Developmental origin of smooth muscle cells in the descending aorta in mice

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Aortic smooth muscle cells (SMCs) have been proposed to derive from lateral plate mesoderm. It has further been suggested that induction of SMC differentiation is confined to the ventral side of the aorta, and that SMCs later migrate to the dorsal side. In this study, we investigate the origin of SMCs in the descending aorta using recombination-based lineage tracing in mice. *Hoxb6-cre* transgenic mice were crossed with *Rosa 26* reporter mice to track cells of lateral plate mesoderm origin. The contribution of lateral plate mesoderm to SMCs in the descending aorta was determined at different stages of development. SMC differentiation was induced in lateral plate mesoderm-derived cells on the ventral side of the aorta at embryonic day (E) 9.0-9.5, as indicated by expression of the SMC-specific reporter gene *SM22α-lacZ*. There was, however, no migration of SMCs from the ventral to the dorsal side of the vessel. Moreover, the lateral plate mesoderm-derived cells in the ventral wall of the aorta were replaced by somite-derived cells at E10.5, as indicated by reporter gene expression in *Meox1-cre/Rosa 26* double transgenic mice. Examination of reporter gene expression in adult aortas from *Hoxb6-cre/Rosa 26* and *Meox1-cre/Rosa 26* double transgenic mice suggested that all SMCs in the adult descending aorta derive from the somites, whereas no contribution was recorded from lateral plate mesoderm.

KEY WORDS: Vascular smooth muscle cell, Aorta, Lateral plate mesoderm, Paraxial mesoderm, Cell origin

INTRODUCTION

A detailed understanding of the ontogeny of vascular smooth muscle cells (SMCs), and the morphological events that lead to their induction, is crucial for unraveling the molecular mechanisms that regulate their differentiation and for evaluating the importance of SMC origin as a susceptibility factor for vascular disease. The origin of vascular SMCs is complex with contributions from several independent cell lineages (Gittenberger-de Groot et al., 1999; Majesky, 2007). Neural crest cells (NCs) give rise to vascular SMCs in the cephalic region and in the cardiac outflow region, and are required for proper patterning of the great vessels (Hutson and Kirby, 2003; Jiang et al., 2000). The contribution of NCs extends to the fifth somite in chicken and to the ligamentum arteriosus in mice (Jiang et al., 2000; Le Lievre and Le Douarin, 1975). The secondary heart field gives rise to SMCs in the root of the aorta and pulmonary trunk (Waldo et al., 2005). The pro-epicardial organ contributes to SMCs in the coronary arteries (Ali et al., 2003; Gittenberger-de Groot et al., 1998; Mikawa and Gourdie, 1996), and ventrally emigrating neural tube cells contribute to SMCs in the great vessels and the coronary vessels in chicken (Ali et al., 2003). The mesothelium finally gives rise to SMC in mesenteric vessels in mice (Wilm et al., 2005).

In posterior parts of the body, vascular SMCs are assumed to derive primarily from splanchnic lateral plate mesoderm (Gittenberger-de Groot et al., 1999), but the precise source of these cells has not been rigorously determined. Observations of the early pattern of SMC

contractile protein expression in mouse and quail aorta suggest that vascular SMC differentiation is induced in mesenchymal cells that line the endothelium (Hungerford et al., 1996; Takahashi et al., 1996). The contractile proteins are first expressed on the ventral side of the vessel, and it has been hypothesized that SMCs are induced only in the ventral area of the aorta and later migrate to populate the lateral and dorsal areas (Hungerford et al., 1996). This model has gained support from studies of *Edg1* knockout mice, which selectively lack SMCs on the dorsal side of the aorta at embryonic day (E) 12.5 but develop SMCs on the ventral side as normal (Liu et al., 2000). Two recent reports challenge this view. Paraxial mesoderm was shown to contribute to SMCs in the descending aorta in mice and chicken (Esner et al., 2006; Pouget et al., 2006). However, the extent of this contribution is not clear.

In this investigation, *Hoxb6-cre* transgenic mice (Lowe et al., 2000) were crossed with *Rosa26* (*R26*) reporter mice (Soriano, 1999) to track cells of lateral plate mesoderm origin. *Meox1-cre/R26* double transgenic mice were used to track cells of paraxial mesoderm origin (Jukkola et al., 2005), and *SM22α-lacZ* mice were used to determine SMC differentiation by expression of the SMC marker *SM22α* (Zhang et al., 2001). We specifically focused on the contribution of lateral plate mesoderm to SMCs in the descending aorta.

MATERIALS AND METHODS

Animals

Hoxb6-cre mice (Lowe et al., 2000) were provided by Michael R. Keuhn (Division of Basic Sciences, National Cancer Institute, National Institutes of Health, Bethesda, MD). *R26* reporter mice (Soriano, 1999) were provided by Henrik Semb (Section of Endocrinology, Stem Cell Center, Lund University, Sweden). Animals were cross-bred to generate *Hoxb6-cre/R26* reporter mice. *SM22α-lacZ* mice (Zhang et al., 2001) were used to track the early presence of SMC. *Meox1-cre/R26* reporter mice (Jukkola et al., 2005) were used to track somite-derived cells. All transgenic lineages have a mixed genetic background. Animals were housed under barrier conditions in the transgenic core facility of University of Gothenburg, in accordance with the local ethical committee guidelines (Dnr. 213-2000, Dnr. 289-2003, Dnr. 230-2006). The morning of vaginal plug detection was counted as E0.5.

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X-gal staining

lacZ expression in E8.5-E10.5 embryos
E8.5-E10.5 embryos were dissected and immediately fixed at 4°C for 2 hours in 0.1 M phosphate buffer (pH 7.3) with 0.2% glutaraldehyde, 1.5% formaldehyde, 5 mM EGTA (pH 7.3) and 2 mM MgCl$_2$. The embryos were washed for 3×10 minutes in phosphate-buffered saline (PBS), whole-mount stained at 37°C overnight in 0.1 M phosphate buffer (pH 7.3) with 2 mM MgCl$_2$, 0.01% sodium deoxycholate, 0.02% IGEPAL CA-630 (Sigma #I-8896), 5 mM potassium hexacyanoferrate(III), 5 mM potassium hexacyanoferrate(II) and X-gal (Fluka #16665, 1 mg/ml staining solution), embedded in paraffin and sectioned at 5 μm.

lacZ expression in E11.5 embryos
E11.5 embryos were fixed at 4°C for 4 hours and washed as previously described, followed by overnight incubation in 30% sucrose in PBS. Specimens were mounted in Tissue-Tek OCT compound (Sakura Finetek, Torrence, CA) at –80°C and cryosectioned at 10 μm. The slides were stained at 37°C for 8 hours in the staining solution previously described, and then either mounted in Mowiol mounting medium with 2.5% (w/v) 1,4-diazabicyclo-[2.2.2]octane (DABCO, Sigma #D-2522) or immunohistochemically stained.

lacZ expression in postnatal animals
Postnatal day 2 (P2) mice were stained using different techniques: organs were dissected, fixed at 4°C for 4 hours in 0.1 M phosphate buffer (pH 7.3) with 0.2% glutaraldehyde, 1.5% formaldehyde, 5 mM EGTA (pH 7.3) and 2 mM MgCl$_2$, and whole mount stained as described for E8.5-E10.5 embryos. Slices of the kidney (1 mm) were fixed and stained en bloc as described for the embryos. Whole pups (unfixed) were mounted in Tissue-Tek OCT compound (Sakura Finetek, Torrence, CA) at –80°C and cryosectioned at 10 μm. The sections were fixed in 0.1 M phosphate buffer (pH 7.3) with 0.2% glutaraldehyde, 1.5% formaldehyde, 5 mM EGTA (pH 7.3) and 2 mM MgCl$_2$ at room temperature for 15 minutes and stained as described for E11.5 embryos.

lacZ expression in dissected adult aortas
Adult mice (n=2 *Hoxb6-cre/R26* double transgenic males, 3-4 months old; n=2 *Meox1-cre/R26* double transgenic females, 2 months old) were anesthetized and the aorta was washed with physiological NaCl solution. Aortas were dissected from fat and surrounding tissues. They were immediately fixed at 4°C for 4 hours, washed, whole-mount stained, paraffin embedded and sectioned, as described for the embryos.

Immunohistochemical staining

E11.5 embryos were triple labeled for Acta2, CD31 and *lacZ*-expressing cells. Embryos were dissected, fixed, mounted, sectioned and stained for *lacZ* activity as described above (*lacZ* expression in E11.5 embryos). Antigen retrieval was accomplished by trypsin incubation [0.025% in 0.05 M Tris-Cl (pH 7.8) at 37°C for 20 minutes (Trypsin EDTA, Gibco #25200)]. The following primary antibodies were used: mouse anti-human Acta2 clone 1A4 (DakoCytomation #M0851) and rat anti-mouse CD31 (PECAM-1) (BD Biosciences Pharmingen #553370). Secondary antibodies used were: rabbit anti-mouse (biotinylated) (DakoCytomation #E0354) and goat anti-rat-alexa$_{568}$ (Molecular Probes #A11077). All antibodies were diluted 1:200 in blocking buffer. Acta2 staining was completed by incubation in FITC-conjugated ExtrAvidin (Sigma #E2761) (1:200 in blocking buffer). Slides were mounted in ProLong Gold antifade reagent (Invitrogen #P36930).

Realtime RT-PCR

Embryos were harvested at indicated time-points and totRNA was extracted according to standard protocols. Meox1 mRNA was amplified with primers from Applied Biosystems (Assay-on-demand Mm00440285_m1) at cycling conditions recommended by the manufacturer. Ct values were normalized for amplification efficacy, and against GAPDH (Assay-on-demand Mm99999915_g1). Data were presented as relative expression compared with the E9.5 level. Error bars represent 95% confidence interval (n=3 per time-point).

Transmission electron microscopy

C57BL/6 mouse embryos at E9.5 were fixed overnight by immersion in 2.5% glutaraldehyde, 2% paraformaldehyde, 0.02% sodium azide in 0.05 M sodium cacodylate buffer (pH 7.2-7.4). After washing in 0.15 M sodium cacodylate buffer, specimens were fixed for 2 hours in 1% osmium tetroxide
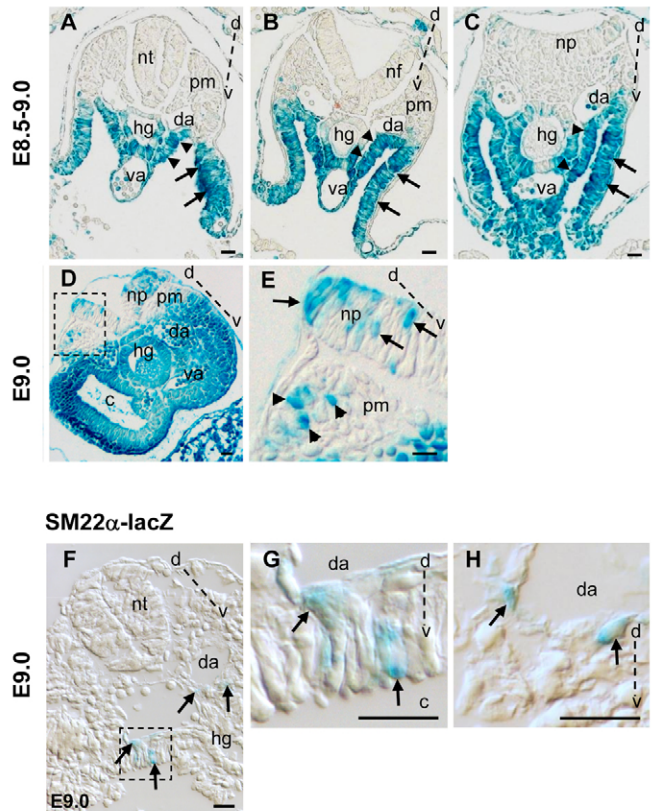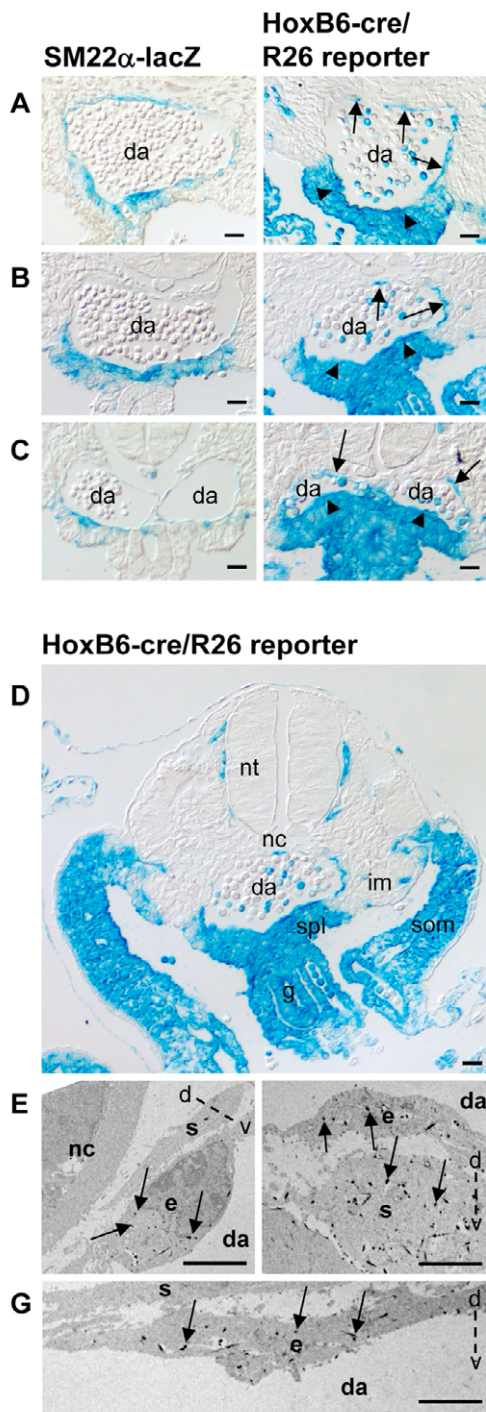

HoxB6-cre/R26 reporter

SM22α-lacZ

Fig. 1. *Hoxb6-cre* activates the *R26* reporter gene in lateral plate mesoderm cells before the first expression of the SMC marker SM22α. (**A-C**) *lacZ* staining for the *Hoxb6-cre*-activated *R26* reporter gene at three levels between the prospective forelimb and hindlimb regions at E8.5-E9.0 showed reporter expression in splanchnic (arrowheads) and somatic (arrows) lateral plate mesoderm. The reporter was also expressed in hindgut endoderm. (**D,E**) In posterior regions of the embryo, *Hoxb6-cre* had a broader expression domain and the reporter was also expressed in paraxial mesoderm cells (arrowheads in E; E shows boxed area in D) and cells in the neural plate (arrows in E). (**F-H**) The first expression of the *SM22α-lacZ* gene in the forelimb bud region was detected at E9.0-E9.5 in splanchnic mesoderm adjacent to the aorta (arrows in F and G), and in cells situated between the dorsal aortas (arrows in H). d-v, dorsal-ventral axis; nt, neural tube; nf, neural folds; np, neural plate; pm, paraxial mesoderm; da, dorsal aorta; hg, hindgut; va, vitelline artery; c, coelomic cavity. Scale bars: 25 μm.

with 1% potassium hexacyanoferrate(II) in 0.1 M cacodylate. The embryos were rinsed in water and contrasted en bloc in 0.5% uranyl acetate in water for 1 hour. They were dehydrated and infiltrated with epoxy resin (Agar100) according to standard protocols.

Hoxb6-cre/R26 mouse E9.5 embryos were prepared for electron microscopy following staining with X-gal, as described above. Transverse body sections were obtained with a Leica VT1000 tissue slicer (Leica Microsystems GmbH, Wetzlar, Germany) at 50 μm. Selected tissue slices were fixed for 2 hours in 1% osmium tetroxide and contrasted en bloc in 0.5% uranyl acetate in water for 1 hour. They were dehydrated in ethanol and infiltrated in plastic resin as described earlier (Masahira et al., 2005), followed by flat embedding in epoxy resin.

Light and electron microscopy sections were obtained with a Reichert Ultracut E ultramicrotome (Reichert AG, Vienna, Austria). Ultrathin sections were contrasted with uranyl acetate and lead citrate before examination in a Zeiss 912AB electron microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany).

SM22α-lacZ   HoxB6-cre/
              R26 reporter

HoxB6-cre/R26 reporter

Fig. 2. SMC differentiation is induced in lateral plate mesoderm-derived cells on the ventral side of the dorsal aorta at E9.5.
(**A-D**) *lacZ* staining of stage- and location-matched sections from E9.5 *Hoxb6-cre/R26* reporter and *SM22α-lacZ* transgenic mice at three levels between the forelimb and hindlimb regions. *SM22α-lacZ* was expressed around the full circumference of the aorta in the anterior sections (A), but was confined to the ventral side in more posterior sections (B,C). The *Hoxb6-cre*-activated *R26* reporter gene was expressed in splanchnic and somatic mesoderm, but not in intermediate mesoderm (D). The reporter gene was expressed in all cells on the ventral side of the aorta, including the endothelial cells (arrowheads in A-C). The reporter was also expressed in a single layer of cells on the lateral and dorsal side of the aorta (arrows in A-C). (**E-G**) TEM of ultrathin sections from *lacZ*-stained E9.5 *Hoxb6-cre/R26* reporter mice. On the dorsal side of the aorta (E,G), the *lacZ* staining, seen as an electron-dense cytoplasmic precipitate (arrows), was confined to endothelial cells. By contrast, the staining was found in endothelium and SMCs on the ventral side (F). The dorsal-ventral axis (indicated as d-v) runs from top to bottom if not otherwise indicated. nt, neural tube; nc, notochord; da, dorsal aorta; im, intermediate mesoderm; spl, splanchnic mesoderm; som, somatic mesoderm; g, gut; e, endothelium; s, SMC. Scale bars: 25 μm in A-D; 5 μm in E-G.

the embryo. In cross-sections, reporter expression was found in both somatic and splanchnic lateral plate mesoderm at all investigated levels (Fig. 1A-C). Ectopic expression was detected in gut epithelium and extra-embryonic mesoderm. At the posterior end of the embryo, the expression was more widespread with staining in paraxial mesoderm cells and in the neural plate (Fig. 1D,E).

As SM22α is one of the earliest SMC markers in mice (Zhang et al., 2001), *SM22α-lacZ* mice were stained for *SM22α*-driven *lacZ* expression to detect the earliest stages of SMC differentiation. *SM22α-lacZ* expression was detected at E8.5 adjacent to the dorsal aorta, but the staining was confined to the anterior part of the embryo. No staining was observed in the regions that stained positive for the *Hoxb6*-cre-activated R26 reporter at this stage. The first SM22α-lacZ-expressing cells in the descending aorta were detected at E9.0, in the splanchnic mesoderm facing the coelom and in cells situated between the initially paired dorsal aortas (Fig. 1F-H).

SMCs of lateral plate mesoderm origin are confined to the ventral wall of the descending aorta

Side-by-side comparison of staining patterns in stage-matched E9.5 *SM22α-lacZ* and *Hoxb6-cre/R26* reporter mouse embryos was performed to map the contribution of lateral plate mesoderm to the SMC population in the descending aorta. *SM22α-lacZ* was expressed in a layer of cells around the circumference of the dorsal aorta in the forelimb bud region and adjacent segments (Fig. 2A). In more posterior positions, the expression was confined to the ventral and lateral walls, and eventually only ventral walls, of the paired dorsal aortas (Fig. 2B and 2C). The *Hoxb6-cre/R26* reporter was expressed in splanchnic and somatic mesoderm but not in intermediate mesoderm (Fig. 2D). All cells on the ventral side of the aorta (or paired aortas in more posterior positions) expressed the reporter, including the *SM22α-lacZ*-expressing cells (Fig. 2A-C). The reporter was also expressed in cells in the lateral wall of the aorta and to a lesser extent in the dorsal wall (arrows in Fig. 2A-C).

RESULTS

Hoxb6-cre activates the *R26* reporter gene in lateral plate mesoderm cells before induction of vascular SMC differentiation

Cre-mediated excision of a loxP-flanked polyadenylation sequence in the *R26* reporter gene activates *lacZ* expression in cre-expressing cells and in the progeny of such cells (Soriano, 1999; Zambrowicz et al., 1997). We investigated the expression pattern of the *Hoxb6-cre*-activated *R26* reporter gene in detail in mouse embryos at E8.5-E9.0. The reporter had an anterior demarcation at the 12th somite (7th cervical somite), and was expressed throughout the length of

Ultra-thin sections were prepared from *lacZ*-stained *Hoxb6-cre/R26* reporter mice at E9.5 to resolve the identity of the lateral and dorsal cells with transmission electron microscopy (TEM). The *lacZ* staining was confined to endothelial cells facing the luminal side of the aorta (Fig. 2E-G). No staining was seen in peri-endothelial cells in the dorsal or lateral wall of the aorta. In the ventral wall, the staining was found in both endothelial cells and in peri-endothelial cells.

TEM of E9.5 embryos showed signs of migration of dorsal mesenchymal cells towards the aortic wall

Alternate semithin and ultrathin sections from the forelimb region of the aorta from E9.5 embryos were examined to explore the morphological prerequisites for induction of SMCs in the dorsal part of the vessel. Scattered mesenchymal cells that were separated by large volumes of unstructured extracellular matrix occupied the zone between the notochord and the aorta (Fig. 3A). The cells exhibited a limited cytoplasmic volume and had a stellate or polygonal shape on transection in the electron microscope. Cells located three cell diameters or less from the aorta occasionally showed signs of migratory activity with an extended shape and an ordered organization of the organelle contents (Fig. 3B,C). The general direction of this cell shape pattern was towards the aorta. Profiles of delicate cytoplasmic extensions bounded by a cell membrane were frequently observed in the matrix, which indicates that the cells form numerous slender processes.

Lateral plate mesoderm-derived SMCs are replaced at E11.5

The expression pattern of the *R26* reporter gene was examined in *Hoxb6-cre/R26* reporter mouse embryos at E11.5 to follow the fate of lateral plate mesoderm-derived cells in the descending aorta. A single layer of *lacZ*-expressing cells was found in the vessel wall, whereas the surrounding tissues were unstained. The lateral plate mesoderm cells that occupied the ventral side of the aorta at E9.5 had been replaced by other cells. The aorta was surrounded by large numbers of *lacZ*-negative cells in the lung bud and liver regions (Fig. 4A), and by four or five layers of *lacZ*-negative cells in more posterior regions (Fig. 4B).

Triple staining for Acta2, the endothelial marker CD31, and the *R26* reporter gene showed consistent *lacZ* expression in endothelial cells (Fig. 4C). SMCs were in general not stained (Fig. 4C,D). A few cases of *lacZ*-expressing SMCs were, however, detected in a discrete region where lateral plate mesoderm-derived mesenchymal cells were still found adjacent to the aorta (Fig. 4E).

Lateral plate mesoderm cells do not contribute to the adult descending aorta

We examined the aortas from adult *Hoxb6-cre/R26* reporter mice for contribution of lateral plate mesoderm cells. Endothelial cells throughout the length of the descending aorta expressed the *R26* reporter gene, whereas SMCs in the anterior and middle part of the vessel were unstained (Fig. 5A-D). By contrast, SMCs in the posterior part of the descending aorta expressed the *R26* reporter (Fig. 5E,F). The transition from *lacZ*-negative to *lacZ*-expressing SMCs was gradual and occurred in a region anterior to the renal arteries. The results could indicate that lateral plate mesoderm contributes to the SMCs in the posterior part of the descending aorta, but the significance of this finding is not clear. *Hoxb6-cre* activates the *R26* reporter gene in some ectopic tissues in the
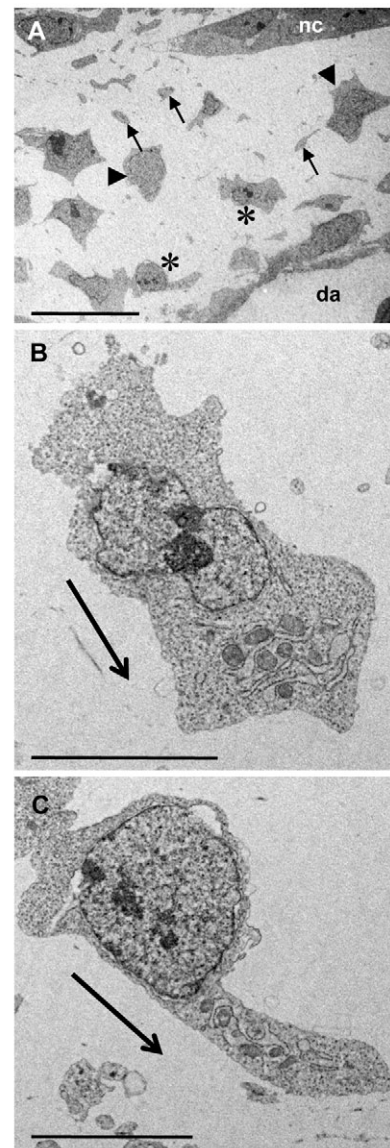

Fig. 3. Migration of dorsal mesenchymal cells towards the aortic wall. (**A**) Ultrastructure analysis of the space between the notochord and the aorta. Mesenchymal cells were widely spaced in a voluminous matrix. Cells that were located at greater distance from the vessel were polygonal (arrowheads), whereas cells closer to the aorta were more elongated (asterisks). Arrows indicate profiles of cytoplasmic extensions. (**B,C**) The asterisk-labeled cells in A displayed elongation in the direction of the arrows with cytoplasmic organelles distributed to the aorta-facing side of the nucleus. Scale bars: 20 μm in A; 5 μm in B,C.

posterior part of the embryo at E9.0-E9.5, including paraxial mesoderm (Fig. 1G,H), and the *lacZ*-expressing cells may derive from these tissues.

Somite-derived cells replace lateral plate mesoderm-derived SMCs in the ventral wall of the aorta at E10.5

Paraxial mesoderm contributes to SMCs in the aorta in mice and chicken (Esner et al., 2006; Pouget et al., 2006). *Meox1-cre/R26* double transgenic mice were investigated for somite contribution to the dorsal aorta in order to identify the cells that replace the lateral plate
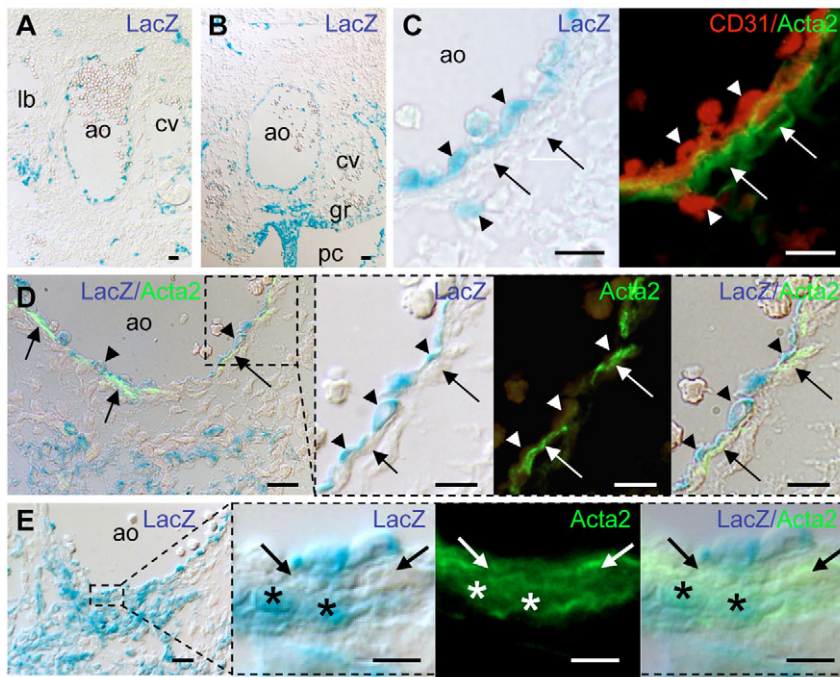
Fig. 4. Lateral plate mesoderm contributes to endothelial cells but not to SMCs in the descending aorta at E11.5. (**A**,**B**) A single layer of *lacZ*-expressing cells was detected in the aortic wall of *Hoxb6-cre/R26* reporter mice. The aorta was surrounded by large areas of *lacZ*-negative cells in the anterior part of the descending aorta, shown here in the lung bud region (A), and by four or five layers of *lacZ*-negative cells in posterior parts, shown here in the gonadal ridge region (B). (**C**) Triple staining against Acta2 (green), CD31 (red) and *lacZ* (blue) showed that endothelial cells, but not SMCs, expressed the *R26* reporter gene. (**D**,**E**) Similarly, double-staining against *lacZ* and Acta2 showed *lacZ* staining in endothelial cells but not in SMCs (high-power magnification of boxed area). (E) Cells that were double-labeled for *lacZ* and Acta2 were detected in a discrete region in which lateral plate mesoderm-derived cells were still found in proximity to the aorta (high-power magnification of boxed area). Arrows indicate *lacZ*-negative SMCs; arrowheads, *lacZ*-positive endothelial cells; asterisks, *lacZ*-positive SMCs; ao, aorta; lb, lung bud; cv, cardinal vein; gr, gonadal ridge; pc, peritoneal cavity. Scale bars: 25 μm in A-E; 10 μm in higher magnification views.

mesoderm-derived SMCs at E11.5. *Meox1* is expressed in pre-somitic paraxial mesoderm and in the somites (Candia et al., 1992), and *Meox1-cre* has been shown to activate the *R26* reporter gene in somites and somite-derived tissues from E8.5-E9.5 in mice (Jukkola et al., 2005).

At E9.5, the *Meox1-cre/R26* reporter gene was expressed in the dermamyotome and sclerotome (Fig. 6A-D). Reporter-expressing cells were found in, and adjacent to, the dorsal and dorsolateral walls of the aorta. Cells in the ventral and ventrolateral walls did not express the reporter.

At E10.5, the expression domain of the reporter had expanded towards the ventral side of the embryo and reporter-expressing cells had migrated along the ventrolateral wall of the aorta to circumvent the vessel (Fig. 6E-J). The *lacZ*-negative cells that were situated on the ventral side of the aorta at E9.5 had been replaced by three or four layers of *lacZ*-expressing cells in the *Meox1-cre/R26* reporter

mice (Fig. 6E-H). We conclude that the invasion of *Meox1-cre/R26*-expressing cells in the area ventral to the aorta at E10.5 correlates with the loss of lateral plate mesoderm-derived cells at E11.5. In the posterior part of the embryo, *Meox1-cre/R26*-expressing cells were still confined to the dorsal and dorsolateral wall of the aorta (Fig. 6I,J), which suggests that the dorsal-to-ventral migration of somite-derived cells is initiated in the anterior part of the embryo and progresses towards the posterior end.

Ectopic expression of the *Meox1-cre/R26* reporter in cardiac outflow tract and kidneys in postnatal mice

The expression pattern of the endogenous *Meox1* gene has not been systematically documented after E10.5. In order to investigate somite contribution to the postnatal and adult aorta, the temporal pattern of *Meox1* expression after somitogenesis and the spatial
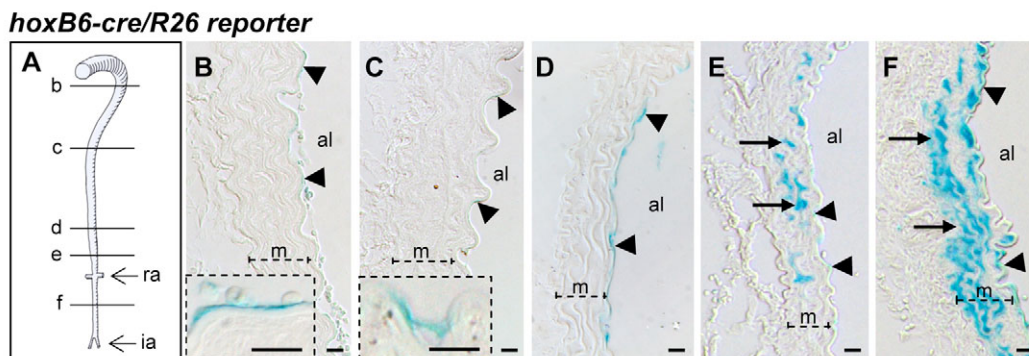

Fig. 5. Lateral plate mesoderm does not contribute to SMCs in the anterior and middle part of the descending aorta in adult mice. (**A-F**) Adult descending aorta from *Hoxb6-cre/R26* reporter mice was stained for *lacZ* activity at different anterior-posterior levels (levels are indicated in A). *lacZ* staining was restricted to endothelial cells in the anterior and middle part of the vessel (B-D). In posterior parts of the vessel, expression was frequently found in SMCs and endothelial cells (E,F). Boxed areas in B,C show *lacZ*-expressing endothelial cells at higher magnification to confirm successful staining. Arrows, SMC; arrowheads, endothelial cells; al, aortic lumen; ra, renal artery; ia, iliac artery; m, media. Scale bars: 25 μm.
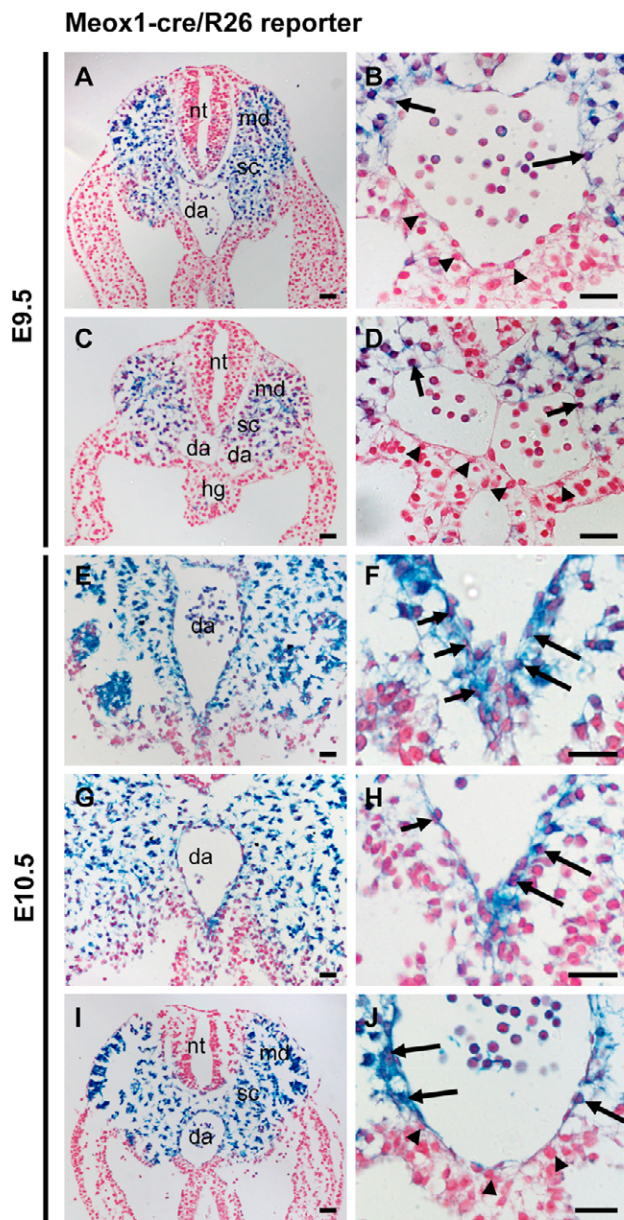
Meox1-cre/R26 reporter

Fig. 6. Somite-derived cells replace lateral-plate mesoderm-derived SMCs at E10.5. (**A-D**) *lacZ* staining of E9.5 *Meox1-cre/R26* reporter mice at two anterior-posterior levels between the forelimb and hindlimb regions. The reporter gene was expressed in myodermatome and in sclerotome in cells surrounding the dorsal part of the aorta. *lacZ*-expressing cells were found in close proximity to the aortic lumen on the dorsal and lateral sides of the vessel but not on the ventral or ventrolateral sides. (**E-J**) *lacZ* staining of E10.5 *Meox1-cre/R26* reporter mice at three anterior-posterior levels between the forelimb and hindlimb regions. The reporter gene was homogenously expressed in dorsal mesoderm, but *lacZ*-expressing cells had also migrated along the ventrolateral walls of the aorta and replaced the *lacZ*-negative cells that were found in the ventral wall of the aorta at E9.5 (E-H). *Meox1-cre/R26*-expressing cells were still confined to the dorsal and lateral walls of the aorta in the posterior part of the embryo (I,J), which indicates that the migration of somite-derived cells along the ventrolateral walls of the aorta is initiated in the anterior part of the animal and progresses towards the posterior end. Arrows, *Meox1-cre/R26*-expressing peri-endothelial cells; arrowheads, *lacZ*-negative peri-endothelial cells; nt, neural tube; md, myodermatome; sc, sclerotome; da, dorsal aorta; hg, hindgut. Scale bars: 50 μm.

pattern of *Meox1-cre/R26* reporter gene activation in postnatal mice were examined to monitor activation of the reporter in non-somite-derived cell lineages.

Quantitative RT-PCR on whole-embryo extracts showed that Meox1 transcription peaked during somite formation, but was sustained at measurable levels throughout embryogenesis (Fig. 7A). X-gal staining of neonatal *Meox1-cre/R26* reporter mice confirmed that expression was largely confined to somite-derived tissues such as dermis, skeletal muscle, cartilage, bone and endothelial cells in various organs (Fig. 7B-E). The gastrointestinal epithelium could not be investigated owing to unspecific staining.

Ectopic expression was recorded in the cardiac outflow tract, in which SMCs in the ascending aorta and aortic arch expressed the reporter (Fig. 7F-I). These structures are known to derive from cardiac neural crest cells. Staining was also seen in cells that form a branched network of fine caliber on the heart surface (Fig. 7H). Double staining against *lacZ* and Acta2 showed that this structure is separate from the coronary arteries (Fig. 7I), and we propose that it represents cardiac nerves that also have neural crest origin (Jiang et al., 2000). However, the staining pattern indicated expression in additional cells in the valve plane, including a small number of atrial myocardial cells, SMCs in the aortic root and SMCs in the pulmonary trunk (Fig. 7F,G). These cells probably originate from the secondary heart field (Waldo et al., 2005).

Expression was also detected in the cortex of the kidney (Fig. 7J,K). The staining encompassed mature and developing nephrons, undifferentiated metanephric mesenchyme, and intra-renal blood vessels, whereas collection ducts lacked expression. No expression was observed in the pelvic region of the kidney, or in the ureter (data not shown). The pattern of reporter expression indicates that the reporter was activated in a subset of intermediate mesoderm, i.e. the metanephrogenic blastema (Sariola and Sainio, 1998). Other intermediate mesoderm-derived structures such as adrenal cortex and gonads were not stained (Fig. 7L,M).

SMCs in the adult descending aorta originate from the somites

We finally examined the aortas from postnatal and adult *Meox1-cre/R26* double transgenic mice for contribution of somite-derived cells. The reporter gene was consistently expressed in adult aortic SMCs from the cardiac outflow tract to the iliac arteries (Fig. 8A-G). The homogenous staining of SMCs in the posterior part of the vessel suggests that the *lacZ*-expressing SMCs that were present in the posterior part of the aorta in *Hoxb6-cre/R26* reporter mice reflect ectopic expression of *Hoxb6-cre* in paraxial mesoderm rather than a contribution from lateral plate mesoderm.

Postnatal *Meox1-cre/R26* mice were investigated for somite contribution to SMCs in the major branches of the descending aorta. The reporter gene was not expressed in coeliac or superior mesenteric arteries, but it was expressed in renal arteries and intercostal arteries (Fig. 8H-J, and data not shown). Somite-derived SMCs occasionally extended a short distance into the coeliac and superior mesenteric vessels, but the seam between the lineages was in principle located to the branch points and SMCs of different origin were not mixed (Fig. 8K,L). SMCs in the renal arteries expressed the reporter from the branch point to the kidney (Fig. 8M). The distal boundary of somite contribution to the intercostal arteries was not determined.

DISCUSSION

Using *Hoxb6-cre/R26* reporter double-transgenic mice to track cells of lateral plate mesoderm origin, we showed that *Hoxb6-cre* activated the *R26* reporter gene in all lateral plate mesoderm cells before the

Fig. 7. Ectopic expression of the *Meox1-cre/R26* reporter gene in cardiac outflow tract and renal cortex. (**A**) Real-time RT-PCR of *Meox1* mRNA expression at E9.5-E18.5 showed that *Meox1* expression peaked during somitogenesis at E9.5-E10.5 and thereafter declined rapidly. (**B-E**) X-gal staining of cryosections (B-D) and whole-mount preparations of organs (E) from P2 mice confirmed reporter gene expression in somite-derived tissues, including dermis, vertebrae, skeletal muscle and microvessels. Arrows in B indicate dermal papilla and arrowheads indicate epidermal follicular epithelium. Thin arrow in C indicates chondrocytes, thick arrow indicates ossification centre in ventral part of vertebrae, arrowhead indicates an intervertebral disc that derive from the notochord. Arrows in D indicate the external oblique muscle surrounded by white adipose tissue. Arrowheads in E indicate blood vessels that spread across the surface of the unlabelled uterus. (**F-I**) Ectopic expression was detected in the arterial pole of the heart: whole-mount staining showed expression in ascending aorta (white arrow in F) and aortic arch, but also in patches of atrial myocardium (black arrow in F). Paraffin sections of whole-mount stained hearts confirmed expression in SMCs in proximal aorta (ao) and pulmonary trunk (pt) (G). Expression was also detected in a fine caliber branched network (arrowheads in H) on the heart surface that seemed to be distinct from blood vessels (arrows in H). Double staining against *lacZ* and Acta2 on paraffin sections confirmed that the network on the heart surface (arrowheads in I) were distinct from the coronary arteries (arrows in I). (**J-M**) Ectopic expression in kidney: en bloc X-gal staining of kidney slices revealed reporter expression in the cortex (white asterisk in J) but not in the pelvic region (black asterisk). Detailed inspection of the cortex in paraffin sections revealed that collection ducts (arrows in K) lacked expression, whereas other components were homogenously stained (asterisk indicates s-shaped nephron anlagen). Other derivates of intermediate mesoderm such as adrenal cortex (asterisk in L) and gonads (white arrow in M) lacked staining. Arrows in L indicate reporter-expressing cells in the adrenal capsule, arrowheads in M indicate blood vessels; ed, epidermis; de, dermis.
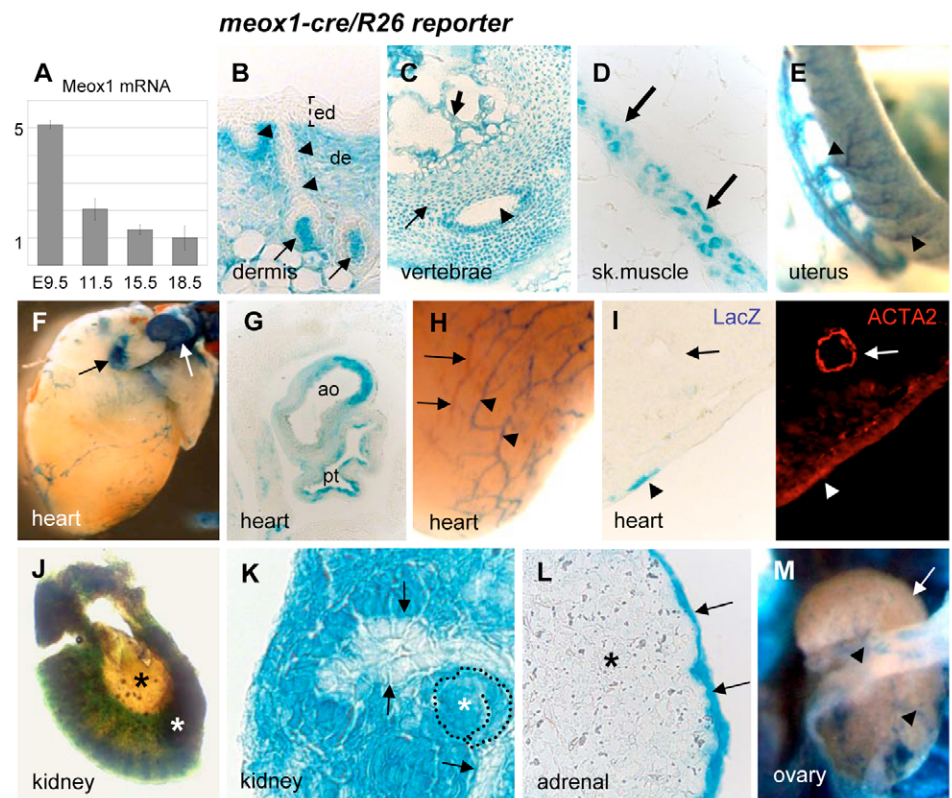
emergence of the first aortic SMCs in posterior regions of the embryo. SMC differentiation was initiated in lateral plate mesoderm cells on the ventral side of the dorsal aortas at E9.5, as indicated by expression of the SMC surrogate marker *SM22α-lacZ*. These cells were, however, replaced by somite-derived cells at E10.5, as indicated by reporter gene expression in *Meox1-cre/R26* double-transgenic mice. Examination of reporter gene expression in aortas from adult *Hoxb6-cre/R26* and *Meox1-cre/R26* transgenic mice revealed that all SMCs derive from the somites and not from lateral plate mesoderm.

Lateral plate mesoderm has generally been regarded as a major source of aortic SMCs, but experimental evidence to support this view is lacking (Gittenberger-de Groot et al., 1999). It has been suggested that SMCs in the descending aorta derive from lateral plate mesoderm on the basis of the early expression pattern of SMC markers (Hungerford et al., 1996; Takahashi et al., 1996). SMC induction has further been proposed to be confined to the ventral side of the vessel, after which the cells migrate to the dorsal side (Hungerford et al., 1996). Our results confirmed that SMC differentiation was induced in lateral plate mesoderm-derived cells on the ventral side of the aorta at E9.0-E9.5. There was, however, no migration of SMCs from the ventral to the dorsal side of the vessel at this time point. Moreover, the lateral plate mesoderm-derived *SM22α-lacZ*-expressing cells in the ventral wall of the aorta were replaced by somite-derived cells at

E11.5. We could not confirm any contribution of lateral plate mesoderm to SMCs in the descending aorta in adult mice, and we conclude that lateral plate mesoderm is not a source of mature SMCs in the descending aorta.

The fate of the early lateral plate mesoderm-derived SMC was not investigated. They could die or migrate away. Endothelial cells retained expression of the *Hoxb6-cre/R26* reporter gene in adult animals, which argues against a concomitant shift from lateral-plate mesoderm-derived to somite-derived cells in the endothelial population. However, the adult endothelial cells were not investigated in sufficient detail to exclude minor changes.

Two recent papers show that paraxial mesoderm contributes to aortic SMCs. Segmental plate mesoderm (presomitic mesoderm) was grafted from quail to chicken, and the host aorta was investigated for quail-derived cells (Pouget et al., 2006). Quail cells were preferentially found in the endothelium, but they also differentiated to SMCs in the trunk region. Genetic recombination experiments in mice showed that skeletal muscle and aortic SMCs share a common progenitor cell (Esner et al., 2006). Moreover, cells from the somite were shown to migrate into the space between the aorta and the gut at E10.5, and contribute to the aortic SMC population on both the ventral and dorsal sides of the vessel. This is in agreement with our finding that somite-derived cells replaced the lateral plate mesoderm-derived *SM22α*-expressing cells in the ventral wall of the aorta at E10.5. The

Fig. 8. SMCs in the adult descending aorta originate from the somites.
(**A-F**) Descending aorta from adult *Meox1-cre/R26* reporter mice was stained for *lacZ* activity at different anterior-posterior levels (levels are indicated in A). SMCs stained positive for the reporter gene at all investigated levels, also in the posterior part of the aorta. (**G**) Double staining against *lacZ* and Acta2 confirmed reporter gene expression in SMCs. (**H-M**) SMCs in the major branches of the descending aorta were investigated in *Meox1-cre/R26* reporter mice at P2 to determine the distal borders of somite contribution. Whole-mount staining of internal organs with attached aorta showed lack of reporter expression in the coeliac artery (arrowheads in H) and superior mesenteric artery (arrows in H and I). The renal arteries were homogenously stained (arrows in J). The aorta was displaced to expose the branch points. X-gal staining of cryosections confirmed lack of somite contribution to the coeliac and superior mesenteric arteries (K,L). The transition from *lacZ*-positive to negative SMCs occurred at the branch points (thin arrows in K and L), but not in a strict manner. Asterisk in L indicates somite-derived SMCs that extended into the superior mesenteric artery (sm). Arrowheads in K and L indicate the walls of the coeliac artery and superior mesenteric artery, respectively. The renal artery was entirely occupied by somite-derived SMCs (M). Boxed area in M shows renal arterial wall at higher magnification. Arrows in M indicate *lacZ*-positive SMCs, arrowheads indicate *lacZ*-negative endothelial cells. al, aortic lumen; ao, aorta; ca, coeliac artery; dm, dorsal mesentery; ia, iliac artery; in, intestines; m, tunica media; ra, renal artery; sm, superior mesenteric artery; asterisks, endogenous X-gal activity. Scale bars: 25 µm in B-G; 50 µm in K-M.
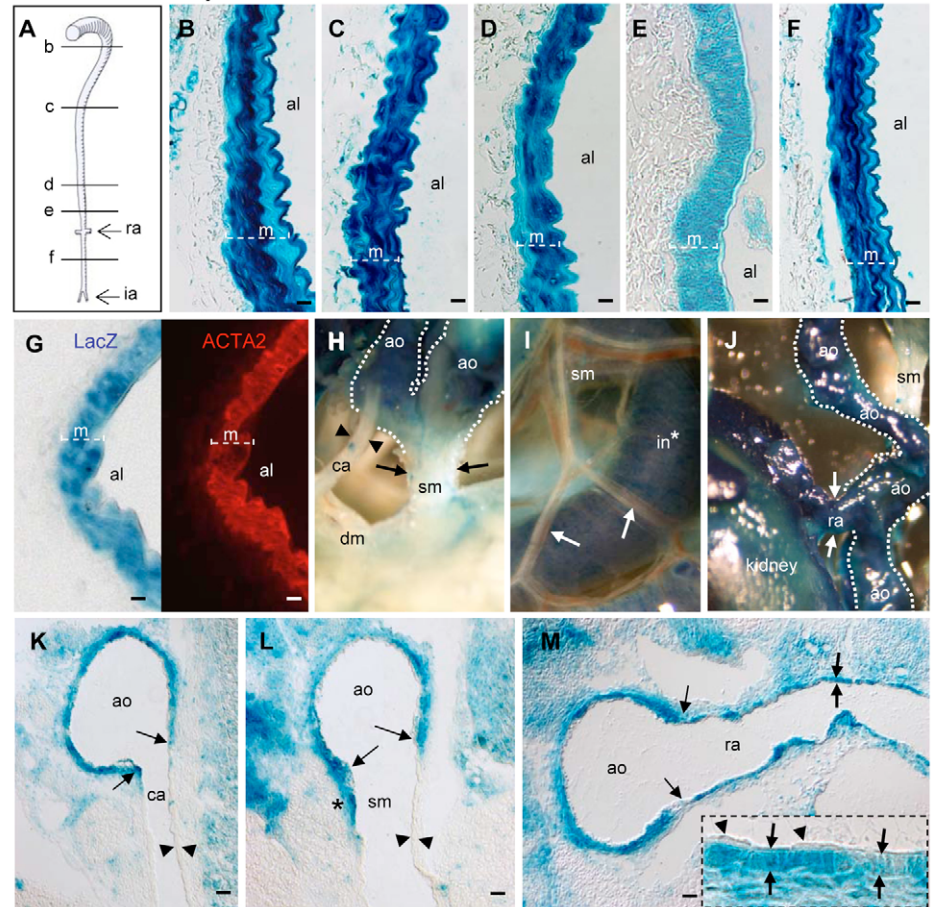
homogenous expression of the *Meox1-cre/R26* reporter gene in SMCs in the adult aorta suggests that somites are the sole contributors to SMCs in the adult aorta in mice.

Cre-recombinase is active in trace amounts, and low level expression in ectopic tissues is a potential source of misinterpretation of all lineage tracing experiments. The *Hoxb6-cre* expression is controlled by a 2.6 kb promoter fragment from the *Hoxb6* gene that contains the lateral plate mesoderm enhancer (Lowe et al., 2000). The expression is restricted to a subregion of the endogenous *Hoxb6* expression domain. In contrast to the *Hoxb6* gene, *Hoxb6-cre* is not expressed in intermediate mesoderm, thoracic paraxial mesoderm (sclerotome), lung and stomach mesenchyme, or CNS, apart from a segment in the midbrain hindbrain junction (Eid et al., 1993). As *Hoxb6-cre* is not a lineage marker for lateral plate mesoderm, it was crucial to establish the exact pattern of reporter gene activation. Our investigation showed that the *R26* reporter was activated in both somatic and splanchnic mesoderm with an anterior expression border at the 12th somite (7th cervical somite) at E8.5-E9.0. The expression was homogenous and seemed to encompass all cells in these compartments. The *Hoxb6-cre/R26* reporter mouse model is therefore suitable for investigating the contribution of lateral plate mesoderm to the aorta posterior of the 12th somite. Ectopic expression was observed in gut epithelium and in extra-embryonic

tissues, but these structures are not likely to contribute to aortic SMCs. In the most posterior part of E9.0 embryos, we observed ectopic expression in paraxial mesoderm, which makes it difficult to determine the origin of *Hoxb6-cre/R26* reporter-expressing cells in the posterior part of the animal. The overlapping expression of *Hoxb6-cre/R26* and *Meox1-cre/R26* reporter genes in SMCs in the posterior part of the adult aorta is, however, consistent with a somite origin of these cells.

The *Meox1-cre* transgene was inserted in the first intron of the endogenous *Meox1* gene, and its expression is controlled by the endogenous *Meox1* promoter (Jukkola et al., 2005). Meox1 is a lineage marker for paraxial mesoderm, and expression of the *Meox1-cre/R26* reporter gene was confined to somites and somite-derived tissues at E11.5 (Jukkola et al., 2005). In postnatal mice, the reporter was also expressed in the ascending aorta and aortic arch that derive from cardiac neural crest (Jiang et al., 2000), and in the aortic root, pulmonary trunk and structures in the heart valve plane that probably derive from the secondary heart field (Waldo et al., 2005). Reporter gene activity in these sites can be traced back to expression of *Meox1* in branchial arch mesenchyme, cardiac cushions and truncus arteriosus at E11.5 (Candia et al., 1992). Ectopic expression in cardiac neural crest cells precludes attempts to determine the anterior border of somite-derived SMCs in the aorta in *Meox1-cre/R26* reporter mice. This border has, however, been
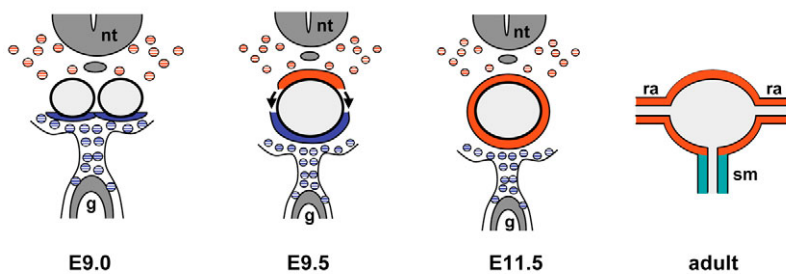
Fig. 9. A model for the dynamics of SMC-lineages during aortic development. Blue stripe indicates lateral plate mesoderm; red stripe, sclerotome; solid blue, lateral plate mesoderm-derived SMCs; solid red, somite-derived SMCs; solid green, serosal mesothelium-derived SMC; nt, neural tube; g, gut; ra, renal artery; sm, superior mesenteric artery.

mapped in detail in *Wnt1-cre/R26* transgenic mice that label neural crest cells, and occurs just distal to the ligamentum arteriosus (Jiang et al., 2000).

Ectopic expression was also detected in renal cortex but not in collecting ducts, renal medulla or ureter. The staining pattern suggests that Meox1 is expressed in the metanephrogenic blastema before or during kidney induction, but not in the nephric duct or derivates of the nephric duct (Sariola and Sainio, 1998). Other derivates of intermediate mesoderm did not express the reporter. The metanephric kidney develops in a posterior position in the embryo at level with the bifurcation of the common iliac arteries at E12, and subsequently ascends to a more anterior position in relation to other structures. It is therefore less likely that *lacZ*-positive SMCs in the descending aorta derive from the metanephrogenic blastema, but contribution from this lineage can not be formally ruled out.

SMCs in the mesenteric blood vessels were recently shown to derive from the mesothelium, but the proximal limit of this contribution was not defined (Wilm et al., 2005). The lack of *Meox1-cre/R26* reporter staining in the superior mesenteric and coeliac arteries suggests that the seam between somite- and mesothelium-derived SMCs is located at the root of the vessels. However, the transition from one lineage to the other is not strictly bound to the branch points, as fields of somite-derived cells sometime extended a short distance into the superior mesenteric and coeliac arteries. By contrast, SMCs in the renal arteries and intercostal arteries consistently expressed the *Meox1-cre/R26* reporter. The recruitment of somite-derived SMCs to the renal and intercostal arteries, but not to the mesenteric arteries, may relate to the developmental origin of the vessels. The renal and intercostal arteries develop by angiogenic sprouting from the aorta, whereas the mesenteric and coeliac vessels are formed by remodeling of the vitelline artery (Gest and Carron, 2003).

Our lineage-tracing experiments and earlier reports on somite-derived SMCs call for a new model concerning the origin of SMCs in the descending aorta (Fig. 9). Lateral-plate mesoderm cells on the ventral side of the aorta express SMC marker genes at E9.0-E9.5. Shortly thereafter, SMC induction occurs in somite-derived cells on the dorsal side of the aorta. At E10.5, somite-derived cells migrate along the lateral and ventral walls of the aorta into the zone between the aorta and the gut, and replace the lateral plate mesoderm-derived SMCs in the ventral wall of the aorta. The adult descending aorta is entirely populated by somite-derived SMC. The new view on the ontogeny of aortic SMCs has implications for vascular biology. The *Edg1* knockout phenotype and proposed function of Edg1 requires re-interpretation. Mice deficient for *Edg1* lack SMCs in the most dorsal aspect of the aorta, and the phenotype has been assigned to defective migration from the ventral wall (Liu et al., 2000). This explanation is not consistent with our findings as we failed to detect any movements of SMCs from the ventral to the dorsal part of the aorta during aortic wall development. An alternative explanation is

that dorsal SMCs, or dorsal SMC progenitor cells, are more sensitive to the loss of *Edg1* compared with ventral SMCs. The Edg1 example illustrates that detailed morphological information is necessary to work out the molecular mechanisms that mediate SMC differentiation.

In summary, we show that SMC differentiation is induced in lateral plate mesoderm-derived cells in the ventral wall of the descending aorta at E9.5 in mice. However, this population is replaced by somite-derived cells at E10.5, and there is no contribution of lateral plate mesoderm cells to SMCs in the adult descending aorta.

Note added in proof

After this work was submitted, a report was published that describes a similar population of transient primitive SMC in the avian aorta. Transplantation experiments using the quail-chick system showed that sclerotomal cells replaced primitive SMCs in the ventral wall of the vessel (Wiegreffe et al., 2007). This indicates that the formation of a transient SMC population is evolutionary conserved.

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