

Chapter 1

Emergence of Endothelial Cells During Vascular Development

Anne Eichmann and Luc Pardanaud

Abstract Arteries, capillaries, and veins form the vascular system that supplies oxygen and nutrients to all tissues and removes waste products. In the embryo the vascular system is the first system to emerge during vasculogenesis, and the factors that initiate the patterning of the endothelial network are, for the most part, involved in the adult angiogenesis. Dysfunctions of the vascular system cause numerous pathologies, including arteriosclerosis, cancer, and ocular diseases. Understanding how endothelial cells differentiate and deciphering the cellular, molecular, and physical clues that drive blood vessel formation in the embryo may therefore provide means to develop therapies against vascular diseases in the adult. In this review, we present recent findings that identify new candidates controlling vascular system development.

A. Eichmann (✉)

Department of Cardiology, Yale University School of Medicine,
300 George Street, New Haven, CT 06510-3221, USA

Collège de France, Center for Interdisciplinary Research in Biology (CIRB),
Paris 75005, France

INSERM U1050, Paris 75005, France

CNRS UMR 7241, Paris 75005, France

e-mail: anne.eichmann@yale.edu

L. Pardanaud

Collège de France, Center for Interdisciplinary Research in Biology (CIRB),
Paris 75005, France

INSERM U1050, Paris 75005, France

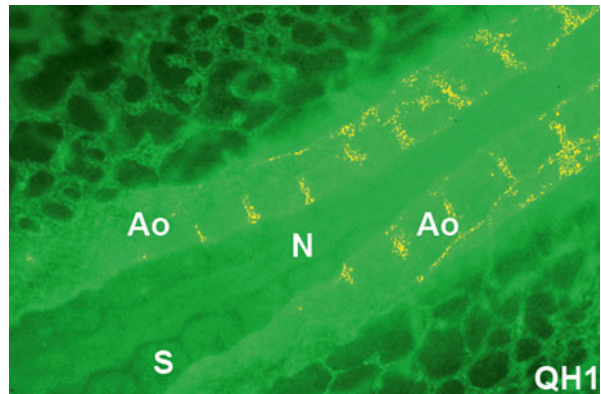
CNRS UMR 7241, Paris 75005, France

1.1 Introduction

The vertebrate vascular system forms a highly branched tubular network of arteries, capillaries, and veins that supplies oxygen and nutrients to all tissues and removes waste products. Blood, which carries oxygen, carbon dioxide, and metabolic products, is pumped from the heart through the arterial system into the tissue capillary bed, where exchanges occur, and is channeled back into the heart through the venous system. The capillary bed, which comprises the largest surface of the vascular system, is composed solely of endothelial cells (EC), occasionally associated with external pericytes. These simple capillary tubes are surrounded by a basement membrane. Larger vessels have additional layers constituting the vessel wall, which are composed of a muscular layer, the tunica media, and an outer connective tissue layer called the tunica adventitia containing vasa vasorum and nerves (Wheater et al. 1978). The size of the vessel wall is adapted to the vessel size and type. The lymphatic system drains extravasated fluid, the lymph, from the extracellular space and returns it into the venous circulation. The lymphatic vasculature is also essential for the immune defense, as lymph and any foreign material present in it, such as microbial antigens, are filtered through the chain of lymph nodes (Alitalo and Detmar 2012 for review). Dysfunction of the vascular and lymphatic systems cause numerous pathologies, including arteriosclerosis, cancer, and ocular diseases (Chung and Ferrara 2011; Libby et al. 2011; Potente et al. 2011; Weis and Cheresh 2011 for reviews). Understanding how blood vessels form may therefore provide means to treat vascular disease.

Blood vessels form during embryogenesis in two successive processes, called vasculogenesis and angiogenesis (Risau 1997; Coultas et al. 2005). The term vasculogenesis describes the *de novo* specification of endothelial precursor cells or angioblasts from the mesoderm. These newly formed cells coalesce into lumenized tubes of the primary vascular plexus, which consists of the central axial vessels (i.e., the dorsal aortae and the cardinal veins), as well as of a meshwork of homogenously sized capillaries (Fig. 1.1). Lumenization of forming capillary tubes has been

Fig. 1.1 Vasculogenesis in a 36 h-old quail embryo stained with QH1, an antibody specific for quail EC and hematopoietic cells. On this ventral view, EC have coalesced axially to form two lumenized aortae (Ao), while laterally, the vascular network appears undifferentiated. *N* Notochord, *S* somite



studied almost 100 years ago by observations of living chick embryos cultured on glass coverslips (Sabin 1920) and was thought to involve “liquefaction” of intracellular compartments of individual endothelial cells. Intracellular vacuolization drives lumen formation in cultured EC in 3D collagen gels (Davis et al. 2013) and in zebrafish intersegmental vessels (Kamei et al. 2006). However, other mechanisms contribute to lumen formation of multicellular vessels including the dorsal aorta and following anastomosis of adjacent blood vessels (Lenard et al. 2013; Xu and Cleaver 2011 for review).

The primary vascular plexus is established before the onset of heartbeat and is ready to receive the first circulatory output. This primitive tubular network subsequently expands via angiogenesis, i.e., sprouting and branching of preexisting vessels. Angiogenesis leads to remodeling of the primary vascular plexus into a highly branched hierarchical vascular tree, composed of arteries and veins. Intussusception, a process of vessel splitting by insertion of transcapillary pillars, leads to rapid expansion of the vascular surface and contributes to vascular remodeling in various tissues (De Spiegelaere et al. 2012 for review). Recruitment of mural cells (pericytes in medium-sized and smooth muscle cells in large vessels) around the endothelial layer completes the formation of a functional network. Later in development vascular networks acquire functional specializations depending on the tissue they have to irrigate, for example, brain vessels form a blood brain barrier, while liver vessels develop a fenestrated network.

1.2 Vasculogenesis

As the diffusion distance of oxygen is limited (100–200 μm), the vascular system in any organ and tissue has to be established early during development. EC differentiation is first observed during gastrulation, when cells invaginate through the primitive streak to form the mesoderm. Newly formed mesodermal cells soon organize into axial mesoderm (notochord), paraxial mesoderm (somites), intermediate mesoderm (kidney and gonads), and lateral plate mesoderm. The lateral plate mesoderm will split into two layers after the formation of the coelom: a dorsal sheet, the somatopleural mesoderm, and a ventral sheet, the splanchnopleural mesoderm. The dorsal sheet is in contact with the ectoderm and will form the body wall and limbs, while the ventral sheet is in contact with the endoderm and will form the visceral organs. The posterior part of the mesoderm, which occupies about half of the embryo during early gastrulation stages, will give rise to the extraembryonic mesoderm.

The first EC that form in the gastrulating embryo originate from lateral and posterior mesoderm (Murray 1932). Their specification is induced by soluble signals, as well as by specific transcription factors. Signaling proteins including fibroblast growth factor FGF-2 and bone morphogenetic proteins BMP 2 and 4, as well as Indian hedgehog (IHH), have been implicated in induction of endothelial differentiation from mesoderm (Marcelo et al. 2013a for review). However, since these

factors also regulate global mesodermal patterning, the precise nature of the soluble signal(s) required to induce endothelial specification remains unclear.

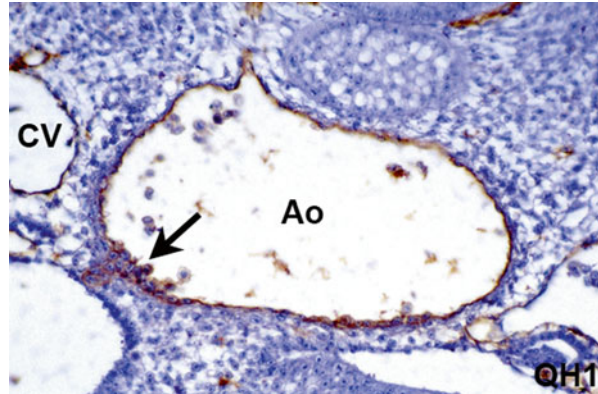
In contrast, transcription factors inducing endothelial specification have been identified (DeVal et al. 2008). Coexpression of the Forkhead protein FoxC2 and the Ets protein Etv2 induces ectopic expression of vascular genes in *Xenopus* embryos, and combinatorial knockdown of the orthologous genes in zebrafish embryos disrupts vascular development. FoxC2 and Etv2 synergistically trans-activate endothelial enhancers as Tie2, Tal1, NOTCH4, VE-CADHERIN/CDH5, and the vascular endothelial growth factor receptor 2 (VEGFR2).

Vascular endothelial growth factor (VEGF) and its receptor VEGFR2 are the most critical drivers of embryonic vessel formation (Olsson et al. 2006, for review). VEGF is expressed in spatial and temporal association with almost all physiological events of vascular formation in vivo. VEGFR2 expression is already observed at very early stages of development and subsequently becomes mainly restricted to EC of all types of blood vessels as well as lymphatic vessels (Chung and Ferrara 2011; Simons and Eichmann 2013 for reviews). Mice deficient in VEGFR2 (VEGFR2^{-/-}) die in utero between 8.5 and 9.5 days post-coitum, as a result of an early defect in the development of hematopoietic cells (HC) and EC. Yolk-sac blood islands were absent at 7.5 days, organized blood vessels could not be observed in the embryo or yolk sac at any stage, and hematopoietic progenitors were absent (Shalaby et al. 1995). VEGF-deficient mouse embryos also die at E8.5 to E9.5 and exhibit severe phenotypes similar to that of the VEGFR2^{-/-} mice; this phenotype was also observed in the VEGF^{+/-} embryos (Carmeliet et al. 1996; Ferrara et al. 1996). The lethality resulting from the loss of a single allele is indicative of a tight dose-dependent regulation of embryonic vessel development by VEGF. Taken together, the results described above confirm the major position of the VEGF/VEGFR2 system in vascular formation.

1.3 Hemangioblast

The simultaneous emergence of EC and HC in the blood islands led to the hypothesis that they were derived from a common precursor, the hemangioblast (Sabin 1920). VEGFR2 expression during successive stages of hemangioblast differentiation shows that gastrulating precursors as well as hemangioblastic aggregates are VEGFR2 positive, while in the differentiated islands, only the EC express this gene and no expression is detected in HC. These observations are compatible with the hypothesis that VEGFR2 labels a bipotent progenitor and that after lineage commitment, only one of the two daughter cells maintains expression of this gene. In support of this idea, isolated VEGFR2⁺ cells from posterior territories of chick embryos at the gastrulation stage cultured in semisolid medium in vitro differentiated to HC of different lineages. In the presence of VEGF, EC differentiation of the VEGFR2⁺ precursors was induced (Eichmann et al. 1997). These experiments showed that VEGFR2⁺ precursors could indeed give rise to EC as well as HC, consistent with

Fig. 1.2 A QH1⁺ cell cluster (arrow) emerges ventrally in the aorta (Ao): these cells will give rise to the definitive hematopoietic cell population. CV cardinal vein



the hypothesis that this receptor is expressed by a common precursor. However, at the single-cell level, an individual VEGFR2⁺ cell would either differentiate to an EC or an HC, but not both, precluding a direct demonstration of the existence of a “hemangioblast.” A recent study shows that *Xenopus* precursor cell blood islands do not normally differentiate into EC and provides evidence that commitment to the erythroid lineage induced by BMP limits development of bipotential precursors toward an endothelial fate (Myers and Krieg 2013).

The concept of an intraembryonic hemangioblast was postulated 30 years ago in the avian model when the aortic hemogenic endothelium (Fig. 1.2) was identified as the site of the definitive hematopoiesis (Le Douarin and Dieterlen-Lièvre 2013 for review). At this level, HC arise from the ventral endothelium and are released in the aortic lumen. In mammals, the emergence of definitive HC from the aortic endothelium was a subject of controversy, some findings showing that definitive HC can also come from the mesenchyme underlining the aorta. Recently, new technologies, as the use of conditional mutant mice carrying VE-cadherin-Cre gene with a ROSA26R Cre reporter line, permit to follow the progeny of the hemogenic endothelium (Zovein et al. 2008) and to demonstrate that, indeed in the mammalian system, much like the avian, amphibian, and zebrafish models, definitive HC emerge from the endothelium.

Concerning the molecular control of the hematopoietic emergence in the aorta, the transcription factor Runx1 is found to be crucial. Runx1 is required in the endothelium, and not in the hematopoietic compartment. When this transcription factor is specifically deleted either in EC or HC using a VE-cadherin-Cre and VAV-Cre tool, respectively, its activation is restricted to the endothelial compartment, thus showing evidence to the hypothesis of endothelial-derived hematopoiesis (Chen et al. 2009). Runx1 expression in ventral aortic EC is induced by the subaortic mesenchyme that collaborates with Notch dynamics to control aortic hematopoiesis (Richard et al. 2013). The hemogenic EC specification also requires retinoic acid (RA) as well as cell-cycle control of endothelium during embryogenesis; indeed, RA regulation requires c-Kit, Notch signaling, and p27-mediated cell-cycle control (Marcelo et al. 2013b).

Two different models are postulated to explain the aortic hematopoietic emergence. Observations in zebrafish suggest that the endothelium enters a hematopoietic transition, where an EC will round off the vessel wall and become an EC in the circulation, while in the mouse, HC appear to be in direct contact, and possible continuance, with the underlying endothelium, which postulates a possible asymmetric divisional process (Zape and Zovein 2011 for review).

In cultures derived from mouse ES cells, single-cell-derived colonies were found to be able to give rise to both EC and HC (Choi et al. 1998; Nishikawa et al. 1998; Schuh et al. 1999; Fehling et al. 2003; Huber et al. 2004; D'Souza et al. 2005). In these conditions, an endothelial-like phenotype stage is observed, then endothelial-specific markers disappear and hematopoietic antigens or factors are acquired as Runx1 and Scl transcription factors (Lancrin et al. 2009; Eilken et al. 2009). These results again support to the existence of a common precursor for both lineages. However, additional studies have shown that ES cell-derived VEGFR2+ cells could also give rise to smooth muscle cells in the presence of platelet-derived growth factor (PDGF) (Yamashita et al. 2000), indicating that rather than being strictly committed to only the EC and the HC lineages, these cells may be pluri- or multipotent progenitors. Cell-tracking experiments in zebrafish embryos have revealed bipotential hemangioblastic precursors present in the ventral mesoderm of gastrulation-stage embryos. Interestingly, the data suggest that hemangioblasts represent a distinct subpopulation of endothelial and hematopoietic precursors and that not all EC and HC are derived from common precursors in zebrafish (Vogeli et al. 2006) or mouse embryos (Ueno and Weissman 2006).

To conclude, while defined *in vitro*, the hemangioblast cannot be detected *in vivo* and remains an unsolved mystery (Nishikawa 2012 for review). Recently, the hemangioblast paradigm was discussed and its identity rethought: this entity may be a state of competence rather than a bipotential progenitor state that exists *in vivo* (Myers and Krieg 2013).

1.4 Remodeling of the Primary Capillary Plexus into Arteries and Veins

Vasculogenesis events described above lead to the formation of the primary vascular plexus, which is completed before the onset of heartbeat. Inside the embryo proper, one major vessel, the dorsal aorta, and numerous capillaries have differentiated, while a meshwork of homogenously sized capillaries is present in the yolk sac. After the onset of heartbeat and of blood flow, the primary plexus is rapidly remodeled into arteries and veins and a circulatory loop is established. Arteriovenous differentiation and flow-induced remodeling are critical for the embryo's survival, and indeed, many mouse mutants for genes involved in vascular development die during this remodeling phase (Coultas et al. 2005, for review).

Based on classic studies, it was believed that EC of the primary capillary plexus constitute a homogenous group of cells and that differentiation into arteries and

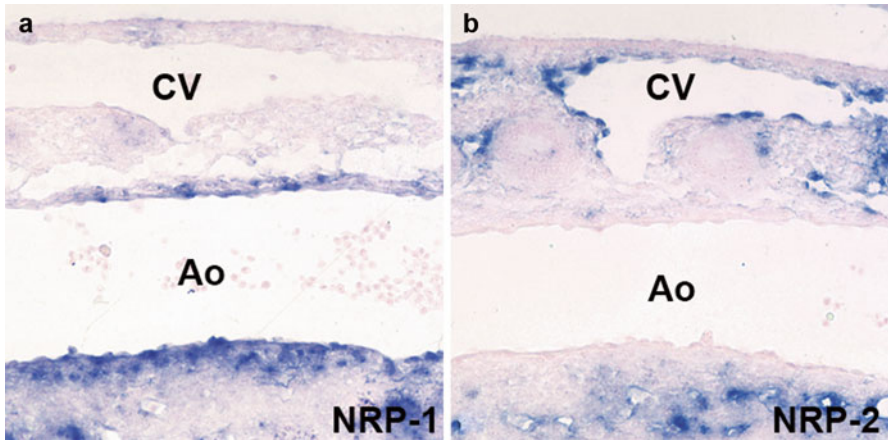


Fig. 1.3 Complementary expression of NRP-1 (a) and NRP-2 (b) mRNAs in arteries and veins respectively. On these two consecutive longitudinal sections of a 13-day-old mouse embryo, NRP-1 is only transcribed along the aorta (Ao) and is absent in the cardinal vein (CV), while NRP-2 messengers surround the cardinal vein but not the aorta

veins occurred due to the influence of hemodynamic forces (Thoma 1893). Over the last decade, however, several signaling molecules were discovered, which labeled arterial or venous EC from early developmental stages onward, prior to the onset of blood flow and the assembly of a vascular wall. Arterial EC in chick, mouse, and zebrafish selectively express members of the Notch pathway, including Notch receptors, ligands and downstream effectors, as well as ephrin-B2 and neuropilin-1 (NRP-1, Fig. 1.3a), which are thought to be induced downstream of Notch (Klein 2012; Swift and Weinstein 2009 for reviews). Other molecules are specifically expressed in the venous system, including the transcription factor COUPTFII and EphB4, the receptor for arterial ephrin-B2 (Swift and Weinstein 2009 for review). The neuropilin-2 (NRP-2, Fig. 1.3b) receptor is expressed by veins and, at later developmental stages, becomes restricted to lymphatic vessels in chick and mice (Herzog et al. 2001; Yuan et al. 2002). In chick embryos, NRP-1 and NRP-2 receptors are expressed on separate but mixed populations of cells in the yolk-sac blood islands. They become segregated prior to the onset of flow to arterial (NRP-1, posterior) and venous (NRP-2, anterior) poles of the embryo (Herzog et al. 2005). Based on these specific expression patterns and on lineage studies in the zebrafish embryo (Zhong et al. 2001), it was proposed that arterial and venous fates are genetically predetermined. A possible role for these signaling molecules in arteriovenous differentiation was suggested by the phenotypes of mouse and zebrafish mutants: ephrin-B2 and EphB4 knockout mouse embryos displayed arrested remodeling of the primary vascular plexus into arteries and veins during early development, leading to death around E9.5 (Wang et al. 1998; Adams et al. 1999; Gerety et al. 1999). Endothelial-specific NRP-1 mouse mutants failed to express arterial markers in the arteries of the embryonic dermis, although these vessels were positioned properly (Gu et al. 2003; Mukouyama et al. 2005).

Zebrafish mutant studies have shown a requirement for Notch signaling to induce arterial fate: inhibition of the Notch signaling pathway using a dominant negative form of suppressor of hairless (SuH), a downstream effector of Notch, leads to decreased expression of arterial markers and ectopic expression of venous markers in arteries (Lawson et al. 2001). Disruption of the Notch signaling pathway in mice also leads to significant vascular defects, ascribed to defective arteriovenous differentiation. Recently we showed that the ALK1 receptor cooperates with the Notch pathway to inhibit angiogenesis. Mechanistically, ALK1-dependent SMAD signaling synergizes with activated Notch in stalk cells to induce expression of the Notch targets HEY1 and HEY2, thereby repressing VEGF signaling and endothelial sprouting. Blocking Alk1 signaling during postnatal development in mice leads to retinal hypervascularization and the appearance of arteriovenous malformations; this direct link between ALK1 and Notch signaling during vascular morphogenesis may be relevant to the pathogenesis of hereditary hemorrhagic telangiectasia vascular lesions characterized by arteriovenous shunts (Larrivée et al. 2012).

Mutation of *dll4*, a Notch ligand selectively expressed in arteries, but not in veins, leads to defective development of the dorsal aorta and cardinal veins, with formation of arteriovenous shunts (Duarte et al. 2004; Gale et al. 2004; Krebs et al. 2004). Interestingly, these defects are already apparent when a single *dll4* allele is lost. Arterial markers such as ephrin-B2 are downregulated, and venous markers are ectopically expressed in the dorsal aorta of *dll4* mutants and of several other mutants of genes in the Notch pathway, including double mutants of Notch1 and Notch4, endothelial knockout of RBP, the SuH orthologue, and double mutants of the downstream targets Hes and Hey (Fischer et al. 2004; Krebs et al. 2004). Recently, we showed that Dll4-Notch signaling modulates embryonic arteriogenesis formation (collateral formation between arteries) and affects tissue perfusion by acting on arterial function and structure. Loss of Dll4 stimulates arteriogenesis and angiogenesis, but not in the context of ischemic diseases (Cristofaro et al. 2013). Among the upstream regulators of Dll4, nuclear factor κ B is a key regulator of adult and developmental arteriogenesis and collateral formation (Tirziu et al. 2012).

Conversely, endothelial-specific mutation of the nuclear receptor COUPTFII, expressed in veins, leads to ectopic activation of arterial markers in veins (You et al. 2005). Taken together, these studies suggest that the specification of angioblasts into arterial or venous lineages is genetically determined and occurs already before the onset of blood circulation. Failure in the specification of arterial and venous identities or in the establishment of the arteriovenous boundaries leads to vascular fusions and dysplasia.

1.5 Role of Hemodynamic Forces in Remodeling

The presence of blood flow is known to be essential for remodeling of the primary vascular plexus into arteries and veins to occur. Nearly 100 years ago, Chapman showed by surgically removing the heart of chick embryos before the onset of

circulation that the peripheral vasculature formed, but failed to remodel without blood flow and pressure (Chapman 1918). Remodeling of the vasculature also did not occur after surgical removal of the heart of young chicken embryos and incubation of the embryos in high levels of oxygen to remove the effects of hypoxia (Manner et al. 1995).

Using *in vivo* time-lapse imaging of developing chick embryos, we showed that small arterial capillary side branches disconnected from the main arterial network to reconnect to the venous plexus. These capillaries lose their arterial identity and start to express venous markers (Le Noble et al. 2004). The relatively high pressure in the arteries repels the expanding disconnected segments, which avoid the arteries and can only reconnect to lower pressure veins. Such avoidance of the arterial segments is also observed in the zebrafish parachordal vessel, which sprouts from the posterior cardinal vein and crosses the intersegmental artery without fusing to it (Isogai et al. 2003). Rerouting flow by artificially obstructing arteries results in perfusion of the arterial tree with blood of venous origin, which transforms the arteries into veins, both morphologically and genetically. Veins perfused with arterial blood can likewise transform them into arteries (le Noble et al. 2004).

Mechanical cues are also essential for vascular remodeling in the mouse. An experimental creation of low shear stress in the young embryo induces the inhibition of vascular remodeling and shows that the viscosity of the fluid, but not the erythroblasts themselves, is important for normal vascular remodeling (Lucitti et al. 2007).

Depending on the type of flow to which EC are exposed, EC behavior varies. Arteries are exposed to pulsatile blood flow and not constant velocity laminar flow. The pulsatile nature of blood flow progressively diminishes throughout the vasculature and disappears in the veins. By exposing human umbilical arterial EC to pulsatile but not to flow of constant velocity, the expression of arterial genes is induced. In contrast, human umbilical vein EC submitted to a pulsatile flow continue to express venous genes, but when exposed to a constant velocity flow, the expression of venous markers is increased (Buschmann et al. 2010).

While it is clear that there must be blood flow in an embryo for remodeling and arteriovenous differentiation to occur, the essential signals induced by flow begin to be identified. During the hematopoietic development, blood flow mediates the emergence of definitive stem cells by activating the nitric oxide pathway, a molecule that plays an important role in the cardiovascular system (Adamo et al. 2009; North et al. 2009, Fig. 1.4). *In vitro*, fluid shear stress, such as exerted by flowing blood, attenuates EC sprouting in a nitric oxide-dependent manner (Song and Munn 2011). The *klf2a* expression during the formation of cardiac valves depends on intracardiac hemodynamic forces (Vermot et al. 2009). Mechanical forces are also involved in the lymphatic system development and in diseases (Planas-Paz and Lammert 2013, for review).

Thus, blood flow carries nutrients, oxygen, and signaling molecules to the vessels and creates physical forces acting on the EC and cells of the forming vessel wall. Therefore, the initiation of blood flow brings many different signals to the embryo.

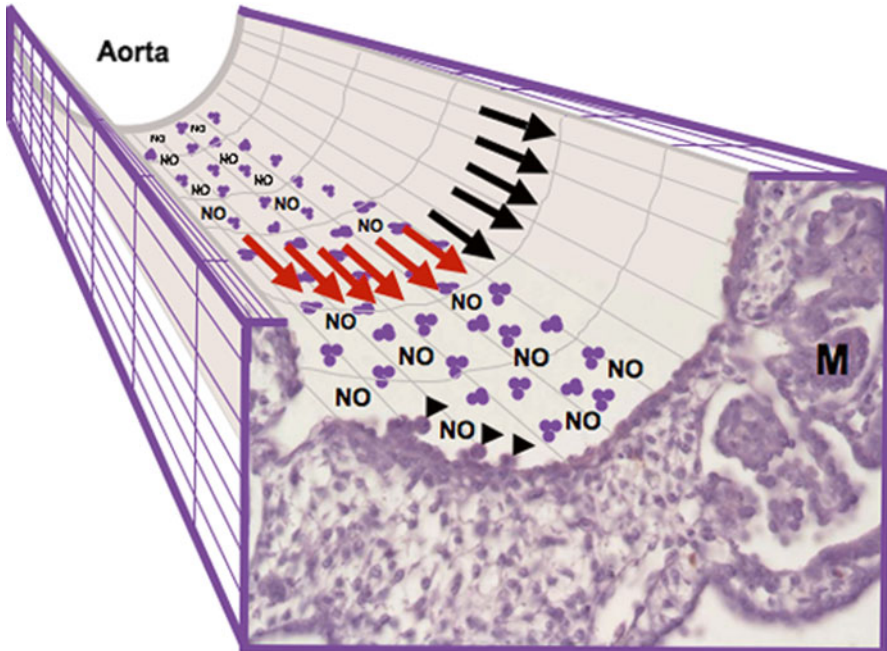
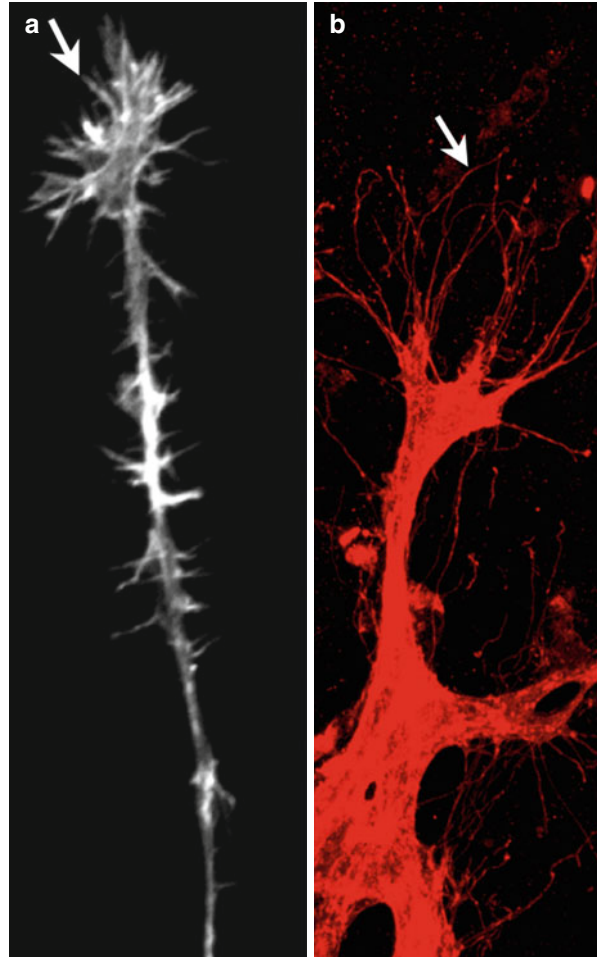


Fig. 1.4 Blood flow promotes development of HC. HC and the aortic endothelium sense blood-flow-induced shear stress. HC only bud from the ventral aortic endothelium (*arrowheads*), although shear stress is sensed throughout the aortic endothelium – ventrally (*red arrows*), laterally (*black arrows*), and dorsally (not shown). Nitric oxide (NO) producing-EC cooperates with shear stress to induce HC emergence. *M* mesonephros

1.6 Guidance of Capillaries by Endothelial Tip Cells

Despite the crucial role of hemodynamic forces in shaping vascular pattern, the gross vascular anatomy of developing mouse, chick, or zebrafish embryos is characterized by highly reproducible branching patterns, suggesting the existence of additional patterning mechanisms. Indeed, during development, blood vessels navigate along stereotyped paths toward their targets – similar to axonal growth cones (Eichmann and Thomas 2013 for review, Fig. 1.5). The mechanisms regulating vessel navigation remain incompletely understood. It was only recently discovered that specialized EC termed “tip cells” are located at the leading front of growing vessels. These tip cells respond to chemoattractant and repellent guidance cues that act over short or long range, similar to axonal growth cones. The existence of such endothelial “growth cones” highlights the anatomical similarities between the nervous and vascular systems (Eichmann and Thomas 2013 for review). Several receptors for axon guidance cues are expressing on growing vessels and were shown to regulate vessel pathfinding, including PlexinD1, Robo4, and the Netrin receptor UNC5B (Adams and Eichmann 2010 for review).

Fig. 1.5 Morphological similarities between an axonal growth cone (a) and a tip cell (b), especially at the level of filopodia (arrows) that permit the navigation of neural and endothelial cells



Endothelial tip cells extend numerous thin filopodia that explore their environment and regulate extension of capillary sprouts. Using multiphoton time-lapse imaging of transgenic $Tg(fli1:EGFP)^{y1}$ zebrafish, specifically expressing enhanced green fluorescent protein in EC, Isogai et al. (2003) documented the dynamic assembly of the intersegmental vessels (ISVs) in embryos. ISV formation is initiated by angioblast migration from the dorsal aorta into the intersomitic space (Swift and Weinstein 2009 for review). These angioblasts form sprouts that grow dorsally between the somites and the neural tube, tracking along vertical myotomal boundaries. The sprouts grow in a saltatory fashion with numerous active filopodia extending and retracting, particularly in the dorsal-most leading extension. ISVs are formed before perfusion, and filopodial movement of tip cells ceases as perfusion of these vessels is initiated. Endothelial tip cells are also seen at the front of the growing postnatal retinal vasculature in mice and in the early chick embryo yolk sac prior to the onset

of flow. Similar to zebrafish, tip cells are far less numerous in perfused vascular beds suggesting a correlation between flow and filopodial extension that remains to be fully explored. However, it is clear that tip cell guidance of growing blood vessels is a general phenomenon in vascular development that is currently being intensely studied in pathological angiogenesis as well.

Tip cells exhibit a characteristic gene expression profile that includes high levels of PDGFB, the Netrin receptor UNC5B, and the Notch ligand DLL4. Using transcriptome analysis of retinal EC or laser capture microdissected retina tip cells isolated from *DLL4*^{-/-} and wild-type mice, clusters of tip cell-enriched genes were identified (Table 1.1), encoding extracellular matrix degrading enzymes, basement membrane components, secreted molecules, and receptors. Secreted molecules endothelial-specific molecule 1, angiopoietin 2, and apelin bind to cognate receptors on endothelial stalk cells. Knockout mice and zebrafish morpholino knockdown of apelin showed delayed angiogenesis and reduced proliferation of stalk cells expressing the apelin receptor APJ. Thus, tip cells may regulate angiogenesis via matrix remodeling, production of basement membrane, and release of secreted molecules, some of which also regulating stalk cell behavior (Del Toro et al. 2010). CXCR4, a receptor for the chemokine stromal-cell derived factor-1 (SDF-1), was also identified as a tip cell-enriched gene; in the developing arteries, apparent coexpression of SDF-1 and CXCR4 suggests an autocrine and/or paracrine signaling mechanism (Strasser et al. 2010). Conversely, the synaptojanin-2 binding protein preferentially expressed in stalk cells, known to enhance DLL1 and DLL4 protein stability and to promote Notch signaling in EC, was recently identified as an inhibitor of tip cell formation, executing its functions predominately by promoting Delta-Notch signaling (Adam et al. 2013).

Tip and stalk cell positioning is coordinated by the interplay between VEGF and Notch signaling. VEGF promotes tip cell selection, while Notch inhibits tip cell formation and promotes the stalk cell phenotype. Notch activation decreases VEGFR2 and 3 levels but increases VEGFR1 (Eichmann and Simons 2012 for review). The VEGF-C receptor VEGFR3, which is critical for lymphangiogenesis, also contributes to coordinate tip cell sprouting and its activation occurs both in a ligand-dependent and ligand-independent manner (Tammela et al. 2011). Mechanistically, VEGFR-3 induces the expression of Notch target genes and restricts the formation of new tip cells (Tammela et al. 2011). In mouse retinas, at vessel branch points, macrophages produce VEGF-C (Tammela et al. 2011) and promote anastomosis of newly formed vessel branches (Fantin et al. 2010). In zebrafish EC, the VEGF-C/VEGFR3 pathway is activated by the Wnt signaling regulator R-spondin1 and promotes intersegmental vessel sprouting (Gore et al. 2011). In the mouse embryo, but not at postnatal stages, Wnt/ β catenin signaling can also influence angiogenic sprouting by upregulating Dll4-Notch pathway (Corada et al. 2010).

Vascular guidance receptors contribute to angiogenic sprouting by regulating the VEGF-Notch balance. PlexinD1 signaling is linked to VEGF signaling to modulate Notch activation and to regulate tip cell formation (Zygmunt et al. 2011). However, its effect depends on the cellular context. The Netrin receptor UNC5B also

Table 1.1 Examples of genes upregulated in retinal tip cells/stalk cells and verified by ISH or immunohistochemistry

Gene name	Protein name	Fold change	Method
<i>ESM1</i>	Endothelial-specific molecule 1	11.07	IHC/ISH
<i>Gcn1ll</i>	GCN1 general control of amino-acid synthesis 1-like 1 (yeast)	5.50	
<i>LAMB1</i>	Laminin β 1	5.44	
<i>PLAUR</i>	uPAR	5.23	ISH
–	MYST histone acetyltransferase (monocytic leukemia) 3	4.68	
<i>ANGPT2</i>	Angiopoietin-2	4.64	ISH
<i>NID1</i>	Nidogen-1	4.26	IHC/ISH
<i>ITGB1</i>	Integrin β 1	4.02	IHC
<i>Trp53i11</i>	Trp53 inducible protein 11	3.96	
<i>MGI:1930803</i>	Tescalcin	3.87	
<i>APLN</i>	Apelin	3.72	ISH
–	Sidekick cell adhesion molecule 1	3.65	
<i>March1</i>	Membrane-associated ring finger (C3HC4) 1	3.54	
–	Adrenomedullin	3.45	
<i>Syt16</i>	Synaptotagmin XVI	3.12	
<i>Sema3a</i>	Semaphorin 3A	3.00	
<i>Cxcr4</i>	Chemokine (C-X-C motif) receptor 4	2.81	
<i>Igf1</i>	Insulin-like growth factor 1	2.77	
<i>Vldlr</i>	Very low density lipoprotein receptor	2.63	
<i>Vegfa</i>	Vascular endothelial growth factor A	2.52	
<i>Il1b</i>	Interleukin1 b	2.42	
<i>Igf1</i>	Insulin-like growth factor 1	2.37	
–	Integrin α V	2.36	
–	DCC	2.33	
<i>Klf5</i>	Kruppel-like factor 5	2.32	
–	Integrin β 3	2.27	
<i>Bmp7</i>	Bone morphogenetic protein 7	2.21	
<i>Gls2</i>	Glutaminase 2 (liver, mitochondrial)	2.19	
<i>Ccnd2</i>	Cyclin D2	2.13	
<i>Tuba3</i>	Tubulin alpha 3	2.13	
<i>Igfbp3</i>	Insulin-like growth factor-binding protein 3	2.11	
<i>Igfbp4</i>	Insulin-like growth factor-binding protein 4	2.09	
<i>Sema3f</i>	Semaphorin 3F	2.04	
<i>Hck</i>	Hemopoietic cell kinase	2.02	

Adapted from Del Toro et al. (2010) and Strasser et al. (2010)

modulates VEGF-induced neovascularization. UNC5B interacts with a number of binding partners in addition to Netrin, including the vascular guidance receptor Robo4. Robo4-UNC5B signaling counteracts VEGF-driven angiogenesis and vascular permeability, a mechanism driven at least in part by competition for downstream activating targets including Src family kinases (Koch et al. 2011).

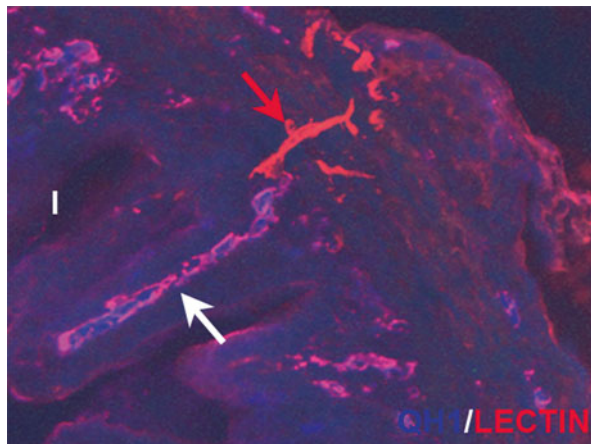
1.7 Circulating Endothelial Cells in the Embryo

In the adult, once the definitive vascular network is established, EC remain essentially quiescent with neovascularization only occurring during physiological or pathological events. However, the existence of adult circulating EC (CEC) is now well established (Urbich and Dimmeler 2004, for review). These cells could have important potential therapeutic applications, as their administration could stimulate blood vessel growth in conditions of hypovascularization (hind limb ischemia, myocardial infarction, stroke, wound healing). Genetic manipulation of CEC could also allow to inhibit blood vessel growth in conditions of hypervascularization (diabetic retinopathy and tumorigenesis).

The origin of CEC was recently investigated in the avian embryo, using the quail-chick parabiosis model in which a quail embryo is added into a chick egg during the second day of development (Pardanaud and Eichmann 2006). From the eighth day, the chorioallantoic membranes (CAM) of the two embryos fused, vascular anastomoses were established, and cells could travel from one species to the other. CEC colonizing the chick embryos could be recognized using the QH1 monoclonal antibody specific for quail cells (Pardanaud et al. 1987). The emergence of CEC was observed early in ontogeny, at day 2 of development, long before the formation of the bone marrow. CEC could colonize all tissues of the chick, but their number always remained low. However, CEC could efficiently be mobilized by wounding or grafting of an organ on the chick CAM, resulting in a significant participation of QH1⁺ CEC to the endothelial network of the grafted organs. However, only a minority of CEC ($\pm 5\%$) were integrated in chick endothelia, while the majority were located interstitially as isolated cells or integrated into chick endothelial cords. It is possible that these cells serve as a structural bridging role or alternatively that they secrete paracrine growth factors. Interestingly, when a chick CAM from a parabiosis was stimulated with VEGF during 2 days, while the vascular density was upgraded by comparison with PBS-treated CAM, the mobilization of QH1⁺ CEC did not occur. Indeed, VEGF-stimulated CEC seemed to act indirectly on angiogenesis via the recruitment of bone marrow-derived circulating cells (Grunewald et al. 2006; Zentilin et al. 2006). In our model, CEC appeared to participate preferentially to angiogenic responses related to ischemia rather than to sprouting angiogenesis.

To define the territory generating CEC in the embryo, we constructed yolk-sac chimera model in which the embryonic territory of quail/chick species is replaced by its equivalent of the other species directly in the egg. Using QH1 and specific endothelial markers, we identify the yolk sac as the source of CEC. These cells integrate vessels but remained scarce. In older developmental stages, CEC are identified in the bone marrow, but their number does not dramatically increase (Pardanaud and Eichmann 2011, Fig. 1.6). In our model, the embryonic territory does not produce CEC, while another study using time-lapse videomicroscopy on transgenic quail expressing GFP in EC nuclei detected a few (Cui et al. 2013). We also showed that the allantois, an extraembryonic appendage rich in vessels and known to produce hematopoietic stem cells, is also able to produce CEC (Pardanaud and Eichmann 2011).

Fig. 1.6 Transverse section at the level of the intestine (I) in a yolk-sac chimera in which a quail territory developed on a chick yolk sac. In this condition, CEC coming from the chick yolk sac colonizes the quail organs and can integrate vessels. On the picture, quail vessels are stained by both QH1 and a lectin (*white arrow*), while chick vessel-forming CEC are only identified with the lectin (*red arrow*)



If the existence of CEC is demonstrated, the high hopes placed in their therapeutic use few years ago are being questioned by recent clinical studies, which have shown at best modestly encouraging results (Pearson 2009; Pasquier and Dias 2010, for reviews).

1.8 Perspectives

Research carried out over 15 years has provided major insights into the mechanisms regulating the emergence of endothelial progenitors from the mesoderm, their coalescence into the primary vascular system, and the remodeling of this system into arteries and veins. The molecules implicated in these different developmental processes are also essential for the maintenance of the adult vascular system. Elucidation of the precise function and interaction of the different molecular players will thus certainly lead to the development of novel treatments for vascular disorders.

The observation that arteriovenous differentiation is a flow-driven highly dynamic process that exhibits a high degree of EC plasticity is an important finding, and understanding the regulation of EC plasticity with respect to vessel identity has obvious important implications for the use of veins in coronary bypass surgery, restenoses, and therapeutic arteriogenesis.

A particularly interesting aspect of recent research carried out on the vascular system is the identification of neural guidance receptors on blood vessels, in particular on endothelial tip cells. Identification of factors able to “guide” developing blood vessels has obvious implications for pro- and antiangiogenic therapies that remain to be fully explored in the future. The close relation between the nervous and the vascular system is moreover highlighted by the finding that the patterning of developing arteries in the limb skin of mouse embryos has been shown to depend on

interactions with nerves (Mukouyama et al. 2002) that produce CXCL12 and VEGF-A (Li et al. 2013). In the avian embryos, neurovascular congruence is also observed in limbs (Bates et al. 2002, 2003; Bentley and Poole 2009). Future studies will be directed at exploring the precise interactions between blood vessels and nerves during development as well as in pathologies; indeed, a recent study reports that autonomic nerve development contributes to prostate cancer progression (Magnon et al. 2013).

References

- Adam MG, Berger C, Feldner A, Yang WJ, Wüsthube-Lausch J, Herberich SE, Pinder M, Gesierich S, Hammes HP, Augustin HG, Fischer A (2013) Synaptotagmin-2 binding protein stabilizes the Notch ligands DLL1 and DLL4 and inhibits sprouting angiogenesis. *Circ Res* 113(11):1206–1218
- Adamo L, Naveiras O, Wenzel PL, McKinney-Freeman S, Mack PJ, Gracia-Sancho J, Suchy-Dacey A, Yoshimoto M, Lensch MW, Yoder MC, García-Cardena G, Daley GQ (2009) Biomechanical forces promote embryonic haematopoiesis. *Nature* 459(7250):1131–1135
- Adams RH, Eichmann A (2010) Axon guidance molecules in vascular patterning. *Cold Spring Harb Perspect Biol* 2(5):a001875
- Adams RH, Wilkinson GA, Weiss C, Diella F, Gale NW, Deutsch U, Risau W, Klein R (1999) Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. *Genes Dev* 13(3):295–306
- Alitalo A, Detmar M (2012) Interaction of tumor cells and lymphatic vessels in cancer progression. *Oncogene* 31(42):4499–4508
- Bates D, Taylor GI, Newgreen DF (2002) The pattern of neurovascular development in the forelimb of the quail embryo. *Dev Biol* 249(2):300–320
- Bates D, Taylor GI, Minichiello J, Farlie P, Cichowitz A, Watson N, Klagsbrun M, Mamluk R, Newgreen DF (2003) Neurovascular congruence results from a shared patterning mechanism that utilizes Semaphorin3A and Neuropilin-1. *Dev Biol* 255(1):77–98
- Bentley MT, Poole TJ (2009) Neurovascular anatomy of the embryonic quail hindlimb. *Anat Rec* 292(10):1559–1568
- Buschmann I, Pries A, Styp-Rekowska B, Hillmeister P, Loufrani L, Henrion D, Shi Y, Duelsner A, Hoefler I, Gatzke N, Wang H, Lehmann K, Ulm L, Ritter Z, Hauff P, Hlushchuk R, Djonov V, van Veen T, le Noble F (2010) Pulsatile shear and Gja5 modulate arterial identity and remodeling events during flow-driven arteriogenesis. *Development* 137(13):2187–2196
- Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoek A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380(6573):435–439
- Chapman WB (1918) The effect of the heart-beat upon the development of the vascular system in the chick. *Am J Anat* 23:175–203
- Chen MJ, Yokomizo T, Zeigler BM, Dzierzak E, Speck NA (2009) Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. *Nature* 457:887–891
- Choi K, Kennedy M, Kazarov A, Papadimitriou JC, Keller G (1998) A common precursor for hematopoietic and endothelial cells. *Development* 125(4):725–732
- Chung AS, Ferrara N (2011) Developmental and pathological angiogenesis. *Annu Rev Cell Dev Biol* 27:563–584
- Corada M, Nyqvist D, Orsenigo F, Caprini A, Giampietro C, Taketo MM, Iruela-Arispe ML, Adams RH, Dejana E (2010) The Wnt/beta-catenin pathway modulates vascular remodeling and specification by upregulating Dll4/Notch signaling. *Dev Cell* 18(6):938–949

- Coultas L, Chawengsaksophak K, Rossant J (2005) Endothelial cells and VEGF in vascular development. *Nature* 438(7070):937–945
- Cristofaro B, Shi Y, Faria M, Suchting S, Leroyer AS, Trindade A, Duarte A, Zovein AC, Iruela-Arispe ML, Nih LR, Kubis N, Henrion D, Loufrani L, Todiras M, Schleifenbaum J, Gollasch M, Zhuang ZW, Simons M, Eichmann A, le Noble F (2013) Dll4-Notch signaling determines the formation of native arterial collateral networks and arterial function in mouse ischemia models. *Development* 140(8):1720–1729
- Cui C, Filla MB, Jones EA, Lansford R, Chevront T, Al-Roubaie S, Rongish BJ, Little CD (2013) Embryogenesis of the first circulating endothelial cells. *PLoS One* 8(5):e60841
- Davis GE, Kim DJ, Meng CX, Norden PR, Speichinger KR, Davis MT, Smith AO, Bowers SL, Stratman AN (2013) Control of vascular tube morphogenesis and maturation in 3D extracellular matrices by endothelial cells and pericytes. *Methods Mol Biol* 1066:17–28
- De Spiegelaere W, Casteleyn C, Van den Broeck W, Plendl J, Bahramsoltani M, Simoens P, Djonov V, Cornillie P (2012) Intussusceptive angiogenesis: a biologically relevant form of angiogenesis. *J Vasc Res* 49(5):390–404
- del Toro R, Prahst C, Mathivet T, Siegfried G, Kaminker JS, Larrivée B, Bréant C, Duarte A, Takakura N, Fukamizu A, Penninger J, Eichmann A (2010) Identification and functional analysis of endothelial tip cell-enriched genes. *Blood* 116(19):4025–4033
- De Val S, Chi NC, Meadows SM, Minovitsky S, Anderson JP, Harris IS, Ehlers ML, Agarwal P, Visel A, Xu SM, Pennacchio LA, Dubchak I, Krieg PA, Stainier DY, Black BL (2008) Combinatorial regulation of endothelial gene expression by ets and forkhead transcription factors. *Cell* 135(6):1053–1064
- D'Souza SL, Elefanty AG, Keller G (2005) SCL/Tal-1 is essential for hematopoietic commitment of the hemangioblast but not for its development. *Blood* 105(10):3862–3870
- Duarte A, Hirashima M, Benedito R, Trindade A, Diniz P, Bekman E, Costa L, Henrique D, Rossant J (2004) Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes Dev* 18(20):2474–2478
- Eichmann A, Simons M (2012) VEGF signaling inside vascular endothelial cells and beyond. *Curr Opin Cell Biol* 24(2):188–193
- Eichmann A, Thomas JL (2013) Molecular parallels between neural and vascular development. *Cold Spring Harb Perspect Med* 3(1):a006551
- Eichmann A, Corbel C, Nataf V, Vaigot P, Bréant C, Le Douarin NM (1997) Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2. *Proc Natl Acad Sci U S A* 94(10):5141–5146
- Eilken HM, Nishikawa S-I, Schroeder T (2009) Continuous single-cell imaging of blood generation from haemogenic endothelium. *Nature* 457:896–900
- Fantin A, Vieira JM, Gestri G, Denti L, Schwarz Q, Prykhozij S, Peri F, Wilson SW, Ruhrberg C (2010) Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction. *Blood* 116(5):829–840
- Fehling HJ, Lacaud G, Kubo A, Kennedy M, Robertson S, Keller G, Kouskoff V (2003) Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation. *Development* 130(17):4217–4227
- Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 380(6573):439–442
- Fischer A, Schumacher N, Maier M, Sendtner M, Gessler M (2004) The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. *Genes Dev* 18(8):901–911
- Gale NW, Dominguez MG, Noguera I, Pan L, Hughes V, Valenzuela DM, Murphy AJ, Adams NC, Lin HC, Holash J, Thurston G, Yancopoulos GD (2004) Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. *Proc Natl Acad Sci U S A* 101(45):15949–15954
- Gerety SS, Wang HU, Chen ZF, Anderson DJ (1999) Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. *Mol Cell* 4(3):403–414

- Gore AV, Swift MR, Cha YR, Lo B, McKinney MC, Li W, Castranova D, Davis A, Mukoyama YS, Weinstein BM (2011) Rspo1/Wnt signaling promotes angiogenesis via Vegfc/Vegfr3. *Development* 138(22):4875–4886
- Grunewald M, Avraham I, Dor Y, Bachar-Lustig E, Itin A, Jung S, Chimenti S, Landsman L, Abramovitch R, Keshet E (2006) VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. *Cell* 124(1):175–189
- Gu C, Rodriguez ER, Reimert DV, Shu T, Fritsch B, Richards LJ, Kolodkin AL, Ginty DD (2003) Neuropilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. *Dev Cell* 5(1):45–57
- Herzog Y, Kalcheim C, Kahane N, Reshef R, Neufeld G (2001) Differential expression of neuropilin-1 and neuropilin-2 in arteries and veins. *Mech Dev* 109(1):115–119
- Herzog Y, Guttmann-Raviv N, Neufeld G (2005) Segregation of arterial and venous markers in subpopulations of blood islands before vessel formation. *Dev Dyn* 232(4):1047–1055
- Huber TL, Kouskoff V, Fehling HJ, Palis J, Keller G (2004) Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. *Nature* 432(7017):625–630
- Isogai S, Lawson ND, Torrealday S, Horiguchi M, Weinstein BM (2003) Angiogenic network formation in the developing vertebrate trunk. *Development* 130(21):5281–5290
- Kamei M, Saunders WB, Bayless KJ, Dye L, Davis GE, Weinstein BM (2006) Endothelial tubes assemble from intracellular vacuoles in vivo. *Nature* 442(7101):453–456
- Klein R (2012) Eph/ephrin signalling during development. *Development* 139(22):4105–4109
- Koch AW, Mathivet T, Larrivée B, Tong RK, Kowalski J, Pibouin-Fragner L, Bouvrée K, Stawicki S, Nicholes K, Rathore N, Scales SJ, Luis E, del Toro R, Freitas C, Bréant C, Michaud A, Corvol P, Thomas JL, Wu Y, Peale F, Watts RJ, Tessier-Lavigne M, Bagri A, Eichmann A (2011) Robo4 maintains vessel integrity and inhibits angiogenesis by interacting with UNC5B. *Dev Cell* 20(1):33–46
- Krebs LT, Shutter JR, Tanigaki K, Honjo T, Stark KL, Gridley T (2004) Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. *Genes Dev* 18(20):2469–2473
- Lancrin C, Sroczynska P, Stephenson C, Allen T, Kouskoff V, Lacaud G (2009) The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. *Nature* 457:892–895
- Larrivée B, Praht C, Gordon E, del Toro R, Mathivet T, Duarte A, Simons M, Eichmann A (2012) ALK1 signaling inhibits angiogenesis by cooperating with the Notch pathway. *Dev Cell* 22(3):489–500
- Lawson ND, Scheer N, Pham VN, Kim CH, Chitnis AB, Campos-Ortega JA, Weinstein BM (2001) Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development* 128(19):3675–3683
- Le Douarin NM, Dieterlen-Lièvre F (2013) How studies on the avian embryo have opened new avenues in the understanding of development: a view about the neural and hematopoietic systems. *Develop Growth Differ* 55:1–14
- le Noble F, Moyon D, Pardanaud L, Yuan L, Djonov V, Matthijsen R, Bréant C, Fleury V, Eichmann A (2004) Flow regulates arterial-venous differentiation in the chick embryo yolk sac. *Development* 131(2):361–375
- Lenard A, Ellertsdottir E, Herwig L, Krudewig A, Sauter L, Belting HG, Affolter M (2013) In vivo analysis reveals a highly stereotypic morphogenetic pathway of vascular anastomosis. *Dev Cell* 25(5):492–506
- Li W, Kohara H, Uchida Y, James JM, Soneji K, Cronshaw DG, Zou YR, Nagasawa T, Mukoyama YS (2013) Peripheral nerve-derived CXCL12 and VEGF-A regulate the patterning of arterial vessel branching in developing limb skin. *Dev Cell* 24(4):359–371
- Libby P, Ridker PM, Hansson GK (2011) Progress and challenges in translating the biology of atherosclerosis. *Nature* 473(7347):317–325
- Lucitti JL, Jones EA, Huang C, Chen J, Fraser SE, Dickinson ME (2007) Vascular remodeling of the mouse yolk sac requires hemodynamic force. *Development* 134(18):3317–3326

- Magnon C, Hall SJ, Lin J, Xue X, Gerber L, Freedland SJ, Frenette PS (2013) Autonomic nerve development contributes to prostate cancer progression. *Science* 341(6142)
- Manner J, Seidl W, Steding G (1995) Formation of the cervical flexure: an experimental study on chick embryos. *Acta Anat* 152:1–10
- Marcelo KL, Goldie LC, Hirschi KK (2013a) Regulation of endothelial cell differentiation and specification. *Circ Res* 112(9):1272–1287
- Marcelo KL, Sills TM, Coskun S, Vasavada H, Sanglikar S, Goldie LC, Hirschi KK (2013b) Hemogenic endothelial cell specification requires c-Kit, Notch signaling, and p27-mediated cell-cycle control. *Dev Cell* 27(5):504–515
- Mukoyama YS, Shin D, Britsch S, Taniguchi M, Anderson DJ (2002) Sensory nerves determine the pattern of arterial differentiation and blood vessel branching in the skin. *Cell* 109(6):693–705
- Mukoyama YS, Gerber HP, Ferrara N, Gu C, Anderson DJ (2005) Peripheral nerve-derived VEGF promotes arterial differentiation via neuropilin 1-mediated positive feedback. *Development* 132(5):941–952
- Murray PDF (1932) The development “in vitro” of blood of the early chick embryo. *Proc Roy Soc Lond B* 111:497–521
- Myers CT, Krieg PA (2013) BMP-mediated specification of the erythroid lineage suppresses endothelial development in blood island precursors. *Blood*, 7 Oct 2013 [Epub ahead of print]
- Nishikawa S (2012) Hemangioblast: an in vitro phantom. *Wiley Interdiscip Rev Dev Biol* 1(4):603–608
- Nishikawa SI, Nishikawa S, Hirashima M, Matsuyoshi N, Kodama H (1998) Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. *Development* 125(9):1747–1757
- North TE, Goessling W, Peeters M, Li P, Ceol C, Lord AM, Weber GJ, Harris J, Cutting CC, Huang P, Dzierzak E, Zon LI (2009) Hematopoietic stem cell development is dependent on blood flow. *Cell* 137(4):736–748
- Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L (2006) VEGF receptor signalling – in control of vascular function. *Nat Rev Mol Cell Biol* 7(5):359–371
- Pardanaud L, Eichmann A (2006) Identification, emergence and mobilization of circulating endothelial cells or progenitors in the embryo. *Development* 133(13):2527–2537
- Pardanaud L, Eichmann A (2011) Extraembryonic origin of circulating endothelial cells. *PLoS One* 6(10):e25889
- Pardanaud L, Altmann C, Kitos P, Dieterlen-Lièvre F, Buck CA (1987) Vasculogenesis in the early quail blastodisc as studied with a monoclonal antibody recognizing endothelial cells. *Development* 100(2):339–349
- Pasquier E, Dias S (2010) Endothelial progenitor cells: hope beyond controversy. *Curr Cancer Drug Targets* 10(8):914–921
- Pearson JD (2009) Endothelial progenitor cells – hype or hope? *J Thromb Haemost* 7(2): 255–262
- Planas-Paz L, Lammert E (2013) Mechanical forces in lymphatic vascular development and disease. *Cell Mol Life Sci* 70(22):4341–4354
- Potente M, Gerhardt H, Carmeliet P (2011) Basic and therapeutic aspects of angiogenesis. *Cell* 146(6):873–887
- Richard C, Drevon C, Canto PY, Villain G, Bollérot K, Lempereur A, Teillet MA, Vincent C, Rosselló Castillo C, Torres M, Piwarzyk E, Speck NA, Souyri M, Jaffredo T (2013) Endothelium-mesenchymal interaction controls runx1 expression and modulates the Notch pathway to initiate aortic hematopoiesis. *Dev Cell* 24(6):600–611
- Risau W (1997) Mechanisms of angiogenesis. *Nature* 386(6626):671–674
- Sabin FR (1920) Studies on the origin of blood-vessels and of red blood corpuscles as seen in the living blastoderm of chicks during the second day of incubation. *Carnegie Contrib Embryol* 272:214–262

- Schuh AC, Faloon P, Hu QL, Bhimani M, Choi K (1999) In vitro hematopoietic and endothelial potential of flk-1(-/-) embryonic stem cells and embryos. *Proc Natl Acad Sci U S A* 96(5):2159–2164
- Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376(6535):62–66
- Simons M, Eichmann A (2013) Lymphatics are in my veins. *Science* 341(6146):622–624
- Song JW, Munn LL (2011) Fluid forces control endothelial sprouting. *Proc Natl Acad Sci U S A* 108(37):15342–15347
- Strasser GA, Kaminker JS, Tessier-Lavigne M (2010) Microarray analysis of retinal endothelial tip cells identifies CXCR4 as a mediator of tip cell morphology and branching. *Blood* 115(24):5102–5110
- Swift MR, Weinstein BM (2009) Arterial-venous specification during development. *Circ Res* 104(5):576–588
- Tammela T, Zarkada G, Nurmi H, Jakobsson L, Heinolainen K, Tvorogov D, Zheng W, Franco CA, Murtomäki A, Aranda E, Miura N, Ylä-Herttua S, Fruttiger M, Mäkinen T, Eichmann A, Pollard JW, Gerhardt H, Alitalo K (2011) VEGFR-3 controls tip to stalk conversion at vessel fusion sites by reinforcing Notch signalling. *Nat Cell Biol* 13(10):1202–1213
- Thoma R (1893) Untersuchungen über die Histogenese und Histomechanik des Gefässsystems. Ferdinand Enke, Stuttgart
- Tirziu D, Jaba IM, Yu P, Larrivée B, Coon BG, Cristofaro B, Zhuang ZW, Lanahan AA, Schwartz MA, Eichmann A, Simons M (2012) Endothelial nuclear factor- κ B-dependent regulation of arteriogenesis and branching. *Circulation* 126(22):2589–2600
- Ueno H, Weissman IL (2006) Clonal analysis of mouse development reveals a polyclonal origin for yolk sac blood islands. *Dev Cell* 11(4):519–533
- Urbich C, Dimmeler S (2004) Endothelial progenitor cells functional characterization. *Trends Cardiovasc Med* 14(8):318–322
- Vermot J, Forouhar AS, Liebling M, Wu D, Plummer D, Gharib M, Fraser SE (2009) Reversing blood flows act through *klf2a* to ensure normal valvulogenesis in the developing heart. *PLoS Biol* 7(11):e1000246
- Vogeli KM, Jin SW, Martin GR, Stainier DY (2006) A common progenitor for haematopoietic and endothelial lineages in the zebrafish gastrula. *Nature* 443(7109):337–339
- Wang HU, Chen ZF, Anderson DJ (1998) Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* 93(5):741–753
- Weis SM, Cheresh DA (2011) Tumor angiogenesis: molecular pathways and therapeutic targets. *Nat Med* 17(11):1359–1370
- Wheater PR, Burkitt HG, Daniels VG (1978) Functional histology: a text and colour atlas. Churchill Livingstone, Edinburgh
- Xu K, Cleaver O (2011) Tubulogenesis during blood vessel formation. *Semin Cell Dev Biol* 22(9):993–1004
- Yamashita J, Itoh H, Hirashima M, Ogawa M, Nishikawa S, Yurugi T, Naito M, Nakao K (2000) Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature* 408(6808):92–96
- You LR, Lin FJ, Lee CT, DeMayo FJ, Tsai MJ, Tsai SY (2005) Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. *Nature* 435(7038):98–104
- Yuan L, Moyon D, Pardanaud L, Bréant C, Karkkainen MJ, Alitalo K, Eichmann A (2002) Abnormal lymphatic vessel development in neuropilin 2 mutant mice. *Development* 129(20):4797–4806
- Zape JP, Zovein AC (2011) Hemogenic endothelium: origins, regulation, and implications for vascular biology. *Semin Cell Dev Biol* 22(9):1036–1047

- Zentilin L, Tafuro S, Zacchigna S, Arsic N, Pattarini L, Sinigaglia M, Giacca M (2006) Bone marrow mononuclear cells are recruited to the sites of VEGF-induced neovascularization but are not incorporated into the newly formed vessels. *Blood* 107(9):3546–3554
- Zhong TP, Childs S, Leu JP, Fishman MC (2001) Gridlock signalling pathway fashions the first embryonic artery. *Nature* 414(6860):216–220
- Zovein AC, Hofmann JJ, Lynch M, French WJ, Turlo KA, Yang Y, Becker MS, Zanetta L, Dejana E, Gasson JC, Tallquist MD, Iruela-Arispe ML (2008) Fate tracing reveals the endothelial origin of hematopoietic stem cells. *Cell Stem Cell* 3:625–636
- Zygmunt T, Gay CM, Blondelle J, Singh MK, Flaherty KM, Means PC, Herwig L, Krudewig A, Belting HG, Affolter M, Epstein JA, Torres-Vázquez J (2011) Semaphorin-PlexinD1 signaling limits angiogenic potential via the VEGF decoy receptor sFlt1. *Dev Cell* 21(2):301–314