Development 133, 1013-1022 doi:10.1242/dev.02269

# Somite-derived cells replace ventral aortic hemangioblasts and provide aortic smooth muscle cells of the trunk

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We have previously shown that endothelial cells of the aortic floor give rise to hematopoietic cells, revealing the existence of an aortic hemangioblast. It has been proposed that the restriction of hematopoiesis to the aortic floor is based on the existence of two different and complementary endothelial lineages that form the vessel: one originating from the somite would contribute to the roof and sides, another from the splanchnopleura would contribute to the floor. Using quail/chick orthotopic transplantations of paraxial mesoderm, we have traced the distribution of somite-derived endothelial cells during aortic hematopoiesis. We show that the aortic endothelium undergoes two successive waves of remodeling by somitic cells: one when the aortae are still paired, during which the initial roof and sides of the vessels are renewed; and a second, associated to aortic hematopoiesis, in which the hemogenic floor is replaced by somite endothelial cells. This floor thus appears as a temporary structure, spent out and replaced. In addition, the somite contributes to smooth muscle cells of the aorta. In vivo lineage tracing experiments with non-replicative retroviral vectors showed that endothelial cells do not give rise to smooth muscle cells. However, in vitro, purified endothelial cells acquire smooth muscle cells characteristics. Taken together, these data point to the crucial role of the somite in shaping the aorta and also give an explanation for the short life of aortic hematopoiesis.

**KEY WORDS: Avian embryo, Aorta, Hemangioblasts, Hematopoiesis, Endothelium, Somite, Vascular smooth muscle**

## INTRODUCTION

The embryonic aorta is now recognized as a site that produces adult-type hematopoietic stem cells. Intra-aortic hematopoiesis is present in all vertebrate species examined so far and exhibits stereotyped aspects: production of hematopoietic cells (HCs) is restricted to the floor of the vessel; hematopoietic production is characterized by the presence of small groups of HCs, the intra-aortic clusters, protruding into the aortic lumen; HCs appear in close association with vascular endothelial cells (ECs) lining the vessel; aortic hematopoiesis is extremely transient, being detected between embryonic days (E) 3 to 4 in birds, 9 to 11 in the mouse and 27 to 40 in the human embryo (Jaffredo et al., 2005b; Tavian and Péault, 2005).

Hematopoietic clusters were proposed to originate from the aortic ventral endothelium via a specialized cell, the aortic hemangioblast (Murray, 1932). However, unlike the yolk sac where ECs and HCs appear in a coordinated manner, the aorta is formed at least 1 day before hematopoietic clusters became visible, suggesting the existence of an aorta-specific type of hemangioblast. Before hematopoiesis, however, these cells ensure endothelial-specific functions and display a gene repertoire characteristic of ECs (Jaffredo et al., 1998; Jaffredo et al., 2005a). With the exception of the *Runx1* transcription factor (previously known as *cbfa2* or *AML1*) (North et al., 1999; North et al., 2002), aortic hemangioblasts are indistinguishable from the other bona fide ECs and, in particular those of the aortic roof and sides (North et al., 1999; Bollérot et al., 2005). This sequence of events is more evocative of a 'hemogenic endothelium', a term coined by Jordan (Jordan, 1917).

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**Accepted 3 January 2006**

A corpus of data in birds and mammals demonstrates that, as hemopoiesis initiates, this hemogenic endothelium looses its endothelial phenotype and acquires a hematopoietic-specific gene repertoire (Nishikawa et al., 1998; de Bruijn et al., 2002; Fraser et al., 2002; Oberlin et al., 2002; Hirai et al., 2003; Sugiyama et al., 2003; Jaffredo et al., 2005a) (K. Bollérot and T.J., unpublished). Newly born HCs are either released into the circulation or ingress into the ventral mesentery (Jaffredo et al., 1998) before definitive hematopoietic organs become open to colonization.

Transplantation experiments in birds have demonstrated that embryonic ECs originate from two different mesodermal lineages (Pardanaud et al., 1996). One, from the splanchnic mesoderm, gives rise to the endothelial network of the visceral organs. This EC production arises in situ (Pardanaud et al., 1989) and exhibits a dual hemangiopoietic potential, i.e. it gives rise to both ECs and HCs (Pardanaud et al., 1996). The other, from the somites, is purely endothelial, colonizes the somatopleural mesoderm and gives the vasculature of the body wall (Pardanaud et al., 1996; Ambler et al., 2001) and lymphatic vessels (Wilting et al., 2000). This mapping also reveals that the aortic endothelium has a dual origin: roof and sides being contributed by somite-derived ECs, floor by splanchnopleura-derived ECs (Pardanaud et al., 1996). As only splanchnopleura-born ECs display hemogenic capacities, intra-aortic clusters are restricted to the ventral aspect of the aorta.

Blood vessels are typically formed by one layer of ECs, one of pericytes and one or several layers of smooth muscle cells, the importance of which varies according to the size and identity (arterial versus venous) of the vessel. ECs originate from the mesoderm whatever the region of the body concerned. By contrast, vascular smooth muscle cells and pericytes, hereafter referred as VSM cells, exhibit different origins according to the region of the body being considered. In the forebrain, face, neck and truncus arteriosus, VSM cells derive from the cephalic neural crest (Le Lièvre and Le Douarin, 1975; Jiang et al., 2000; Etchevers et al., 2001). In the heart, the septum separating the aorta from the pulmonary trunk (Waldo et al., 1998) and the VSM cells of the

proximal cardiac artery (Bergwerff et al., 1998; Etchevers et al., 2001) are derived from neural crest. Coronary vein VSM cells originate from the myocardium and coronary arteries VSM cells from the epicardium (Mikawa and Gourdie, 1996; Vrancken Peeters et al., 1999; Perez-Pomares et al., 2002). The origins of VSM cells in the trunk, however, still remain elusive. ECs have been proposed either to influence mesodermal cells differentiation (Drake et al., 1998; Hungerford and Little, 1999) or to generate VSM cells directly (DeRuiter et al., 1997; Drab et al., 1997; Yamashita et al., 2000; Ema et al., 2003). Platelet derived growth factor (PDGF) B and its receptor PDGFRβ have been shown to promote smooth muscle cell differentiation; PDGFB- and PDGFRβ-deficient mice exhibit impaired VSM cell recruitment (Lindahl et al., 1997). Chick and mouse embryos have been shown to harbor multipotent progenitor cells, the mesoangioblasts, that are able to give rise to ECs and VSM cells (Cossu and Bianco, 2003). Smooth muscle cells are also thought to derive from the somites, although this assertion has not been documented in detail (Brand-Saberi and Christ, 2000).

We report here the sequence of events contributing to vascularization of the embryo with a special interest for the aorta, i.e. the fate of the hemogenic endothelium and the role of the somite in the origins of the aortic wall from E1.5 to E6 in the chick embryo. Orthotopic and isochronic grafts at E1.5 of segmental plate show that the initial roof and sides of the aorta are replaced by new roof and sides from the somite. Cardinal veins, vasculature of the back, body wall and kidney are also of somitic origin. As hematopoiesis proceeds, the hemogenic endothelium also disappears from the aortic floor and is replaced by somitic ECs. Thus, the aortic floor appears as a transitory structure spent out and replaced. Aortic ECs are thus totally renewed during the first 4 days of development. In addition, we have characterized somite EC precursors at the molecular level. We also demonstrate that somites give rise to smooth muscle cells of the aorta. Finally, by using cell lineage tracing experiments in the aortic endothelium, we show that vascular ECs do not generate VSM cells during in ovo avian development, but do in vitro under appropriate conditions

## MATERIALS AND METHODS

### Segmental plate grafting

Orthotopic, isochronic, unilateral or bilateral grafts of segmental plates were performed between chick host (*Gallus gallus* JA57 strain) and quail donor (*Coturnix coturnix japonica*) according to the scheme in Fig. 1A. Eggs were incubated for 36-45 hours at 37±1°C in a humidified atmosphere to reach 10-18 somite pairs. Microsurgery was performed as previously described (Pardanaud et al., 1996). The segmental plate attached to the last formed somite was removed over a length corresponding to 5-10 somites. Segmental plates were rinsed in DMEM (PAA Laboratories)/10% fetal calf serum (Eurobio) and transplanted into a host submitted to the same ablation. Grafts were performed according to the original dorsoventral and anteroposterior orientations. The chimeric embryos were incubated for an additional period of 24 to 120 hours.

### Immunohistology

#### Antibodies

QH1 monoclonal antibody (mAb, kind gift of Dr Luc Pardanaud), specific for quail ECs and HCs (Pardanaud et al., 1987), was revealed with a goat anti mouse (GAM) IgM coupled to either Horse Radish Peroxydase (Southern Biotechnology Associated) or Alexa Fluor 488 (Molecular Probes). QCPN (Quail non-chick perinuclear antigen) recognizes all quail cell nuclei. It was developed by Carlson and Carlson, and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa (Department of Biological Sciences, Iowa City, IA 52242). QCPN was visualized with a GAM IgG1-Alexa Fluor 555 (Molecular Probes). Anti α-smooth muscle actin (αSMA) was purchased from Sigma (clone 1A4) and revealed with a GAM IgG2a-Alexa Fluor 350 (Molecular Probes). Anti-chick CD 45, anti 68-1 and 74-2, supplied by ID-DLO (The Netherlands), were used to identified macrophages (Jeurissen and Janse, 1998). They were revealed with a GAM IgG2a-Alexa 350 or -Alexa 488 for CD45 or GAM IgG1-Alexa 555 for 68-1 and 74-2. MEP21 (generous gift of Dr K. McNagny) recognized chick ECs and HCs (McNagny et al., 1997). MEP21 was revealed with a biotinylated GAM IgG1 followed by an amplification using the Tyramide Signal Amplification system (TSA, NEN Life Science) using Cyanin3 as a fluorescent probe. When needed, sections were counterstained with DAPI.

#### Histology

For cryostat sections, embryos were fixed in 4% paraformaldehyde and processed as described previously (Jaffredo et al., 1998). For Paraplast sections, embryos were fixed overnight in modified Formoy's solution. They were embedded in Paraplast (Sigma) and transversally sectioned at 5-7 μm. After rehydration, sections were submitted to PBS/$H_2O_2$ 3% to remove endogenous peroxydases followed by a PBS/FCS 5% incubation for 30 minutes at room temperature. Primary and secondary antibodies were deposited in PBS/FCS 5% 1 hour at room temperature. Sections were washed in PBS four times between each incubation. HRP was revealed with 3,3′-diaminobenzidine (DAB, Sigma).

### RNA probes

The chicken GATA2 plasmid was a gift from Dr Engel (Northwestern University, Evanston, IL). The chicken SCL/TAL1 was provided from Dr Green (University of Cambridge, UK). Sense probes were obtained as reported in (Minko et al., 2003). The chick VEGFR2 extracellular domain was isolated as follows (details available upon request). Total RNA was extracted from yolk sacs of HH17 chick embryos using the RNAble kit (Eurobio). RT-PCR was performed with the Enhanced Avian HS RT-PCR 100 kit using the two-step method (Sigma). First step: specific RT-PCR primer 5′ GAGGAACGCCATTTACTGTG. Second step: 40 PCR cycles with 5′ primer (TCGGCACGGCTCGGCTTC) and 3′ primer (TCCAGATTTCCCACAAGACG). A 2045 bp fragment was purified from agarose gel using QIAEX Gel Extraction kit (Qiagen) and subcloned into the pCR2.1 vector (InVitrogen) using Rapid DNA ligation kit (Roche). The antisense VEGFR2 probe was obtained by digesting the plasmid with *Sac*I followed by transcription with T7.

### In situ hybridization on sections

Embryos were collected and fixed overnight at 4°C in Formoy's solution. Paraplast sections were treated as described previously (Wilting et al., 1997; Minko et al., 2003).

### Retroviral constructs and cells lines

The D17.2G cell line was obtained from The American Type Culture Collection (CRL-8468). D17.2G is a REV-A-based packaging cell line obtained from D17 canine cells. D17.2G was co-transfected with SNV-hPLAP (human Placental Alkaline Phosphatase) and pUT626-containing phleomycin as a selection vector (Cayla, Toulouse) as previously described (Gautier et al., 2000; Jaffredo, 2000). Harvesting and concentration were performed as previously described (Gautier et al., 1996; San Clemente et al., 1996; Jaffredo et al., 2000). Retroviral concentrations were evaluated on QT-6 cells, ATCC number CRL-1708 (Moscovici et al., 1977). PLAP, was estimated to be $1\times10^6$ infectious units/ml and CXL, $2\times10^4$ infectious units/ml.

### *In ovo* inoculation

#### AcLDL-DiI

Human acetylated low density lipoprotein coupled to DiI (AcLDL-DiI, Molecular Probes) was inoculated into the heart of E2 or E4 chick embryos as described previously (Bollérot et al., 2006). Two hours after inoculation, embryos were either photographed or processed for Flow Cytometry Analysis.

#### Retroviral vectors

Intracardiac inoculations of viral supernatant were performed on E4 embryos as described (Jaffredo et al., 2000). Embryos were sacrificed at E6, beheaded, fixed in PFA 4% for 2 hours at room temperature and

stained in toto for PLAP and *lacZ* expression with respectively NBT/BCIP and X-Gal. Embryos were embedded for cryostat and sectioned at 7 μm.

### Flow cytometry analysis

Heads, hearts, legs, allantois and extra-embryonic membranes of AcLDL-DiI-inoculated embryos were removed. Embryo bodies were minced on ice in a Petri dish and dissociated for 15 minutes at room temperature in sterile 1×PBS/dispase (2 U/ml) (Gibco) supplemented with 1% penicillin-streptomycin. Enzymatic dissociation was stopped with DMEM/10% FCS. After several washes with DMEM/10%FCS, cells were incubated for 20 minutes with the anti-CD45 mAb and for 20 minutes with a GAM IgG2a-Alexa 488. Non-inoculated embryos were used as controls. AcLDL-DiI$^+$/CD45$^-$ cells were sorted using a Coulter Epics Elite ESP FACS.

### Cell culture

Sorted AcLDL-DiI$^+$/CD45$^-$ cells and unsorted cells (control) were seeded in Lab-Tek Chamber Slides (Nunc) coated with laminin at 20 μg/ml (InVitrogen) at 2500 cells per well in EGM2-MV medium (kind gift of Dr G. Uzan). This day was considered as day 0. The day after, EGM2-MV medium was supplemented with either VEGF 50 ng/ml (R&D System), TGFβ 5 ng/ml (R&D System), or without growth factor. Growth factors were renewed every 2 days. Cells were fixed at day 0, 1, 4 and 7 in PFA4% for 20 min at RT and analyzed for MEP 21 and αSMA expression.

## RESULTS

## Somite contribution to aorta organogenesis

### Controls

To avoid unwanted contaminating tissues in transplantation experiments (Fig. 1A), we first examined the localization of the aorta and associated vascular network at the level of the last formed somite and the segmental plate in chick and quail embryos ranging from 9 to 20 somite pairs. AcLDL-DiI were specifically and rapidly endocytosed by chick ECs, thus enabling the whole vascular network to be visualized under UV (Fig. 1B). In 9-18 somite pair embryos, aortae and the associated vascular network are positioned lateral to the graft (Fig. 1B). This is also visible on a 13-somite pairs quail embryo following QH1 staining (Fig. 1C,D). From 19 somite pairs onwards, the bilateral aortae become positioned ventral to the last formed somite and the anterior aspect of the segmental plate. On the basis of this pattern, we decided to use embryos ranging from 9-18 somite pairs for transplantation experiments.

To make sure that no ECs were left at the surface of quail segmental plates, isolated grafts were submitted to QH1 immunohistochemistry. As a control, isolated quail somite strips attached to the lateral plate mesoderm, displayed strong QH1 staining (not shown). Out of 20 segmental plates examined, 18 were free of contaminating cells (Fig. 1E), whereas two displayed one to three positive cells in the anterior part of the graft. Conversely, in chick embryos submitted to segmental plate ablation, the aortic anlage and the associated vascular network appeared intact (not shown).

### Unilateral grafts

Forty-six grafts were performed on the right side of chick hosts, staged 9-18 somite pairs. In rare cases, grafts were performed on the left side with no difference in results. Embryos were sacrificed 1-5 days after grafting, fixed and transversely sectioned. Analysis was performed either with the QH1 mAb alone or by double staining with QH1 and QCPN or QH1 and MEP21 mAbs.

One day after the operation (E2.5, *n*=22), the contribution of quail somitic ECs varied along the axis, followed somite maturation but was restricted to the grafted side. At caudal-most levels where the aortae were still paired, a few QH1$^+$ cells were detected around the neural tube. The roof of the aorta was found to be colonized by somitic ECs (Fig. 2A). This contribution, one of the earliest of somite ECs to the vascular system, also agreed with the QH1 pattern in non-operated quail embryos (see Fig. 5E). At more cephalic levels, dermatome and myotome have separated, the sclerotome began to dissociate and the aortae have fused. The cardinal vein and vascular networks of the mesonephros, the neural tube, inter-somitic arteries and the lateral plate vascular network contained numerous quail ECs (Fig. 2B). This pattern is consistent with a centrifugal emigration of somite ECs to the lateral plate already reported (Pardanaud et al., 1996; Ambler et al., 2001). In keeping with the results at more caudal levels, the roof and sides of the aorta on the grafted side were formed entirely by quail ECs (Fig. 2B). Complete replacement was preferentially observed at the medial level of the graft and obtained only when grafts longer than five-somite equivalents are used. With shorter grafts, vascularization of the

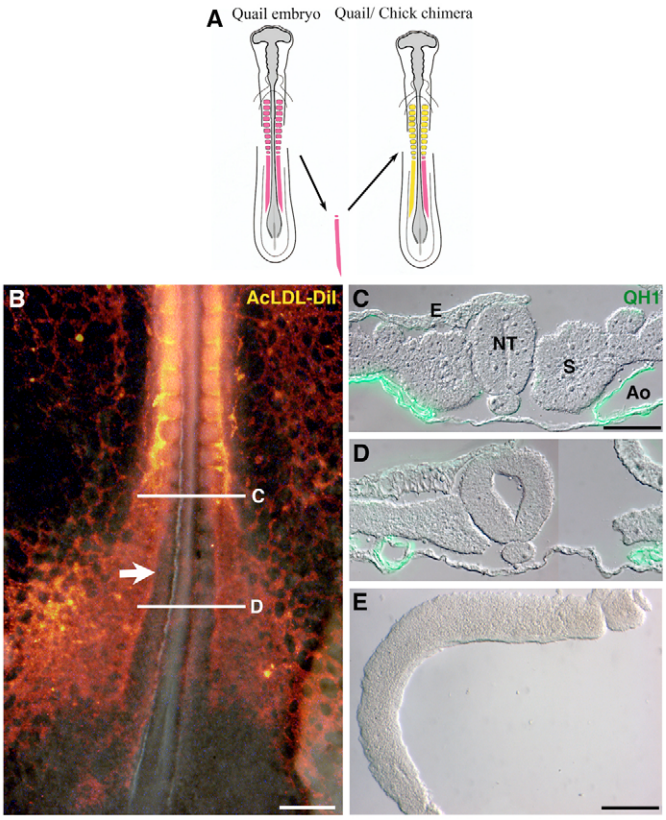


**Fig. 1. Isochronic and orthotopic graft of segmental plate from quail to chick.** (**A**) Scheme of a graft. (**B**) AcLDL-DiI labeling of the vascular network; 13 somite-pair embryo; ventral view, truncal level. The whole vascular network is labeled. The arrow indicates the level of the last-formed somite pair. At this level, the paired aortae are positioned lateral to the somites, whereas they are in more central positions at more cephalic levels. Lines indicate the levels of sections in C,D. Scale bar: 800 μm. (**C**,**D**) Unilateral last somite + segmental plate removal. 13 somite-pair embryo; cross-sections, QH1 staining. (C) Level of the third last-formed somite. The ectoderm has been removed. (D) Segmental plate level. The right segmental plate has been removed with no damage to the aorta. The aorta is positioned lateral to the segmental plate. Scale bar: 70 μm. (**E**) Isolated last somite + lateral plate mesoderm, QH1 staining. No QH1$^+$ cell was detected in 18 out of 20 grafts analyzed. Scale bar: 100 μm. Ao, aorta; E, ectoderm; NT, neural tube; S, somite.

structures cited above remained unchanged but the aorta was found to contain rare quail cells in its dorsal aspect. QH1$^+$ ECs never crossed the dorsal midline (cf. Klessinger and Christ, 1996; Pardanaud et al., 1996). This aorta-associated QH1 pattern persisted until aortic hematopoiesis initiated, i.e. the beginning of E3.

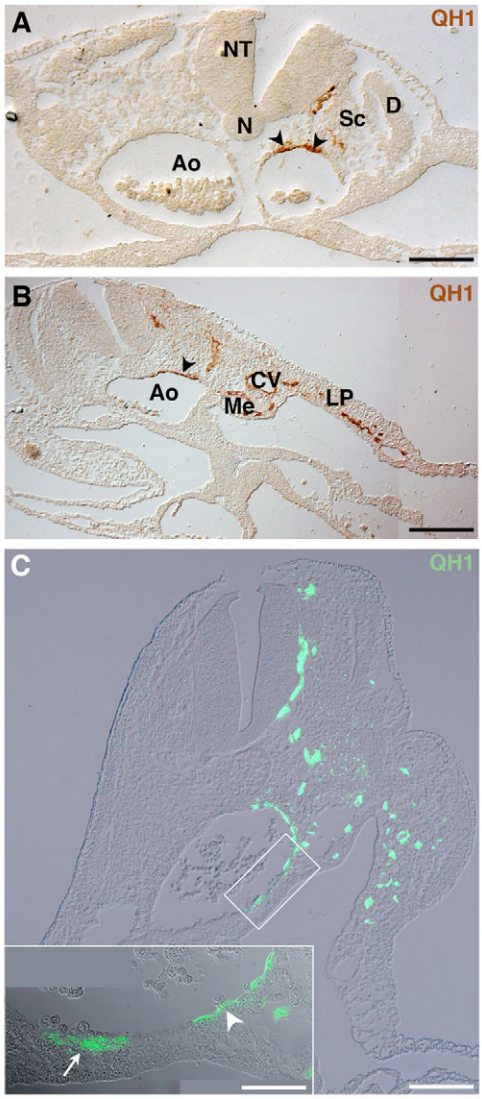


**Fig. 2. Somite contribution to aortic endothelium remodeling and vascularization of the body wall.** (A,B) E2.5 chick host, 1 day post-grafting. QH1 immunohistochemistry DAB stained cross-sections. (**A**) Caudal region of the grafted embryo. The roof of the right aortic rudiment is colonized with QH1$^+$ cells (arrowheads). QH1$^+$ cells around the neural tube organize into the perineural plexus. No QH1$^+$ signal is visible lateral to the somite. (**B**) Medial level of the graft. The roof of the aorta (Ao) is entirely of quail origin on the grafted side. QH1$^+$ cells have also colonized the ipsilateral chick host body wall where they give rise to the vascularization of the neural tube, mesonephros (Me), lateral plate (LP) and cardinal vein (CV). Scale bar: 150 μm. (**C**) E4 chick host, 2 days post-grafting. QH1 immunofluorescence (green) overlaid with Nomarski's interferential contrast. QH1$^+$ cells form a conspicuous plexus around the neural tube. The lateral plate contains numerous QH1$^+$ vessels. The QH1 aortic domain has extended ventrally to the hematopoietic clusters. Scale bar: 150 μm. (Inset) A prominent QH1$^-$ hematopoietic cluster of host (chick) origin is underlined by QH1$^+$ ECs (arrow). Some QH1$^+$ cells are integrated in the vascular endothelium (arrowhead). Scale bar: 30 μm. D, dermomyotome; Sc, sclerotome.

Two days after grafting, at E3.5, as hematopoiesis proceeds ($n$=7), QH1$^+$ vascular cells began to colonize the aortic floor (Fig. 2C). Two different aspects of colonization were observed. In the first, QH1$^+$ cells lining the aorta, the limits of which being initially lateral, extended to the ventral side. In the second, QH1$^+$ angioblasts were detected immediately underneath, or in the vicinity of, intra-aortic clusters in the ventral mesenchyme (Fig. 2C, inset). In several cases, quail angioblasts appeared wedged at the base of the clusters (Fig. 2C, inset). Additionally, a few QH1$^+$ cells were found integrated between the clusters, all of host origin.

Three to five days after the operation (E4.5, 5.5, 6.5; $n$=7, 8 and 2 respectively), intra-aortic hematopoiesis has ceased and the embryo has substantially developed. The contribution of somite ECs has dramatically increased. QH1$^+$ vascular plexuses were found around and inside the neural tube and nerve ganglia, and in the dermis, wing, body wall mesenchyme and kidney. Double staining of an E6.5 grafted embryo with the EC-specific MEP21 (chick) (McNagny et al., 1997) and QH1 (quail) mAbs showed that most, if not all, vessels on the grafted side were quail. QH1 was mostly restricted to the grafted side (Fig. 3A). On the dorsal aspect of the grafted embryo, however, some QH1$^+$ cells, either organized into vascular structures or scattered as single cells, had crossed the midline. These structures remained within 250 μm of the midline (not shown). In addition to the roof and sides, the aortic floor was now found to be colonized by QH1$^+$ cells (Fig. 3A, inset). Contrary to the distribution observed for the roof, QH1$^+$ cells in the floor significantly colonized the contralateral side (not shown). However, no QH1$^+$ cells were detected in visceral organs of splanchnopleural origin. Angioblasts participating to this second remodeling are probably recruited among the numerous migrating angioblasts present in the body wall. They may also originate from the limb bud vessels by centripetal migration as previously demonstrated (Christ et al., 1990).

To evaluate the actual contribution of the somitic compartment, we performed six bilateral grafts of segmental plate. Three samples were sacrificed at E4 (two days after the graft) and three at E5. Embryos were sectioned and double stained for QCPN and QH1 expression. At the graft level, vascular networks of the neural tube, dermis and body wall were of quail origin (Fig. 3B). Aortic ECs were entirely from quail, confirming the results obtained with unilateral grafts (Fig. 3B).

## Somites contribute to the formation of vascular smooth muscle cells of the aorta

QCPN staining revealed the presence of numerous QH1$^-$/QCPN$^+$ cells around the aorta. The first QH1$^-$/QCPN$^+$ cells reached the aorta at the time sclerotomal cells began to colonize the notochord region. By E5, the vessel was completely wrapped by a QCPN$^+$ tunica media (Fig. 3C). These QCPN$^+$ cells express the αSMA protein demonstrating the smooth muscle nature of the cells (Fig. 3C, arrows). Cells contributing vascular smooth muscle, however, displayed at least two major differences compared with QH1 angioblasts. First, angioblasts are highly motile and dissociated from their original tissue, whereas QCPN$^+$/QH1$^-$ cells contributing to the vascular tunica media are tightly associated with the movement of other somitic cells in the body wall. Second, contrary to angioblasts that display QH1 expression early during development, smooth muscle cell progenitors never expressed αSMA, considered as one of the earliest marker for VSM cells (Hungerford and Little, 1999), during their migration. αSMA expression was switched on when cells reached the vicinity of the aorta. Identity and contribution of the two cell lineages were clearly dissociated during aorta

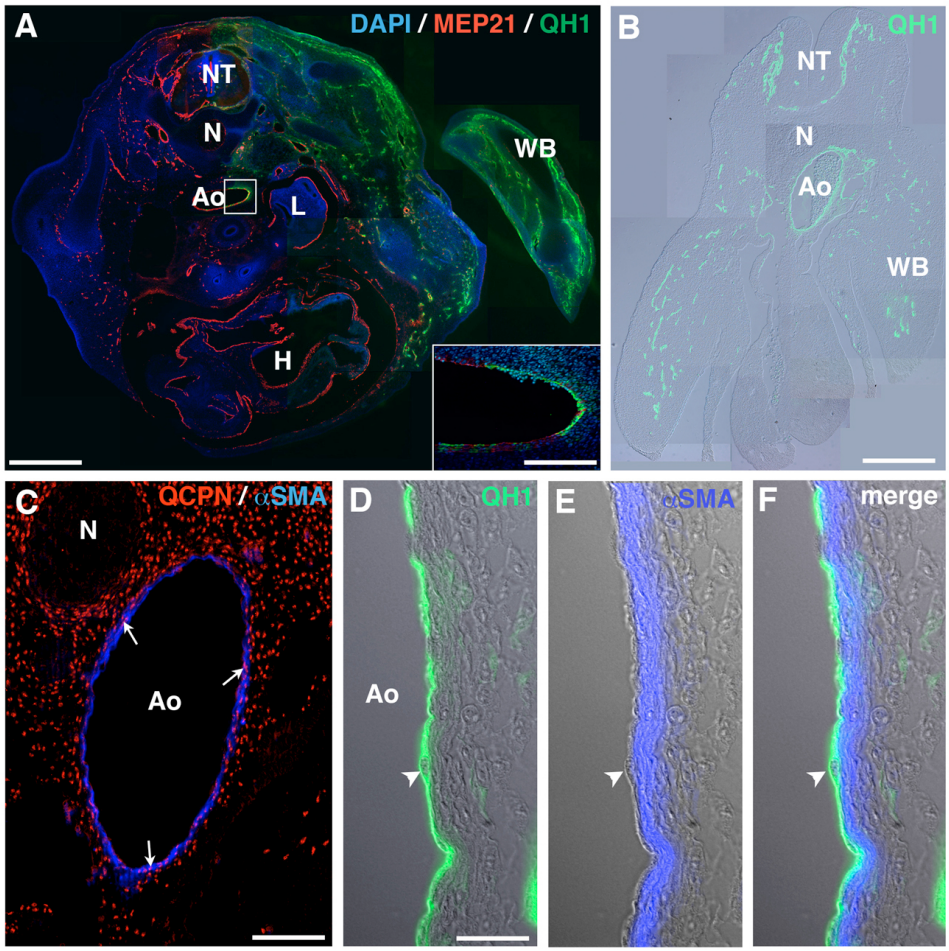


**Fig. 3. Contribution of the somite to the vascularization of the host and to formation of the smooth muscle tunicae.** (**A**) E6.5 chick host, 5 days after the graft. Cross-section at the trunk level. Triple labeling with QH1 (green)/MEP21 (red)/DAPI (blue). The vascular networks of the neural tube, dorsal root ganglia, dermis, mesonephros, body wall and limb bud derive entirely from the graft. Hematopoiesis has ceased at that stage. The MEP21 immunoreactivity with the coelomic epithelium around the lungs and the heart is a staining artifact. Scale bar: 200 μm. Inset: closer view of the right side of the aorta. The aortic endothelium on the grafted side mostly derives from the graft. Scale bar: 50 μm. (**B**) E5 chick host, 3.5 days after a bilateral graft of segmental plates. QH1 immunofluorescence overlaid with Nomarski's interferential contrast. Wing bud level. The quail perineural vasculature is conspicuous. ECs of the aorta are entirely quail. Quail ECs are not found in internal organs. Scale bar: 150 μm. (**C**) Section immediately adjacent to B. Double QCPN (red)/αSMA (blue) immunofluorescence. QCPN shows numerous quail cells that surround the notochord and the aorta. Quail cells closest to the aortic lumen express αSMA (arrows). Many QCPN$^+$/αSMA$^-$ cells organize around the αSMA$^+$ layer. Scale bar: 70 μm. (**D-F**) Detail of the ventrolateral side of an E4.5 chick aorta, 3 days after a unilateral graft. QH1 (D) and αSMA (E) immunohistochemistry merged to Nomarski's interferential contrast. (F) Overlay of QH1 and αSMA signals merged to the Nomarski's picture. The endothelial (green) and smooth muscle (blue) expression domains are clearly separated. The arrowhead indicates the same endothelial cell. Scale bar: 30 μm. H, heart; L, lungs; WB, wing bud.

morphogenesis as analyzed with segmental plates grafts. QH1 staining remained restricted to the luminal, i.e. endothelial, layer, whereas αSMA was expressed by abluminal cells and never overlap with QH1$^+$ cells (Fig. 3D-F).

## Clonal analysis of EC progeny within the vascular wall

Although somite contribution to the formation of aortic VSM cells was apparent, the existence of other sources of VSM cells could not be ruled out. One potential source is the ECs that were shown to generate VSM cells.

We have performed a clonal analysis of vascular EC derivatives with non-replicative retroviral vectors carrying either lacZ or PLAP reporter genes (Fig. 4A). Vectors were inoculated into the heart thus in close contact to ECs lining the vessels (Jaffredo et al., 2000). E4 inoculated embryos were sacrificed at E6 ($n$=5) and stained for *lacZ* and PLAP expression. Data were collected at the level of the aorta. Reporter gene detection was combined to anti MEP21 and αSMA immunohistochemistry (Fig. 4B). A clone refers to either a single cell or a small group of cells expressing one or the other reporter gene. None of the clones was found to be expressed in more than one cell layer, i.e. endothelium or smooth muscle layer. Most were distributed in vascular ECs. Out of 297 cells or cell groups counted, 233 (78.4%) were found in the endothelial layer (Fig. 4B and inset) and 64 (21.5%) in the smooth muscle cell layer. Among the latter, most cells displayed the characteristics of tissue macrophages (large cells with numerous filopodia) as detected with the combined use of 68-1 and 74-2 mAbs (not shown) that, respectively, recognize avian monocytes and mature macrophages (Jeurissen and Janse, 1998) (T.J., unpublished). Out of 812 'macrophage-like' clones, 740 (90.3%) were of the monocyte/macrophage lineage. However, 72 could not be identified as macrophages and, on the basis of their position and shape, were identified as VSM cells.

## Purified endothelial cells transdifferentiate into smooth muscle cells in culture

Vascular ECs were FACS purified on the basis of AcLDL-DiI uptake and exclusion of CD45 antigen expression (Fig. 4C). Purity was 99% at the time of plating. Cells were cultured in the endothelium-specific medium EGM2-MV supplemented or not with 50 ng/ml VEGF or 5 ng/ml TGFβ. Cultures were analyzed at different time points for MEP21 and αSMA expression. Twenty-four hours after VEGF addition, cells displayed a uniform, spindle-shape phenotype, expressed MEP21 but not αSMA (Fig. 4D, top). Similar antigenic characteristics were observed in TGFβ conditions, except that cells displayed a flat phenotype. Seventy-two hours after growth factor addition (Fig. 4D, bottom), cells cultured with VEGF retained the spindle-shape aspect but co-expressed MEP21 and αSMA antigens.

The flat cells in TGFβ conditions were enlarged and displayed a conspicuous αSMA expression. However, some cells retained signs of MEP21 expression. The αSMA phenotype was even more striking in the absence of growth factors; MEP21 expression was totally absent. Taken together, these results demonstrate that embryonic ECs retain the capacity to transdifferentiate into smooth muscle cells. This capacity is not, however, revealed in vivo, suggesting a tight environmental control.

## Formation of the aorta and molecular characterization of somite ECs

We have established the developmental patterns of the aorta from deposition of the mesoderm to the paired vessel stage. We have also studied the molecular characteristics of the endothelial somite compartment in quail and chick embryos ranging from 10 to 25 somite pairs. $QH1^+$ aggregates, probably angioblasts, were detected at the primitive streak level in a lateral position, in close contact with the embryonic endoderm (Fig. 5A). At the segmental plate level, $QH1^+$ vascular structures have formed but remained positioned lateral to the segmental plate. These structures were segregated from the overlying tissues and situated between the splanchnopleural mesoderm and the endoderm (Fig. 5B). At the level of the nascent somites, the paired aortae have formed and begin to position underneath the somites. A few $QH1^+$ cells were detected in a dorsal position between the ectoderm and the somatopleural mesoderm (Fig. 5C). This QH1 pattern was even more visible at a more anterior level when the somite has epithelialized (Fig. 5D). $QH1^+$ cells appeared to be associated with the Wolffian duct and, in some cases, in close contact with the roof of the aorta (Fig. 5E). Somites were analyzed for the expression of several markers known to recognize endothelial cells, i.e. GATA2, VEGFR2 and SCL/TAL1. As previously reported for VEGFR2 (Eichmann et al., 1993; Nimmagadda et al., 2004), the two last-formed somites never expressed EC-specific markers. Expression of GATA2, VEGFR2 and SCL/TAL1 first appeared in a few cells visible as a triangle-like area localized in

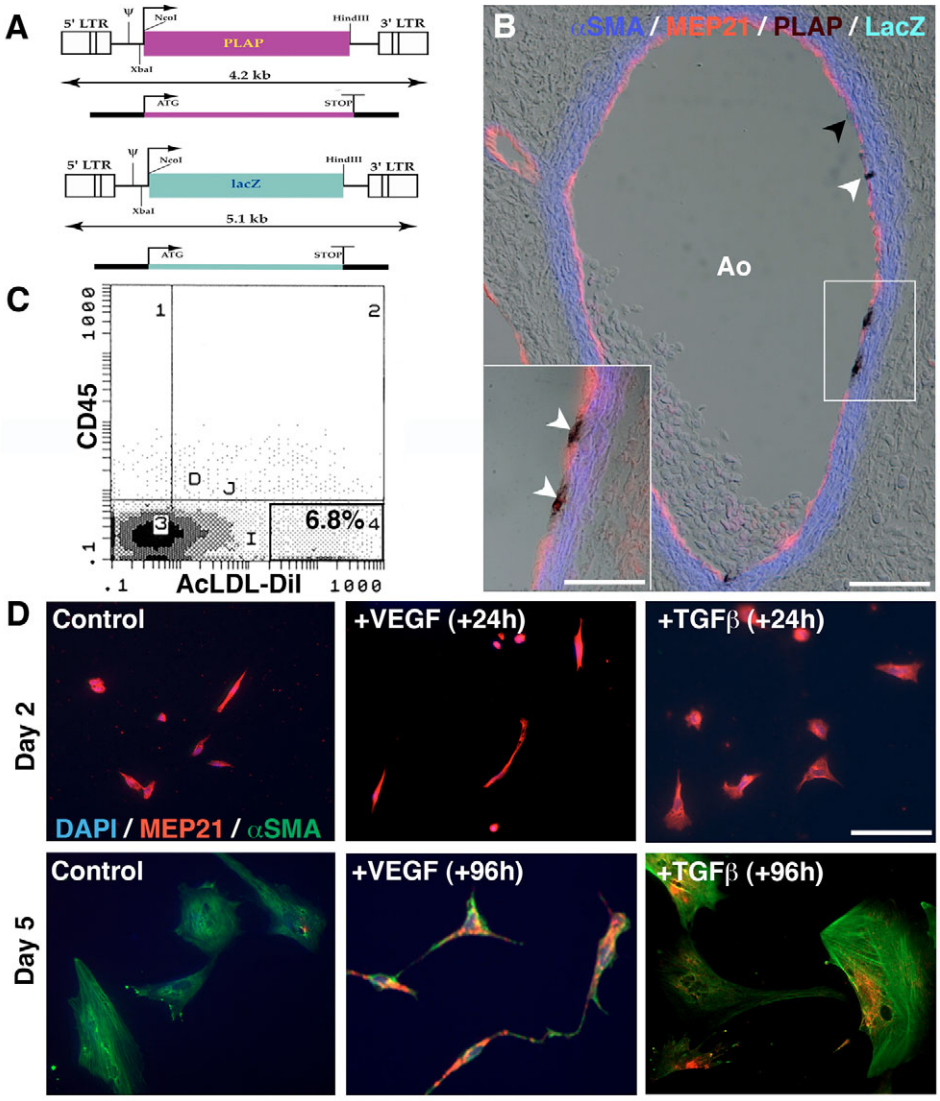


**Fig. 4. Clonal analysis of the vascular endothelial lineage and culture of purified endothelial cells.** (**A**) SNV-based, non-replicative retroviral vectors. 5′ and 3′ long terminal repeats (LTR) are shown as open boxes. An arrow indicates the direction of transcription from the promoter in the left-hand LTR. Bold lines between LTRs denote SNV sequences. Encapsidation sequences required for the packaging of virions are indicated as ψ. Provirus sizes and putative translations of the reporter genes are indicated below the viral structure. (**B**) Six-day-old embryo inoculated at E4 with a mix of lacZ- and PLAP-carrying vectors. Double histochemistry to reveal the reporter genes and double immunofluorescence with anti-MEP21 (orange) and anti-αSMA (blue) mAbs. Cross-section through the aorta. *lacZ* (black arrowhead) or PLAP (white arrowhead) infections are restricted to the endothelium. (Inset) Two infected cells expressing PLAP framed in B. Scale bars: 50 μm and 20 μm in inset. (**C**) FACS analysis after AcLDL-DiI and CD45 immunostaining. The frame points to the $AcLDL\text{-}DiI^+/CD45^-$ cell population, i.e. the endothelial cells, selected for culture. (**D**) Cultures of purified endothelial cells without growth factors (left column), supplemented with VEGF (middle column) or TGFβ (right column) at two different time points. Upper line, second day of culture; lower line, fifth day of culture. Triple staining with MEP21 (red)/αSMA (green)/DAPI (blue). Two days of culture: purified endothelial cells uniformly express MEP21, although cell sizes in the different culture conditions may vary. Five days of culture: MEP21 expression has decreased and αSMA becomes expressed. Disappearance of MEP21 is total in the control medium without growth factors. The VEGF supplementation produces numerous cells co-expressing MEP21 and αSMA. With TGFβ supplementation, most of the cells have lost MEP21 expression. Some cells, however, retain a MEP21 signal and thus express both markers. Scale bar: 25 μm.

the dorsolateral quadrant of the epithelialized somite (Fig. 5F,G,H). These markers appeared at the same time and follow a similar pattern, probably labeling the same cell population in both chick and quail species. QH1 expression was generally very weak at the epithelialized somite stage but a few QH1$^+$ cells could be revealed (Fig. 5I). At a slightly later stage, QH1 expression became conspicuous (Fig. 5E).

## DISCUSSION

We report here the sequence of events contributing to aorta construction as detected by segmental plate + last somite grafting from the quail into the chick. By replacing either one to three somites or the segmental plate equivalent, Pardanaud et al. (Pardanaud et al., 1996) demonstrated that the embryo is vascularized by two pools of endothelial precursors – one from the splanchnopleural mesoderm and another from the somites. Based on the homing territories of quail cells, the aorta was proposed to be a chimeric vessel, the roof and sides being constituted by somitic ECs and the floor by splanchnopleural mesoderm-derived ECs endowed with a hemangiopoietic potential. The reported contributions of the somite to the aortic roof were, however, generally weak. Here, using a renewed version of the same experimental approach, we provide a dynamic view of aorta construction. Our approaches differ in two crucial aspects: (1) the grafted material involved the whole segmental plate (~10 future somites) plus the last segmented somite; (2) the host embryos were examined at different time points.

### Contribution of the somite-derived ECs to the maturation of the aorta

#### First remodeling: new roof and sides

Replacement of the aortic roof is one of the earliest contributions of somite ECs to vascular development. It occurs when the aortae that are splanchnopleural in origin are still paired and when no somitic ECs have yet seeded the lateral plate (Fig. 6A,B). The seeding of intermediate and lateral plate mesoderm as well as formation of the cardinal vein by somitic ECs is in agreement with previous reports (Wilting et al., 1995; Pardanaud et al., 1996; Ambler et al., 2001). At E3, vessels surrounding the neural tube, intersomitic arteries, cardinal veins, kidney vasculature, body wall and limb bud vessels are quail derived at the level of the graft. The only exception is the aorta that displays a chimeric and complementary pattern: the roof and sides originated from the grafted somites, whereas the floor remains of host origin (Fig. 6C). Cooperation between the somite and the splanchnopleural mesoderm is thus required at this stage to construct the aorta. As only splanchnopeural-derived ECs have the capacity to generate hematopoietic cells, restriction of hematopoiesis to the aortic floor is obvious.

#### Second remodeling: the hemogenic endothelium disappears from the aortic floor and a new floor is contributed to by somite-derived ECs

A new finding is that the hemogenic endothelium lining the aortic floor disappears and is replaced by somite-derived ECs. Replacement is tightly associated with the production of intra-aortic clusters, always of host origin (Fig. 6D,E). When intra-aortic hematopoiesis has ceased, aortic ECs are totally of quail origin, and thus from the somite (Fig. 6F). Contrary to the first remodeling phase, ECs are now able to cross the ventral midline to settle in the contralateral side of the host. However, they never enter visceral organs, suggesting a tight spatiotemporal control of EC distribution in the embryo.

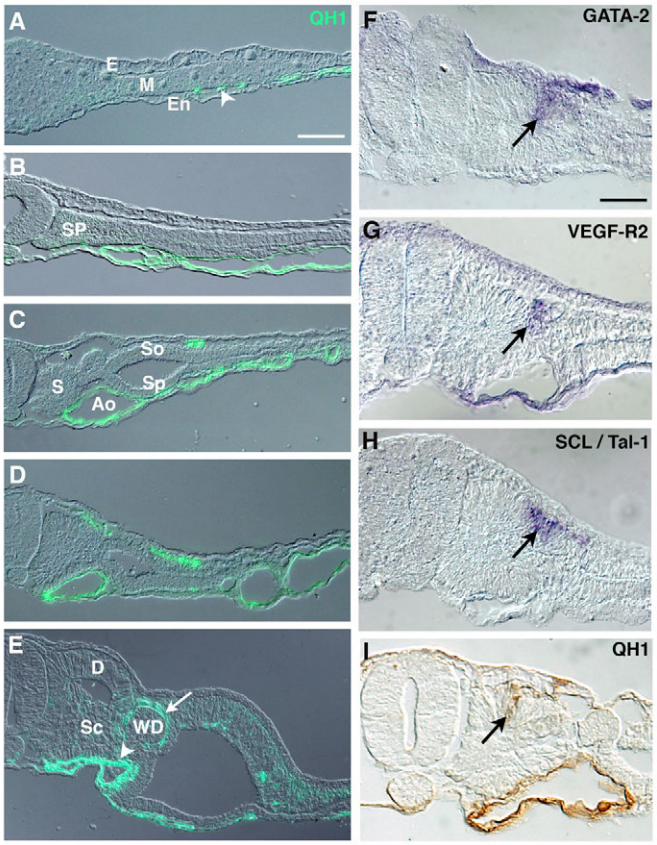


**Fig. 5. Formation of the aorta and molecular characterization of somite angioblasts.** (A-E) Cross-sections from caudal (A) to cephalic (E) levels in an 18-somite quail embryo. QH1 staining merged to Nomarski's interferential contrast. (**A**) Primitive streak level. QH1$^+$ angioblasts visible at a lateral position (arrowhead) aggregate against the endoderm and organize into groups of cells. (**B**) Segmental plate level. QH1$^+$ groups organize into vessels that progressively fuse. These structures remain positioned lateral to the segmental plate. (**C**) Nascent somite level. The paired aortae have formed. A few QH1$^+$ angioblasts are now detected in a dorsal position. (**D**) Epithelialized somite level. The paired aortae are now underneath the somites. Dorsal QH1$^+$ angioblasts become more numerous. (**E**) Dermomyotome and sclerotome have separated. Conspicuous QH1$^+$ cells are around the Wolffian duct (arrow) and in close association with the aortic roof (arrowhead). Scale bar: 100 μm. (**F-I**). EC-specific markers in chick (F-H) and quail (I) somites. (F-H) GATA2, VEGFR2 and SCL/TAL1 in situ hybridization. All markers delineate a quadrant of cells in the dorsolateral aspect of the epithelial somite (arrow). GATA2 is also present in the epidermis (Sheng and Stern, 1999; Minko et al., 2003). (I) QH1 immunohistochemistry. Scale bar: 50 μm. D, dermomyotome; E, ectoderm; En, endoderm; M, mesoderm; Sc, sclerotome; So, somatopleural mesoderm; Sp, splanchnopleural mesoderm; SP, segmental plate; WD, Wolffian duct.

In all vertebrates species described so far, intra-aortic hematopoietic production is transient. Our finding that the aortic floor is renewed during development explains the short-lived intra-aortic hematopoiesis. Hematopoietic production in this site thus depends on a limited, non-renewable, pool of hemogenic ECs. Here, floor replacement is total, meaning that all hemogenic ECs undergo the phenotypic change. As these cells also ensure endothelial functions, the need for a new vessel lining is obvious and is provided by somite-derived ECs. These latter are, however, restricted to

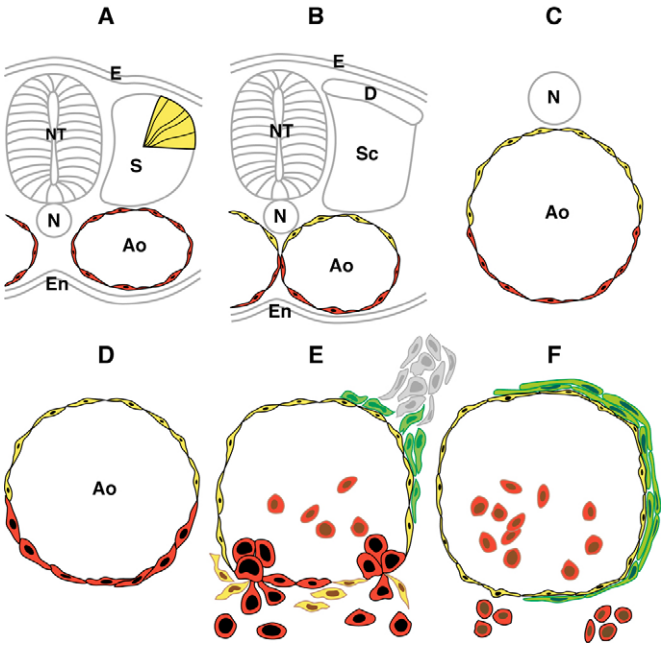


**Fig. 6. Developmental history of the aorta in relationship with endothelial remodeling and smooth muscle formation.** (**A**) Before fusion of the aortae, aortic ECs (red) derive from the splanchnopleura. The dorsolateral quadrant of the somite displays a population of ECs (yellow). (**B**) Immediately before fusion, the initial roof of splanchnopleural origin (red) has been replaced by ECs from the somite (yellow). (**C**) After fusion, the roof is of somite origin, the sides and floor remain of splanchnopleural origin. (**D**) During early hematopoiesis, the floor begins to loose endothelial markers and acquires of hematopoietic traits (Jaffredo et al., 1998). (**E**) At the hematopoietic clusters stage, clusters are budding into the lumen, whereas some HCs ingress into the mesentery. At the same time, somitic ECs replace the initial floor, whereas numerous somitic ECs are found in the floor either underneath the clusters. At this time, cells of somite origin reach the aorta (gray). When these cells reach the aorta, they begin to express αSMA (green). (**F**) Completion of hematopoiesis. The aortic floor has disappeared and is replaced by somitic ECs. Aortic ECs are entirely of somite origin. Somite cells in abluminal position now express αSMA (green).

angiopoiesis (Pardanaud et al., 1996) and therefore incapable of producing blood. The change in floor identity abolishes its capacity to contribute to hematopoiesis. The somite also gives rise to vascularization of the body wall. Comparison of QH1 and MEP21 patterns in grafts indicates that most, if not all, the vessels of the body wall and limbs are somite derived. The only exception is the viscera that were previously shown to contain their own ECs (Pardanaud and Dieterlen-Lièvre, 1995) and were not colonized in our experiments.

## Patterns of aorta formation and molecular characterization of somite-derived ECs

Angioblasts that gives rise to the aorta are first detected immediately after gastrulation at a slightly lateral position. As development proceeds, these forerunners form a vascular network that progressively moves to a more central position. These cells become segregated from the overlying splanchnopleural mesoderm, in close contact with the endoderm. The specific patterns of GATA2, SCL/TAL1 and chick VEGFR2 in the somite confirm and extend a previous study using QUEK1 (the quail VEGFR2 gene) as a probe (Eichmann et al., 1993). However, by grafting either dorsal, ventral or lateral somite quadrants, Wilting et al. showed that the angiogenic potential was not restricted to this dorsolateral region but was equally carried by each part of the somite. These data suggest that the somite cells have great plasticity and that the specific in situ emergence of angioblast is probably influenced by signaling molecules, the nature of which remains to be determined. As somites mature along the cephalo-caudal axis, GATA2, SCL/TAL1, VEGFR2 and QH1 become localized in a more lateral and ventral position. In some cases, angioblasts are in direct contact with the aortic roof and, laterally, surround the Wolffian duct. We interprete these dynamic patterns as resulting from the migration of the somite ECs to the lateral mesoderm.

## The somite: a source of smooth muscle cells

This origin had been proposed some years ago (Brand-Saberi and Christ, 2000) but has never been demonstrated experimentally. Our unpublished results show that, contrary to head and heart (Le Douarin et al., 2004), trunk neural crest cells do not contribute to VSM cells. Several origins for trunk VSM cells have been proposed. One privileged hypothesis is that EC-secreted factors locally recruit mesodermal cells surrounding the vessels (Drake et al., 1998; Hungerford and Little, 1999). The fact that QCPN$^+$ cells initiate αSMA expression in the vicinity of the aorta is in keeping with this hypothesis. It has also been proposed that ECs may generate smooth muscle cells (DeRuiter et al., 1997). This hypothesis has been revived to experiments with ES cells and lineage tracing. In vitro and in vivo approaches reported the generation of ECs and VSM cells from a single mesodermal precursor expressing VEGFR2 or TIE2 (Ema et al., 2003; Yamashita et al., 2000; Marchetti et al., 2002). However, VSM cell traits are also obtained in vitro during establishment of hematopoietic-supportive stromal cell lines (Charbord et al., 2002). Another hypothesis is the presence of pluripotent precursors, designated as mesoangioblasts (Minasi et al., 2002; Cossu and Bianco, 2003). Our tracing experiments show that retroviral expression is mainly restricted to ECs, identified with MEP21 expression, and is rarely found in the tunica media. Thus, ECs do not make a major contribution to VSM cell when integrated into a vascular structure. However, derivation of VSM cells from ECs at earlier stages cannot be ruled out. When ECs were labeled with wheat germ agglutinin (WGA) coupled to gold particles, labeled VSM cells were found. WGA is not, however, a specific marker for ECs but binds N-acetylglucosamine oligomers at the surface of the cells. Leakage to subendothelial layers is thus possible. When the aortic endothelium was traced earlier, at E2 or E3, with either AcLDL-DI or a non-replicative retroviral vector, derivatives of the tagged cells were restricted to the hematopoietic lineage and were never found in other cell lineages (Jaffredo et al., 1998; Jaffredo et al., 2000). In keeping with this, genetic analyses suggest that EC and VSM cell differentiation is uncoupled as mice lacking PDGFB or PDGFRβ have functional endothelial cells but lack pericytes (Lindahl et al., 1997; Enge et al., 2002). A model based on our results, of the different steps from the paired aorta to the end of hematopoiesis, is presented in (Fig. 6). In contrast to these in vivo approaches, we now provide evidence that ECs purified from the embryo change their phenotype and acquire SMA expression. This lineage switch is striking in the absence of growth factor or in the presence of TGBβ. In the presence of VEGF, ECs retain endothelial traits and, at the same time, express SMA. Taken together, these result point out the major differences between in vivo and vitro approaches and at the same time shed a new light on some yet unexplained results obtained after in vitro culture.

## The somite: a source of pluripotent cells?

Although our data demonstrate that the somite contributes to ECs and VSM cells of the aorta, the allocation of these cells to a specific somite compartment remains elusive. A clonal retroviral approach (Kardon et al., 2002) showed that striated muscle cell precursors of the myotome and ECs in the limb share a common progenitor in the dermomyotome of the somite. More recently, the existence of dermomyotomal progenitors endowed with dermal and muscle potential was demonstrated (Ben-Yair and Kalcheim, 2005), leading to the suggestion that the dermomyotome harbored an homogeneous population of self-renewing progenitors able to give rise to different lineages. Do VSM cells originate from the same stem cell compartment or do they differentiate from an alternative source of cells? Our experimental approach with transplantation of the entire somite, do not allow us to identify which compartment contributes to VSM cells. However, our immunohistological analyses show that VSM cell precursors colonize the aorta from the medioventral aspect of the somite, in close association with the sclerotomal compartment. Whether VSM cell precursors actually originate from the sclerotome or from the dermomyotome and cross the sclerotome is currently under investigation. In addition, molecular markers of VSM cell precursors are required. αSMA is expressed in differentiated smooth muscle and furthermore its expression is shared with the striated muscle lineage. The recent identification of myocardin and myocardin-like molecules as master regulators for smooth muscle cell differentiation may provide better identification of smooth muscle cell progenitors and their fates during embryogenesis. In addition, an eventual link with mesoangioblasts stem cells can be tested as smooth muscle differentiation of these cells depends on different regulatory genes.

We thank Margaret Buckingham, Giulio Cossu, Pierre Charbord, Françoise Dieterlen-Lièvre and Heather Etchevers for helpful comments and critical reading of the manuscript. J.-L. Duband is acknowledged for stimulating discussions. We thank Sophie Gournet for excellent drawing assistance and Marie-Claude Gendron for FACS analysis. This study is supported by the Centre National pour la Recherche Scientifique; by the University Pierre et Marie Curie-Paris VI; by grants from the Association pour la Recherche contre le Cancer 3312 (T.J.) and 4815 (M.-A.T.), and the Ligue Régionale contre le Cancer R04/75-158; and by an MERT ACI 22-2002-296. C.P. is a recipient of a French MERT fellowship and an ARC fellowship.

### References

**Ambler, C. A., Nowicki, J. L., Burke, A. C. and Bautch, V. L.** (2001). Assembly of trunk and limb blood vessels involves extensive migration and vasculogenesis of somite-derived angioblasts. *Dev. Biol.* **234**, 352-364.

**Ben-Yair, R. and Kalcheim, C.** (2005). Lineage analysis of the avian dermomyotome sheet reveals the existence of single cells with both dermal and muscle progenitor fates. *Development* **132**, 689-701.

**Bergwerff, M., Verberne, M. E., DeRuiter, M. C., Poelmann, R. E. and Gittenberger-de Groot, A. C.** (1998). Neural crest cell contribution to the developing circulatory system: implications for vascular morphology? *Circ. Res.* **82**, 221-231.

**Bollérot, K., Romero, S., Dunon, D. and Jaffredo, T.** (2005). Core binding factor in the early avian embryo: cloning of Cbfbeta and combinatorial expression patterns with Runx1. *Gene Expr. Patterns* **6**, 1-11.

**Bollérot, K., Sugiyama, D., Escriou, V., Gautier, R., Tozer, S., Scherman, D. and Jaffredo, T.** (2006). Widespread lipoplex-mediated gene transfer to vascular endothelial cells and hemangioblasts in the vertebrate embryo. *Dev. Dyn.* **235**, 105-114.

**Brand-Saberi, B. and Christ, B.** (2000). Evolution and development of distinct cell lineages derived from somites. *Curr. Top. Dev. Biol.* **48**, 1-42.

**Charbord, P., Oostendorp, R., Pang, W., Herault, O., Noel, F., Tsuji, T., Dzierzak, E. and Péault, B.** (2002). Comparative study of stromal cell lines derived from embryonic, fetal, and postnatal mouse blood-forming tissues. *Exp. Hematol.* **30**, 1202-1210.

**Christ, B., Poelmann, R. E., Mentink, M. M. and Gittenberger-de Groot, A. C.** (1990). Vascular endothelial cells migrate centripetally within embryonic arteries. *Anat. Embryol. (Berl.)* **181**, 333-339.

**Cossu, G. and Bianco, P.** (2003). Mesoangioblasts – vascular progenitors for extravascular mesodermal tissues. *Curr. Opin. Genet. Dev.* **13**, 537-542.

**de Bruijn, M., Ma, X., Robin, C., Ottersbach, K., Sanchez, M. J. and Dzierzak, E.** (2002). Hematopoietic stem cells localise to the endothelial cell layer in the midgestation mouse aorta. *Immunity* **16**, 673-683.

**DeRuiter, M. C., Poelmann, R. E., VanMunsteren, J. C., Mironov, V., Markwald, R. R. and Gittenberger-de Groot, A. C.** (1997). Embryonic endothelial cells transdifferentiate into mesenchymal cells expressing smooth muscle actins in vivo and in vitro. *Circ. Res.* **80**, 444-451.

**Drab, M., Haller, H., Bychkov, R., Erdmann, B., Lindschau, C., Haase, H., Morano, I., Luft, F. C. and Wobus, A. M.** (1997). From totipotent embryonic stem cells to spontaneously contracting smooth muscle cells: a retinoic acid and db-cAMP in vitro differentiation model. *FASEB J.* **11**, 905-915.

**Drake, C. J., Hungerford, J. E. and Little, C. D.** (1998). Morphogenesis of the first blood vessels. *Ann. New York Acad. Sci* **857**, 155-179.

**Eichmann, A., Marcelle, C., Breant, C. and Le Douarin, N. M.** (1993). Two molecules related to the VEGF receptor are expressed in early endothelial cells during avian embryonic development. *Mech. Dev.* **42**, 33-48.

**Ema, M., Faloon, P., Zhang, W. J., Hirashima, M., Reid, T., Stanford, W. L., Orkin, S., Choi, K. and Rossant, J.** (2003). Combinatorial effects of Flk1 and Tal1 on vascular and hematopoietic development in the mouse. *Genes Dev.* **17**, 380-393.

**Enge, M., Bjarnegard, M., Gerhardt, H., Gustafsson, E., Kalen, M., Asker, N., Hammes, H. P., Shani, M., Fassler, R. and Betsholtz, C.** (2002). Endothelium-specific platelet-derived growth factor-B ablation mimics diabetic retinopathy. *EMBO J.* **21**, 4307-4316.

**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.

**Fraser, S. T., Ogawa, M., Yu, R. T., Nishikawa, S. and Yoder, M. C.** (2002). Definitive hematopoietic commitment within the embryonic vascular endothelial-cadherin(+) population. *Exp. Hematol.* **30**, 1070-1078.

**Gautier, R., Drocourt, D. and Jaffredo, T.** (1996). Generation of small fusion genes carrying phleomycin resistance and Drosophila alcohol dehydrogenase reporter properties: their application in retroviral vectors. *Exp. Cell Res.* **224**, 291-301.

**Gautier, R., Jiang, A., Rousseau, V., Dornburg, R. and Jaffredo, T.** (2000). Avian reticuloendotheliosis virus strain A and spleen necrosis virus do not infect human cells. *J. Virol.* **74**, 518-522.

**Hirai, H., Ogawa, M., Suzuki, N., Yamamoto, M., Breier, G., Mazda, O., Imanishi, J. and Nishikawa, S.** (2003). Hemogenic and nonhemogenic endothelium can be distinguished by the activity of fetal liver kinase (Flk)-1 promoter/enhancer during mouse embryogenesis. *Blood* **101**, 886-893.

**Hungerford, J. E. and Little, C. D.** (1999). Developmental biology of the vascular smooth muscle cell: building a multilayered vessel wall. *J. Vasc. Res.* **36**, 2-27.

**Jaffredo, T., Gautier, R., Eichmann, A. and Dieterlen-Lièvre, F.** (1998). Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. *Development* **125**, 4575-4583.

**Jaffredo, T., Gautier, R., Brajeul, V. and Dieterlen-Lièvre, F.** (2000). Tracing the progeny of the aortic hemangioblast in the avian embryo. *Dev. Biol.* **224**, 204-214.

**Jaffredo, T., Bollérot, K., Sugiyama, D., Gautier, R. and Drevon, C.** (2005a). Tracing the hemangioblast during embryogenesis: developmental relationships between endothelial and hematopoietic cells. *Int. J. Dev. Biol.* **49**, 269-277.

**Jaffredo, T., Nottingham, W., Liddiard, K., Bollérot, K., Pouget, C. and de Bruijn, M.** (2005b). From hemangioblast to hematopoietic stem cell: an endothelial connection? *Exp. Hematol.* **33**, 1029-1040.

**Jeurissen, S. H. and Janse, E. M.** (1998). The use of chicken-specific antibodies in veterinary research involving three other avian species. *Vet. Q.* **20**, 140-143.

**Jiang, X., Rowitch, D. H., Soriano, P., McMahon, A. P. and Sucov, H. M.** (2000). Fate of the mammalian cardiac neural crest. *Development* **127**, 1607-1616.

**Jordan, H. E.** (1917). Aortic cell clusters in vertebrate embryos. *Proc. Natl. Acad. Sci. USA* **3**, 149-156.

**Kardon, G., Campbell, J. K. and Tabin, C. J.** (2002). Local extrinsic signals determine muscle and endothelial cell fate and patterning in the vertebrate limb. *Dev. Cell* **3**, 533-545.

**Klessinger, S. and Christ, B.** (1996). Axial structures control laterality in the distribution pattern of endothelial cells. *Anat. Embryol. (Berl.)* **193**, 319-330.

**Le Douarin, N. M., Creuzet, S., Couly, G. and Dupin, E.** (2004). Neural crest cell plasticity and its limits. *Development* **131**, 4637-4650.

**Le Lièvre, C. S. and Le Douarin, N. M.** (1975). Mesenchymal derivatives of the neural crest: analysis of chimaeric quail and chick embryos. *J. Embryol. Exp. Morphol.* **34**, 125-154.

**Lindahl, P., Johansson, B. R., Leveen, P. and Betsholtz, C.** (1997). Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science* **277**, 242-245.

**Marchetti, S., Gimond, C., Iljin, K., Bourcier, C., Alitalo, K., Pouyssegur, J. and Pages, G.** (2002). Endothelial cells genetically selected from differentiating mouse embryonic stem cells incorporate at sites of neovascularization in vivo. *J. Cell Sci.* **115**, 2075-2085.

**McNagny, K. M., Pettersson, I., Rossi, F., Flamme, I., Shevchenko, A., Mann,**

**M. and Graf, T.** (1997). Thrombomucin, a novel cell surface protein that defines thrombocytes and multipotent hematopoietic progenitors. *J. Cell Biol.* **138**, 1395-1407.
**Mikawa, T. and Gourdie, R. G.** (1996). Pericardial mesoderm generates a population of coronary smooth muscle cells migrating into the heart along with ingrowth of the epicardial organ. *Dev. Biol.* **174**, 221-232.
**Minasi, M. G., Riminucci, M., De Angelis, L., Borello, U., Berarducci, B., Innocenzi, A., Caprioli, A., Sirabella, D., Baiocchi, M., De Maria, R. et al.** (2002). The meso-angioblast: a multipotent, self-renewing cell that originates from the dorsal aorta and differentiates into most mesodermal tissues. *Development* **129**, 2773-2783.
**Minko, K., Bollérot, K., Drevon, C., Hallais, M. F. and Jaffredo, T.** (2003). From mesoderm to blood islands: patterns of key molecules during yolk sac erythropoiesis. *Gene Expr. Patterns* **3**, 261-272.
**Moscovici, C., Moscovici, M. G., Jimenez, H., Lai, M. M., Hayman, M. J. and Vogt, P. K.** (1977). Continuous tissue culture cell lines derived from chemically induced tumors of Japanese quail. *Cell* **11**, 95-103.
**Murray, P. D. F.** (1932). *The Development "In Vitro" of Blood of the Early Chick Embryo*, pp. 497-521.Cambridge: Strangeways Research Laboratory.
**Nimmagadda, S., Loganathan, P. G., Wilting, J., Christ, B. and Huang, R.** (2004). Expression pattern of VEGFR-2 (Quek1) during quail development. *Anat. Embryol. (Berl.)* **208**, 219-224.
**Nishikawa, S. I., Nishikawa, S., Hirashima, M., Matsuyoshi, N. and Kodama, H.** (1998). Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. *Development* **125**, 1747-1757.
**North, T., Gu, T. L., Stacy, T., Wang, Q., Howard, L., Binder, M., Marin-Padilla, M. and Speck, N. A.** (1999). Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. *Development* **126**, 2563-2575.
**North, T. E., de Bruijn, M. F., Stacy, T., Talebian, L., Lind, E., Robin, C., Binder, M., Dzierzak, E. and Speck, N. A.** (2002). Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. *Immunity* **16**, 661-672.
**Oberlin, E., Tavian, M., Blazsek, I. and Péault, B.** (2002). Blood-forming potential of vascular endothelium in the human embryo. *Development* **129**, 4147-4157.
**Pardanaud, L. and Dieterlen-Lièvre, F.** (1995). Does the paraxial mesoderm of the avian embryo have hemangioblastic capacity? *Anat. Embryol. (Berl.)* **192**, 301-308.
**Pardanaud, L., Altmann, C., Kitos, P., Dieterlen-Lièvre, F. and Buck, C. A.** (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.
**Perez-Pomares, J. M., Carmona, R., Gonzalez-Iriarte, M., Atencia, G., Wessels, A. and Munoz-Chapuli, R.** (2002). Origin of coronary endothelial cells from epicardial mesothelium in avian embryos. *Int. J. Dev. Biol.* **46**, 1005-1013.
**San Clemente, H., Dieterlen-Lièvre, F. and Jaffredo, T.** (1996). in vivo diversification and migration of chick embryo heart muscle cells: a morphometric analysis with ALV- and SNV-based non-replicative vectors. *Dev. Genes Evol.* **206**, 169-179.
**Sheng, G. and Stern, C. D.** (1999). Gata2 and Gata3: novel markers for early embryonic polarity and for non-neural ectoderm in the chick embryo. *Mech. Dev.* **87**, 213-216.
**Sugiyama, D., Ogawa, M., Hirose, I., Jaffredo, T., Arai, K. and Tsuji, K.** (2003). Erythropoiesis from acetyl LDL incorporating endothelial cells at the preliver stage. *Blood* **101**, 4733-4738.
**Tavian, M. and Péault, B.** (2005). Embryonic development of the human hematopoietic system. *Int. J. Dev. Biol.* **49**, 243-250.
**Vrancken Peeters, M. P., Gittenberger-de Groot, A. C., Mentink, M. M. and Poelmann, R. E.** (1999). Smooth muscle cells and fibroblasts of the coronary arteries derive from epithelial-mesenchymal transformation of the epicardium. *Anat. Embryol. (Berl.)* **199**, 367-378.
**Wilting, J., Brand-Saberi, B., Huang, R., Zhi, Q., Kontges, G., Ordahl, C. P. and Christ, B.** (1995). Angiogenic potential of the avian somite. *Dev. Dyn.* **202**, 165-171.
**Wilting, J., Eichmann, A. and Christ, B.** (1997). Expression of 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.
**Wilting, J., Papoutsi, M., Schneider, M. and Christ, B.** (2000). The lymphatic endothelium of the avian wing is of somitic origin. *Dev. Dyn.* **217**, 271-278.
**Yamashita, J., Itoh, H., Hirashima, M., Ogawa, M., Nishikawa, S., Yurugi, T., Naito, M. and Nakao, K.** (2000). Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature* **408**, 92-96.