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# Vascular development: from precursor cells to branched arterial and venous networks

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**ABSTRACT The adult vascular system is composed of an arterial, a venous and a lymphatic compartment. These different compartments respectively provide oxygen and nutrients to peripheral organs, remove carbon dioxide and waste products and maintain an immune barrier to defend the host against foreign organisms. Malfunctions of the vascular system represent a major cause of mortality and disease in developed countries. Understanding of the molecular mechanisms regulating vascular system development and maintenance is thus crucial for the design of therapies to cure vascular diseases. The molecules implicated in the control of physiological and pathological angiogenesis in the adult already function during embryonic development. Indeed, the survival of the embryo also critically depends on the establishment of a functional circulatory loop. Here we review our current knowledge about the emergence of endothelial precursor cells in the embryo, of their assembly into the primary vascular plexus and of the remodeling of this plexus into arteries and veins. We also focus on the molecular mechanisms controlling the development of arteries, veins and lymphatic vessels.**

KEY WORDS: *endothelial cell, vasculogenesis, angiogenesis, arterio-venous differentiation, growth factor receptor, plasticity, flow*

## Introduction

All vertebrates require a mechanism to distribute oxygen and nutrients to tissues and to remove carbon dioxide and other metabolic waste products, which have to be transmitted to the excretory organs. The circulatory system carries out these vital functions via its two main components, the blood-vascular and the lymphatic system (Fig. 1). Blood, which is the carrier of oxygen, carbon dioxide and metabolic products, is pumped from the heart through the arterial system into the tissue capillary bed, where exchanges occur. The blood is then channeled through the venous system back into the heart. The blood-vascular system is affected by numerous pathologies, including artherosclerosis and cancer, the two major causes of death in developed countries (Carmeliet and Jain, 2000; Cines *et al.,* 1998; Ferrara and Alitalo, 1999; Folkman, 1995, for reviews). 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 (Fig. 1). Defects in lymphatic development or damage to the lymphatics provoke lymphedema, a disabling and disfiguring swelling of the extremities. In addition, many cancer cell types use the lymphatic vessels for their metastatic spread (Alitalo and Carmeliet, 2002, for review).

Histologically, the structure of blood vessels is rather simple. 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 tunica adventitia containing vasa vasorum and nerves (Wheater *et al.,* 1978). The size of the vessel wall varies according to the vessel size and type. The main focus of this review will be on EC, which represent the major cellular compartment of the vascular system and which are the first to form during embryonic development.

*Abbreviations used in this paper:* EC, endothelial cell; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor.

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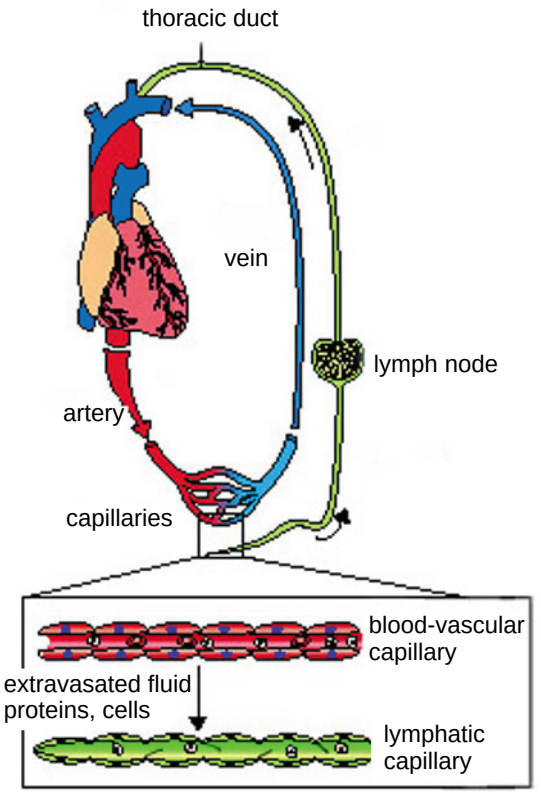


**Fig. 1. Schematic representation of the circulatory system.** *(Modified from Karkkainen* et al., *2002). Blood is pumped from the heart through arteries, arterioles and capillaries to the tissues, where exchanges occur. Blood is returned to the heart via the venous circulation. The lymphatic system, composed of lymphatic capillaries and vessels, drains excess fluid, the lymph, containing proteins, lipids and immune cells, from the extracellular space and returns it to the venous circulation.*

## Emergence of EC during embryonic development

As the diffusion distance of molecules is limited (100-200 μm for $O_2$ for example), the vascular system in any organ and tissue has to be established early during development. The cardiovascular system is actually the first organ system formed during early embryonic 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) and intermediate mesoderm (kidney and gonads) (Fig. 2A). The lateral plate mesoderm is situated on both sides of the intermediate mesoderm and will split into two layers after the formation of the coelome: 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 endothelial cells that form in the gastrulating embryo originate from lateral and posterior mesoderm, as shown by Murray (Murray, 1932). Murray dissected 24-hour old chick embryos into small pieces, which were cultured in vitro to obtain blood cells, visible by their hemoglobin content. The posterior two thirds of the embryo, corresponding to the presumptive territories of lateral and posterior mesoderm (Fig. 2A) were found to give rise to blood cells, as well as to EC. More recently, we have shown that these territories expressed the vascular endothelial growth factor receptor –2 (VEGFR2) (Fig. 2B) (Eichmann *et al.,* 1993).

Vascular endothelial growth factor (VEGF) and its receptor VEGFR2 are the most critical drivers of embryonic vessel formation (Yancopoulos *et al.,* 2000 for review). VEGF is expressed in spatial and temporal association with almost all physiological events of vascular formation in vivo (Jakeman *et al.,* 1993; Shweiki *et al.,* 1993). VEGFR2 expression is already observed at very early stages of development (Fig. 2B) and subsequently becomes mainly restricted to EC of all types of blood vessels as well as lymphatic vessels (Eichmann *et al.,* 1993; Wilting *et al.,* 1997; Yamaguchi *et al.,* 1993). Mice deficient in VEGFR2 ($VEGFR2^{-/-}$) died in utero between 8.5 and 9.5 days post-coitum, as a result of an early defect in the development of 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.

The newly formed lateral and posterior mesodermal cells migrate toward the yolk sac, where they will differentiate to EC and to HC of the blood islands. During their migration, the precursors aggregate to clusters, termed hemangioblastic aggregates. These were first described by F. Sabin (Sabin, 1920), who studied their migration under the light-microscope. She could distinguish the hemangioblastic aggregates from the remaining mesodermal cells by their increased refringence. The peripheral cells of these aggregates subsequently

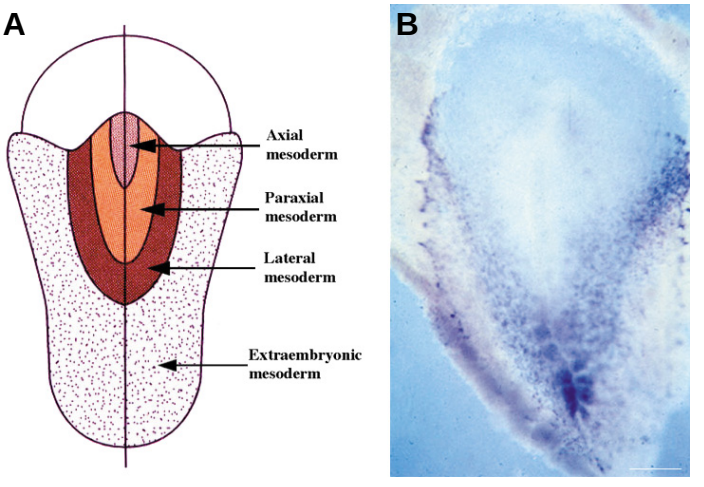


**Fig. 2. Origin of endothelial cells in the gastrulating chick embryo. (A)** *Map of presumptive embryonic territories (from Vakaet, 1985).* **(B)** *Labeling of a chick embryo at stage 4 of Hamburger and Hamilton (Hamburger and Hamilton, 1951), corresponding to the stage shown in A, with an antisense riboprobe specific for VEGFR2. The presumptive extraembryonic and lateral mesoderm expresses this receptor.*

flatten and differentiate to EC, while the centrally located cells differentiate to HC (Fig. 3A). 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 positive, while in the differentiated islands only the EC express this gene and no expression is detected in HC (Fig. 3B). These observations are compatible with the hypothesis that VEGFR2 labels a bipotent progenitor and that after lineage diversification; only one of the two daughter cells maintains expression of this gene.

To test this idea, we prepared a monoclonal antibody directed against the extracellular domain of quail VEGFR2 (Eichmann *et al.,* 1997). Using this antibody, we isolated cells from posterior territories of embryos at the gastrulation stage (Fig. 2A), which were subsequently cultured in semi-solid medium in vitro. In the absence of added VEGF, the VEGFR2+, but not the VEGFR2- precursors differentiated to HC of different lineages. In the presence of VEGF, EC differentiation of the VEGFR2+ precursors was induced. These experiments showed that VEGFR2+ precursors could indeed give rise to EC as well as HC, consistent with 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'. In cultures derived from mouse ES cells, a single VEGFR2+ cell was found to be able to give rise to both EC as well as HC (Choi *et al.,* 1998; Nishikawa *et al.,* 1998). This cell is currently referred to as the 'hemangioblast' in the literature. However, additional studies have shown that ES cell-derived VEGFR2+ cells can 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 lineage, these cells may be pluri-or multipotent progenitors.

## Formation of the primary capillary plexus

Following the differentiation of the yolk sac blood islands, EC surrounding these blood islands soon anastomose to form a capillary meshwork, which serves as a scaffold for the beginnings of circulation (Fig. 3, 4A). The embryo has thus laid down the rudiments of its extraembryonic vascular system before the onset of heart beat around the 12-somite stage in the chick. Inside the embryo proper, one major vessel, the dorsal aorta, as well as numerous capillaries have differentiated (Fig. 4A). EC differentiation in the embryo proper during this developmental time window occurs in the absence of associated hematopoiesis. After the onset of heartbeat and of blood flow, the yolk sac capillary plexus is rapidly remodeled into arteries and veins and a functional circulatory loop essential for survival is established (Fig. 4B). The newly formed blood island HC are channeled through this primitive circulation. These yolk sac hematopoietic precursors mostly differentiate into primitive erythrocytes, which are replaced, as development proceeds, by definitive hematopoietic precursors generated in the embryo proper (Cumano *et al.,* 2001; Dieterlen-Lievre, 1975). These definitive precursors are again observed to develop in close association with the endothelium of the dorsal aorta (Jaffredo *et al.,* 1998; Pardanaud *et al.,* 1996).

Collectively, the in situ differentiation of EC from the mesoderm and their coalescence into tubes of the primary capillary plexus are called vasculogenesis (Risau, 1997). Vasculogenesis results in the formation of the major embryonic vessels, the dorsal aorta and of the primary vascular plexus in the yolk sac. In the chick embryo, both are formed before the 14ss, prior to the onset of perfusion. Fig. 4A shows the structure of the primary capillary plexus at this stage revealed by immunohistochemistry with the QH1 antibody, specific for EC in the quail (Pardanaud *et al.,* 1987).

## Remodeling of the capillary plexus into arteries and veins

Over the next few hours, the primary vascular plexus has to be remodeled into a system with arteries and veins, to accommodate the output of the heart and to establish the primary circulation. This step is critical for the embryo's survival and indeed, many mouse mutants for genes involved in vascular development die during this 'remodeling' phase (Roman and Weinstein, 2000, for review). We have recently performed a detailed analysis of the remodeling of the primary vascular plexus into arteries and veins in the chick embryo yolk sac, using time-lapse video-microscopy (LeNoble *et al.,* 2004). Fig. 4B shows a chick embryo about 24 hours older than the embryo in Fig. 4A, it is obvious that the primary capillary plexus has been remodeled into larger and smaller vessels. The chick

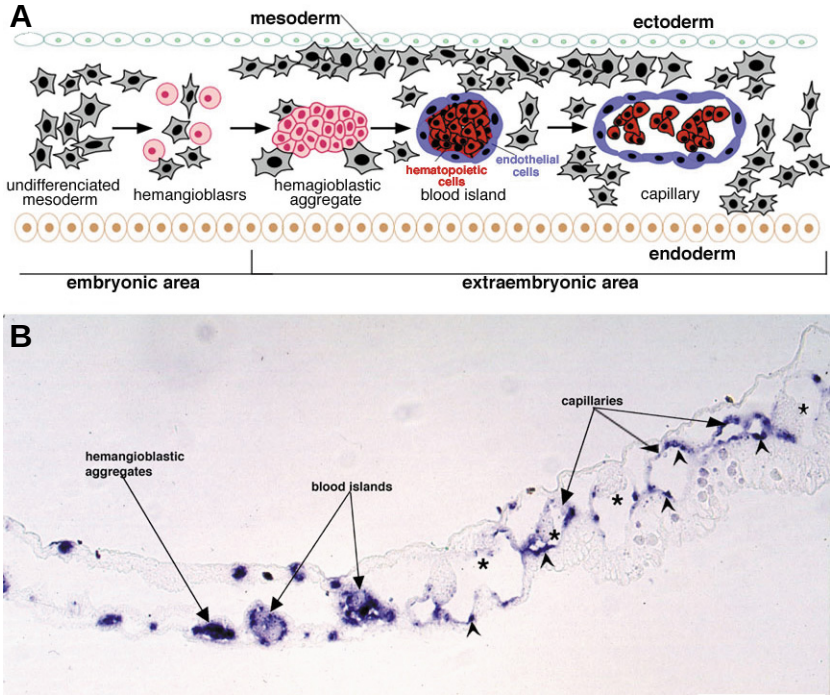


**Fig. 3. Differentiation of yolk sac blood islands. (A)** *Schematic representation: see text for details.* **(B)** *Transverse section through the extraembryonic area of a 15-somite stage embryo, labeled with an antisense riboprobe specific for VEGFR2. Hemangioblastic aggregates are positive. In the blood islands and capillaries, expression of the receptor decreases in the hematopoietic cells (asterisks), but is maintained in the endothelial cells (arrowheads). Bar, 75 μm.*

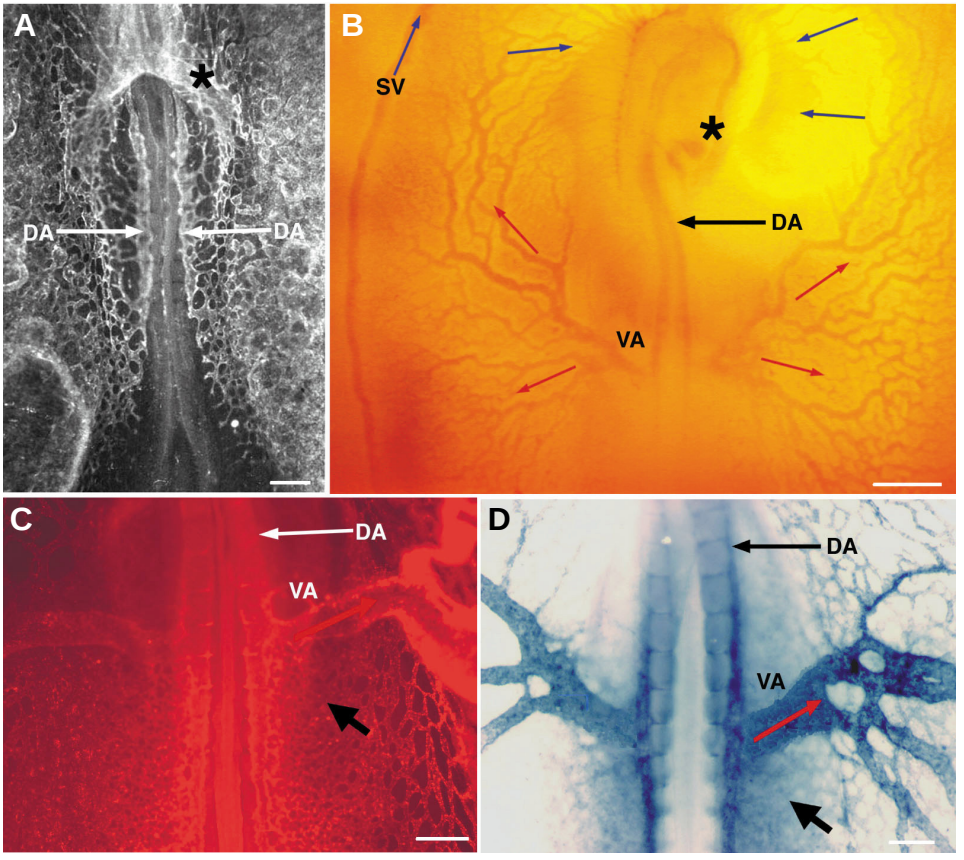


**Fig. 4. Arterial-venous differentiation in the yolk sac. (A)** *Primary capillary plexus stage. Labeling of a 10-somite stage quail embryo with the QH1 antibody. Note labeling of the endocardium (asterisk) and the paired dorsal aorta (DA, arrows). At this stage, only these two major vessels are formed, the remainder of the vascular system is seen as a capillary plexus.* **(B)** *Photo-micrograph of a living chick embryo 24 hours older than the embryo in A. The heart is indicated (asterisk). Note that in the yolk sac, two main arteries, the vitelline arteries (VA) have formed. Arterial blood (red arrows) is pumped from the heart through the dorsal aorta (DA) and the vitelline arteries into the yolk sac and is returned to the heart via the venous circulation (blue arrows) either directly or through the sinus vein (SV).* **(C)** *Onset of formation of the posterior vitelline vein. Embryo at the same stage as in B. Intracardiac injection of DiI-Ac-LDL, which specifically recognizes endothelial cells. Note the dorsal aorta and vitelline artery. As more flow passes through these vessels, they enlarge. Smaller vessels of the initial arterial capillary plexus (black arrow) carry progressively less flow and are disconnected from the main branch of the dorsal aorta. As shown by the intracardiac injection, these vessels are still perfused through more distal parts of the capillary plexus. They will be used to form the posterior vitelline vein.* **(D)** *Arterial marker expression.* In situ *hybridization with an antisense riboprobe specific for NRP-1. Note strong expression in the vitelline artery (VA), but reduced expression in the disconnected capillaries that will form the vitelline vein (black arrow). Bars 250 μm.*

offers the possibility of direct visual inspection of the blood flow and the direction of circulation can thus be easily visualized in living embryos. As indicated in Fig. 4B, arterial blood pumped from the heart through the dorsal aorta enters the yolk sac through the vitelline artery and its branches. To be returned towards the heart, blood either reaches the peripheral sinus vein and is returned through the anterior venous plexus (Fig. 4B), or blood can flow directly back towards the heart. Indeed, close visual inspection of the circulatory system at this stage reveals that arterial and venous blood actually flows through the same vascular channel. The definition of arterial and venous blood is given in this case simply by the direction of the blood flow away from (arterial) or towards (venous) the heart. This embryonic vessel configuration is in contrast to the adult situation where blood flows through arteries into successively smaller arterioles, a capillary bed and through successively larger venules and veins back to the heart. In fact, if the embryonic arterial-venous vessel configuration would persist, large arterial-venous shunts would develop and distal parts of the yolk sac would not be perfused, resulting in death of the embryo. The embryo has solved this 'plumbing' problem in a simple manner: during the formation of the large vitelline artery from the primary capillary plexus, not all capillaries are integrated into this tube: some small side branches become disconnected (Fig. 4C, D). The disconnection of side branches is regulated by perfusion: the increased flow in the main branch leads to a diameter decrease and subsequent obstruction of the lumen of the side branch (LeNoble, F., Eichmann, A., Nguyen, T.H., Fleury, V., submitted). These disconnected vessels are subsequently used to fashion the embryonic veins of the secondary circulation, which come to lie dorsally and parallel to the arteries (LeNoble *et al.,* 2004). To become reconnected to the venous circulation, the disconnected side branches sprout dorsally and perpendicular to the arteries. The molecular basis underlying the directed sprouting is currently unknown but may involve some of the neuronal guidance receptors expressed in developing arterial and venous EC (see below).

## Molecular markers specific for arteries and veins

Based on classic studies, it was believed that EC of the primary capillary plexus constitute a rather homogenous group of cells and that differentiation into arteries and veins occurred due to the influence of hemodynamic forces (Thoma, 1893). Over the last few years, however, several signaling molecules were discovered, which labeled arterial or venous EC from early developmental stages onward, prior to the assembly of a vascular wall. Interestingly, most of these molecules are also expressed in the nervous system, where they regulate cell fate decisions and guidance of migration of neuronal precursors as well as of developing axons (Artavanis-Tsakonas *et al.,* 1999; Flanagan and Vanderhaeghen, 1998; Kullander and Klein, 2002; Neufeld *et*

*al.,* 2002; Raper, 2000). It is thus tempting to speculate that these molecules regulate similar cell behaviors in the developing nervous and vascular system. Arterial EC in chick, mouse and zebrafish selectively express ephrin-B2, neuropilin-1 (NRP-1) and members of the Notch pathway, including notch3, DLL4 and gridlock (Herzog *et al.,* 2001; Lawson *et al.,* 2001; Moyon *et al.,* 2001a; Moyon *et al.,* 2001b; Shutter *et al.,* 2000; Villa *et al.,* 2001; Wang *et al.,* 1998; Zhong *et al.,* 2000). Other molecules are specifically expressed in the venous system, most notably EphB4, the receptor for arterial ephrin-B2 (Gerety *et al.,* 1999). The neuropilin-2 (NRP-2) 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). Based on these specific expression patterns and on mutant studies in zebrafish and mouse, it has been suggested that embryonic arterial-venous differentiation is actually genetically predetermined (Wang *et al.,* 1998). In support for this idea, tracing of individual fluorescently labeled angioblasts in the zebrafish embryo showed that a single EC precursor will give rise to an arterial or a venous, but not to mixed clones (Zhong *et al.,* 2001).

A possible role for these signaling molecules in arterial-venous differentiation was suggested by the phenotypes of ephrinB2 and EphB4 knockout mice: embryos displayed symmetrical phenotypes, remodeling of the primary vascular plexus into arteries and veins was arrested during early development, leading to death around E9.5 (Adams *et al.,* 1999; Gerety *et al.,* 1999; Wang *et al.,* 1998). Inactivation or other signaling molecules specifically expressed by arteries, such as NRP-1 and members of the Notch family also leads to embryonic lethality due to failure of vascular system remodeling (Kawasaki *et al.,* 1999; Swiatek *et al.,* 1994; Xue *et al.,* 1999). In these latter mutants, a specific effect on arteries has not been described, rather a general failure to form large and small vessels in the yolk sac has been noted. Zebrafish mutant studies have shown the requirement for Notch signaling to repress venous fate in arteries: inhibition of the Notch signaling pathway using a dominant negative form of suppressor of hairless (SuH), a downstream effector of Notch, leads to ectopic expression of venous markers in arteries and to vascular malformations (Lawson *et al.,* 2001). These observations led to the hypothesis that the embryonic vascular system could actually be pre-determined to an arterial or venous fate from early developmental stages onward.

## Arterial-venous differentiation involves EC plasticity

Our own work has shown that a considerable degree of EC plasticity is observed during arterial-venous differentiation. In a first series of experiments, we have isolated arterial or venous vessel fragments from quail embryos at different developmental stages, together with their vessel wall. These fragments were grafted into the coelome or in place of a somite in an E2 chick host (Moyon *et al.,* 2001a). Progeny of the grafted EC could be traced in the host embryo using the QH1 antibody, specific for quail EC but not recognizing host EC (Pardanaud *et al.,* 1987). To determine if the grafted EC colonized arteries or veins of the host, we used in situ hybridization with ephrinB2 or NRP-1 probes, which recognize arterial, but not venous EC of both species. We could then ask if EC from a grafted quail artery would only colonize chick arteries or also veins. These experiments showed that until late developmental stages (E11), quail EC from arteries or veins colonized both host arteries and veins with equal efficiency. Moreover, the expression of arterial markers changed according to the novel environment: when the quail cells colonized an artery, they expressed arterial markers, when they colonized a vein, they did not, irrespective of their origin. Thus, EC are plastic with respect to arterial-venous differentiation until E11. After E11, EC derived from a grafted artery would only colonize host arteries, while those from a vein would only colonize host veins. Quantification of the number of grafted EC showed that this loss of plasticity affected the vast majority (98%) of all grafted EC. These experiments suggested some intrinsic changes in the grafted arteries and veins after E11 that restricted their capacity to colonize respectively host veins and arteries. To determine the source of the plasticity-restricting signal, we removed the vessel wall from E14 quail aortic fragments, this fully restored their

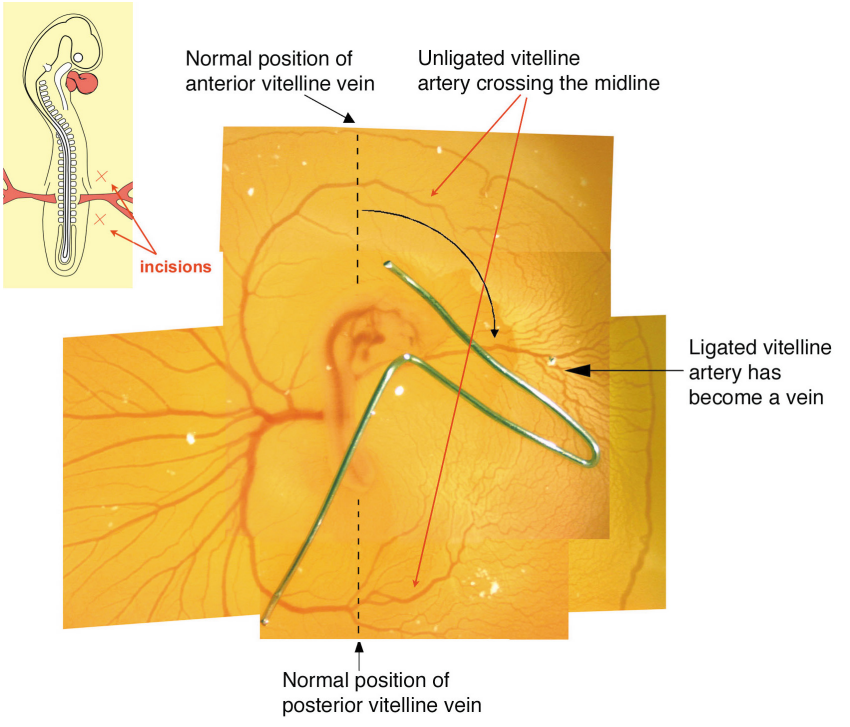


**Fig. 5. Flow manipulations transform arteries into veins and veins into arteries.** *Ligation of the right vitelline artery of a chick embryo (Stephan, 1952). The schematic drawing shows the experimental manipulation: two small incisions are made on each side of the vitelline artery, a metal clip is then inserted underneath this artery. The clip is devised such that it lifts the right vitelline artery and blocks blood flowing through it. The photomicrograph shows such an embryo 24 hours after ligation: note that the right vitelline artery has been transformed into a vein. The normally present anterior and posterior vitelline veins are absent: they have been incorporated into the unligated left vitelline artery, which crosses the midline of the embryo/yolk sac axis.*

capacity to colonize veins: about 50% of grafted cells were found in arteries and 50% in veins. The precise nature of the plasticity-restricting signal coming from the vessel wall is yet unknown.

## Yolk sac arterial-venous differentiation is flow-regulated

To determine if EC plasticity with respect to arterial-venous differentiation also occurred during normal embryonic development, we examined yolk sacs of embryos at different stages using a combination of time-lapse video-microscopy on living embryos and in situ hybridization with arterial and vein-specific markers (LeNoble *et al.,* 2004). We observed that prior to the onset of circulation, arterial markers are expressed in the arterial capillary plexus of the posterior pole of the embryo, the territory where the vitelline artery will form. As described above, many small capillary branches of this arterial plexus become disconnected from the main branch of the vitelline artery during its formation. Arterial marker expression in these disconnected branches is rapidly down-regulated (Fig. 4D). In contrast, arterial marker expression is maintained in the newly formed vitelline artery (Fig. 4D). The disconnected capillaries that have down-regulated arterial markers now serve to form the vitelline vein. These observations show that the posterior vitelline vein is formed by incorporating previously arterial capillary segments, demonstrating that EC plasticity is required during normal arterial-venous differentiation. Moreover, these experiments suggested that hemodynamic forces play a major role during arterial-venous differentiation and patterning.

To test this idea directly, we performed several alterations in the flow pattern of developing chick embryos (LeNoble *et al.,* 2004). We first generated embryos devoid of a circulatory system by destroying the embryonic heart. In these embryos, the yolk sac continues to grow for at least 7 days, in spite of the degeneration of the embryo and the lack of perfusion. However, the yolk sac does not develop arteries or veins and remains in the configuration of a primary vascular plexus. In situ hybridization with arterial markers showed that some regions of this yolk sac expressed the arterial marker ephrinB2, while others did not. Thus, initiation of arterial marker expression occurs independently of flow.

We next performed ligations of the vitelline artery on one side of the yolk sac (Fig. 5) (Stephan, 1952). In these embryos, the entire ligated side becomes venularized over a period of 24 hours, as judged first by the direction of blood flow, which becomes reversed in the ligated vessels and second by the expression of arterial markers, which decreases rapidly following ligation (LeNoble *et al.,* 2004). Thus, manipulation of the flow pattern can morphologically and genetically transform arteries into veins. The same flow manipulation can also transform veins into arteries. Indeed, the vitelline artery on the unligated side significantly enlarges after ligation, since it receives more flow. Subsequently, this artery crosses over the midline of the embryo-yolk sac axis both on the anterior and on the posterior pole of the embryo (Fig. 5). Time-lapse video-microscopy shows that branches of the anterior vitelline vein are integrated into the vitelline artery as it crosses the midline. Flow therefore appears as the master-regulator of arterial-venous differentiation in the yolk sac. Rather than being pre-determined, the yolk sac vessel EC appear as 'bricks' that can be used and re-used by the developing vascular system to fashion arteries or veins.

## Formation of the lymphatic vascular system

Lymphatics are the last vascular compartment to be formed during development. Florence Sabin proposed the first theory on lymphatic vessel formation. Based on observation of ink-injected chick embryos, she concluded that the earliest primitive lymphatics arise as a result of EC sprouting from the cardinal veins. Subsequently, these cells proliferate and migrate toward the organs of the embryo and form the lymphatic system (Sabin, 1902). Other studies suggested that the connection of the lymphatic and venous systems was secondary. They proposed that lymphatic stem cells, called lymphangioblasts, invaded the mesenchyme in order to form primitive lymphatic sacs (Huntington and McClure, 1908; Kampmeier, 1912). Recent work using chick embryo limb buds has shown that lymphatic vessels can be derived both by sprouting of vessels from the cardinal vein and by mesodermal lymphangioblasts (Schneider *et al.,* 1999; Wilting *et al.,* 2001; Wilting *et al.,* 2000).

Specific markers for lymphatic EC have recently been identified, providing new tools for the study of lymphangiogenesis. In addition, gene inactivation experiments have identified different molecules implicated in successive steps of the development of the lymphatic system (Fig. 6).

The earliest marker for developing lymphatic EC is the homeobox transcription factor Prox-1 (prospero-related homeobox protein-1) (Wigle and Oliver, 1999). Prox-1 first becomes expressed in the vascular system in the lateral aspect of the posterior cardinal vein at E9, shortly before the onset of sprouting of

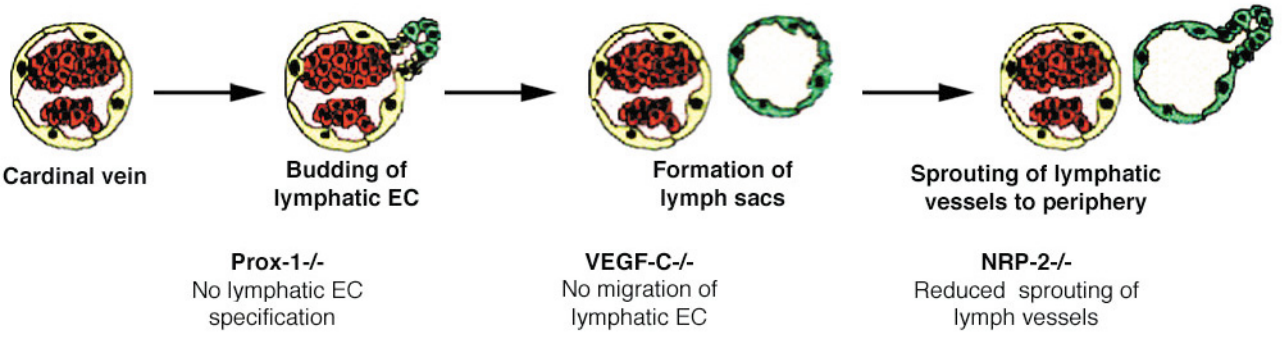


**Fig. 6. Formation of the lymphatic vascular system.** *Schematic drawing representing the initial steps of lymphatic vessel formation. Mouse mutants implicated in these steps are indicated. See text for details.*

lymphatic EC from this vein. Inactivation of Prox-1 results in the complete absence of formation of lymphatic EC. Detailed examination of the phenotype of Prox-1-/- mice has shown that budding of lymphatic EC from the lateral aspect of the cardinal vein occurs. However, the budding cells fail to acquire expression of lymphatic-specific markers and instead continue to express blood-vascular markers, which normally are down-regulated after budding (Wigle *et al.,* 2002). Lymphatic EC specification thus depends on the presence of Prox-1.

After specification of lymphatic EC has occurred, these cells normally migrate to form the first embryonic lymph sacs. Migration of the lymphatic EC towards the lymph sacs critically depends on the presence of the growth factor VEGF-C: in mice deficient for this growth factor, Prox-1 expressing lymphatic EC are formed, but fail to migrate towards the lymph sacs and subsequently die (Karkkainen *et al.*, 2004). VEGF-C-/- embryos die at around E17 of development due to formation of massive oedema, since they do not develop any lymphatic vessels. VEGF-C specifically binds to its high affinity tyrosine kinase receptor VEGFR3 (Alitalo and Carmeliet, 2002 for review). During embryogenesis, VEGFR3 is first expressed in a subset of blood vascular EC and subsequently becomes restricted to lymphatic EC (Kaipainen *et al.,* 1995; Wilting *et al.,* 1997). Consistent with the early expression, VEGFR3 involvement in embryonic angiogenesis has been confirmed by targeted gene inactivation (Dumont *et al.,* 1998). The VEGFR3 deficient mice showed defective blood vessel development at early embryonic stages and the embryos died at E9.5. Vasculogenesis and angiogenesis occurred, but large vessels became abnormally organized with defective lumens, leading to fluid accumulation in the pericardial cavity and cardiovascular failure (Dumont *et al.,* 1998). Thus VEGFR3 has an essential function in the remodeling of the primary capillary vasculature prior to formation of the lymphatic vessels.

VEGF-C also binds to the NRP-2 receptor, which is specifically expressed by veins and subsequently becomes restricted to lymphatic vessels (Karkkainen *et al.,* 2001; Yuan *et al.,* 2002). In accordance with this specific expression, we have shown that mice deficient for NRP-2 show selective defects in the formation of lymphatic vessels (Yuan *et al.,* 2002). Interestingly, however, the phenotypes of VEGF-C and NRP-2 deficient mice appear distinct. In NRP-2-/- mice, formation of lymph sacs occurs normally, while sprouting of lymphatic vessels from the lymph sacs to the periphery is reduced (Fig. 6). The precise molecular mechanisms regulating the different steps of embryonic lymphangiogenesis thus remain to be fully elucidated.

## Perspectives

Research carried out over the past decade has provided major insights into the mechanisms regulating the emergence of endothelial progenitors from the mesoderm, their coalescence into the primary vascular system, the remodeling of this system into arteries and veins as well as into the formation of lymphatic endothelium. The molecules implicated in these different developmental processes are also essential for the maintenance of the adult vascular system. For example, patients suffering from hereditary lymphedema have been found to carry a mutation in the tyrosine kinase domain of VEGFR3 (Karkkainen *et al.,* 2000). 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. A particularly interesting aspect of recent research carried out on the vascular system is the identification of neural guidance receptors such as ephrins and neuropilins, which are expressed on arteries, veins and lymphatic vessels. In the nervous system, these molecules are implicated in the establishment of cell boundaries and in the guidance of developing axons. It is thus tempting to speculate that these receptors may also play a role in vessel guidance during embryonic development. Indeed, recent studies have shown that specialized cells termed 'tip cells' are present at the ends of developing vessel sprouts, which extend filipodia that explore their environment in much the same way as the growth cone of a developing axon (Gerhardt *et al.,* 2003; Ruhrberg *et al.,* 2002). Moreover, 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), emphasizing the close interaction between the two systems. Future studies will be directed at exploring the precise interactions between blood vessels and nerves during development as well as in pathologies.

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