

Flow regulates arterial-venous differentiation in the chick embryo yolk sac

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Summary

Formation of the yolk sac vascular system and its connection to the embryonic circulation is crucial for embryo survival in both mammals and birds. Most mice with mutations in genes involved in vascular development die because of a failure to establish this circulatory loop. Surprisingly, formation of yolk sac arteries and veins has not been well described in the recent literature. Using time-lapse video-microscopy, we have studied arterial-venous differentiation in the yolk sac of chick embryos. Immediately after the onset of perfusion, the yolk sac exhibits a posterior arterial and an anterior venous pole, which are connected to each other by cis-cis endothelial interactions. To form the paired and interlaced arterial-venous pattern characteristic of mature yolk sac vessels, small caliber vessels of the arterial domain are selectively disconnected from the growing arterial tree and subsequently reconnected to the venous system, implying that endothelial plasticity is needed to fashion normal

growth of veins. Arterial-venous differentiation and patterning are controlled by hemodynamic forces, as shown by flow manipulation and *in situ* hybridization with arterial markers ephrinB2 and neuropilin 1, which show that expression of both mRNAs is not genetically determined but plastic and regulated by flow. *In vivo* application of ephrinB2 or EphB4 in the developing yolk sac failed to produce any morphological effects. By contrast, ephrinB2 and EphB4 application in the allantois of older embryos resulted in the rapid formation of arterial-venous shunts. In conclusion, we show that flow shapes the global patterning of the arterial tree and regulates the activation of the arterial markers ephrinB2 and neuropilin 1.

Movies available online

Key words: Arterial-venous differentiation, Hemodynamic forces, Growth factors, Receptors, Ephrin, Neuropilin

Introduction

In the adult vascular system, arterial and venous endothelial cells (EC) show marked phenotypic differences in their capacity to generate vasoactive substances, to adapt to hemodynamic changes, the hemostatic responses or the immune responses to inflammatory stimuli. This raises the question if the differences in phenotype relate to the origin of the EC used for making these different compartments, and how these arise during the ontogeny of the embryonic cardiovascular system.

During development, blood vessels in the embryo and in the yolk sac are initially formed by a process termed vasculogenesis, which denotes the *in situ* differentiation of EC from the mesoderm and their coalescence into primary vessels (Risau, 1997). Vasculogenesis leads to the formation of the first major intra-embryonic blood vessel, the aorta, and to the formation of the primary vascular plexus in the yolk sac. With the onset of embryonic circulation, these primary vessels have to be remodeled into arteries and veins, in order to develop a functional vascular loop and to accommodate cardiac output. Remodeling of the primary vascular plexus into a more mature

vascular system is thought to occur by a process termed angiogenesis. Angiogenesis in the yolk sac involves capillary sprouting, splitting and remodeling, which leads to the reorganization of the primary vessels into large and small vessels (Risau, 1997). Surprisingly, the formation of yolk sac arteries and veins has not been specifically addressed in the recent literature.

EC were long considered as a homogenous population of cells (Risau, 1997), and differentiation of arteries and veins was thought to be governed by hemodynamic forces, molding out these vessels from the primary vascular plexus. This idea was based on classic studies carried out by Thoma in the chick embryo yolk sac (Thoma, 1893). Thoma observed that vessels that carry a lot of blood flow widen, while those that carry little flow regress. Murray subsequently postulated that vessels adapt to flow in order to optimize the shear stress to which they are subjected (Murray, 1926). These studies have shown that flow can alter lumen dimensions of arterial segments. However, they did not address if or how flow contributes to arterial-venous differentiation and patterning. Studies subsequently carried out in adult vessels showed that vessel segments can adapt to the

amount of flow carried (Peirce and Skalak, 2003; Skalak and Price, 1996). Flow can alter the expression and release of growth factors implicated in vascular remodeling including nitric oxide, endothelin 1, FGF and PDGF.

Recently, specific markers for arteries and veins were discovered, which labeled EC from early developmental stages onwards, before the assembly of a vascular wall. For the arterial system, ephrinB2, neuropilin 1 (NRP1), and members of the Notch pathway, including notch 3, Dll4 and gridlock have been described in zebrafish, chick and mouse (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., 2001). Other molecules are specifically expressed in the venous system, most notably EphB4, the receptor for arterial ephrinB2 (Gerety et al., 1999). The neuropilin 2 (NRP2) 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, the Tie2 receptor is expressed at higher levels in veins compared with arteries (Moyon et al., 2001b). Based on these specific expression patterns and on mutant studies in zebrafish and mouse, it has been suggested that arterial-venous differentiation during embryogenesis is actually genetically predetermined (Wang et al., 1998). Interestingly, ephrins and neuropilins are also expressed in the developing nervous system where they are implicated in the establishment of cell boundaries and axon guidance (Flanagan and Vanderhaeghen, 1998). It is tempting to speculate that the growth control processes in the nervous system, exerted through repulsive and attractant signaling, may also apply to the vasculature (Kullander and Klein, 2002).

Mouse mutants for ephrinB2 and EphB4 die at embryonic day (E) 9.5 because of defective remodeling of the primary vascular plexus into branched arteries and veins. The specific effects on arteries and veins were recognized because of the restricted expression of these molecules (Adams et al., 2001; Gerety et al., 1999; Wang et al., 1998). Interestingly, phenotypes of mouse mutants defective for the ligand (ephrinB2) and the receptor (EphB4) are symmetrical. Both molecules are transmembrane-spanning, raising the question of how and where they interact during the formation of arteries and veins. Moreover, both ligand and receptor are able to elicit a signaling cascade. In the nervous system, this bi-directional signaling is involved in the formation of cell boundaries in the rhombomeres (Klein, 1999). In the vascular system, it has been shown that reverse signaling initiated by EphB4 binding to its ligand ephrinB2 is responsible for the observed remodeling failure (Adams et al., 2001). Many other mouse mutants for genes involved in vascular development die because of failure in vessel remodeling, including angiopoietin 1 (Suri et al., 1996), HIF1 α (Ryan et al., 1998; Iyer et al., 1998), VE-cadherin (Carmeliet et al., 1999), activin receptor-like kinase 1 (Urness et al., 2000) and connexin 45 (Krüger et al., 2000), as well as signaling molecules specifically expressed by arteries, such as NRP1 and members of the Notch family (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.

It remains currently unknown if the remodeling of the primary vascular plexus into arteries and veins is governed by flow, as suggested by the classic studies (Murray, 1932;

Thoma, 1893), or genetically predetermined, as suggested by the early expression of arterial and venous markers and zebrafish studies. Zhong et al. (Zhong et al., 2001) have shown that individual fluorescent angioblasts give rise to arterial or venous, but not mixed clones, which is suggestive of a genetic predetermination. Inactivation of the Notch pathway by dominant-negative expression of suppressor of Hairless led to ectopic expression of venous markers in arteries (Lawson et al., 2001), suggesting that the Notch pathway is required to suppress venous fate in arterial endothelium. In spite of a possible genetic predetermination, two recent studies reported that EC can still become incorporated into the venous system after acquiring the expression of the arterial-specific genes ephrinB2 or NRP1 (Moyon et al., 2001a; Othman-Hassan et al., 2001). These studies suggest that EC may be genetically programmed for a certain phenotype but can display plasticity with respect to local cues, probably hemodynamics, the vascular wall or oxygen.

The aim of this study was to explain the regulation of the morphogenesis of the arterial and venous system with respect to the activation of genes specifically implicated in arterial-venous differentiation. We show that arterial-venous differentiation in the chick yolk sac is achieved after the onset of blood flow, and requires endothelial plasticity. We describe four crucial steps for the formation and patterning of arteries and veins resulting in paired and interlaced arteries and veins.

(1) Prior to the onset of flow, EC expressing arterial or venous specific genes are localized to a posterior (arterial) and anterior (venous) pole. EC of both vessel types are initially interacting in a cis-cis configuration, the border of arteries and veins is located in the same vascular tube (Yancopoulos et al., 1998).

(2) Shortly after the onset of the heart beat and perfusion, the vitelline artery plexus forms in the posterior arterial pole.

(3) Distal progression of arterial tube formation is achieved by disconnection of small side branches.

(4) These side branches are subsequently used to make veins.

To achieve this, the disconnected segments make sprouts growing dorsal to arteries, projecting to the primary veins. Thus, vessels originally part of the arterial tree contribute to the formation of the venous tree. This secondary venous plexus now lies dorsally to the arteries and EC of both vessel types are now in a cis-trans configuration. The process of disconnection and reconnection allows the formation of paired and interlaced arteries and veins. Expression of the arterial markers ephrinB2 and NRP1 is regulated by blood flow and becomes rapidly downregulated in the disconnected segments. Instead, the plexus located dorsal to the arteries starts to express the venous markers NRP2 and Tie2, genetically confirming their venous identity. Similarly, experimental manipulation of the flow pattern changed global patterning of the yolk sac arteries and veins and regulated expression of arterial and venous markers accordingly.

The observation that ephrinB2 and NRP1 were regulated by flow prior to the subsequent changes in the vascular system suggested that they might be 'markers' instead of 'makers'. To test their function, we applied recombinant proteins in the yolk sac and allantois of chick embryos. Ephrin B2 and EphB4 application on yolk sac vessels failed to induce any obvious morphological changes of arteries and veins. By contrast,

application on more mature arteries and veins in the allantois induced the formation of arterial-venous shunts. These results suggest that ephrinB2/EphB4 interaction may play a role in the maintenance of the mature vessel configuration rather than in its establishment.

Materials and methods

Embryonic material

Fertilized quail (*Coturnix coturnix japonica*) and chick (*Gallus gallus*, brown leghorn) embryos were purchased from commercial sources and incubated at 38°C in a humidified atmosphere. Embryonic stages were determined according to the number of somites formed (ss).

Flow manipulation: no-flow and ligation model

To generate no-flow embryos, three different approaches were used to obliterate the formation of a functional loop between heart and yolk sac. Similar results were obtained with all three methods. In the first method, we excised the head at the 10 ss, which effectively impeded formation of inflow to the heart. In the second method we excised the heart at the 13-14 ss. The third method mainly used in this work consists of slight dehydration of the embryo, generated by opening a window in the egg-shell prior to incubation. The egg is then sealed with Scotch tape into which holes are made and incubated at 38°C until the desired stage.

Ligation of the right vitelline artery was performed using a tungsten wire tool (0.5 mm diameter) as previously described (Stephan, 1952). Briefly, the tool (see Fig. 8) contains one extending part, which is introduced underneath the artery and subsequently lifts it. This lifting results in a mechanical obstruction of the artery lumen, which blocks flow through the artery. Unmanipulated, age-matched embryos were used as controls.

In toto protein binding

Recombinant mouse ephrinB2-Fc and EphB4-Fc were purchased from R&D Systems (UK). Whole-mount protein binding in chick embryos was adapted from a previously described protocol (Gale et al., 1996). Briefly, embryos were dissected in cold PBS, transferred into culture medium [Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and 15% chick serum] for 1 hour, and incubated in culture medium containing 5 µg/ml of the fusion protein ephrinB2-Fc or EphB4-Fc, overnight at 4°C. Embryos were washed three times with PBS, post-fixed with 4% paraformaldehyde overnight at 4°C, and washed with PBS. Endogenous phosphatases were inactivated by incubation at 70°C for 1 hour. Embryos were incubated in culture medium containing 0.1% Triton X-100, overnight at 4°C followed by incubation with an anti-Fc antibody (Goat anti-human Fc-AP bound, dilution 1/5000, Jackson ImmunoResearch) overnight at 4°C. Finally, embryos were washed with TBST (20 mM Tris, pH 8, 140 mM NaCl, 0.1-0.2% Triton) during 1 day. Color development was performed as described for whole-mount in situ hybridization (Moyon et al., 2001a).

In vivo application of ephrinB2-Fc and EphB4-Fc protein

Recombinant ephrinB2-Fc and EphB4-Fc protein were dissolved in sterile PBS, and applied at a final concentration of 2 µg or 4 µg of protein per 5 µl of PBS. For treatment of the yolk sac in ovo, the vitelline membrane was removed and protein was applied into a silicon ring placed on the yolk sac. For treatment of New cultures, proteins were applied without rings. For treatment of the allantois membrane, protein was applied within a silicon ring at E4 (90 hours incubation). Application on older embryos had no effect. Pre-clustering of ephrinB2-Fc or EphB4-Fc was achieved by pre-incubation with anti-Fc antibody (rabbit anti-human IgG, Fc fragment specific, Jackson ImmunoResearch, USA) at a concentration ratio of 1:8, for 1 hour at 4°C.

Time-lapse video-microscopy

New cultures were prepared as previously described (Chapman et al., 2001). For time-lapse video-microscopy, embryo New cultures or eggs containing a window (LeNoble et al., 1993), were placed between two glass heating plates (Minitüb HT300, Germany), the lower one at 37°C, the upper one at 39°C to avoid condensation. Embryos were observed using a Leica MZFLIII stereo-microscope equipped with a digital camera (Princeton Coolsnap cf). Images were acquired using Metaview software (Princeton, version 5.0r6, 2002, US).

An intravital microscope setup (Leica) was used to obtain vessel images at a high magnification and high resolving power. The vasculature was visualized with a 4× (N.A.=0.12) or 11× objective (N.A.=0.25) using polarized epi-illumination from a 100W Hg lamp. Images were projected onto a Hamamatsu camera attached to an intensifying unit. The camera was connected to a video recorder (Panasonic) and images were stored on S-VHS tape for off-line analysis of vessel dimensions, and blood flow characteristics (Rouwet et al., 2000).

In situ hybridization

In toto in situ hybridization using antisense mRNA probes for ephrinB2 and NRP1 were performed as described previously (Moyon et al., 2001a). 2114 bp of coding sequence of chick NRP2 (Accession Numbers, AF417235.1 and AF417236) were amplified by RT-PCR using two primers (forward, CTC AAC TTC AAC CCT CAC TTC; reverse, ATC CGG TAC TCC ATG TCG TAG) from E8 chick embryo RNA. The fragment was subcloned into T/A TOPOII vector (Invitrogen).

DiI-ac-LDL, FITC-dextran, QH1 and TUNEL staining

DiI-ac-LDL (Biomedical Technologies 200 µg/ml in PBS) and FITC-Dextran (Sigma, M_r 2000 kDa, 8 mg/ml in PBS) were injected intracardially using a micropipette. Embryos were observed immediately (Fitc dextran) or after a 2 hours re-incubation period (DiI-Ac-LDL). TUNEL stainings were done on paraffin sections (7.5 µm) prepared from chick embryos at the 25 and 35 ss, using an in situ cell death detection kit with peroxidase (Roche Diagnostics) according to the manufacturer's instructions. Whole-mount QH1 stainings were done as previously described (Pardanaud et al., 1987), using undiluted hybridoma supernatant and GAM IgM coupled to Texas Red (Southern Biotechnology Associates).

Results

Development of the yolk sac vascular tree: from a capillary plexus to arteries and veins

At the end of the vasculogenesis period and prior to the onset of perfusion, yolk sac vessels are present in the form of a capillary plexus. To understand how this primary capillary plexus gets remodeled into a paired, branched and interlaced structure of arteries and veins, we initially examined normal chick or quail embryo yolk sacs after the onset of perfusion, between the 15 somite stage (ss) and the 35 ss. We observed two stages of arterial-venous differentiation shown in Fig. 1. Shortly after the onset of perfusion, a posterior arterial and an anterior venous pole are present in the yolk sac: arteries and veins at this stage are present in a two-dimensional cis-cis configuration (Fig. 1A,B), the primary circulation. This configuration is transient and remodeled over a period of only 24 hours into a three dimensional structure with paired or interlaced arteries and veins (Fig. 1C,D), the secondary circulation, which allows cis-trans interactions. These rapid and dramatic changes in the vascular system were subsequently investigated in more detail by time-lapse videomicroscopy.

Development of the vitelline artery

The formation of the vitelline artery was studied by time-lapse video-microscopy of chick embryos in ovo or in New cultures (Chapman et al., 2001) (Fig. 2). Starting around the 15 ss, the future vitelline artery appears as a plexus of small caliber sized vessels located laterally to the segmental plate (Fig. 2A,B). Perfusion of the yolk sac vascular plexus starts around this stage by blood flowing from the heart through the dorsal aorta and entering the yolk sac through this arterial plexus (red arrows, Fig. 2). With the establishment of perfusion, the plexus is rapidly remodeled into a larger vessel, the vitelline artery, the proximal part of which becomes clearly distinguishable at the 21 ss, when it branches off the aorta at the level of somite 21 (Fig. 2C). Detailed in vivo time-lapse recording of the remodeling of the arterial plexus into a tube shows that this process is achieved by fusion of individual capillary segments. Not all capillary segments are integrated into the tube, some become disconnected (see below) and conspicuous capillary-free zones appear around the newly formed vitelline artery. Over the next 24 hours, the vitelline artery significantly enlarges and extends to progressively more distal areas of the yolk sac (Fig. 2C-H). Remodeling of the capillary plexus into the vitelline artery and its branches in the more distal regions proceeds exactly as in the proximal part, by fusion of some capillary segments and disconnection of others (not shown).

Analysis of the blood-flow using intravital microscopy shows a two-dimensional vessel plexus carrying only arterial flow, venous flow is absent in the posterior region of the embryo at this stage (not shown). Blood carried through the vitelline artery into the yolk sac reaches either the peripheral sinus vein or more anterior regions of the yolk sac and is then

carried back to the heart. Thus, blood flow, visualized either directly or by injection of FITC-dextran into the heart, initially proceeds directly from arteries into veins, without passing through a true capillary network (Fig. 3C). This is in contrast to later developmental stages and the adult circulatory system, where blood flows through arteries, arterioles and capillaries into venules and veins. In situ hybridization with the arterial markers NR1P1 and ephrinB2 shows that prior to perfusion of the yolk sac, expression of both mRNAs is observed in the lateral mesoderm at the segmental plate level as well as in the primitive capillary plexus in the posterior (arterial) part of the plexus (Fig. 3A,B). Significantly less expression is observed in the anterior (venous) part of the plexus. With the establishment of perfusion, arterial marker expression rapidly becomes restricted to the vitelline artery and its branches (Fig. 3D,E). Given that arterial and venous blood flow in the same vascular segment (Fig. 3C), the limit of arteries and veins at this stage can only be defined by the direction of blood flow away from (arterial) and towards (venous) the heart. Expression of the arterial markers indeed declines at this 'border'. At later stages, we can see the interlaced pattern of veins and arteries as shown in Fig. 3F by the protein binding of EphB4-Fc, which stains the arteries expressing its ligand ephrinB2.

Development of the vitelline veins

The next step in the remodeling of the primary yolk sac vascular plexus is the formation of veins and ultimately of paired and interlaced arteries and veins in a three-dimensional configuration. We followed this process by time-lapse videomicroscopy (Fig. 4, Movie 1 at <http://dev.biologists.org/supplemental>). The first major veins to arise are the anterior (not shown) and the posterior vitelline veins, which run from the sinus vein to the heart at the cranial and caudal pole of the embryo, respectively. These veins never get paired with arteries, even after experimental manipulation (see below). The posterior vitelline vein arises in a territory that was previously arterial, as determined first by the

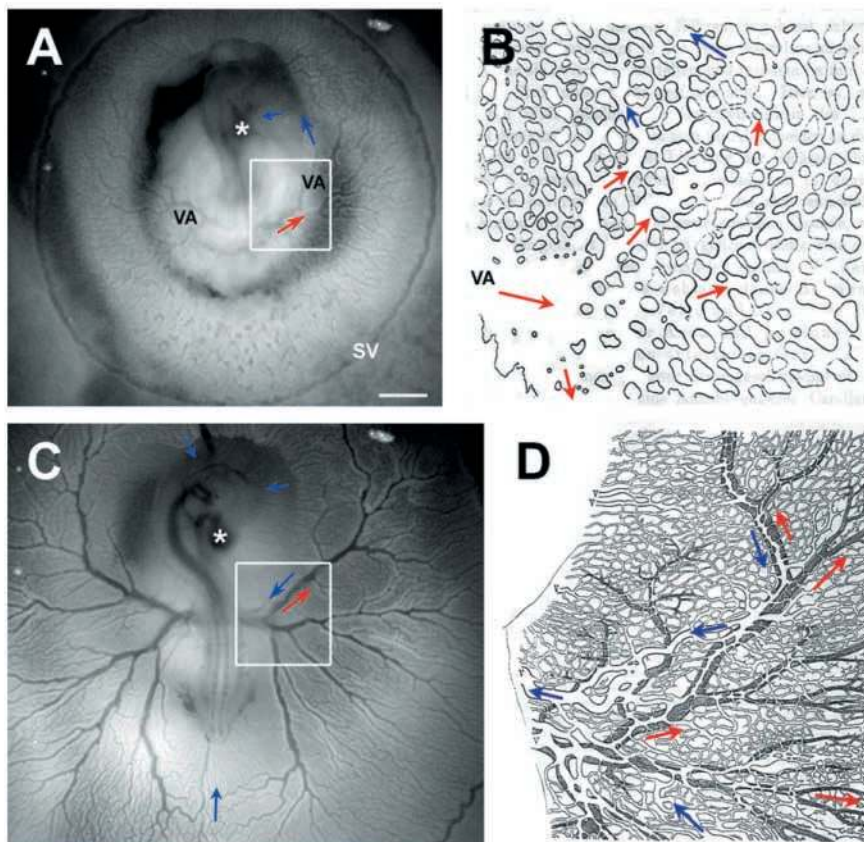


Fig. 1. Overview of arterial-venous differentiation in the yolk sac. (A) Yolk sac vessels just after the onset of perfusion. The vitelline artery (VA) is just beginning to form. Direction of arterial and venous blood flow is indicated by red and blue arrows respectively. The heart is indicated (*). (B) Schematic representation of vascular system as seen in the boxed area in A. Arteries (red arrows) carry blood away from the heart and veins (blue arrows) carry blood back toward the heart. Note that arteries and veins are in a cis-cis configuration. (C) Embryo 26 hours later than in A. (D) Schematic representation of the vascular system as seen in the boxed area in C. Veins are drawn in white, the direction of venous flow is shown by blue arrows. Arteries are drawn in black, arterial flow is indicated by the red arrows. Note that veins have come to lie parallel to arteries, a venous network is covering the arterial network dorsally. SV, sinus vein. Scale bar: 1100 μm .

direction of blood flow (Fig. 2; data not shown) and second by the expression of arterial markers (Fig. 3A,B). In situ hybridization with arterial markers ephrinB2 and NRP1 shows that just prior to formation of the vein, the capillary plexus has downregulated expression of these markers (Fig. 3D,E). The formation of the posterior vein proceeds at great velocity: only 3 hours elapse between the time the first venous flow can be observed in this region (Fig. 4F) and the formation of the posterior vitelline vein (Fig. 4I). Time-lapse studies suggest that the major mechanisms accounting for the formation of this vein is the fusion of capillary segments within this region (Fig. 4, Movie 1 at <http://dev.biologists.org/supplemental>). We subsequently examined whether these vessels were pre-existing or generated through sprouting. To determine if sprouting vessels could be observed during this period, we injected DiI-ac-LDL into the heart of embryos at this stage. DiI-ac-LDL binds to endothelial surface receptors, is taken up into the cells and subsequently labels the entire EC surface, including sprouts. Although intersegmental endothelial sprouts could be readily visualized, very few sprouts were seen in the yolk sac vascular plexus, including the area of the forming posterior vitelline vein (not shown). Similar observations were made by whole-mount staining of quail embryos with QH1, an antibody specific for quail EC (Fig. 6C and data not shown). TUNEL staining failed to reveal endothelial apoptosis (data not shown). EC divisions in the area of the forming posterior vitelline vein are as frequent as in other regions of the yolk sac (Fabian and Wilt, 1973) (data not shown). In combination with the *in vivo* time-lapse images, these observations suggest that the embryo uses already existing capillary segments to fashion the posterior vitelline vein, as explained in detail below.

The first embryonic veins that are paired with arteries are the lateral vitelline veins, which run parallel and dorsal to the vitelline artery (Fig. 5, Movies 2 and 3 at <http://dev.biologists.org/supplemental>). High-magnification time-lapse video-microscopy shows that these veins are also formed by fusion of pre-existing capillary segments. During the formation of the vitelline artery, some arteriolar branches are disconnected from the main branch. This process of disconnection is first observed in the proximal part of the vitelline artery (Figs 5, 6), and progresses to more distal parts as development proceeds. The disconnected vessel segments in this region are still connected to the vitelline artery through more distal parts of the arterial tree. This was confirmed first by intravital microscopic observations in this region, which revealed that hemodynamic characteristics of the arterial system [pulsatility (systole-diastole)] could still be observed in these disconnected vessel segments (Movie 3 at <http://dev.biologists.org/supplemental>). Arterial blood is 'pushing' in these segments; however, there is no net displacement of blood because there is no outflow. The absence of outflow in the presence of the

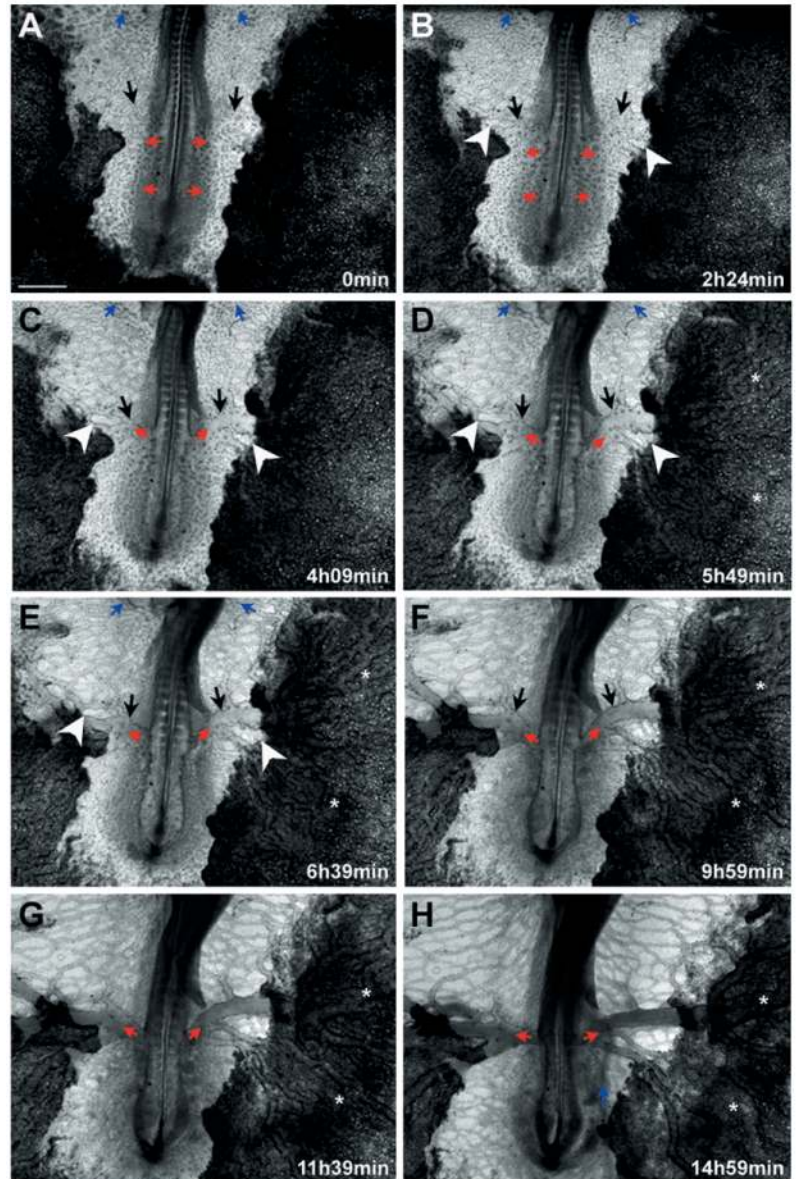


Fig. 2. Formation of the vitelline artery. Time-lapse video-microscopy of a 19 ss chick embryo in *New culture*. Endoderm is facing up. Still images at the indicated times are shown. Note the formation of the vitelline artery (black arrows), which becomes clearly visible at the 21ss (C). Red and blue arrows indicate arterial and venous blood flow, respectively. White arrowheads indicate capillary free-zones. Asterisks show distal progression of vitelline artery formation. (H) The posterior vitelline vein (blue arrow) is formed. Scale bar: 1500 μ m.

'push' leads to the appearance of the blood-filled 'spots' apparent in Fig. 5 and Movies 2 and 3 (at <http://dev.biologists.org/supplemental>). Second, to visualize the disconnection process in more detail at high magnification, we prepared Mercox casts of the yolk sac vasculature at the 35 ss, corresponding to Fig. 5 and Movies 2 and 3 (at <http://dev.biologists.org/supplemental>), that were subsequently observed by scanning electron microscopy (Fig. 6). Casts showed small capillary-free zones on both sides of the vitelline artery (Fig. 6A). Indeed, when one follows the vitelline artery branches distally, one can observe EC protrusions at both sides

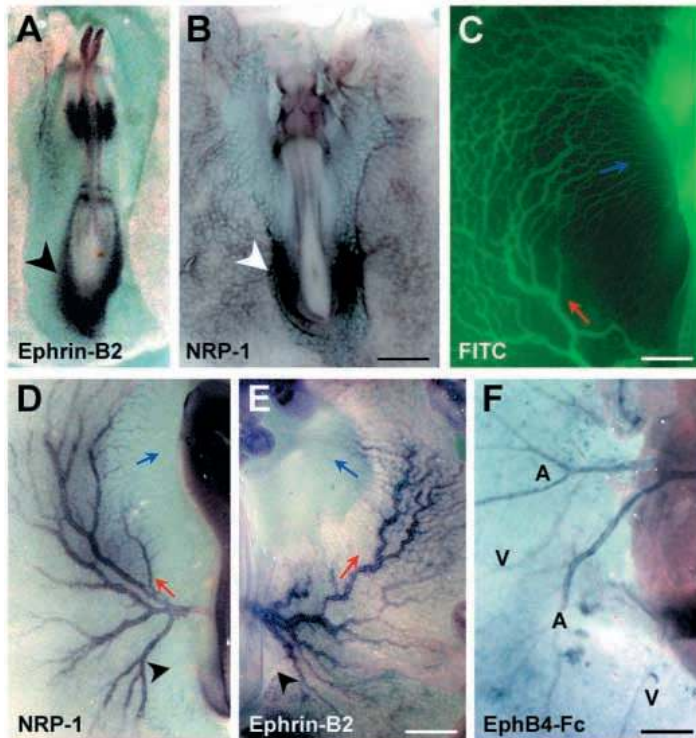


Fig. 3. Yolk sac vessel configuration and arterial marker expression following perfusion. (A) 12 ss and (B) 19 ss embryos. Whole-mount in situ hybridization with the indicated markers. (A,B) Prior to (A) and just after (B) perfusion, ephrinB2 (A) and NRP1 (B) label the posterior (arterial) pole (arrowheads; A,B). (C-E) 30 ss embryos. (C) Intracardiac FITC-Dextran injection. The left side of the yolk sac is shown. Blood from the vitelline artery (red arrow) flows directly into veins (blue arrow), without passing through a true capillary bed. (D) NRP1 labels the vitelline artery (red arrow), but not the vein (blue arrow). (E) The right side of the yolk sac is shown. Ephrin-B2 expression closely resembles NRP1 expression. The future territory of the posterior vitelline vein has downregulated NRP1 and Ephrin-B2 expression (arrowheads, D and E). (F) 40 ss embryo, the left side of the yolk sac is shown. In vivo protein binding of EphB4-Fc to arteries (A) expressing ephrinB2. Veins (V) are negative and are already interlaced with arteries at this stage. Scale bars: 420 μ m in A,B; 400 μ m in C-E; 190 μ m in F.

of the vitelline artery, which appear as blind ending sacs (Fig. 6B,D). Similar blind-ending sacs are observed on the adjacent capillary segments, strongly suggesting that these segments had been disconnected immediately before. Third, similar results were obtained by QH1 whole-mount staining on quail embryos, which also showed the blind ending sacs (Fig. 6D).

The blood-filled 'spots' represent the origin of the veins of the secondary circulation, including the lateral vitelline veins.

When followed over time, it can be seen that these blood filled 'spots' make sprouts perpendicular and dorsal to the arteries (Fig. 5, Fig. 6D and Movies 2 and 3 at <http://dev.biologists.org/supplemental>). Subsequently, these sprouts are actually reconnected to the venous system: the blood contained within the disconnected segments is 'sucked up' into the newly formed vein (Fig. 5F-I, Movie 3 at <http://dev.biologists.org/supplemental>), and the disconnected segments are reperused. In comparison to the initial situation, the disconnected segments now show a venous flow direction (see Movie 3 at <http://dev.biologists.org/supplemental>). Reconnection of sprouts to the arterial system was never observed. Because of the position of the disconnected segments within the arterial network, the disconnection-

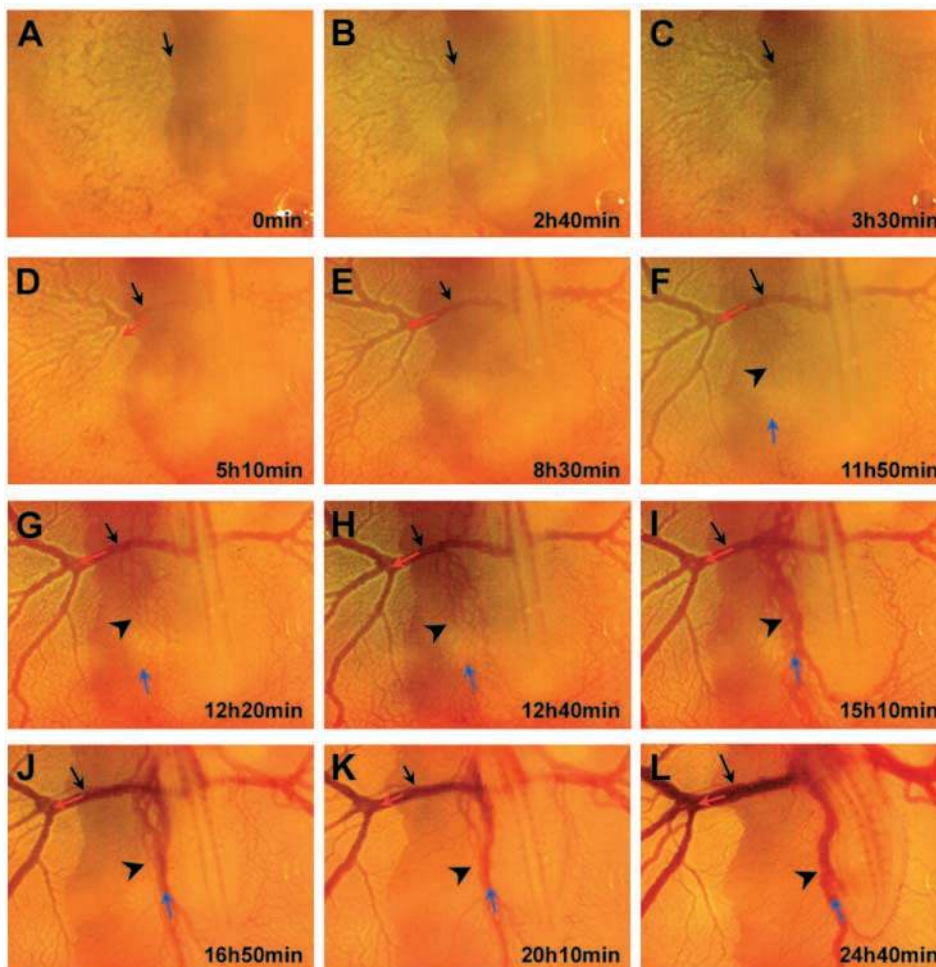


Fig. 4. Formation of the posterior vitelline vein. Time-lapse video-microscopy of a 19 ss chick embryo in ovo (dorsal side upwards). Still images taken at the indicated time points. Black arrows show the vitelline artery, black arrowheads the vitelline vein. Red and blue arrows (D-L) indicate direction of arterial and venous flow, respectively. Venous flow is first detected in F; there is rapid formation of the vitelline vein (arrowheads, F-L).

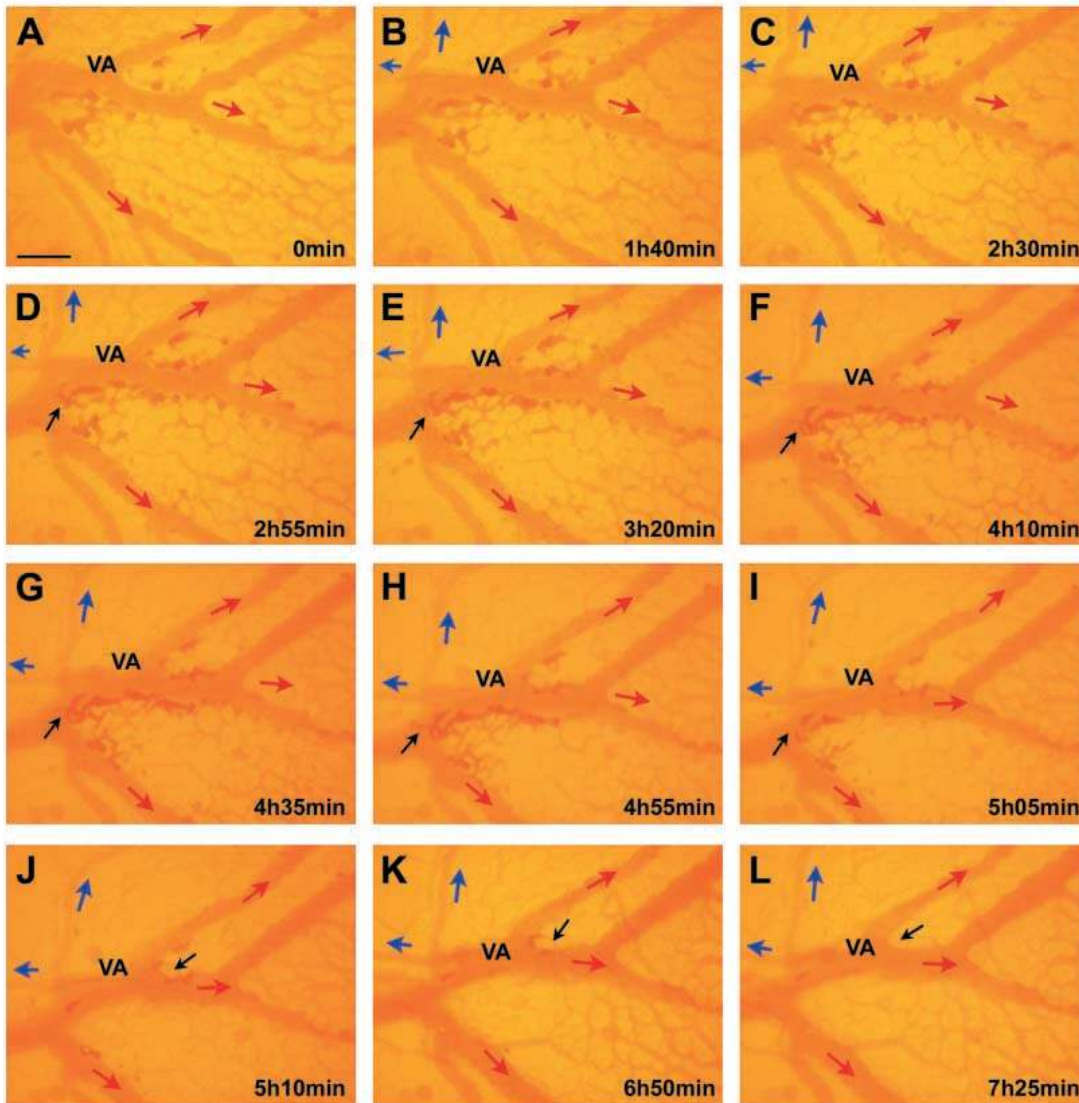


Fig. 5. Reconnection of disconnected arterial capillaries to the venous system. Still images of time-lapse video microscopy of a 30 ss chick embryo in ovo taken at the indicated time points. Red and blue arrows indicate direction of arterial and venous flow, respectively. (A-I) There are blood filled 'spots' at both side of the vitelline artery (VA). Venous flow at this stage is only detected in two small veins (blue arrows). Sprouts originating from these 'spots' cross over the VA (D-H, black arrows). As soon as the sprouts connect to the vein, the blood filled 'spots' are flushed out (compare H with I, black arrows). The same process can be observed more distally (compare J-L, black arrows). Scale bar: 455 μ m.

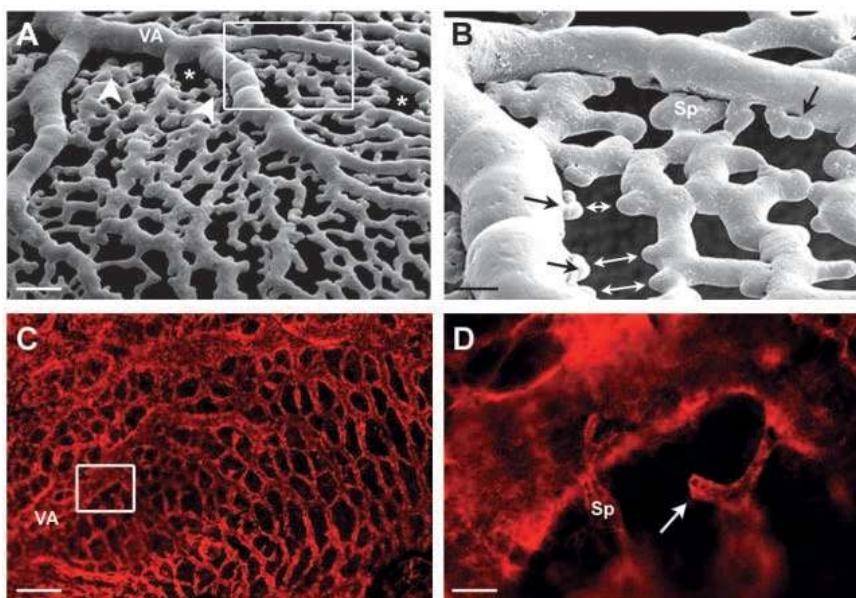


Fig. 6. Mercocast and QH1 whole-mount staining of the developing vitelline artery. (A,B) Scanning electron micrographs of mercocast-filled vitelline artery (VA). (C,D) QH1 staining. QH1 endothelial surface staining shows the same morphology as the mercocast of the vessel lumen. (A) Asterisks show capillary free-zones. Arrowheads indicate disconnected arterial capillaries that are perfused from distal parts and that correspond to the blood filled 'spots' in Fig. 5. (B,D) Higher magnification of the boxed areas in A, C, respectively. Blind ending sacs (B,D, arrows) are present on both sides of the vitelline artery and there are corresponding protrusions on opposite capillary segments (B, double arrows). Note the presence of a capillary sprout (Sp) dorsal to the vitelline artery (VA). Scale bars: 160 μ m in A; 70 μ m in B; 125 μ m in C; 20 μ m in D.

reconnection process results in interlacing and pairing of arteries and veins. Subsequent enlargement of the veins is achieved by fusion of the capillary segments.

Theoretically, another possibility for the existence of the blood-filled ‘spots’ is that there may be degenerating vascular segments through apoptosis, as previously suggested (Risau, 1997). To test this possibility, we sectioned yolk sacs at these stages and examined apoptosis of EC by TUNEL staining. We could not detect any significant number of apoptotic EC in yolk sac blood vessels, while some endodermal cells showed TUNEL staining (not shown). Thus, pruning of the vitelline arterial tree and remodeling of the veins may not involve apoptosis.

In summary, we have observed that during vitelline artery development, small arteriolar side-branches are selectively disconnected and broken off the arterial tree. These disconnected vessels may not carry flow for a period of 5-6 hours, after which, through a process of directed sprouting, they reconnect to the primary venous system, and are again perfused. Thus, the separation of the arterial and venous system is achieved by breakage whereas the venous system grows by incorporating previously existing small arterioles. During normal development, EC plasticity thus seems to be an essential requirement for the growth of the veins.

During this process the system evolves from a two-dimensional architecture allowing cis-cis interactions between EC, the primary circulation, to a three-dimensional architecture, the secondary circulation, which allows cis-trans interaction between EC of the arterial and venous system, respectively. Mercox casts clearly showed that the vitelline artery comes to lie ventrally to the capillary plexus. This transition from a two-dimensional system to a three dimensional system with a dorsoventral polarity, is not restricted to chick embryos, it can also be observed in mouse embryo yolk sac during the period E10-E13 (data not shown). In fact, the patterning of the mouse and chick embryo yolk sac vessels show marked similarities.

Manipulations of blood flow and the effects on arterial-venous differentiation and patterning

No flow model

In the absence of perfusion in no-flow embryos (see Materials and methods), the yolk sac develops a distinct vascular plexus, which contains blood islands filled with erythrocytes in all embryos (Fig. 7). This plexus can continue to grow until at least E7 in the absence of perfusion. In spite of this growth, its morphology continues to resemble the yolk sac of a 13-15 ss embryo and no signs of arterial-venous differentiation are detected (Fig. 7A). In situ hybridization with ephrinB2 shows that part of the no-flow yolk sac vascular plexus expresses this gene, whereas other regions are clearly negative (Fig. 7B,C). The initiation of arterial marker expression does thus not depend on perfusion of the yolk sac.

Incision/ligation model

We subsequently examined the effects of flow perturbations on arterial-venous patterning and expression of arterial markers. To test if formation of the vitelline artery was dependent on perfusion, we performed a longitudinal incision through all germ layers on the left side of the yolk sac of 10-12 ss embryos from the head to the tail (incision model, Fig. 7D). In this model,

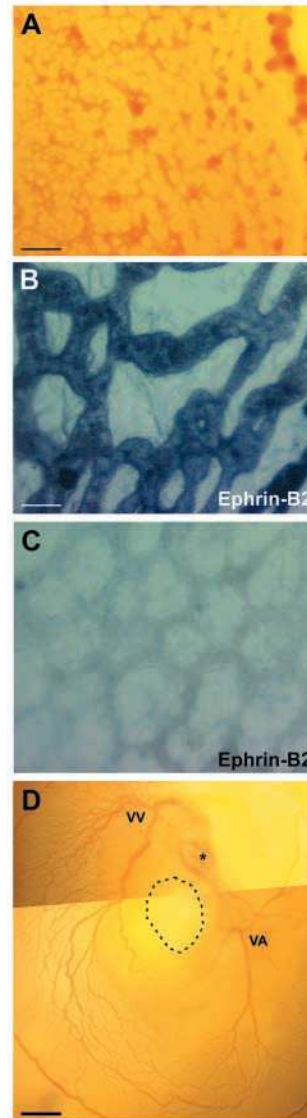


Fig. 7. No-flow and incision models. (A) Bright field photomicrograph of a no-flow yolk sac of an E5 quail embryo. Note the absence of arteries and veins. (B,C) Whole-mount in situ hybridization of an E5 quail embryo no-flow yolk sac with an ephrinB2 anti-sense riboprobe. Part of the capillary plexus expresses ephrinB2 (B), other parts are ephrinB2 negative (C). (D) Incision model. 35 ss embryo 24 hours after incision. The incision site separating the embryo from the left side of the yolk sac is indicated (stippled area). A single vitelline vein (VV) has developed on the left side. The left vitelline artery (VA) is absent. The right VA shows increased growth. Asterisk indicates the heart. Scale bars: 300 μ m in A; 50 μ m in B,C; 600 μ m in D.

blood flowing through the left dorsal aorta cannot enter the yolk sac vascular plexus because it has been spatially separated. Because the incision is performed at an early developmental stage, prior to the onset of perfusion of the yolk sac, minimal bleeding is observed. Embryos ($n=50$) were analyzed 24 hours after the incision, no increase in their mortality rate was observed over this time period. In this experimental condition, the left vitelline artery completely failed to develop (Fig. 7D). By contrast, the right vitelline artery developed to twice its normal size and usually crossed the midline of the embryo/yolk sac axis (Fig. 7D). On the left side of the embryo, a large vitelline vein developed. These experiments show that formation of the vitelline artery depends on perfusion.

To further test this idea, we performed ligation experiments of the already formed vitelline artery. Ligation of the right vitelline artery was achieved by introducing a metal clip in ovo underneath the artery, the clip was devised such that it gently lifts the vitelline artery and arrests blood flow distal to the clip without perturbing general embryonic development. Embryos treated in this way can survive at least until E8, they do not exhibit overt morphological abnormalities and can develop a

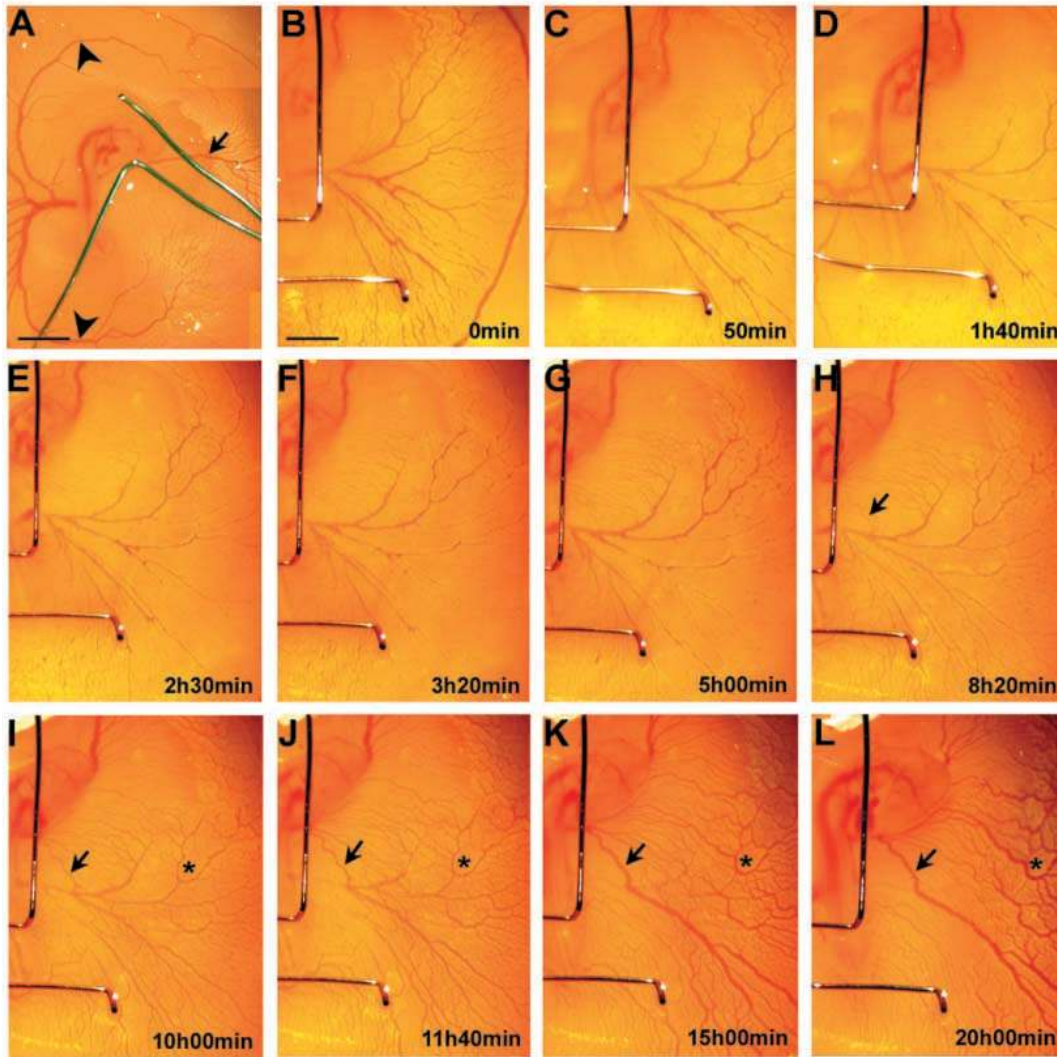


Fig. 8. Ligation model. Ligation of the right vitelline artery of a 30 ss chick embryo in ovo. (A) Overview of an embryo 24 hours after ligation. Note venularization of the ligated vitelline artery (arrow). The unligated vitelline artery shows enhanced growth across the midline (arrowheads). (B-L) Still images of time-lapse videomicroscopy of another ligated embryo taken at the indicated time points. The future major venous branch can be seen in H (arrow). Note incorporation of an entire branch of the vitelline artery into this vein (arrows, H-L). Asterisks in I-L indicate another arterial segment that becomes venularized. Scale bars: 945 μm in A; 715 μm in B-L.

chorioallantoic membrane. We routinely incubated ligated embryos for 3 days and found morphology to proceed normally, except for the changes in the vascular patterning of the yolk sac described below.

Immediately after the ligation of the right vitelline artery, flow was attracted from the marginal vein and, hence, part of the arterial system was perfused in a retrograde manner. In 100% of the embryos ($n=70$), ligation resulted within 24 hours in complete venularization of the right side of the yolk sac (Fig. 8, Movie 4 at <http://dev.biologists.org/supplemental>). We observed that many previously existing arterial segments were incorporated into the new venular tree (compare Fig. 8B with 8I-L). The chance of a previously existing arterial segment to be incorporated into the venous tree appeared dependent on its orientation relative to the new preferential flow direction. Vessel segments oriented in the preferential flow direction (Fig. 8B,I-L) became incorporated into the venular tree. Other segments located immediately downstream of the ligation, more perpendicular to the new preferential flow direction, were not incorporated into the new venous tree but remained visible as unperfused tubes.

The ligation also affected growth of the left vitelline artery, and the anterior and posterior vitelline veins (Fig. 8A). About

24 hours after the ligation, large arteries branching off the left vitelline artery were observed to project to the right side of the yolk sac, and crossed the midline of the embryo/yolk sac axis (Fig. 8A, Fig. 9I,J), thus crossing the areas normally occupied by the anterior and posterior vitelline veins. Detailed examination of the fate of the anterior vitelline vein after ligation showed that it was remodeled and became partly integrated into the arteries that expand from the left side (Fig. 8A, Movie 5 at <http://dev.biologists.org/supplemental>), indicating that originally venous EC can be transformed into arteries. Thus, manipulation of the flow pattern can transform arteries into veins and veins into arteries.

To determine if expression of arterial markers would be influenced by alterations in the flow pattern, we performed whole-mount in situ hybridizations on ligated embryos (Fig. 9). Expression of NRP1 starts to be downregulated on the ligated side 10 minutes after ligation (Fig. 9A). After 30 minutes and 1 hour of ligation, expression levels have decreased further and are undetectable after 4 hours (Fig. 9B-D). Ephrin B2 mRNA and protein levels decrease over a similar time period following ligation (Fig. 9E,F). To determine if venous markers were upregulated in the ligated artery, we examined expression of NRP2 and Tie2 (Moyon et al., 2001b;

Herzog et al., 2001) at different time points after ligation. One hour after ligation, neither Tie2 nor NRP2 showed upregulation in the ligated side (Fig. 9G,H). Upregulation of both markers was, however, readily apparent after 12 hours of ligation (Fig. 9I,J). Although the left anterior and posterior vitelline artery crossing over the midline were clearly negative for Tie2 (Fig. 9I) and NRP2 (Fig. 9J), the venularized branches of the ligated right vitelline artery expressed both markers abundantly. These observations suggest that the downregulation of arterial

markers is more rapid than the upregulation of venous markers. The precise time point of onset of expression of venous markers has not yet been determined; however, we failed to detect any increase in expression of Tie2 5 hours after ligation (not shown).

The downregulation of arterial marker expression could also be due simply to degeneration of the vessels. To test for this possibility, we first injected FITC-dextran intravenously into embryos at different time points after ligation. Four hours after ligation, fluorescent signals could be readily detected in all vessels both on the unligated and on the ligated side (Fig. 10A). Time-lapse video-microscopy showed that perfusion of vessels on the ligated side originated from the peripheral sinus vein and the flow direction in the proximal part of the ligated vitelline artery was now reversed (see Movie 6 at <http://dev.biologists.org/supplemental>). No bleedings or plasma extravasations were detected, indicating that the experimental manipulation had not disturbed vessel integrity. We next performed ligations for different periods of time (30 minutes–8 hours), after which the ligation tools were removed and arterial flow was allowed to re-perfuse the right vitelline artery. The embryos were fixed 6 hours after removal of the ligation tools and hybridized with ephrinB2 (not shown) or NRP1 (Fig. 10B,C,F). Removal of the ligation tools after 30 minutes or 4 hours resulted in the re-expression of NRP1 mRNA in arterial vessels on the ligated side (Fig. 10B,C). Expression levels of NRP1 on the ligated side after 4 hours of ligation appeared low compared with the unligated side (Fig. 10C). However, *in vivo* examination of the right vitelline arterial tree after this 4 hour period of ligation followed by 12 hours of reperfusion revealed that the arterial tree on the ligated side was much smaller compared with the control side (Fig. 10D,E). Indeed, during the period of ligation, all the arterial blood flow passed through the left vitelline artery, which enlarged, allowing faster growth in comparison with the right side. After reperfusion, the growth of the right vitelline artery is therefore delayed (Fig. 10D,E). Reperfusion thus completely restores arterial marker expression, albeit in a reduced territory. After 6 hours of ligation, reperfusion was no longer obtained because of definitive obstruction of the vitelline artery and re-expression of arterial markers was not observed (Fig. 10F).

Role of ephrinB2-EphB4 in arterial-venous differentiation

The observations described above suggest that flow is a major driving force responsible for arterial patterning. Moreover,

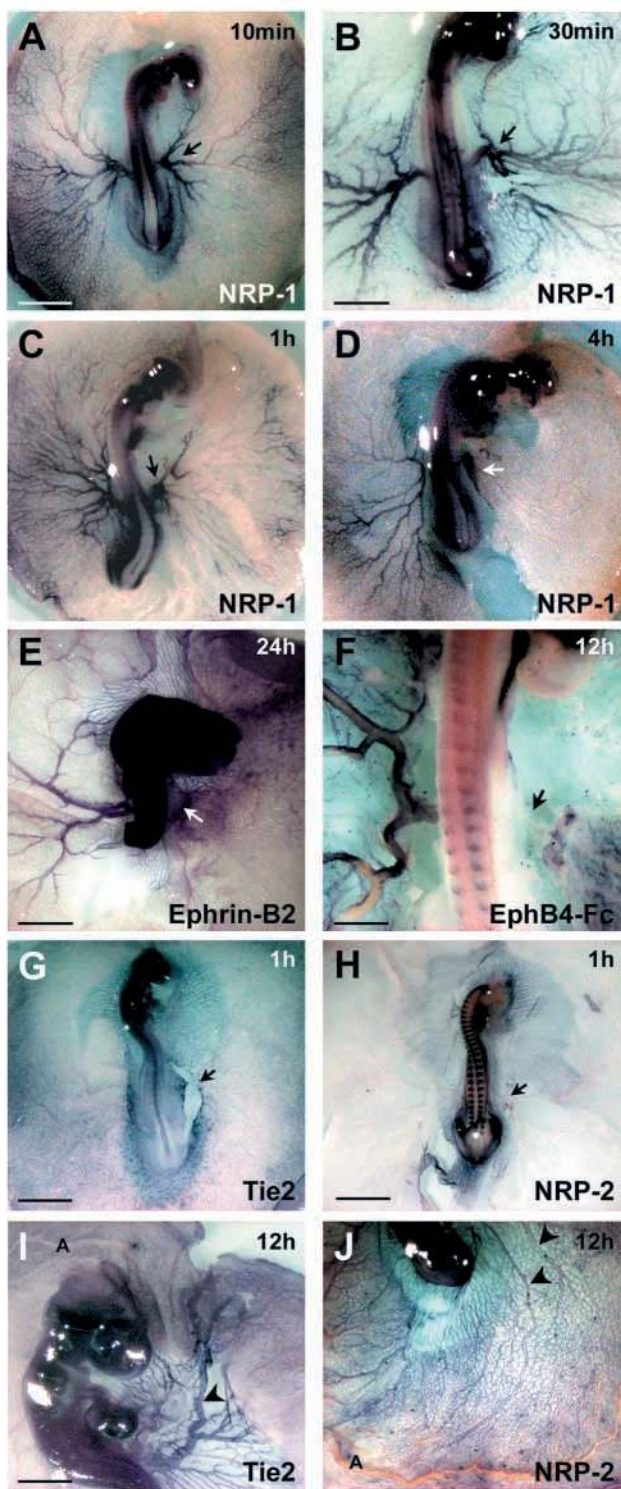


Fig. 9. Whole-mount in situ hybridization of ligated embryos with arterial markers. Ligations were performed for the indicated time periods, and embryos were hybridized with the indicated antisense riboprobes. Arrows (A–H) show ligation site. (A–F) Arterial markers. Note arterial expression on the left side of all embryos and progressive downregulation of NRP1 (A–D) and ephrinB2 (E), as well as the absence of binding of EphB4-Fc (F) on the ligated side. (G–J) Venous markers. (G,H) No upregulation of venous marker expression is detectable after 1 hour of ligation. (I,J) 12 hours after ligation. Tie2 and NRP2 mRNA are not expressed in the anterior (I) or posterior (J) left vitelline arteries (A) that cross over the midline, although they are both expressed in venularized arteries (arrowheads) on the ligated side. Scale bars: 530 μ m in A,C,D; 510 μ m in B; 725 μ m in E; 230 μ m in F; 700 μ m in G,J; 880 μ m in H; 435 μ m in I.

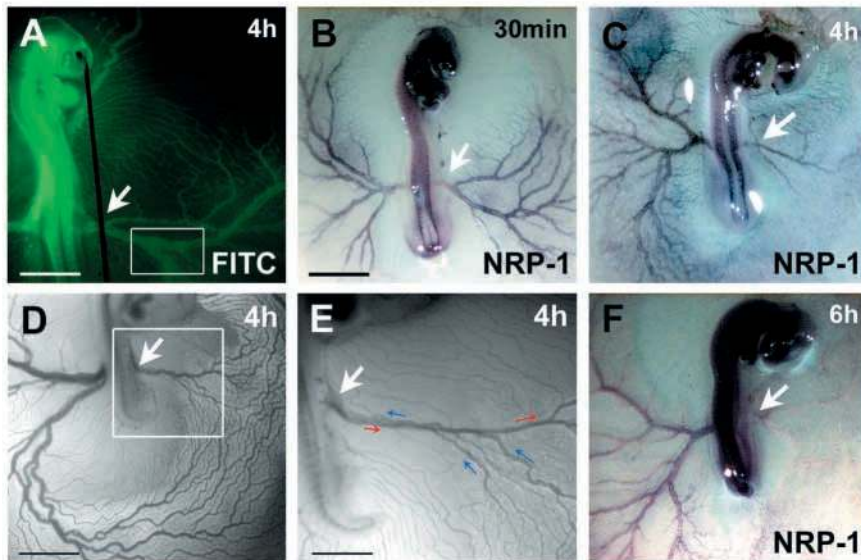


Fig. 10. Removal of the ligation tool restores arterial marker expression. (A) Vessel integrity 4 hours after ligation demonstrated by FITC-dextran injection. Note retrograde perfusion of the ligated vitelline artery. Boxed region is shown in Movie 6 at <http://dev.biologists.org/supplemental>. (B,C,F) The ligation tool was removed after the indicated time points, and embryos were fixed 6 hours later. Whole-mount in situ hybridization with NRP1 antisense riboprobe shows re-expression after 30 minutes (A) and 4 hours (B), but not after 6 hours (F). (D,E) Photomicrographs of a live embryo treated as the embryo in B. Note decreased size of the vascular plexus on the ligated side. (E) Higher magnification of the boxed region in D. Arterial blood flow is shown in red, venous blood flow in blue. Arrows indicate ligation site. Scale bars: 760 μm in A; 725 μm in B,C,F; 1400 μm in D; 780 μm in E.

after experimental manipulation, arterial marker expression is regulated by flow. Flow regulation also appears to play a major role in expression of these markers in normal embryos: the posterior arterial territory expressing ephrinB2 and NRP1 (Fig. 3A,B) becomes negative for ephrinB2 and NRP1 mRNA expression just prior to the formation of the posterior vitelline vein (Fig. 3D,E). These findings then raise the question of whether these molecules are ‘markers’ or ‘makers’, as suggested by their role as growth factor receptors. To address this question, we applied ephrinB2-Fc and EphB4-Fc proteins topically to the yolk sac. In toto receptor-binding studies showed that ephrinB2 bound to veins, while EphB4 bound to arteries (Fig. 3F, Fig. 9F and data not shown), indicating that the applied proteins could reach their respective targets on EC. Indeed, protein-binding studies closely resemble the images obtained by in situ hybridization. We then applied recombinant proteins on chicken yolk sacs during the stages described above. Ephrin B2 was applied to both the anterior (venous) and posterior (arterial) pole of the embryo, no effects on yolk sac remodeling could be observed. Addition of pre-clustered ephrinB2 to the yolk sac showed no obvious effects either (not shown). Similar results were obtained with unclustered and clustered EphB4 protein, effects were not detectable. Thus, in spite of binding, neither clustered nor unclustered ephrinB2 or EphB4 recombinant protein induced a major change in arterial-venous patterning or yolk sac remodeling.

We next examined the in vivo effects of ephrinB2 and EphB4 in the allantois membrane vessels at E4. Proteins were applied into silicon rings placed onto the allantois. Control application of the vehicle PBS showed no effects on vessel patterning (not shown). Strikingly, ephrinB2 overexpression in the allantois induced major changes in the vascular patterning (Fig. 11). In 5/5 embryos, the first changes in vessel patterning were observed several minutes after application and concerned veins, which appeared to increase the number of their branches. This was rapidly followed by the enlargement of both veins and arteries (compare Fig. 11A with 11I). Some arteries displayed a huge enlargement and increased their size more than 10-fold when compared with their initial diameter. Concomitant to the diameter changes, we observed progression

into the formation of arterial-venous shunts (Fig. 11E-I). Although PBS-treated embryos survived, three out of five ephrinB2 treated animals died 8-10 hours after treatment. Given that ephrinB2 application on the yolk sac did not affect the survival of the embryos, we presume that they may die because of formation of the arterial-venous shunts.

Application of EphB4 protein ($n=3$) resulted in comparable morphological effects, formation of arterial-venous shunts (Fig. 11L). The magnitude of response with respect to changes in vessel diameters seemed less when compared with ephrinB2 (Fig. 11J-L). More detailed examination of the morphological alterations revealed that the allantois arteries started to expand into regions previously occupied by venules. Although in control situations, venules expand distally, application of EphB4 resulted in retraction or regression of the venules after which these regions were invested by arteries.

Discussion

In the present study, we describe the morphogenic events necessary to separate the arterial plexus from the venous plexus within the yolk sac of the chick embryo. We then tested if arterial-venous differentiation and patterning is genetically predetermined or plastic depending on flow, and finally we investigated the in vivo function of ephrinB2 and EphB4 during arterial-venous differentiation.

Careful inspection of the morphological events underlying the separation of the arterial and venous system within the chick embryo yolk-sac plexus revealed a novel key event necessary for arterial-venous differentiation: the disconnection of small caliber sized vessels from the growing arterial tree starting at the most proximal part and progressing distally. Using detailed in vivo time-lapse observations of the initial arterial plexus starting at 15 ss for 24 hours, we observed that small caliber vessels connected to the stem of the forming vitelline artery are disconnected. On casts obtained from embryos at 21-35 ss, these disconnected vessels are visible as blind-ending sacs connected to the main branch. We subsequently observed that this process repeated itself towards the more distal parts of the vascular tree. The small vessels

disconnected from the arterial system are subsequently reconnected to the venular plexus through specific projections, apparent as sprouting towards the developing venular plexus. After establishing connection with the primary venous system, they will start to carry flow again. To our knowledge, such a morphogenic event with potential relevance for arterial/venous growth has not been previously shown *in vivo*.

Our observations raise several important issues with respect to the understanding of arterial-venous differentiation. First, we noted the remarkable speed at which arterial-venous differentiation is achieved: only 12 hours elapse between the formation of the vitelline artery and the formation of the vitelline vein. TUNEL staining failed to reveal significant cell death in the yolk sac vessels during arterial-venous differentiation. Endothelial divisions are uniform in the yolk sac and are thus unlikely to account for the remarkably speed of the formation of the major vessels such as the posterior vitelline vein (Fabian and Wilt, 1973). According to the current literature (Risau, 1997), several possibilities for vessel remodeling have been described: sprouting, intussusception, angioblast differentiation and migration, and pruning. DiI injections and QH1 staining labeling EC membranes of

developing yolk sac vessels have not revealed a significant number of sprouts in yolk sac vessels, while such sprouts were readily observed in intersomitic vessels. Sprouting angiogenesis does therefore not seem to constitute the major operational mode during yolk sac remodeling. Intussusception may better account for some aspects of the process we describe here. Djonov has described intussusceptive arborization (IAR) of vessels in the chorioallantoic membrane (Djonov et al., 2002). During IAR, a capillary plexus is transformed into large and small vessels by the formation and alignment of tissue pillars alongside the future large vessels, which are subsequently split off the capillary bed. This process may apply for the formation of the most proximal part of the vitelline artery between 15 ss and 22 ss. However, actual physical disconnection of small vessel segments has not been described in IAR, or any other angiogenic remodeling process. Disconnection differs from shear driven vessel regression as neither the main branch nor the disconnected vessel diameters have significantly changed. However, it can be explained based on physical principles. This form of pruning is not a reduction of diameter as a function of shear inside the small side branch, but direct breakage of the connection in the region of the intersection of the main branch

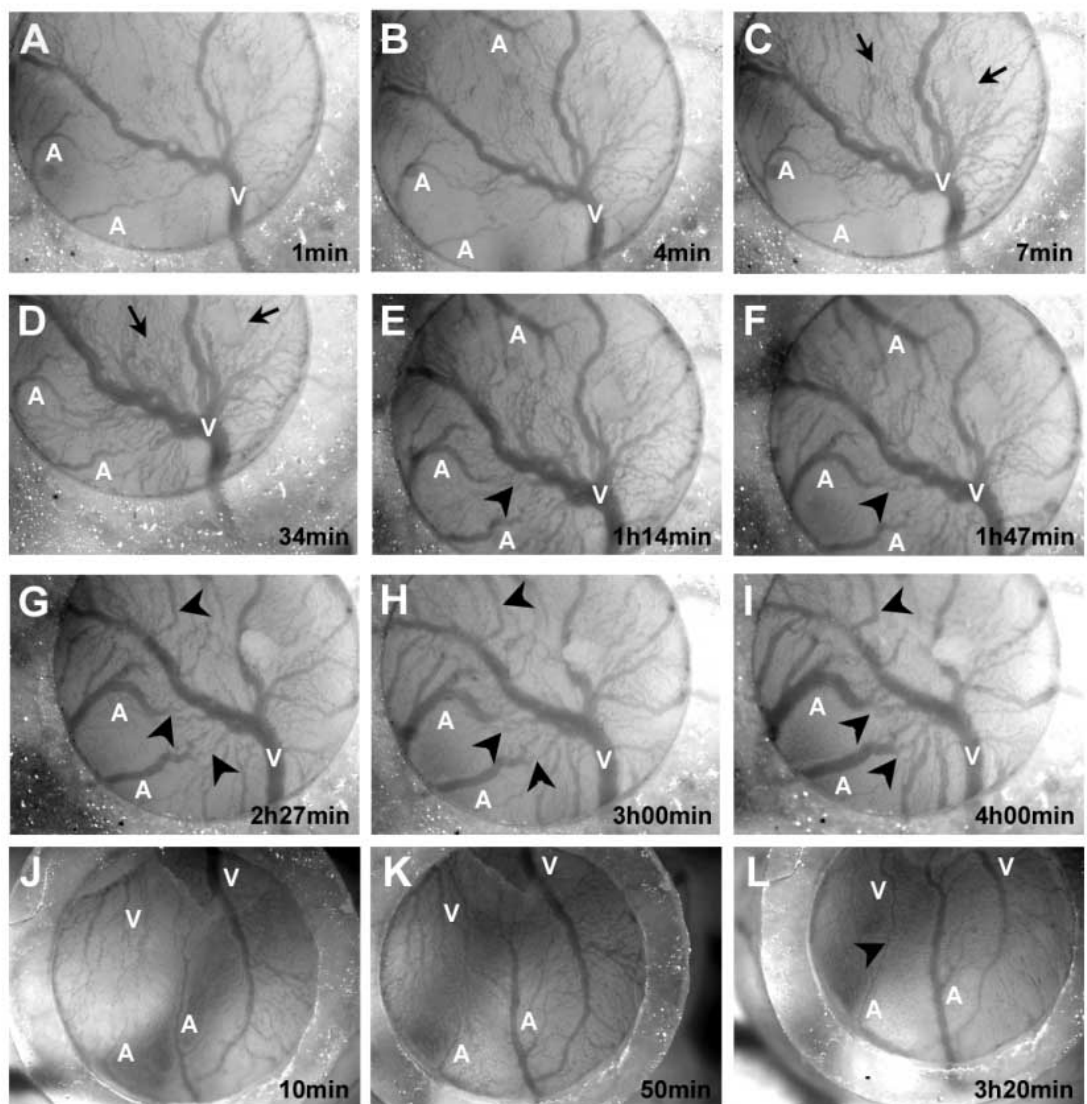


Fig. 11. Application of ephrinB2-Fc and EphB4-Fc on the allantois.

(A-I) Ephrin-B2 application. (J-L) EphB4 application. Still images of time-lapse video-microscopy taken at the indicated time points. Arteries (A) and veins (V) in the application area are indicated. Note increased venous branching (C,D, arrows), and rapid formation of arterial-venous shunts after ephrinB2-Fc application (arrowheads in F-I). Note enlargement of the artery in EphB4-Fc treated embryos (J-L) as well as formation of arterial-venous shunts (arrowhead, L). Scale bar: 830 μ m.

and the smaller caliber side branch, as a function of flow in the larger vessel (Fleury and Schwartz, 2001). The limited amount of apoptosis in yolk-sac vessels, together with the observation that disconnected vessel are re-used to make veins, indeed supports the idea that not vessel regression, but disconnection is the main pruning mechanism, enabling use and re-use of vascular segments.

We have not yet addressed the possible role of angioblast migration in yolk sac remodeling. It remains possible that vessel disconnection or reconnection is achieved by the directed incorporation of migrating angioblasts into these sites. QH1 stainings have failed to detect isolated angioblasts at sites of vessel disconnection. Quail-chick chimera studies should allow to directly test if migrating angioblasts are incorporated into vessel breakage sites. Nevertheless, the observations we describe suggest that yolk sac vessels can be considered as 'bricks' that are used and re-used by the growing vascular system according to its needs.

This view implies that yolk sac arteries and veins are plastic and regulated by external cues, such as flow. To address this question, we altered the perfusion pattern using physical approaches. Complete inhibition of flow to the yolk sac was achieved in the no-flow model. No arterial-venous differentiation and patterning was obtained in the absence of perfusion, in spite of continued growth of the unpatterned yolk sac blood islands over a considerable period of development (at least 7 days). A functional perfused loop between heart and yolk sac vessels therefore appears necessary to initiate formation of arteries and veins in the yolk sac. Examination of the expression of ephrinB2 in no-flow yolk sacs showed that some areas of the undifferentiated capillary bed expressed this marker, whereas others did not. These data suggest that initiation of arterial marker expression is independent of flow and perhaps genetically predetermined. Indeed, observation of the normal expression pattern of ephrinB2 and NRP1 has already shown that expression is initiated in the embryo prior to the onset of flow (Herzog et al., 2001; Moyon et al., 2001). Second, normal flow patterns in the yolk sac were altered in the incision and ligation models. In the incision model, the vitelline artery on the incised side did not develop; instead, a single vein formed in the area normally occupied by the vitelline artery. In the ligation experiments, the arterial bed on the ligated side was venularized. Moreover, growth of arteries was induced in areas normally devoid of arteries such as the posterior region of the embryo, normally occupied by the posterior vitelline vein and the anterior region, normally occupied by the anterior vitelline vein. These experiments indicate that flow determines both the arteriolar and venular patterning. Pre-existing arterioles were incorporated into the newly developing venular system, if they were oriented in the direction of the newly induced venous flow. Conversely, pre-existing veins could be incorporated into expanding arteries. Expression of mRNA encoding ephrinB2 or NRP1 was rapidly downregulated after ligation of the vitelline artery. Already 10 minutes after ligation, expression of NRP1 is reduced in the distal tips of the arterial tree. After 1 hour, the loss of expression becomes more pronounced and after 4 hours it is completely lost. In the unligated side, normal expression patterns were observed. Subsequently we investigated whether the venularized arterial tree can express NRP2 and Tie2, genetic markers of venous endothelium. Expression of both mRNAs was observed 12 hours after ligation, indicating that EC in the

new plexus acquired a venous identity. Interestingly, the kinetics of induction of venous markers were slower than those of loss of arterial markers. The manipulated vessels therefore seem to undergo a phase of loss of arterio-venous identity.

As it may be argued that the loss of ephrinB2 and NRP1 expression is due to absence of perfusion and subsequent death of the arteries, we examined in close detail the flow changes acutely after ligation. We observed that after ligation the arterial plexus is still perfused but with blood attracted from the marginal vein; hence, blood of venous origin. To investigate the changes in more detail, we also re-opened the ligation allowing reperfusion of the right vitelline arterial tree with arterial blood. Re-opening the ligation and allowing 4 hours of re-perfusion resulted in the re-emergence of NRP1 expression. This clearly shows that flow can control NRP1 and ephrinB2 expression *in vivo*. These data strongly indicate that the yolk sac vasculature patterning is not genetically committed but shows plasticity in the sites of arterial-venous differentiation, and subsequent patterning depending on the local hemodynamic conditions, flow. Indeed, physical modeling data that incorporate the time-lapse observations obtained *in vivo* show that flow can explain and moreover is sufficient to explain arterial and venous patterning (Fleury and Schwartz, 2000; Fleury and Schwartz, 2001). This for the first time clearly shows that flow may be the master regulator of the arterial-venous differentiation and patterning process.

Although the yolk sac remodeling may be plastic and regulated by flow, several steps in the formation of the cardiovascular system may require solely genetic predetermination control. We postulate that formation of a contracting heart, the in- and outflow tract, the projection of arteries from the aorta towards the target organs, the connection to draining veins and the inflow tract, thus all steps needed to make a functionally perfused loop between heart and developing organs, are genetically driven. These postulates are confirmed by the expression pattern of genes specific for arterial endothelium. Gridlock, NRP1 and Delta-C can be expressed in presumptive arterial endothelium before the onset of flow (Moyon et al., 2001a; Smithers et al., 2000; Zhong et al., 2000).

As the flow signal has to be transmitted into morphogenic events we subsequently investigated the role of ephrinB2 and EphB4, molecules previously implied to be crucial for arterial-venous differentiation and patterning. The process of vessel disconnection and reconnection described here indeed shows remarkable similarities with the function of these molecules as described in the nervous system, repulsive and attractant signaling (Kullander and Klein, 2002).

Expression of ephrinB2 in the main arterial branch, in conjunction with expression of EphB4 in the disconnected vessel theoretically could induce separation of arterial and venous endothelium. Such a mechanism would provide a theoretical explanation of the proposed models on ephrin/Eph interaction in the vascular system. To test whether ephrinB2/EphB4 may be involved in micro-events such as vessel disconnection/reconnection, or more macro events like the global patterning of arteries and veins, we applied recombinant ephrinB2 and EphB4, clustered and unclustered, on the chick embryo yolk sac. Neither application of recombinant ephrinB2 or EphB4 protein resulted in major changes in arterial and venous patterning. We were also not

able to demonstrate a significant effect in the disconnection and reconnection process.

Although application of ephrinB2 and EphB4 on the yolk sac did not provoke any overt morphological changes, application on more mature arteries and veins in the E4 allantois induced the formation of large arterial-venous shunts. EphrinB2 induced effects in the venous plexus: more side-branches were detected. Subsequently the arterial plexus responded with huge diameter increases of arterioles, finally progressing in the formation of arterial-venous shunts. Vasodilation could not account for the huge enlargement of the arteries, as previous studies already showed that allantois vessels are always maximally dilated to allow adequate oxygen uptake (Dusseau et al., 1986). These diameter changes probably have a structural nature and have to be explored further. In literature that addresses collateral formation in adult vascular beds, the phenomenon of structural outward remodeling of the arteries is referred to as arteriogenesis (Schaper and Buschmann, 1999). Interestingly, embryonic and adult arteriogenesis are both perfusion driven. In fact, modeling studies have shown that adaptation to flow induced shear stress alone, will result in the formation of large arterial-venous shunts (Hacking et al., 1996). It is therefore tempting to speculate that application of ephrinB2 enhanced shear stress sensing, hence, enabling the formation of shunts. Given that ephrinB2 binds to veins, enhanced shear stress sensing would be mediated by a different molecular mechanism.

At the molecular level, the *in vivo* effects of ephrinB2 suggest that there is active forward signaling through the EphB4 receptor at this stage of development. Application of EphB4 to the allantois also resulted in the formation of arterial-venous shunts; however, these connections appeared smaller in dimension in comparison to ephrinB2. Detailed investigation of the kinetics of this process revealed more differences with respect to ephrinB2. We observed that EphB4 enhanced the distal outgrowth of the arterial tree into areas occupied by veins. The distal tips of the veins regressed, resulting in a smaller venous tree. During subsequent stages, these regions are invaded by arteries. Hence, EphB4 may be needed for arteries to invade presumptive venous domains. This *in vivo* effect of EphB4 in E4 allantois suggests the presence of reversed signaling through ephrinB2. These observations are compatible with the phenotypes observed in mouse mutants for ephrinB2 and EphB4, which suggest that reverse signaling through ephrinB2 is responsible for the observed defects in arterial-venous differentiation (Adams et al., 2001; Gerety et al., 1999; Wang et al., 1998). Further confirmation for these results will await the generation of mutants deficient in the kinase domain of EphB4. The allantois data suggest that ephrin/Eph signaling at this stage of allantois development, is required for the maintenance of this system, by inhibiting the mixing of arterial and venous EC. Interestingly, application of ephrinB2 during the period E5-E10, failed to produce similar effects, suggesting time-dependent changes in the efficacy of this signal transduction pathway.

Such time-dependent changes could also account for the failure to detect any overt morphological changes in the yolk sac after ephrinB2/EphB4 application. Collectively, our data suggest that ephrin application has effects in allantois vessels and not in yolk sac vessels. A theoretical explanation for the observed difference could be that ephrinB2 protein does not

reach the yolk sac vessels. We feel this unlikely, given that protein binding is observed. It is furthermore possible that protein cannot elicit a proper signal transduction cascade in the yolk sac, perhaps because a member of the downstream cascade is absent. This occurs in other receptor/ligand systems during embryonic development (LeNoble et al., 2000). It may also be that ephrin/Eph signaling in the yolk sac is already maximally active, and the added proteins, which were previously described as agonists in both neural and endothelial cells (Wang and Anderson, 1997; Füller et al., 2003) do not increase signal transduction. In this case, application of ephrinB2 monomers or other antagonistic forms should provoke an effect in the yolk sac. Future experiments will answer to these questions.

The observation of impaired yolk sac remodeling in the absence of flow or perfusion may have important impact on the interpretation of yolk sac remodeling events in mice deficient for genes implicated in vascular development. For example, *HiF1 α ^{-/-}*, *VE-cadherin^{-/-}*, *activin receptor-like kinase 1^{-/-}* and *connexin 45^{-/-}* mice all show severe impairment of yolk-sac remodeling. However, these mice also exhibit cardiac defects or failures in outflow tract remodeling (Carmeliet et al., 1999; Iyer et al., 1998; Krüger et al., 2000; Ryan et al., 1998; Urness et al., 2000). Perfusion in these mice is thus certainly perturbed leaving open the possibility that part of the vascular phenotype observed in the yolk-sac of these null mice is in fact due to the absence of perfusion, and not directly due to the targeted deletion of the gene. It has been reported that the yolk sac of the original ephrinB2 knockout mouse is perfused (Wang et al., 1998) and makes a primitive arterial plexus. Hence, a functional perfused loop between heart, aorta and yolk-sac must have been present. However, as both the ephrinB2 knockout, and the conditional *Tie2-Cre ephrinB2* knockout (Gerety and Anderson, 2002) display cardiac malformations as well as malformations of intraembryonic vessels, it cannot be ruled out that the failure to drive the outgrowth of arteries is partly due to a defect in the perpetuation of cardiovascular perfusion.

In conclusion, we show that arterial-venous differentiation is a flow driven highly dynamic process that exhibits a high degree of EC plasticity. 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.

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