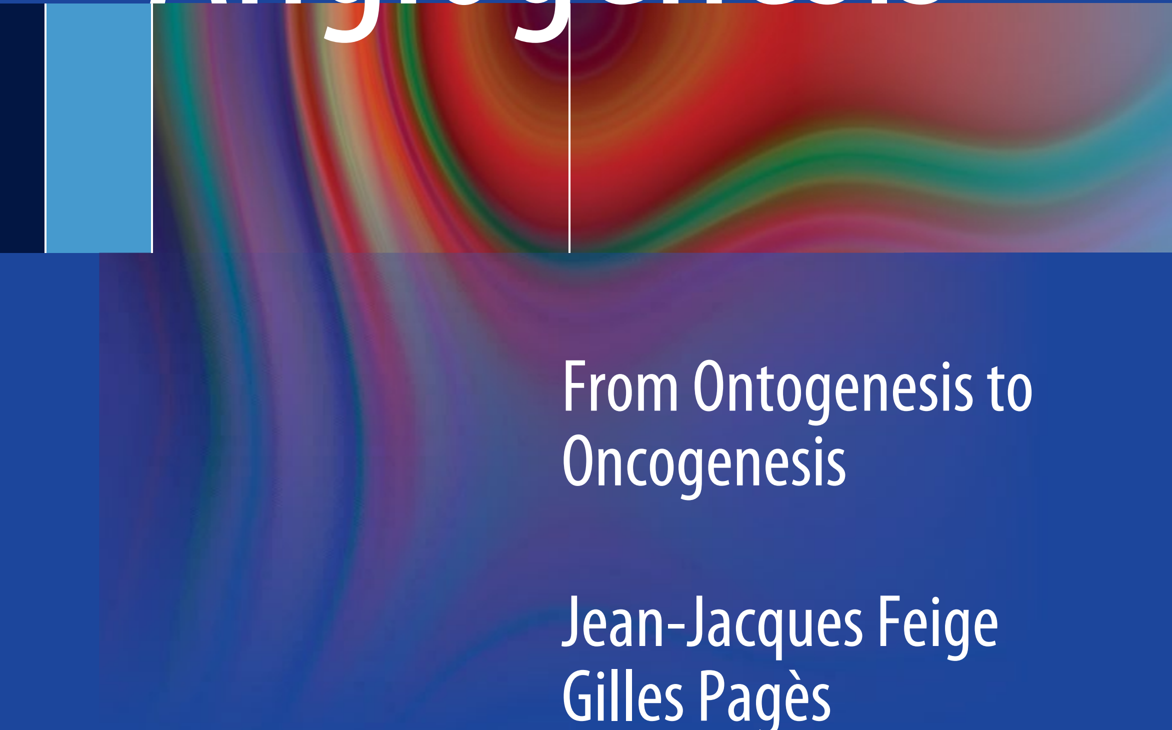
Molecular Mechanisms of Angiogenesis



From Ontogenesis to Oncogenesis

Jean-Jacques Feige
Gilles Pagès
Fabrice Soncin
Editors

Springer

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From Ontogenesis to Oncogenesis

Société Française
d'Angiogenèse

Springer

Editors
Jean-Jacques Feige
UMR 1036, Biologie du Cancer et de l'Infection
Institut de Recherches en Technologies et Sciences pour le Vivant
Université Grenoble-Alpes
Grenoble
France

Fabrice Soncin
CNRS UMR8161, Institut Pasteur de Lille
Institut de Biologie de Lille
Lille
France

Gilles Pagès
Institute for Research on Cancer and aging of Nice (IRCAN)
University of Nice Sophia Antipolis
UMR CNRS 7284/U INSERM 1081
Nice
France

ISBN 978-2-8178-0465-1 ISBN 978-2-8178-0466-8 (eBook)
DOI 10.1007/978-2-8178-0466-8
Springer Paris Heidelberg New York Dordrecht London

Library of Congress Control Number: 2014940751

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Angiogenesis: An Ever-Challenging Research Field

The field of angiogenesis has emerged in the mid-1940s with the first observations of the vascular reaction to wounds and tumor transplants in mice (Algire et al. 1945). In 1968, two independent teams demonstrated the initiating role of tumor cells in the growth of capillaries within tumors (Ehrmann and Knoth 1968; Rijhsinghani et al. 1968). Pr. Judah Folkman then paved the way for future major clinical developments by postulating that tumors, as any other tissue, need oxygen and nutrients to grow and spread. Hence, based on his observation of human tumor samples as a surgeon, he assumed that tumors lie in a dormant phase until they become vascularized and further progress (Folkman 1971). Indeed, following the idea that tumor development requires blood vessels, the search for the so-called tumor angiogenic factors using in vitro and in vivo angiogenic assays and the emergence of cell culture conditions suitable for the growth of endothelial cells allowed the initial discovery of a dozen of angiogenic factors, growth factors for the most part, and, reciprocally, of natural or synthetic anti-angiogenic compounds. The most important of these factors, vascular endothelial growth/permeability factor (VEGF/VPF), was discovered by several independent teams in 1989 (Connolly et al. 1989; Ferrara and Henzel 1989; Keck et al. 1989; Leung et al. 1989; Plouet et al. 1989). For many years afterward, research on angiogenesis has been mostly focused on the role of these factors and their receptors on endothelial cells and the role of the extracellular matrix, with limited access to the processes by which blood vessels form in vivo under normal conditions. In the mid-1990s, gene inactivation techniques using homologous recombination of essential angiogenic factors and endothelial cell genes opened a new era in the understanding of the physiological construction of the vascular tree. Concomitantly, the discovery of the molecular regulation of cellular response to hypoxia allowed a better understanding of the initiation of the angiogenic signal. Clinical applications of these researches paralleled the discovery of the mechanisms of angiogenesis. However, the use of anti-angiogenic compounds in preclinical and clinical assays remained largely deceptive until 2004, when the milestone work of Hurwitz et al. demonstrated the effectiveness of anti-VEGF antibodies (bevacizumab/Avastin) to treat metastatic colon cancer (Hurwitz et al. 2004). Anti-VEGF antibodies revolutionized also the field of ophthalmology, following the work of Rosenfeld et al. focusing on the treatment of wet or vascular macular degeneration (Rosenfeld et al. 2006). One of the pioneers in the discovery of VEGF, Dr. N Ferrara, won the Lasker Award in 2010 for the use of anti-VEGF antibodies to treat ophthalmic disorders. The detailed description of the physiological postnatal development of the retinal vasculature using a well-established mouse model allowed to decipher in more detail the cellular and molecular mechanisms implied in the angiogenic process. It led to the description of very specialized endothelial cells driving angiogenesis (tip cells), of the molecular mechanisms underlying the differentiation of capillaries, arteries, and veins, and to the concept that endothelial cells share families of guidance molecules (semaphorins, neuropilins, plexins) with neural cells. At the same time, the field of lymphangiogenesis emerged from nil to become a rich research playground. The role of circulating endothelial precursor cells and immune cells in the formation of blood vessels also came to light. Multiarray and high-throughput genetic methods have recently allowed the identification of new markers of endothelial and perivascular cells and have, so far, added more complexity to the field.

Thus, over the last 50 years, the study of angiogenesis has evolved through several cycles of exciting steps, and major fundamental discoveries in the field have contributed to the development of effective treatments, especially for particularly aggressive and debilitating diseases, including several cancers. The general enthusiasm generated by these new therapies has however been dampened by the observation of resistance to anti-angiogenic treatments. The development of these targeted therapies, which represent a major progress, should give rise to the development of personalized treatments. The main challenges remain the identification of predictive markers of sensitivity/resistance to current anti-angiogenic treatments and the development of second-generation anti-angiogenic drugs that hit new therapeutic targets.

This book is an overview of the recent progress made in the field of angiogenesis and its associated therapies. It was written under the auspices of the French Angiogenesis Society. This Society (http://www.angiogenese.fr/) was created in 2007 and holds an international meeting every 18 months. Many of the contributors to this book have previously given conferences at these annual meetings. Our aim was to gather the most recent information about the recent discoveries of the basic mechanisms of both physiological and pathological angiogenesis. We intended also to present the recent developments in the success and limitations of pro- and anti-angiogenic therapies. We wish that reading this book will inspire the research community to make this field further progress and enhance the successful translation of basic science toward clinical applications.

Grenoble, France — Jean-Jacques Feige
Nice, France — Gilles Pagès
Lille, France — Fabrice Soncin

Acknowledgment

We dedicate this book to the memory of Jean Plouët, a pioneer in the field of angiogenesis, a founding member of the French Angiogenesis Society, and a wonderful colleague whose scientific input still inspires us.

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Part I
Angiogenesis During Embryonic Development

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 artherosclerosis, 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

J.-J. Feige et al. (eds.), *Molecular Mechanisms of Angiogenesis*,
DOI 10.1007/978-2-8178-0466-8_1, © Springer-Verlag France 2014

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 artherosclerosis, 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

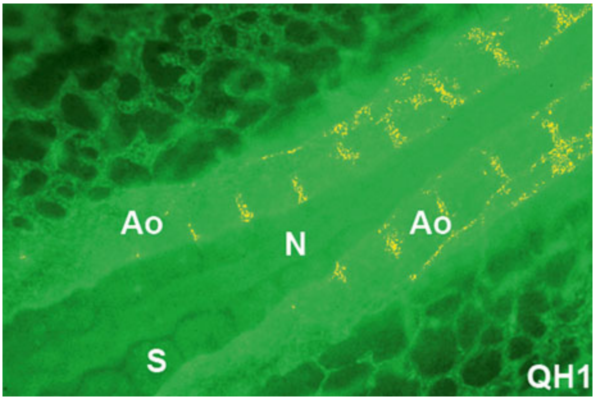

Fig. 1.1 Vasculogenesis in a 36 h-old quail embryo stained with QH1, an antibody specific for quail EC and hematopoietic cells. On this ventral view, EC have coalesced axially to form two lumenized aortae (Ao), while laterally, the vascular network appears undifferentiated. *N* Notochord, *S* somite

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

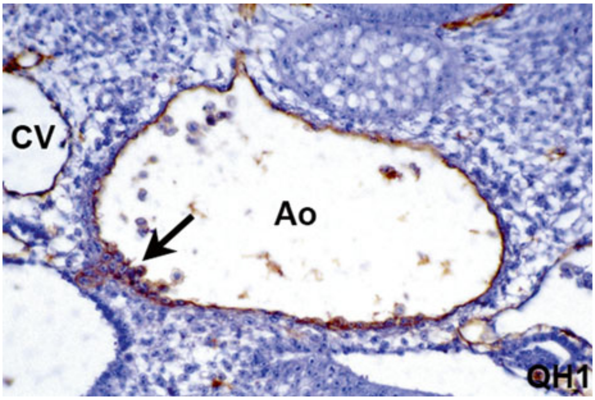

Fig. 1.2 A QH1$^+$ cell cluster (arrow) emerges ventrally in the aorta (Ao): these cells will give rise to the definitive hematopoietic cell population. *CV* cardinal vein

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

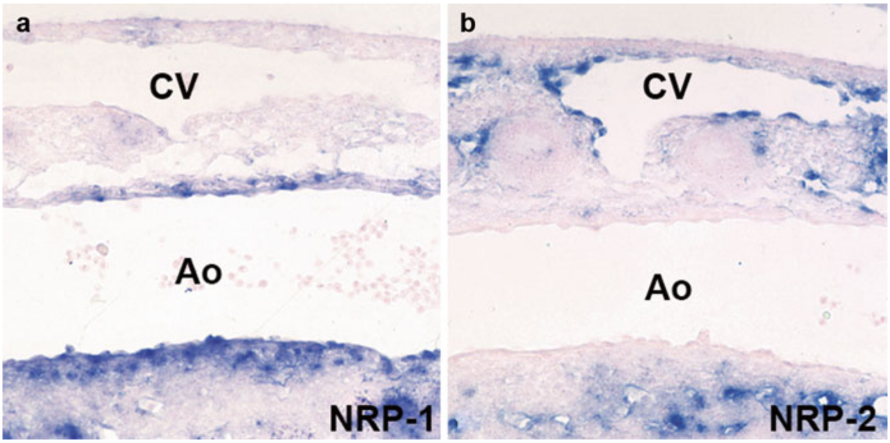

Fig. 1.3 Complementary expression of NRP-1 (**a**) and NRP-2 (**b**) mRNAs in arteries and veins respectively. On these two consecutive longitudinal sections of a 13-day-old mouse embryo, NRP-1 is only transcribed along the aorta (Ao) and is absent in the cardinal vein (CV), while NRP-2 messengers surround 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 κB 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.

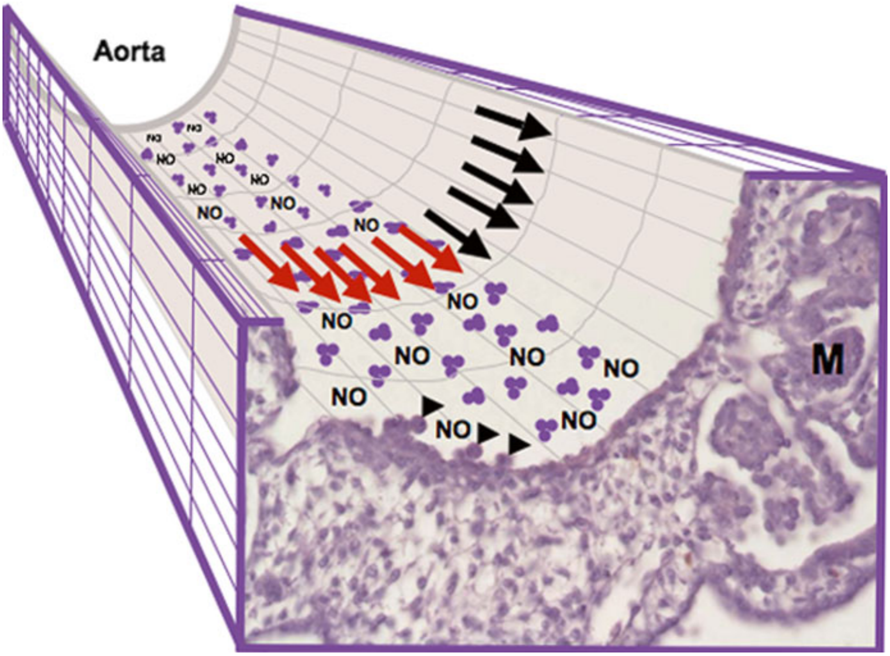

Fig. 1.4 Blood flow promotes development of HC. HC and the aortic endothelium sense blood-flow-induced shear stress. HC only bud from the ventral aortic endothelium (*arrowheads*), although shear stress is sensed throughout the aortic endothelium – ventrally (*red arrows*), laterally (*black arrows*), 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).

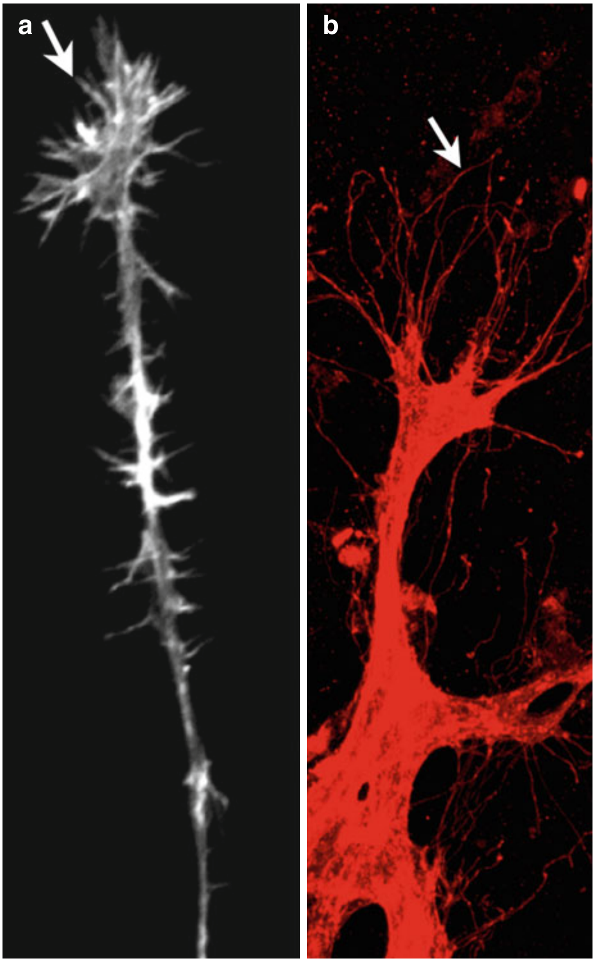

Fig. 1.5 Morphological similarities between an axonal growth cone (**a**) and a tip cell (**b**), especially at the level of filopodia (*arrows*) that permit the navigation of neural and endothelial cells

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/βcatenin 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
ESM1	Endothelial-specific molecule 1	11.07	IHC/ISH
Gcn1l1	GCN1 general control of amino-acid synthesis 1-like 1 (yeast)	5.50	
LAMB1	Laminin β 1	5.44	
PLAUR	uPAR	5.23	ISH
–	MYST histone acetyltransferase (monocytic leukemia) 3	4.68	
ANGPT2	Angiopoietin-2	4.64	ISH
NID1	Nidogen-1	4.26	IHC/ISH
ITGB1	Integrin β 1	4.02	IHC
Trp53i11	Trp53 inducible protein 11	3.96	
MGI:1930803	Tescalcin	3.87	
APLN	Apelin	3.72	ISH
–	Sidekick cell adhesion molecule 1	3.65	
March1	Membrane-associated ring finger (C3HC4) 1	3.54	
–	Adrenomedullin	3.45	
Syt16	Synaptotagmin XVI	3.12	
Sema3a	Semaphorin 3A	3.00	
Cxcr4	Chemokine (C-X-C motif) receptor 4	2.81	
Igf1	Insulin-like growth factor 1	2.77	
Vldlr	Very low density lipoprotein receptor	2.63	
Vegfa	Vascular endothelial growth factor A	2.52	
Il1b	Interleukin1 b	2.42	
Igf1	Insulin-like growth factor 1	2.37	
–	Integrin αV	2.36	
–	DCC	2.33	
Klf5	Kruppel-like factor 5	2.32	
–	Integrin β 3	2.27	
Bmp7	Bone morphogenetic protein 7	2.21	
Gls2	Glutaminase 2 (liver, mitochondrial)	2.19	
Ccnd2	Cyclin D2	2.13	
Tuba3	Tubulin alpha 3	2.13	
Igfbp3	Insulin-like growth factor-binding protein 3	2.11	
Igfbp4	Insulin-like growth factor-binding protein 4	2.09	
Sema3f	Semaphorin 3F	2.04	
Hck	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 (±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).

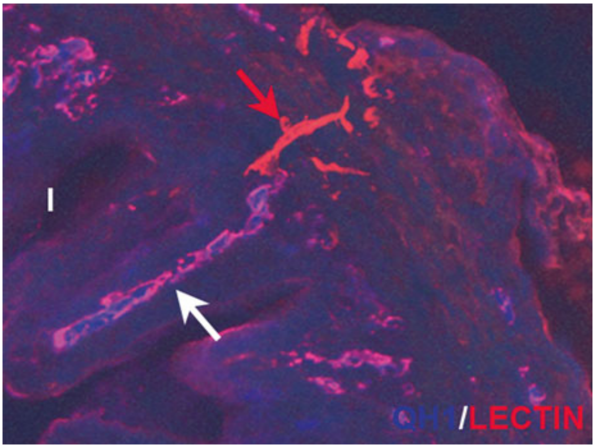

Fig. 1.6 Transverse section at the level of the intestine (I) in a yolk-sac chimera in which a quail territory developed on a chick yolk sac. In this condition, CEC coming from the chick yolk sac colonizes the quail organs and can integrate vessels. On the picture, quail vessels are stained by both QH1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

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