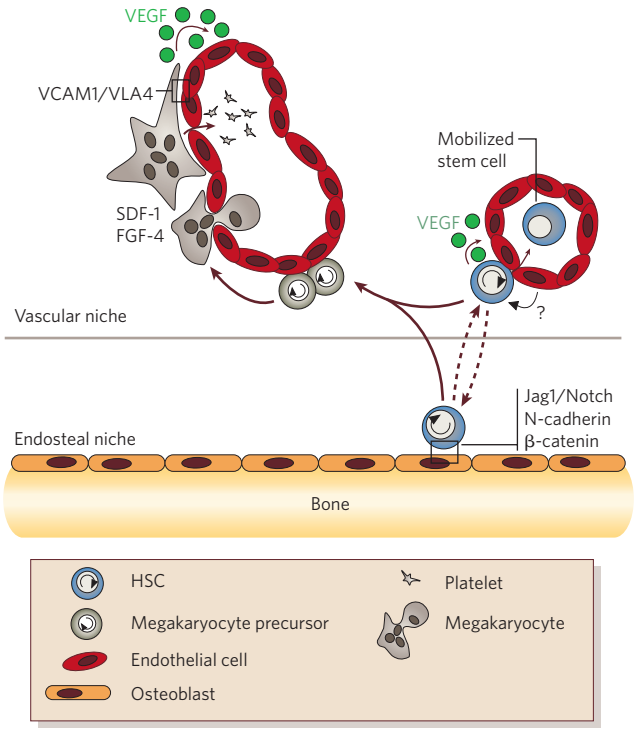

Figure 5 | Endothelial cells provide a niche for haematopoietic stem cells (HSCs). Osteoblasts in the endosteal niche expressing Jagged-1 (Jag1) and N-cadherin contact and maintain HSCs by activation of Notch, and might further regulate HSC activity through N-cadherin and β-catenin signalling. Endothelial cells in the vascular niche also contact HSCs and provide unknown maintenance signals (question mark). HSCs might be transported between niches and could be subject to differential regulation in each niche (dashed lines). Endothelial cells expressing vascular cell-adhesion molecule 1 (VCAM-1) also associate closely with megakaryocytes and their progenitors through very late activation antigen 4 (VLA4) in response to chemotactic factors, stromal cell-derived factor-1 (SDF1) and fibroblast growth factor-4 (FGF4), and provide a niche for megakaryocyte maturation and platelet production. The immediate juxtaposition of HSCs to endothelial cells also facilitates their rapid mobilization and entry into circulation in response to stress and, in the case of megakaryocytes, release of platelets directly into the blood. HSCs and haematopoietic progenitor cells as well as megakaryocytes produce VEGF and other angiogenic factors, which might act in a feedback loop to support endothelial cells in the bone marrow and in the periphery at sites of normal and pathologic angiogenesis.

Endothelial cells and the stem-cell niche

An additional function for endothelial cells in organogenesis might be the regulation of stem cells. This has been proposed for the nervous system. Proliferating endothelial and neural precursors are closely associated, and endothelial-derived soluble factors regulate neuro-genesis (see review in this issue by Greenberg and Jin, p. 954). Given their close proximity, cross-talk between proliferating endothelial and neuronal progenitors is likely to involve more than just secreted factors. Juxtacrine signalling molecules, such as Notch, which are essential for cell-fate choice in progenitors of both neuronal and vascular lineages, are obvious candidates. Endothelial cells are therefore likely to have a key role in providing a niche for regulating neural stem-cell activity.

Endothelial cells are also found in other areas where stem cells reside and might have important roles in promoting their maintenance and survival. Haematopoietic stem cells (HSCs) are well defined in terms of their stem-cell properties, but the stromal environment contributing to their regulation is less well defined. One cell type contributing to the adult HSC niche is the osteoblast, but other stromal cells in the marrow, such as endothelial cells, probably also contribute[76,77] (Fig. 5). As already discussed, there is an intimate association and, indeed, close lineage relationship of endothelial and blood cells, suggesting that they also regulate HSC development from their inception through to their occupation of the bone marrow. Bone-marrow endothelial cells regulate proliferation and differentiation of more-committed progenitors of the myeloid and megakaryocyte lineages[78,79]. As megakaryocytes produce VEGF, the interaction between these progenitors and endothelial cells is probably reciprocal, as it is in other organs[80]. Endothelial cells also promote survival of HSCs in culture, but this seems to be limited to certain populations of endothelial cells[81]. On the basis of this finding, the existence of an endothelial niche for HSCs seems axiomatic; however, only recently has *in vivo* evidence emerged supporting an endothelial niche for HSCs[82]. Significant fractions of HSCs in both adult bone marrow and spleen were found in close association with endothelial sinusoids, suggesting that endothelial cells provide support to HSCs *in vivo*. It will be interesting to further define the respective contributions of endothelial and endosteal niches to HSC behaviour. Furthering the idea of reciprocal signalling between haematopoietic progenitors and endothelial cells, bone-marrow-derived progenitor cells, possibly including haematopoietic progenitor cells and HSCs, localize to sites of active angiogenesis, including tumour angiogenesis, raising the possibility that haematopoietic cells signal back to endothelial cells to regulate angiogenesis[83]. The transport of circulating HSCs or haematopoietic progenitor cells to specific organs might be dependent on adhesion molecules specific to endothelial cells of each organ and could represent another level of regulation.

Further study of the functional roles of endothelial cells in promoting adult organ maintenance, and stem-cell and progenitor-cell proliferation, seems certain to reveal more interesting functions for the vasculature than simply carrying the blood supply.

VEGF-A signalling in non-endothelial cells

Expression of VEGFRs on non-endothelial cells and observations made in VEGF-mutant mice have also implicated VEGF as an essential signalling factor in non-endothelial cells. However, the evidence for a direct effect versus indirect effects needs to be critically evaluated in all cases.

VEGF action on cells of the nervous system

Numerous studies point to a neuroprotective role for VEGF[84]. For instance, mice homozygous for a VEGF-A allele lacking the hypoxia-response element developed a motor neuron disease similar to amyotrophic lateral sclerosis (ALS)[85]. This effect of reduced VEGF was probably partly due to effects on endothelial cells, as reduced neural perfusion was observed; however, a direct effect of VEGF might also be involved, as VEGF protected motor neurons under stress conditions *in vitro*[85] and overexpression of Flk1 in motor neurons was subsequently shown to prolong survival of ALS mice[86]. Indeed, *in vitro*, VEGF displays pro-survival activity for many types of neuron under a range of stress conditions, stimulates axon outgrowth in explant cultures, and promotes the survival, proliferation and migration of Schwann cells, astrocytes and microglia[84]. *In vivo* studies have demonstrated that VEGF-1 (ref. 64) is required for migration of facial motor neurons[87]. Lack of VEGF-A specifically in the neural population resulted in abnormalities in retina and cortex development, accompanied by reduced vascular density and branching in these sites, which, in the retina, resembled some human retinopathies[88]. However, neural development proceeded apparently normally when Flk1 was removed from the neural population, suggesting that the VEGF-A phenotype was caused by secondary effects of loss of appropriate vascular invasion[88]. VEGF might therefore have two roles in nervous-system development and neuroprotection: first, as a direct neurotrophic agent; and second, as an angiogenic factor stimulating endothelial cells to provide adequate perfusion and neurotrophic factors. Regardless of the direct or indirect role, administration of VEGF can reduce the severity of neurological trauma, including spinal-cord injuries, making it a potentially useful adjunct therapy for these patients[89].

Bone

Signalling by VEGF seems to have multiple roles in bone development, promoting vascularization during endochondral bone formation, and regulating survival and activity of chondrogenic and osteogenic cells. Soluble inhibitors first revealed a requirement for VEGF in bone development, suppressing vascular invasion and growth of long bones[90]. Conditional and isoform-specific VEGF mutants have subsequently shown that the heparin-binding isoforms of VEGF produced by chondrocytes are essential for vascular invasion of the metaphysis, cartilage resorption and primary ossification of long bones[91,92]. VEGF, presumably secreted from non-hypertrophic chondrocytes, also seems to be required to promote angiogenesis around the epiphysis of long bones to facilitate secondary ossification. In contrast to metaphyseal vasculature, recruitment of epiphyseal vasculature depends on the soluble VEGF isoforms[93], the absence of which results in massive apoptosis of non-hypertrophic epiphyseal chondrocytes due to hypoxia[93,94]. VEGF might act directly on these chondrocytes to protect them against hypoxia[93,94]. VEGF also has an important role in bone-fracture healing in animal models, in part by stimulation of vascular growth in the region of the injury, but evidence also suggests that it acts on bone-forming cells themselves[95]. VEGF directly stimulated the activity of isolated osteoblasts and osteoclasts *in vitro*, whereas VEGF blockade in explant cultures of embryonic cartilaginous metatarsals inhibited ossification[95,96].

VEGF might signal to other non-endothelial cell types in addition to those described. For example, VEGF deletion in HSCs suggests an autocrine VEGF signalling loop in HSC survival[97], whereas a podocyte-specific *Flk1* mutation points to an autocrine role for VEGF in adult podocytes (S. Quaggin, personal communication). Other non-endothelial targets of VEGF might include myoblasts[98] and pneumocytes[99]. The examples presented above clearly highlight the need for more studies using cell-specific VEGFR mutants to further dissect the autonomous and non-autonomous requirements of VEGF signalling in both development and disease.

Future directions

We have presented an overview of vascular development from the initial specification of vascular cell types through to their role in maintaining homeostasis in adult tissues. Although we have made significant progress towards understanding how blood vessels are formed and patterned to generate the many types of vessel, much work remains to be done. We still do not fully understand the origins of angioblasts or the relationship of endothelial cells to other vascular cell types, such as cardiac, smooth-muscle and blood cells. The complexity and heterogeneity of endothelial cells is becoming increasingly apparent, opening the possibility of refining therapeutic applications to specific subsets of the vasculature. To what extent is this heterogeneity defined by intrinsic programmes or influenced by local environment? The genetic and environmental control of vascular assembly and remodelling is still not well understood, although it is crucial to understanding the states of neoangiogenesis in the adult and defining new angiogenesis-based therapies. The multiple roles of VEGF signalling in endothelial development and function have made it the most popular target for angiogenic therapeutic interventions. Yet, its importance and the increasing evidence of its possible roles in cell types other than the vasculature make the side effects of anti-VEGF therapies a real concern. A more refined understanding of the complex sig-

nalling pathways controlling all aspects of vascular development and remodelling will help define new and more-specific targets for future therapeutic intervention. ■

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