Plasticity of endothelial cells during arterial-venous differentiation in the avian embryo

Delphine Moyon*, Luc Pardanaud*, Li Yuan, Christiane Bréant and Anne Eichmann‡

Institut d'Embryologie Cellulaire et Moléculaire CNRS FRE 2160, 49bis, Avenue de la Belle Gabrielle, Nogent-sur-Marne Cedex 94736, France

*These authors have contributed equally to this work

‡Author for correspondence (e-mail: eichmann@infobiogen.fr)

SUMMARY

Remodeling of the primary vascular system of the embryo into arteries and veins has long been thought to depend largely on the influence of hemodynamic forces. This view was recently challenged by the discovery of several molecules specifically expressed by arterial or venous endothelial cells. We here analysed the expression of neuropilin-1 and TIE2, two transmembrane receptors known to play a role in vascular development. In birds, neuropilin-1 was expressed by arterial endothelium and wall cells, but absent from veins. TIE2 was strongly expressed in embryonic veins, but only weakly transcribed in most arteries. To examine whether endothelial cells are committed to an arterial or venous fate once they express these specific receptors, we constructed quail-chick chimeras. The dorsal aorta, carotid artery and the cardinal and jugular veins were isolated together with the vessel wall from quail embryos between embryonic day 2 to 15 and grafted into the coelom of chick hosts. Until embryonic day

7, all grafts yielded endothelial cells that colonized both host arteries and veins. After embryonic day 7, endothelial plasticity was progressively lost and from embryonic day 11 grafts of arteries yielded endothelial cells that colonized only chick arteries and rarely reached the host veins, while grafts of jugular veins colonized mainly host veins. When isolated from the vessel wall, quail aortic endothelial cells from embryonic day 11 embryos were able to colonize both host arteries and veins. Our results show that despite the expression of arterial or venous markers the endothelium remains plastic with regard to arterial-venous differentiation until late in embryonic development and point to a role for the vessel wall in endothelial plasticity and vessel identity.

Key words: Neuropilin-1, TIE2, Endothelial cell, Artery, Vein, Vessel wall, Chick-quail chimera

INTRODUCTION

Blood vessels in the early embryo arise by de novo formation of endothelial cells (EC) from the mesoderm, a process termed vasculogenesis (Risau and Flamme, 1995, for review). Vasculogenesis leads to the formation of the first major intra-embryonic blood vessel, the aorta, and to the formation of the yolk sac blood islands. Blood island EC anastomose to form primitive vascular channels, which are then transformed into an intricately branched system of vessels resembling the adult vascular pattern. This process, collectively termed angiogenesis (Risau, 1997), involves capillary sprouting, splitting and remodeling, and leads to the reorganization of the embryonic vasculature into large and small vessels. Angiogenesis also involves sprouting of vessel branches into previously avascular regions of the embryo (Pardanaud et al., 1989) and pericyte recruitment to the different segments of the vasculature (Folkman and D'Amore, 1996). Arteries and veins also have to differentiate during the early remodeling phase, in order to form a functional vascular loop between the embryo and the yolk sac necessary to accomodate the output of the first

heart beat. The cellular and molecular mechanisms governing these complex processes are still poorly understood. In particular, the differentiation of arteries and veins has received little attention.

Two growth factor families, VEGFs and angiopoietins, discovered over the last decade, are critical for vasculogenesis and angiogenesis (for reviews see Neufeld et al., 1999; Petrova et al., 1999; Yancopoulos et al., 2000). Moreover, several molecules have recently been shown to be expressed selectively by developing arterial or venous EC. Arterial-specific markers include the transcription factor gridlock (Zhong et al., 2000), and two membrane anchored molecules, the Notch ligand delta-like 4 (DLL4) (Shutter et al., 2000) and the transmembrane growth factor ephrinB2 (Wang et al., 1998; Adams et al., 1999). In contrast, venous EC specifically express the receptor for ephrin B2, EphB4 (Wang et al., 1998; Adams et al., 1999; Gerety et al., 1999). These observations raised, for the first time, the possibility of a genetic predetermination of blood vessel endothelium. Indeed, EC had previously been thought of as a homogeneous cell population, and arterial-venous differentiation was thought to occur

as a consequence of hemodynamic forces (reviewed by Yancopoulos et al., 1998). However, targeted inactivation of the ephrinB2 gene in mice resulted in malformation of arteries as well as veins, suggesting that interaction of this growth-factor-receptor pair must be important for correct vascular patterning (Wang et al., 1998; Adams et al., 1998).

We present results of an expression analysis of two receptors known to be involved in vascular development, neuropilin-1 (NRP1) and TIE2. TIE2 is known as an EC-specific receptor for growth factors of the angiopoietin family, which play a role in angiogenesis and the assembly of the vascular wall (Gale and Yancopoulos, 1999; Yancopoulos et al., 2000, for reviews). NRP1 is a receptor for members of the semaphorin/collapsin family, which are negative mediators of neuronal guidance (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). In EC, NRP1 is a receptor for VEGF$_{165}$ (Soker et al., 1998; Gluzman-Poltorak et al., 2000) and for several other members of the VEGF family, including PlGF, PlGF-2, VEGF-B and the VEGF-like protein from orf virus NZ2 (Migdal et al., 1998; Makinen et al., 1999; Wise et al., 1999). Expression of NRP1 in porcine aortic EC enhanced the ability of VEGF$_{165}$ to bind to VEGFR2 and to stimulate chemotaxis via VEGFR2 (Soker et al., 1998). Overexpression of NRP1 in transgenic mice resulted in excess capillary and blood vessel formation and hemorrhaging in the embryo, contributing to embryonic lethality (Kitsukawa et al., 1995). Production of a *NRP1* null mutant by homologous recombination of the *NRP1* gene induced disorganization of nerve pathways (Kitsukawa et al., 1997) and also vascular defects, resulting in embryonic lethality by embryonic day (E)12.5 (Kawasaki et al., 1999).

We show that in the avian vascular system, both NRP1 and TIE2 are initially expressed in virtually all EC. Once arteries and veins differentiate, the *NRP1* gene is transcribed exclusively in arterial EC and mesenchymal cells surrounding developing arteries. In contrast, the chick TIE2 gene (Jones et al., 1999) is a good marker for venous EC. Downregulation of NRP1 in veins and TIE2 in arteries has not been reported in rodents (Kawasaki et al., 1999; Schnurch and Risau, 1993; Sato et al., 1995) and may thus be specific for birds. To investigate whether EC are determined to an arterial or venous fate once they express specific molecules such as NRP1, TIE2 or the previously described ephrinB2 and EphB4 ligand and receptor, we have grafted embryonic arteries or veins from quail embryos at different stages of development into the coelom of chick hosts. This procedure has previously been shown to allow colonization of the host vasculature by donor EC, which can be visualized by staining with the QH1 monoclonal antibody (mAb) specific for quail EC and white blood cells (Pardanaud et al., 1987; Pardanaud and Dieterlen-Lièvre, 1999). We show that the embryonic aorta, taken from E2, E5 or E7 quail donors, still colonizes both arteries and veins of the host embryo. After E7, plasticity is progressively lost and EC from E11 to E15 quail aortae and carotid arteries are largely restricted to colonizing host arteries. EC from cardinal or jugular veins until E7 also reached both types of host vessels, while they were restricted to host veins after this stage. When E11 quail aortic EC were isolated from the vessel wall before grafting, they remained plastic and colonized both host arteries and veins. These results show that the endothelium remains plastic with regard to arterovenous differentiation long after it has acquired the expression of specific markers and that EC plasticity is restricted by the vessel wall.

MATERIALS AND METHODS

In situ hybridization, antibody staining

Probes were described previously (NRP1: Takagi et al., 1995; TIE2: Jones et al., 1999; VEGFR2, VEGFR3: Eichmann et al., 1998; ephrinB2: Moyon et al., 2001). In situ hybridizations on whole-mounts and on paraffin sections were done as described (Eichmann et al., 2000; Etchevers et al., 2001). For double in situ hybridizations, we simultaneously applied NRP1 labeled with fluorescein-UTP and TIE2 labeled with dig-UTP. TIE2 was developed first, using anti-dig Fab fragments (Boehringer), and development was stopped by rinsing with PBS, glycine/HCl 0.1 M pH 2.2, and MABT (100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.1% Tween 20). The development procedure was repeated using anti-fluorescein Fab fragments and INT/BCIP solution (Boehringer). Antibody staining was done following development of the in situ hybridization, using 1A4 α-SMA (α-smooth muscle actin; Sigma) (IgG2a) at a 1:400 dilution in QH1 hybridoma supernatant (IgM) (Pardanaud et al., 1987). Immunostaining was developed using Texas Red- or HRP-conjugated goat anti-mouse (Gam) IgM and FITC-conjugated Gam-IgG2a (Southern Biotechnology) diluted 1:50 in PBS. Immunohistochemistry of grafted embryos using QH1 mAb followed by a nuclear staining with glychemalun was as described (Pardanaud and Dieterlen-Lièvre, 1999). In some cases, sections were double-stained with QH1 and the QCPN mAb, which labels the nucleus of all quail cells. QH1 staining of cultured EC was done after fixation of the cells for 20 minutes in 3.7% formaldehyde.

Quail-chick grafting

Lateral splanchnopleural mesoderm and somites were retrieved as described (Pardanaud et al., 1996). E2 aorta and cardinal vein were isolated by pancreatin digestion and mechanical dissection. Aortic tubes were peeled off from the ventral part of the somites. Cardinal veins were isolated with the intermediate plate located laterally to the somites. Aortae, carotid arteries and jugular veins from E5 to E15 quail (*Coturnix coturnix japonica*) embryos were dissected together with their wall, and cut into rings of about 50-100 µm in length. For isolation of EC, aortae (16-25 per experiment) were longitudinally sectioned, pinned onto a black support and treated with collagenase (Sigma) for 30 minutes at 37°C. EC were then flushed out with a micropipette, placed in DMEM supplemented with 20% foetal calf serum (FCS), centrifuged and resuspended in 100 µl of the same medium. Two aliquots of 10 µl were cultured for 48 hours in a Petri dish to verify the presence of EC. Aliquots of 20 µl were placed overnight in hanging drop cultures for aggregate formation and grafting. For grafting, Indian ink, diluted 1:1 in PBS, was injected beneath the E2 chick (*Gallus gallus*, JA57) host blastodisc. Two types of grafts were performed. (1) Coelomic grafts, which were as described previously (Pardanaud and Dieterlen-Lièvre, 1999). (2) Dorsal grafts, in which 3 host somites at the wing level (somite 15-20) were substituted by quail tissues. A piece of ectoderm was removed above the somites, which were dissected after brief pancreatic digestion. The graft was inserted inside the cavity and the ectoderm was replaced. The host embryos were incubated for 2 days before autopsy, fixed in Bouin or Serra fluid and 5 µm paraffin sections were prepared.

Quantification of EC migration into host arteries and veins

The number of QH1$^+$ EC reaching host arteries and veins was counted manually on every fifth section (×25 objective, final magnification ×110). A positive cell was scored when it showed a nucleus, visible either by glychemalun or by QCPN labeling, and membrane QH1

staining. The arterial or venous identity of quail EC was obvious when they integrated great vessels (aorta, cardinal vein, omphalomesenteric artery and vein, subclavian and brachial arteries, umbilical vein, segmental arteries and veins). In the limb bud, we considered that all the EC present at the periphery belonged to the venous plexus while arterial EC were centrally located and connected to the brachial artery. QH1+ EC in the perineural vascular plexus were only quantified if we could trace the connection of these capillaries with a known artery or a vein. If we could not classify the arterial or venous identity of a donor EC, it was not counted. The mean number of these undetermined QH1+ EC reached 4.6% for 107 analyzed grafts and exceeded 10% in 8/107 grafts (not shown).

RESULTS

We compared NRP1 and TIE2 expression to two other EC-specific genes: *VEGFR2*, which labels all EC until at least E13, and *VEGFR3*, which becomes restricted to lymphatic EC in avian and mouse embryos (Kaipainen et al., 1995; Wilting et al., 1997). Expression was examined by in situ hybridization on whole-mounts and paraffin sections, using digoxigenin-labeled antisense riboprobes. Sense probes did not give specific hybridization signals (not shown). Quail and chick embryos were examined from presomitic stages to E10. No difference in expression between the two avian species was observed.

Onset of NRP1 and TIE2 expression in the arterial and venous system

NRP1 transcripts were first detected before gastrulation in the hypoblast (not shown). During early somitic stages, NRP1 was expressed in the developing heart region and in yolk sac blood islands (Fig. 1A). With the onset of embryonic circulation at approximately the 12-somite stage (ss), NRP1 expression was detected in the dorsal aorta (Fig. 1B). NRP1 was progressively downregulated in the anterior part of the lateral mesoderm and the yolk sac vasculature (Fig. 1B). By the 18-20 ss, NRP1 expression was observed mainly in the posterior lateral mesoderm and yolk sac, corresponding to the prospective arterial compartment (Fig. 1C). In contrast, VEGFR2 expression was observed in both the posterior and anterior compartments of the lateral mesoderm and yolk sac (Fig. 1D). Following the development of the omphalomesenteric arteries and their connection to the embryo proper at the 21ss, NRP1 was strongly expressed in these vessels and their branches (Fig. 1E,F). Thus, NRP1 appeared to be restricted to arterial EC as soon as these vessels became morphologically distinguishable.

TIE2 expression became detectable during early somitic stages in EC and some hematopoietic cells (not shown) of the yolk sac blood islands. TIE2 remained uniformly expressed in EC of the yolk sac and the embryo until E2.5. After this stage, TIE2 transcripts became down-regulated in arteries, while they remained highly expressed in venous EC both in the yolk sac (Fig. 2A) and in the embryo proper (see below). Conversely, NRP1 expression appeared restricted to yolk sac arteries (Fig. 2B). To extend these observations, adjacent sections of the extraembryonic appendages, including yolk sac and allantois were hybridized with VEGFR2 (Fig. 2C) and both NRP1 and TIE2 (Fig. 2D). VEGFR2 expression was observed in both arterial and venous EC (Fig. 2C). TIE2 was strongly transcribed in venous EC, while few EC were positive in arteries (Fig. 2D). In contrast, NRP1 only labeled arterial EC
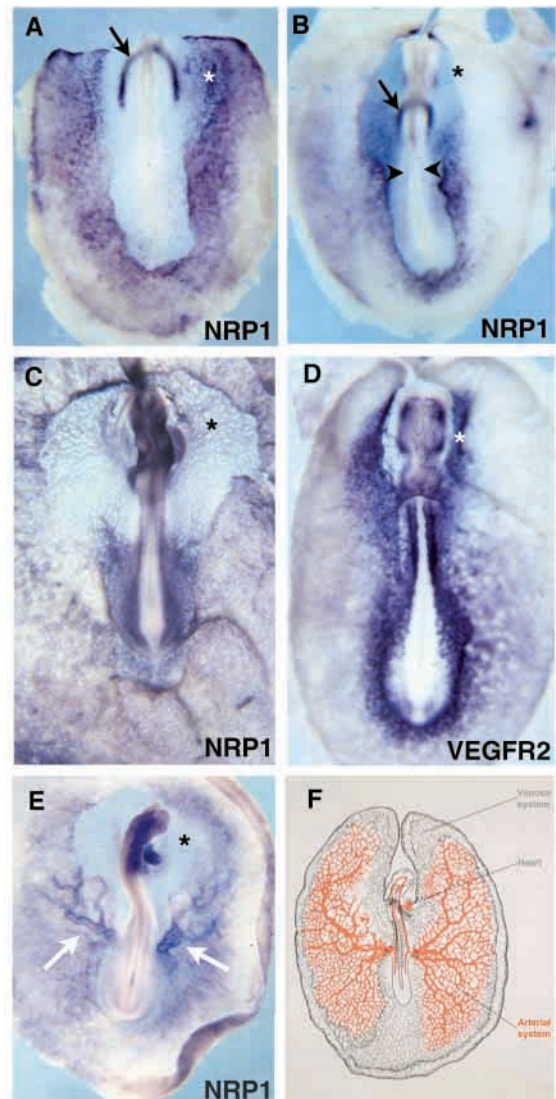

Fig. 1. Expression of NRP1 becomes restricted to the arterial compartment of the blastodisc. Whole-mount in situ hybridizations with the indicated antisense riboprobes. (A) 4ss, NRP1 is expressed throughout yolk sac blood islands and in the heart anlage (arrow). (B) 14ss, (C) 21ss. NRP1 expression is lost from EC in the anterior lateral mesoderm (asterisk in A-E). Arrowheads in B point to the dorsal aorta. (D) VEGFR2 labels both anterior and posterior lateral mesoderm at the 14ss. (E) 25ss. NRP1 expression is observed in the omphalomesenteric arteries (arrows) and their branches.
(F) Schematic representation of the embryonic and extra-embryonic circulation at the 25ss (redrawn from Gilbert, 1994).

(Fig. 2D). Mesenchymal cells surrounding the arteries and, to a lesser extent, veins were also stained with NRP1 (Fig. 2D). The results from the double-labeling studies were confirmed by single-labeling with NRP1 or TIE2 on adjacent sections, which yielded the same results (not shown).

NRP1 specifically labels arterial EC during organogenesis

On truncal sections of E2.5-E3 chick or quail embryos, NRP1 was detected in a variety of non-endothelial cell types, including motoneurons, dorsal root and sympathetic ganglia

Fig. 2. NRP1 and TIE2 label extra-embryonic arterial and venous EC, respectively. (A,B) Whole-mount in situ hybridizations on yolk sacs of E3.5 chick embryos. (A) TIE2 is expressed on veins (arrows), but not on the parallel-running arteries (arrowheads). NRP1 (B) expression is the reciprocal. (C,D) Adjacent sections through the E6 chick allantois. VEGFR2 (C) labels EC of the umbilical artery (A) and vein (V). AS, allantoic stalk. (D) NRP1 (brown staining) is only observed in EC of the umbilical artery, while TIE2 (blue staining) is down-regulated in these EC (arrows) and transcribed in the veins. Mesenchymal cells surrounding arteries are also NRP1 positive (arrowheads). Bar in C, for C and D, 120 μm.
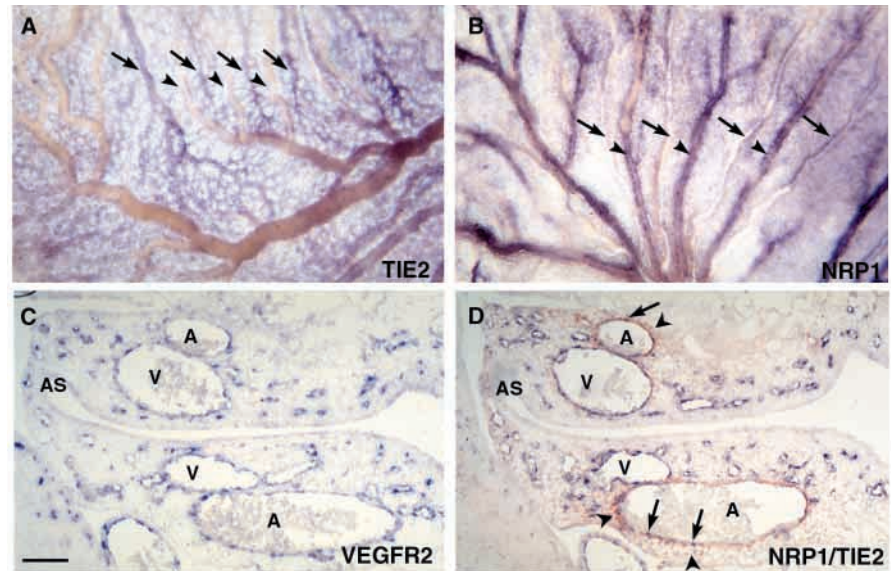
(Fig. 3A,G) (not detailed here). Within the vascular system, NRP1 was expressed in the aortic endothelium but not in the posterior cardinal vein or omphalomesenteric and umbilical veins (Fig. 3A). In the cephalic region, NRP1 was observed in the internal carotid artery, but not in the anterior cardinal vein (Fig. 3C). Adjacent sections hybridized with TIE2 probes showed TIE2 expression in virtually all EC of the trunk region (Fig. 3B). In the cephalic region, TIE2 expression was down-regulated in the internal carotid artery but remained strong in the anterior cardinal vein (Fig. 3D). In older embryos (E4-E10), TIE2 mRNAs were abundant in venous EC, while most arteries showed significantly weaker TIE2 expression (Fig. 3D,F-H and see below). Double in situ hybridization with NRP1 and TIE2 probes thus allowed distinction of arterial (NRP1$^+$) from venous (NRP1$^-$) TIE2-expressing EC. In the aortic arches (Fig. 3F) or the femoral artery (Fig. 3G,H), TIE2$^+$ cells were rare, while strong NRP1 labeling was observed. Capillaries branching from the femoral artery were positive for both genes (Fig. 3H). NRP1 expression in the arteries was not restricted to EC, but was also observed in the developing arterial wall and was strongest in regions of arterial branching (Fig. 3G and data not shown).

Double labeling of E10 quail embryo sections with NRP1 and TIE2 probes showed reciprocal staining patterns of parallel-running vessels in connective tissue (Fig. 4A), skeletal muscle (Fig. 4B) and internal organs such as the developing kidney, gut (not shown) and lung vessels (Fig. 4C). To make sure that NRP1 was specifically expressed in the arterial compartment, we performed triple stainings of quail embryo sections with NRP1 and with α-SMA and QH1 mAbs, which stain the wall of developing arteries and all quail EC, respectively. All the blood vessels surrounded by a thick α-SMA$^+$ wall were NRP1$^+$ (Fig. 4D-F): the triple-labeling of a truncal section of an E10 quail embryo showed that NRP1 labeled EC of the dorsal aorta and intersegmentary artery branching from it. In contrast, QH1$^+$ EC of veins and lymphatic EC of the thoracic duct were NRP1$^-$ (Fig. 4D-F). α-SMA$^+$ lymphatics were positive for VEGFR3 expression and negative for TIE2 (not shown). In the heart muscle, coronary arteries,

but not veins, expressed NRP1 (Fig. 4G-I). Postnatal day 2 heart showed the same expression pattern of NRP1 and TIE2 (data not shown).

Plasticity of the vascular system with respect to arterial-venous differentiation

To determine if EC could still differentiate into venous EC once they expressed arterial-specific genes and vice-versa, we performed quail-chick grafting experiments. Quail rudiments were isolated from embryos at different stages, as detailed below, and grafted into the coelom of E2 chick hosts. This grafting experiment has previously been shown to allow colonization of the host vasculature on the grafted side by quail donor EC (Pardanaud and Dieterlen-Lièvre, 1999). The grafted embryos were incubated for 2 days before autopsy. Quail-derived EC were visualized with the QH1 mAb and the number of quail EC migrating into host arteries and/or veins was counted for each graft (see Materials and Methods). The arterial nature of quail-derived EC was furthermore confirmed by in situ hybridization with NRP1 and ephrinB2, which selectively labels arterial EC in mice and birds (Wang et al., 1998; Adams et al., 1999; Moyon et al., 2001).

Grafts of early mesodermal territories

In situ hybridization with NRP1 (see Fig. 1D) had shown high expression levels in the posterior, but not the anterior lateral mesoderm. We divided the lateral splanchnopleural mesoderm in posterior or anterior regions, corresponding respectively to the presumptive territory of the omphalomesenteric artery and vein (Fig. 5A). The capacity of these mesodermal compartments to give rise to arteries and veins was analyzed after grafting into the chick coelom. We also grafted somites of E2 quail embryos, which contain undifferentiated angioblasts (Pardanaud et al., 1996).

In the host coelom, grafts from all three territories developed in contact with the body wall, the mesonephros and the dorsal mesentery. They were vascularized by QH1$^+$ vessels which connected to the host vascular tree. An important population of QH1$^+$ EC migrated into the chick host and participated in

Fig. 3. Intra-embryonic expression of NRP1, TIE2 and VEGFR2.
(A,B) Adjacent transverse sections of an E3 quail embryo at the level of the limb bud hybridized with the indicated probes. (A) NRP1 is expressed in motoneurons (arrow) of the neural tube (NT), in future dorsal root and sympathetic ganglia (arrowheads), and in EC of the aorta (Ao). EC of the posterior cardinal vein (cv), omphalomesenteric vein (omv) and umbilical vein (uv) are negative. (B) TIE2 labels all EC. (C,D) Adjacent sections through the hindbrain of an E4 embryo at the level of the otic vesicle (OV). (C) NRP1 is expressed in the internal carotid artery (ica), but not in the anterior cardinal vein (cv). (D) TIE2 shows the complementary expression. (E,F) Adjacent sections through aortic arches (AA) 3, 4 and 5 of an E6 chick embryo hybridized with the indicated probes. (E) VEGFR2 is detectable on the majority of EC in both the aortic arches and the anterior cardinal vein (cv). (F) NRP1 (brown staining) is only detectable in aortic arch EC, few of which (arrows) also express TIE2 (blue staining). (G) Section through the leg bud of an E6 chick embryo. NRP1 (brown staining) is expressed in EC and some mesenchymal cells of the aorta and femoral artery (fa) and is strong in regions of arterial branching (arrows). TIE2 (blue staining) is expressed weakly or not at all in the femoral artery, but is expressed in the cardinal vein (cv) and the peripheral venous plexus of the limb (arrowheads). (H) Higher magnification of the boxed region in G shows the femoral artery EC expressing NRP1, while the underlying venous plexus is TIE2 positive. Capillaries branching from the femoral artery express both markers (arrows). N, notochord. Bars, (A,B,E,F) 130 µm; (C,D) 65 µm; (G) 290 µm; (H) 65 µm.
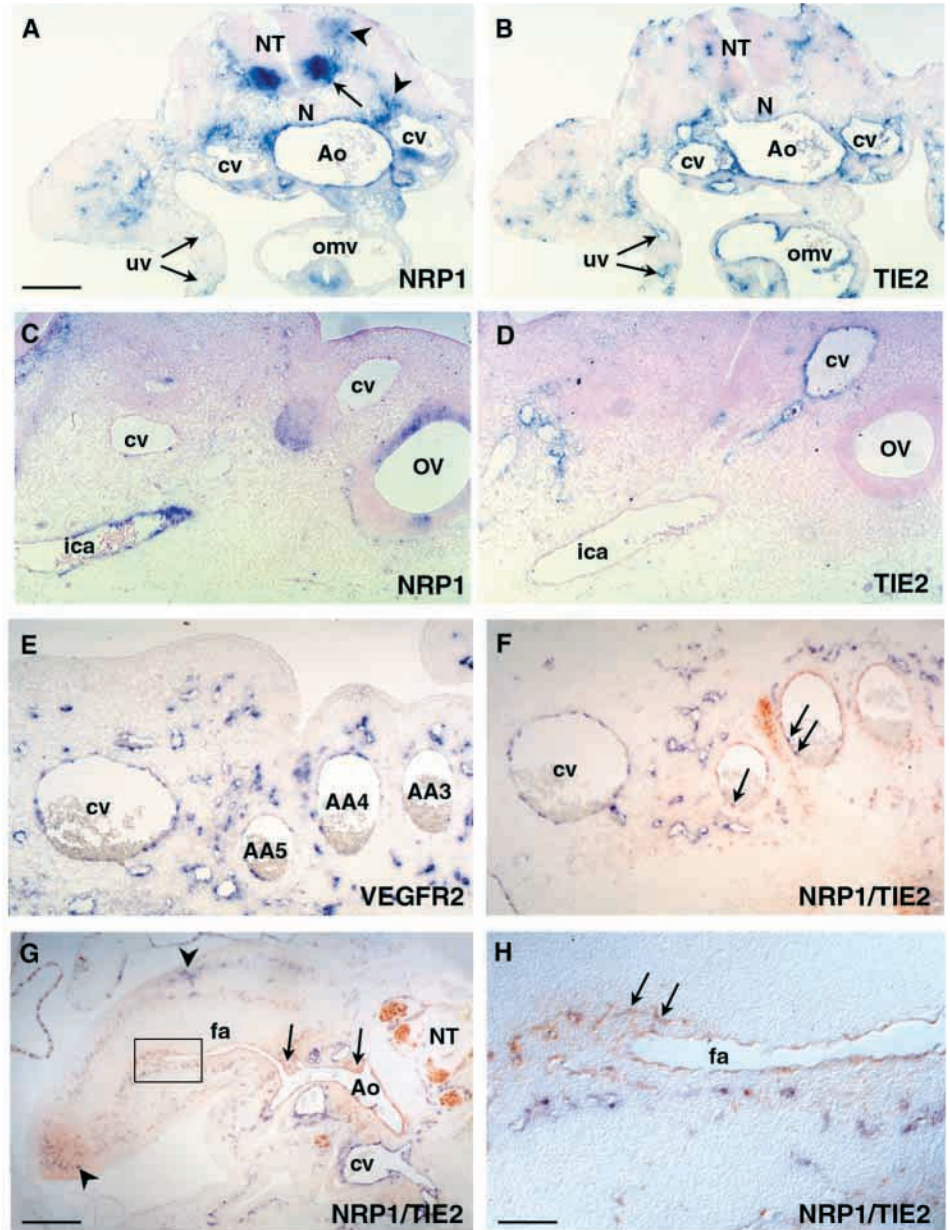

the endothelium of great vessels such as the aorta, brachial artery (Fig. 5B) and subclavian artery. Quail EC were also observed in host veins, including the posterior cardinal vein, omphalomesenteric and umbilical veins as well as veins of the wing bud (Fig. 5B). Quantification of the number of quail EC showed that all three territories behaved similarly with respect to arterovenous differentiation (Table 1A). They gave rise to both arterial and venous EC, with a preference toward the colonization of host veins (Table 1A). The anterior region of the splanchnopleural mesoderm produced less EC than the posterior territory or the somitic mesoderm (Table 1A). In situ hybridization with NRP1 followed by double-labeling of sections with QH1 showed that in the host coelom, the grafted tissues lost NRP1 expression (Fig. 5B), irrespective of their origin. Quail EC that integrated arteries were QH1+ NRP1+, while quail EC that integrated veins were QH1+ but did not express NRP1 (Fig. 5B). Thus, NRP1 expression of emigrated

EC was regulated according to the novel host environment. As described previously (Pardanaud et al., 1996), QH1+ cells migrating from the anterior or posterior splanchnopleural mesoderm colonized the ventral aortic endothelium and gave rise to hematopoietic cells, while QH1+ cells emigrating from somites did not (data not shown).

Grafts of arteries

To determine the plasticity of already formed blood vessels, we isolated dorsal aortae from quail donors at different ontogenic stages, including E2, E5, E7-E11, E14 and E15, i.e. one day before hatching (Table 1B). The isolated vessels were dissected together with their vascular wall and cut into short rings before grafting into the chick coelom. The size of the grafts as well as the number of migrating quail EC and their migration distance varied significantly between different embryos, however, we did not observe any significant age-related
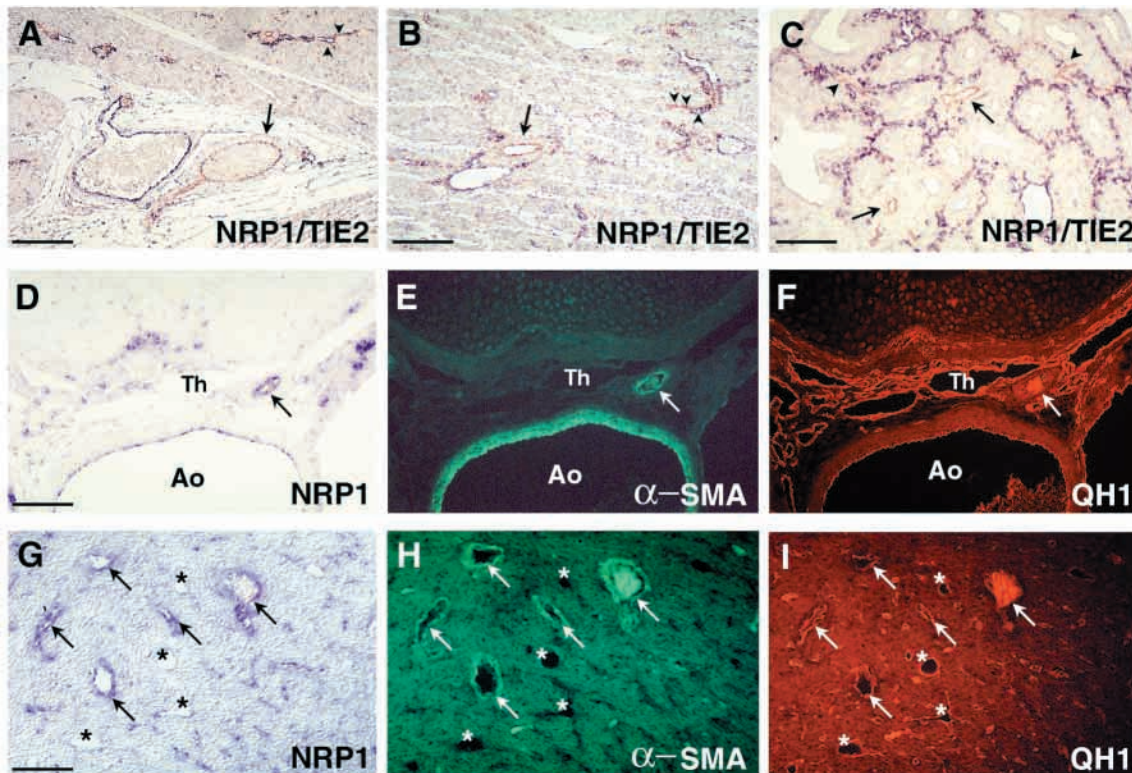
Fig. 4. Expression of NRP1/TIE2 at later developmental stages. Sections through an E10 quail embryo. (A-C) Sections through connective tissue (A), skeletal muscle (B) and lung (C), double-labeled with NRP1 (brown) and TIE2 (blue) antisense riboprobes. Arteries (arrows), surrounded by a thick wall, are NRP1 positive but show weak TIE2 expression. Veins express TIE2 but not NRP1. Parallel-running streams of capillaries expressing NRP1 or TIE2 can be observed (A,B, arrowheads). (D-F) Triple-labeling of a section through the aorta (Ao) and lymphatic thoracic duct (Th) with the indicated markers. (D) Bright-field view, NRP1 is expressed in EC of the aorta and smaller vessel branching from it (arrow). (E) Dark-field view ; FITC-α-SMA staining shows a thick coat of positive cells surrounding the branching NRP1-positive vessel. (F) Dark-field view ; Texas Red-labeled QH1-positive EC of the lymphatic thoracic duct are NRP1 negative. (G-I) Section through the myocardium, treated with the same markers. All NRP1-positive arteries (arrows) are surrounded by α-SMA^{+} cells, while NRP1-negative vessels (asterisks) are also α-SMA negative. QH1 staining (I) shows the corresponding NRP1-positive arterial, and NRP1-negative venous, EC. Bars, (A) 90 μm; (B and C) 180 μm; (D-F) 95 μm; (G-I) 30 μm.

changes in these parameters for the same type of grafting experiment (Table 1B). In some cases, E11 to E14/15 arteries and veins were longitudinally sectioned to directly expose the endothelium, this treatment did not influence the migratory behaviour of EC (not shown). Until E7, QH1^{+} EC from dorsal aortae colonized both arterial and venous host blood vessels, including the dorsal aorta, brachial and subclavian arteries, cardinal, umbilical and omphalomesenteric veins, and wing bud arteries and veins (Fig. 6A,B; Table 1B). In situ hybridization with NRP1 and ephrinB2 probes showed that quail EC which integrated host veins lost expression of both markers, while those colonizing host arteries remained NRP1^{+}/ephrinB2[+] (Fig. 6A,B).

The percentage of arterial QH1^{+} EC colonizing host veins decreased at E7-E10 (Table 1B). Grafts of E11, E14 and E15 quail aorta yielded EC which preferentially migrated into the host aorta, colonisation of host veins such as the posterior cardinal vein was exceptional (Fig. 6C; Table 1B). In situ hybridization with NRP1 (Fig. 6D) and ephrinB2 (not shown) confirmed that donor-derived vessels were positive for both genes. NRP1 expression in the graft itself was strongest in the region where EC started migrating out from the grafted vessel rudiment (Fig. 6D), as observed in a normal embryo in regions

of vessel branching. The colonisation of the host aorta was preferentially directed towards the ventral and lateral aortic endothelium, and positive hematopoietic cells were occasionally formed. QH1^{+} EC also reached the subclavian and brachial arteries and scarcely the omphalomesenteric artery. To examine if the apparent loss of the capacity to colonize veins was observed with aortic EC only or was typical of all arterial EC, we performed grafts of carotid arteries from E4.5 to E14 quail donors. These grafts gave rise to EC, which behaved like aortic EC, i.e. they efficiently colonized both arteries and veins until E10 but were restricted to host arteries from E11 (Fig. 6E; Table 1B).

Grafts of veins

We next examined the behaviour of venous EC grafted in a novel environment. QH1^{+} EC from E2 cardinal vein or E5 to E7 jugular vein remained plastic and colonized host arteries and veins (Fig. 7A; Table 1C). Until E7, the distribution of vein- or artery-derived EC in the host vascular tree was very similar. No significant differences in the number of migrating EC or their migration distance was observed between arterial or venous grafts or between venous grafts at different ages. In situ hybridization with NRP1 (Fig. 7A) and ephrinB2 (not

Table 1. Quantification of host vessels colonization by quail EC

Grafted rudiment	Mean percentage (±s.d.)[1] of QH1+EC in		Total number of QH1+EC (±s.d.)[2]	Graft size (mean value in μm±s.d.)[3]	EC migration (mean value in μm±s.d.)[4]	n[5]
	Arteries	Veins				
A: coelomic grafts with E2 mesoderm						
Anterior splanchnopleural mesoderm	30±13	70±13	214±81	309±139	672±162	5
Posterior splanchnopleural mesoderm	39±11	61±11	1036±963	429±244	1004±318	7
Somites	28±6	72±6	1380±533	407±144	1160±287	3
B: coelomic grafts with arteries						
E2 aorta	52±31	48±31	91±70	85±67	681±292	6
E4.5 carotid	59±4	41±4	277±109	354±104	928±75	4
E5 aorta	60±9	40±9	401±64	415±67	1013±127	3
E7 aorta	71	29	207	410	660	1
E8 aorta	67±21	33±21	368±44	363±128	929±226	4
E8 carotid	65	35	563	510	1260	1
E9 aorta	78±11	22±11	228±118	338±127	785±212	4
E9 carotid	71	29	509	600	1210	1
E10 aorta	82	18	63	385	605	1
E10 carotid	73	27	431	250	970	1
E11 aorta	98±2	2±2	196±12	303±33	953±128	2
E11 carotid	95±6	5±6	369±182	472±172	910±128	3
E14 carotid	98±2	2±2	173±146	328±61	553±257	4
E14-15 aorta	98±2	2±2	162±92	320±96	521±155	4
C: coelomic grafts with veins						
E2 cardinal vein	32±9	68±9	255±202	170±131	565±208	7
E5 jugular vein	43±17	57±17	176±66	408±64	727±66	3
E7 jugular vein	63	37	239	330	880	1
E8 jugular vein	27±13	73±13	215±70	422±88	813±183	3
E9 jugular vein	14±3	86±3	245±89	325±49	750±212	2
E10 jugular vein	21±6	79±6	181±116	385	633±39	2
E11 jugular vein	9±3	91±3	279±133	380±15	670±20	2
E14-15 jugular vein	13±11	87±11	212±105	376±67	614±213	6
D: dorsal grafts						
E7 aorta	68±10	32±10	48±26	191±82	493±161	5
E7 carotid	79±2	21±2	69±17	428±28	875±55	2
E14-15 aorta	96±4	4±4	22±8	243±69	270±90	6
E7 jugular vein	58±16	42±16	142±71	324±151	523±228	5
E14-15 jugular vein	8±8	92±8	21±9	312±28	352±6	3

[1]The mean±s.d. for all parameters was calculated from the total number of grafts for each experiment.
[2]Total number of QH1+ cells was counted on every fifth section then the percentage of grafted EC colonizing arteries and veins was calculated.
[3]The graft size represented the number of sections containing graft.
[4]The EC migration was the distance which separated the first section containing QH1+ cells from the last section.
[5]n, the number of grafting experiments in each case.

shown) showed that vein-derived QH1+ cells integrating arteries acquired expression of both markers, while those colonizing host veins remained NRP1 and ephrinB2 negative. After E7, the great majority of QH1+ EC derived from the jugular endothelium participated in the venous tree, and only a small number of QH1+ EC reached arteries (Fig. 7B; Table 1C). Colonisation of veins was mainly directed towards the umbilical vein and the cardinal vein and more rarely to the omphalomesenteric vein and the venous plexus of the limb.
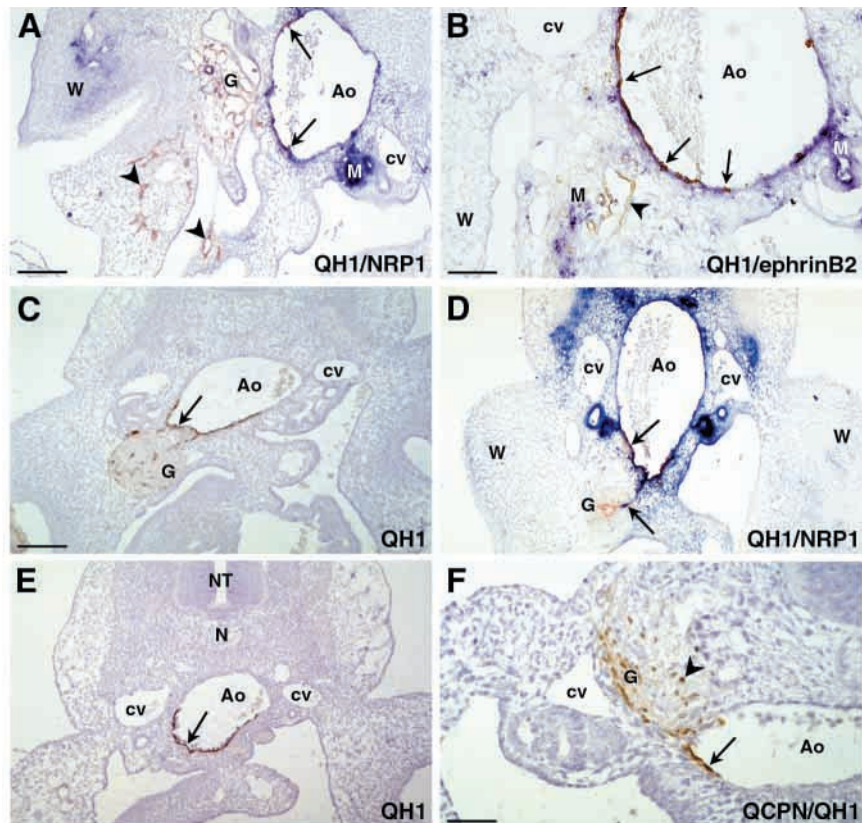
Dorsal grafts

To investigate whether the observed plasticity of arteries and veins was related to their site of engraftment, we performed dorsal grafting experiments in which 3 host somites were substituted by quail vessel rudiments. This protocol had previously been shown to permit migration of EC derived from somitic or splanchnopleural mesoderm (Pardanaud et al.,

1996). We grafted aortae, carotid arteries or jugular veins from E7 and E14/15 quails (Table 1D). The quail explants developed in the somitic territory, ventrolaterally to the neural tube and close to the cardinal vein and the dorsal endothelium of the aorta (Figs 6F, 7C). Owing to the absence of the somites, the cardinal vein and the Wolffian duct on the grafted side were sometimes displaced medially (Fig. 7C) or laterally, but no other morphological abnormalities were observed. Compared to the coelomic grafts, the number of QH1+ EC that migrated from the different quail rudiments as well as their migration distance was reduced (Table 1D). Grafts of E7 arteries or veins colonized mainly the dorsal aortic endothelium and the posterior cardinal vein. They were more rarely observed in the subclavian and brachial arteries or in the peripheral venous plexus of the wing (not shown). Grafts of E11 to E14 arteries or veins colonized the same vessels, however, plasticity was lost and arterial grafts colonized only host arteries (Fig. 6F)

Fig. 5. Grafts of early vascular rudiments. (A) Quail donors were at the 14ss. The boxed areas were dissected and grafted. The box labeled omv corresponds to the presumptive territory of the omphalomesenteric vein, while the box labeled oma corresponds to the territory of the omphalomesenteric artery. (B) QH1 (brown) and NRP1 (blue) double-staining of an E4 chick host that had received a graft (G) of territory oma 2 days before. Asterisks mark the connections of the graft to the host. QH1-positive/NRP1-positive EC are observed in the arterial system (arrows), including the aorta (Ao) and brachial artery (ba). QH1-positive/NRP1-negative cells are present in the venous system (arrowheads), including the posterior cardinal vein (cv), the subcardinal vein (scv), the umbilical vein (uv) and the peripheral plexus of the wing (W). M, mesonephros. Bar, 80 μm.
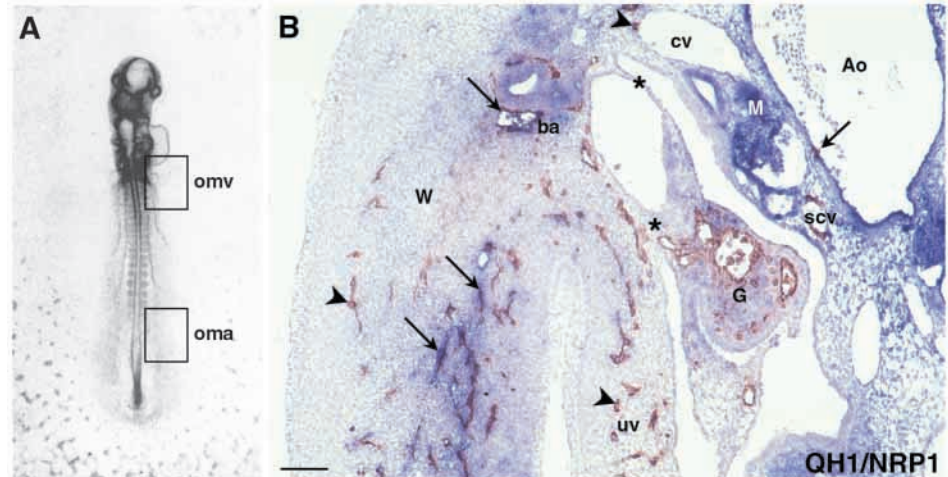
Fig. 6. Grafts of arteries. (A-E) coelomic grafts, (F) dorsal graft. (A,D) Double-staining with QH1 (brown) and NRP1 (blue). (B) Double-staining with QH1 (brown) and ephrinB2 (blue). (C,E) QH1 staining. (F) QCPN/QH1 staining, both the nuclear QCPN and the membranous QH1 labeling are revealed by HRP-staining. (A) Graft (G) of an E5 aorta. QH1-positive/NRP1-positive arterial EC (arrows) have colonized the aorta while QH1-positive/NRP1-negative EC are present in umbilical and omphalomesenteric veins (arrowheads). (B) Section of the same embryo as in A, QH1-positive/ephrinB2-positive EC (arrows) are present in the aorta, while QH1-positive/ephrinB2-negative EC have colonized the subcardinal vein (arrowhead). (C) E15 aorta graft. Only the lateroventral aortic endothelium is colonized. (D) E15 aorta graft. QH1-positive cells in the aorta express NRP1 (arrow). NRP1 expression in the grafted aorta is strongest in the region of EC emigration (Ao, arrow). (E) E11 carotid artery graft. The graft is not visible on this section. QH1-positive EC only colonize the ventrolateral aortic endothelium (arrow). (F) Dorsal graft of an E14 aorta. QH1-positive cells colonize the dorsolateral aortic endothelium (arrow) but appear excluded from the cardinal vein. QCPN-positive/QH1-negative non-endothelial cells (arrowhead) do not migrate. Ao, aorta; cv, cardinal vein; G, graft; M, mesonephros; N, notochord; NT, neural tube; W, wing bud. Bars, (A,D) 140 μm, (B,F) 40 μm, (C,E) 100 μm.


while veins colonized only host veins (Fig. 7C). The results of the dorsal and coelomic grafts were thus equivalent with regard to EC plasticity (Table 1D).

EC plasticity in the absence of a vascular wall

To examine the role of the vascular wall in EC plasticity, we isolated EC from E11 quail aortae by collagenase digestion. Isolated EC were cultured for 48 hours and the efficacy of removal of the vessel wall was tested by QH1 staining of the cultured cells (Fig. 8A,B). Cultures contained mainly QH1$^+$

EC, as well as some other QH1$^-$ cell types. For grafting, isolated EC were cultured overnight in hanging drops to obtain small aggregates. These were introduced into the coelom of chick hosts ($n=6$), where they developed in contact with the body wall, the mesonephros and the dorsal mesentery, as observed for intact vessel rudiments. However, the distribution of grafted EC in the host vessels was strikingly different compared to grafts of intact E11 aortic rings: grafted EC colonized host arteries ($59\pm17\%$) as well as host veins ($41\pm17\%$). QH1$^+$ EC reached the lateroventral endothelium of
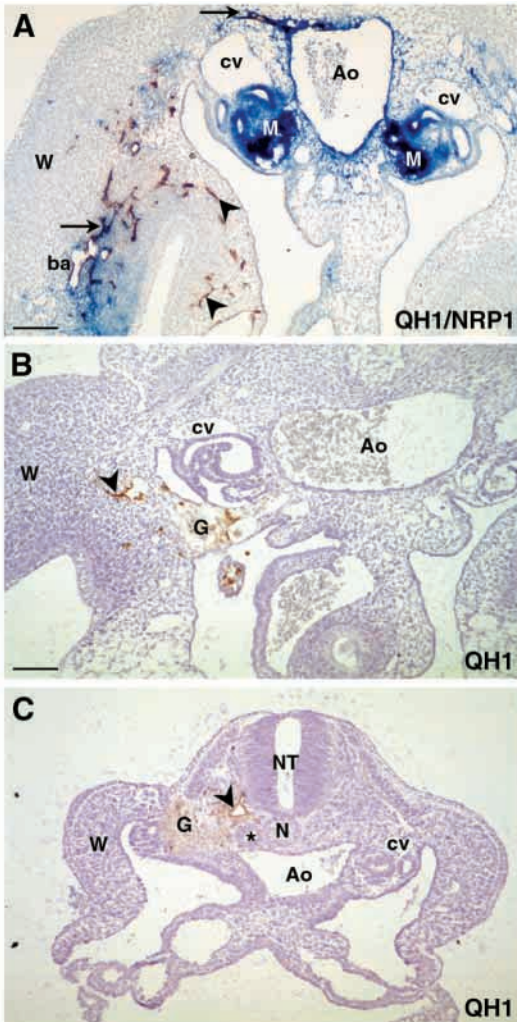
Fig. 7. Grafts of veins. (A,B) Coelomic grafts, (C) dorsal graft.
(A) Double-staining with QH1 (brown) and NRP1 (blue) of an E4
chick host that had received a graft of an E5 jugular vein 2 days
before. The graft is not visible on this section. Quail EC migrated
into the subclavian and brachial artery (ba, arrows), as well as into
the umbilical vein (arrowheads). (B) Graft of an E11 jugular vein.
QH1-positive EC are found in the umbilical vein (arrowhead).
(C) Dorsal graft of an E14 jugular vein. Quail EC have colonized the
posterior cardinal vein (arrowhead), which was displaced medially in
this graft together with the Wolffian duct (asterisk). G, graft. Bars,
(A) 125 µm, (B,C) 90 µm.

the aorta, the brachial artery, the cardinal vein and its branches,
the umbilical and omphalomesenteric veins and the peripheral
venous plexus of the wing bud (Fig. 8C,D).

DISCUSSION

We here report the results of a detailed analysis of NRP1 and
TIE2 expression in the developing vascular system of avian
embryos. NRP1 was found to be expressed specifically by
developing arteries and mesenchymal cells surrounding these
vessels. Venous EC never expressed this growth factor
receptor. In contrast, TIE2 mRNA showed higher expression
levels in venous EC than in arterial EC. Although TIE2

expression persisted in a subset of arterial EC, such as the
dorsal aorta, throughout the developmental period examined,
this receptor was reproducibly expressed at lower levels in
arterial EC of the internal carotid artery, aortic arches,
intersomitic arteries, etc., from E3 onwards. The down-
regulation of TIE2 expression levels in arterial EC
corresponded precisely to the onset of expression of its
ligand angiopoietin-2 in the mesenchyme surrounding these
developing arteries (Moyon et al., 2001). In mouse embryos, a
down-regulation of TIE2 mRNA in the arterial compartment
has not been reported (Schnurch and Risau, 1993; Sato et al.,
1995). This expression pattern may thus be specific for birds.
A specific expression of NRP1 in arterial EC has not been
reported in mice either (Kitsukawa et al., 1995; Kawasaki et
al., 1999). Interestingly, the reported vascular defects in NRP1
null mutant mice all concern the arterial compartment: these
mice exhibit poor nervous system vascularization, agenesis of
branchial arch great vessels and dorsal aorta, transposition of
the aortic arches, insufficient septation of the truncus arteriosus
and disorganized extraembryonic vasculature (Kawasaki et al.,
1999). We have obtained preliminary evidence in E15 and
E17 mouse embryos for preferential expression of NRP1 in
arterial EC (D. M., L. P., C. B. and A. E., unpublished
observations). However, further experiments are required to
establish if NRP1 expression in rodents is restricted to
the arterial compartment and if the NRP1 null mutants
indeed exhibit only arterial malformations. Arterovenous
differentiation in birds and mice may thus be regulated
differently, but involves at least some common sets of
molecules, since ephrinB2 is expressed selectively by arterial
EC in both species (Wang et al., 1998; Adams et al., 1999;
Moyon et al., 2001).

During the phase of vasculogenesis, NRP1 and TIE2 were
expressed throughout the yolk sac blood islands. During the
subsequent remodeling of the primary vascular plexus into
arteries and veins, NRP1 expression became restricted to
arteries and TIE2 to venous EC. This chronology was
reminiscent of the reported expression of ephrinB2 and its
receptor EphB4 in the mouse embryo, both of which appear on
EC only during the phase of remodeling (Wang et al., 1998;
Adams et al., 1999; Gerety et al., 1999). In Zebrafish, two
arterial-specific genes were recently cloned: *gridlock* (Zhong
et al., 2000) and *deltaC* (Smithers et al., 2000), which encodes
a ligand for members of the Notch family of receptors that
control binary cell fate decisions (reviewed in Artavanis-
Tsakonas et al., 1999). Interestingly, both *gridlock* and *deltaC*
were expressed in presumptive arterial EC before the onset of
blood flow. These observations raised the question of whether
the mesoderm is pre-specified at the gastrulation stage to
produce a posterior arterial and anterior venous domain, in
order to achieve a functional vascular circuit with the onset of
heartbeat? To address the question if EC are specified to an
arterial or venous identity once they acquire expression of
specific ligands or receptors, we performed quail-chick
grafting experiments. Blood vessels at different stages of
development were isolated from quail donors and grafted into
the coelom of a chick host. The earliest vessel rudiments we
isolated came from an E2 quail donor and corresponded to the
first two major extraembryonic blood vessels, the
omphalomesenteric artery and vein. At this stage, the posterior
territory corresponding to the future omphalomesenteric artery
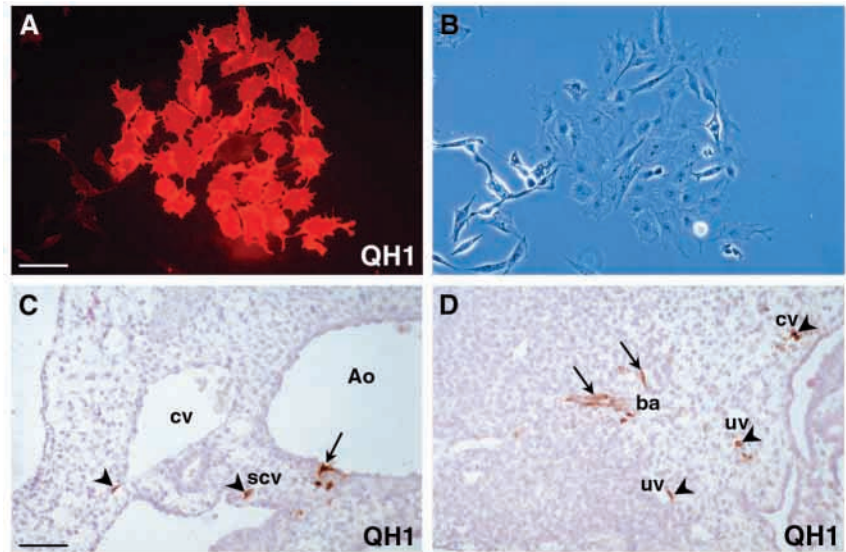
Fig. 8. EC plasticity in the absence of a vascular wall. (A,B) QH1 immunoreactivity (A), and phase contrast image (B) of EC isolated from E11 aortae. The majority of the cells are QH1 positive. (C,D) QH1 staining of a chick host that had received a graft of isolated EC, not visible in these sections. (C) Quail EC are found in the aorta (Ao) and the cardinal (cv) and subcardinal veins (scv, arrowheads). (D) Adjacent section, QH1-positive cells are found in the brachial artery (ba, arrows) and in the cardinal and umbilical veins (uv, arrowheads). Bars, (A,B) 85 µm, (C,D) 40 µm.

strongly expressed NRP1, while the region of the future omphalomesenteric vein had already down-regulated NRP1 expression. EC from both territories could still colonize both arteries and veins of the host embryo. The same result was obtained with grafts from the aorta, the carotid artery or the jugular vein taken from quail embryos between E2 and E7. The expression of NRP1 and ephrinB2 was regulated according to the host vessel, which was colonized by the grafted EC, implying that the plasticity of expression of arterial- or venous-specific genes accompanies endothelial plasticity with regard to arterial-venous differentiation. The adaptation of EC to the novel vascular environment could explain the arterialization of vein grafts currently performed in therapeutic procedures (Henderson et al., 1986; Cahill et al., 1987; Wallner et al., 1999). An interesting question is whether constitutive expression of arterial or venous EC markers would lead to the loss of EC plasticity.

Vessel rudiments isolated from embryos older than E7 were still capable of colonizing the host vasculature. Thus, in spite of the presence of a fully differentiated vascular wall, EC were still able to exit the graft and to migrate into the host vessels. Comparison of the number of migrating quail EC and their migration distance failed to reveal any significant decrease between E7 or E11 aortae, carotid arteries or jugular veins. However, in striking contrast to the results obtained with early EC, plasticity was progressively lost after E7, and from E11 onward aortic EC mainly colonized the host aorta and other arteries. Thus, at late embryonic stages, aortic EC appeared restricted to an arterial fate. This behaviour was not specific to the aorta, since the same result was obtained using E11 and E14 carotid arteries. Venous EC showed a loss of ability to colonize host arteries between E7 and E11 of development, while they were still capable of colonizing host veins. To determine if the loss of plasticity was due to the site of engraftment, we perfomed dorsal grafts, in which 3 host somites were substituted by quail vessel rudiments. It had previously been shown that EC derived from somites selectively colonize only dorsal endothelium of the aorta in both coelomic and dorsal grafts, while splanchnopleural EC selectively colonize ventral aorta (Pardanaud et al., 1996,

Pardanaud and Dieterlen-Lièvre, 1999). The loss of plasticity with respect to arterovenous differentiation was also observed in dorsal grafts. Moreover, these grafts showed that EC colonized the vessels closest to their site of engraftment: in the case of arteries, the dorsal aortic endothelium was colonized in dorsal grafts, while coelomic grafts led to colonization of the ventral aortic endothelium. Taken together, these results indicate that EC become restricted to an arterial or venous identity between E7 and E11 of development.

To understand the mechanisms regulating this loss of plasticity, we investigated the role of the vessel wall. EC from the E11 aorta were isolated from their wall and grafted into the coelom. Strikingly, this procedure fully restored their capacity to colonize host veins; about 60% of EC were found in arteries and 40% in veins of the hosts, as observed at earlier stages. A purely mechanical effect of the vessel wall cannot be definitively excluded, but seems unlikely, since migration distance and number of migrating cells were not significantly affected by the presence of the vessel wall, and longitudinal sectioning of vessels to directly expose the endothelium did not increase their migration. It therefore appears that a signal derived from the vessel wall determines EC identity.

The molecular mechanisms restricting arterial or venous EC identity remain to be explored. Our expression study with NRP1 and TIE2 shows that these arterial- and venous-specific genes are expressed long before restriction of plasticity occurs and thus make a direct role for these genes in the establishment of arterial and venous identity unlikely. It is however possible that these genes could be involved in the development and assembly of the arterial and venous vessel wall. In this respect, it is interesting to note that NRP1 and ephrinB2 are also expressed in cells of the arterial vessel wall in both chick and mouse embryos (Shin et al., 2001; Gale et al., 2001). The experiments reported here provide a framework for the investigation of the molecular mechanisms involved in restricting EC plasticity with respect to arterial-venous differentiation.

REFERENCES

Adams, R. H., Wilkinson, G. A., Weiss, C., Diella, F., Gale, N. W., Deutsch, U., Risau, W. and Klein, R. (1999). Roles of *ephrin*B ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. *Genes Dev*. **13**, 295-306.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-776.

Cahill, P. D., Brown, B. A., Handen, C. E., Kosek, J. C. and Miller, D. C. (1987). Incomplete biochemical adptation of vein grafts to the arterial environment in terms of prostacyclin production. *J. Vasc. Surg*. **6**, 496-503.

Eichmann, A., Corbel, C., Jaffredo, T., Bréant, C., Joukov, V., Kumar,V., Alitalo, K. and Le Douarin, N. M. (1998). Avian VEGF-C: cloning, embryonic expression pattern and stimulation of the differentiation of VEGFR2-expressing endothelial cell precursors. *Development* **125**, 743-752.

Eichmann, A., Yuan, L., Bréant, C., Alitalo, K. and Koskinen, P. J. (2000). Developmental expression of Pim kinases suggests functions also outside of the hematopoietic system. *Oncogene* **19**, 1215-1224.

Etchevers, H. C., Vincent, C., Le Douarin, N. M. and Couly, G. F. (2001). The cephalic neural crest provides pericytes and smooth muscle cells to all blood vessels of the face and forebrain. *Development* **128**, 1059-1068.

Folkman, J. and D'Amore, P. A. (1996). Blood vessel formation: what is its molecular basis ? *Cell* **87**, 1153-1155.

Gerety, S. S., Wang, H. U., Chen, Z. F. and Anderson, D. J. (1999). Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand *ephrin*-B2 in cardiovascular development. *Mol. Cell* **4**, 403-414.

Gale, N. W. and Yancopoulos, G. D. (1999). Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, *angiopoietin*s, and *ephrin*s in vascular development. *Genes Dev*. **13**, 1055-1066.

Gale, N. W., Baluk, P., Pan, L., Kwan, M., Holash, J., DeChiara, T. M., McDonald, D. M. and Yancopoulos, G. D. (2001). *Ephrin*B2 selectively marks arterial vessels and neovascularization sites in the adult, with expression in both endothelial and smooth-muscle cells. *Dev. Biol*. **230**, 151-160.

Gilbert, S. F. (1994). In *Developmental Biology*. 4th edn, p. 351. Sunderland, MA: Sinaur Associates.

Gluzman-Poltorak, Z., Cohen, T., Herzog, Y. and Neufeld, G. (2000). Neuropilin-2 and neuropilin-1 are receptors for the 165-amino acid form of vascular endothelial growth factor (VEGF) and of placenta growth factor-2, but only neuropilin-2 functions as a receptor for the 145-amino acid form of VEGF. *J. Biol. Chem*. **275**, 18040-18045.

He, Z. and Tessier-Lavigne, M. (1997). *Neuropilin* is a receptor for the axonal chemorepellent semaphorin III. *Cell* **90**, 739-751.

Henderson, V. J., Cohen, R. G., Mitchell, R. S., Kosek, J. C. and Miller, D. C. (1986). Biochemical (functional) adaptation of "arterialized" vein grafts. *Ann. Surg*. **203**, 339-345.

Jones, P. F., McClain, J., Robinson, D. M., Sato, T. M. and Yancopoulos, G. D. (1999). Identification and characterisation of chicken cDNAs encoding the endothelial cell-specific receptor tyrosine kinase *Tie*2 and its ligands, the *angiopoietin*s. *Angiogenesis* **2**, 357-364.

Kaipainen, A., Korhoonen, J., Mustonen, T., van Hinsbergh, V., Fong, G. H., Dumont, D., Breitman, M. L. and Alitalo, K. (1995) Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc. Natl. Acad. Sci. USA* **92**, 3566-3570.

Kawasaki, T., Kitsukawa, T., Bekku, Y., Matsuda, Y., Sanbo, M., Yagi, T. and Fujisawa, H. (1999). A requirement for *neuropilin*-1 in embryonic vessel formation. *Development* **126**, 4895-4902.

Kitsukawa, T., Shimono, A., Kawakami, A., Kondoh, H., Fujisawa, H. (1995). Overexpression of a membrane protein, *neuropilin*, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs. *Development* **121**, 4309-4318.

Kitsukawa, T., Shimizu, M., Sanbo, M., Hirata, T., Taniguchi, M., Bekku,

Y., Yagi, T. and Fujisawa, H. (1997). *Neuropilin*-semaphorin III/D-mediated chemorepulsive signals play a crucial role in peripheral nerve projection in mice. *Neuron* **19**, 995-1005.

Kolodkin, A. L., Levengood, D. V., Rowe, E. G., Tai, Y. T., Giger, R. J. and Ginty, D. D. (1997). *Neuropilin* is a semaphorin III receptor. *Cell* **90**, 753-762.

Makinen, T., Olofsson, B., Karpanen, T., Hellman, U., Soker, S., Klagsbrun, M., Eriksson, U. and Alitalo, K. (1999). Differential binding of vascular endothelial growth factor B splice and proteolytic isoforms to *neuropilin*-1. *J. Biol. Chem*. **274**, 21217-21222.

Migdal, M., Huppertz, B., Tessler, S., Comforti, A., Shibuya, M., Reich, R., Baumann, H. and Neufeld, G. (1998). *Neuropilin*-1 is a placenta growth factor-2 receptor. *J. Biol. Chem*. **273**, 22272-22278.

Moyon, D., Pardanaud, L., Yuan, L., Bréant, C. and Eichmann, A. (2001). Selective expression of angiopoietin 1 and 2 in mesenchymal cells surrounding veins and arteries of the avian embryo. *Mech. Dev*. (in press).

Neufeld, G., Cohen, T., Gengrinovitch, S. and Poltorak, Z. (1999). Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J*. **13**, 9-22.

Pardanaud, L., Altmann, C., Kitos, P., Dieterlen-Lièvre, F., and Buck, C. (1987). Vasculogenesis in the early quail blastodisc as studied with a monoclonal antibody recognizing endothelial cells. *Development* **100**, 339-349.

Pardanaud, L., Yassine, F. and Dieterlen-Lièvre, F. (1989). Relationship between vasculogenesis, angiogenesis and haemopoiesis during avian ontogeny. *Development* **105**, 473-485.

Pardanaud, L., Luton, D., Prigent, M., Bourcheix, L. M., Catala, M. and Dieterlen-Lièvre, F. (1996) Two distinct endothelial lineages in ontogeny, one of them related to hemopoiesis. *Development* **122**, 1363-1371.

Pardanaud, L. and Dieterlen-Lièvre, F. (1999) Manipulation of the angiopoietic/hemangiopoietic commitment in the avian embryo. *Development* **126**, 617-627.

Petrova, T. V., Makinen, T. and Alitalo, K. (1999). Signaling via vascular endothelial growth factor receptors. *Exp. Cell Res*. **253**, 117-130.

Risau, W. and Flamme, I. (1995). Vasculogenesis. *Ann. Rev. Cell Dev. Biol*. **11**, 73-91.

Risau, W. (1997). Mechanisms of angiogenesis. *Nature* **386**, 671-674.

Sato, T. N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W. and Qin, Y. (1995). Distinct roles of the receptor tyrosine kinases *Tie*-1 and *Tie*-2 in blood vessel formation. *Nature* **376**, 70-74.

Schnurch, H. and Risau, W. (1993). Expression of *TIE*2, a member of a novel family of receptor tyrosine kinases, in the endothelial cell lineage. *Development* **119**, 957-968.

Shin, D., Garcia-Cardena, G., Hayashi, S-I., gerety, S., Asahara, T., Stavrakis, G., isner, J., Folkman, J., Gimbrone, M. A. and Anderson, D. J. (2001). Expression of *ephrin*B2 identifies a stable genetic difference between arterial and venous vascular smooth muscle as well as endothelial cells, and marks subsets of microvessels at sites of adult neovascularization. *Dev. Biol*. **230**, 139-150.

Shutter, J. R., Scully, S., Fan, W., Richards, W. G., Kitajewski, J., Deblandre, G. A., Kintner, C. R. and Stark, K. L. (2000). Dll4, a novel Notch ligand expressed in arterial endothelium. *Genes Dev*. **14**, 1313-1318.

Soker, S., Takashima, S., Miao, H. Q., Neufeld, G. and Klagsbrun, M. (1998). *Neuropilin*-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* **92**, 735-745.

Smithers, L., Haddon, C., Jiang, Y. and Lewis, J. (2000). Sequence and embryonic expression of deltaC in the zebrafish. *Mech. Dev*. **90**, 119-123.

Takagi, S., Kasuya, Y., Shimizu, M., Matsuura, T., Tsuboi, M., Kawakami, A. and Fujisawa, H. (1995). Expression of a cell adhesion molecule, neuropilin, in the developing chick nervous system. *Dev. Biol*. **170**, 207-222.

Wang, H. U., Chen, Z. F. and Anderson, D. J. (1998). Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by *ephrin*-B2 and its receptor EphB4. *Cell* **93**, 741-753.

Wallner, K., Li, C., Fishbein, M. C., Shah, P. K. and Sharifi, B. G. (1999). Arterialization of human vein grafts is associated with tenascin-C expression. *J. Am. Coll. Cardiol*. **34**, 871-875.

Wilting, J., Eichmann, A. and Christ, B. (1997). The avian VEGF receptor homologues Quek1 and Quek2 in blood-vascular and lymphatic endothelial and non-endothelial cells during quail embryonic development. *Cell. Tissue Res*. **288**, 207-223.

Wise, L. M., Veikkola, T., Mercer, A. A., Savory, L. J., Fleming, S. B., Caesar, C., Vitali, A., Makinen, T., Alitalo, K. and Stacker, S. A. (1999).

Vascular endothelial growth factor (VEGF)-like protein from orf virus NZ2 binds to VEGFR2 and *neuropilin*-1. *Proc. Natl. Acad. Sci. USA* **96**, 3071-3076.

Yancopoulos, G. D., Klagsbrun, M. and Folkman, J. (1998). Vasculogenesis, angiogenesis and growth factors: *ephrin*s enter the fray at the border. *Cell* **93**, 661-664.

Yancopoulos, G. D., Davis, S., Gale, N. W., Rudge, J. S., Wiegand, S. J. and Holash, J. (2000). Vascular-specific growth factors and blood vessel formation. *Nature* **407**, 242-248.

Zhong, T. P., Rosenberg, M., Mohideen, M-A. P. K., Weinstein, B. and Fishman, M. C. (2000). Gridlock, an HLH gene required for assembly of the aorta in zebrafish. *Science* **287**, 1820-1824.