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# Smooth muscle of the dorsal aorta shares a common clonal origin with skeletal muscle of the myotome

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We show that cells of the dorsal aorta, an early blood vessel, and of the myotome, the first skeletal muscle to form within the somite, derive from a common progenitor in the mouse embryo. This conclusion is based on a retrospective clonal analysis, using a *nlaacZ* reporter targeted to the α-cardiac actin gene. A rare intragenic recombination event results in a functional *nlacZ* sequence, giving rise to clones of β-galactosidase-positive cells. Periendothelial and vascular smooth muscle cells of the dorsal aorta are the main cell types labelled, demonstrating that these are clonally related to the paraxial mesoderm-derived cells of skeletal muscle. Rare endothelial cells are also seen in some clones. In younger clones, arising from a recent recombination event, myotomal labelling is predominantly in the hypaxial somite, adjacent to labelled smooth muscle cells in the aorta. Analysis of *Pax3*$^{GFP/+}$ embryos shows that these cells are Pax3 negative but GFP positive, with fluorescent cells in the intervening region between the aorta and the somite. This is consistent with the direct migration of smooth muscle precursor cells that had expressed Pax3. These results are discussed in terms of the paraxial mesoderm contribution to the aorta and of the mesoangioblast stem cells that derive from it.

**KEY WORDS: Dorsal aorta, Skeletal muscle, Smooth muscle, Clonal analysis, LaacZ, Pax3**

## INTRODUCTION

The dorsal aorta in amniotes is an early embryonic blood vessel composed of endothelial and smooth muscle cells. It is also the source of two classes of mesodermal stem cell, hematopoietic stem cells (de Bruijn et al., 2000) and mesoangioblasts (De Angelis et al., 1999). The latter are stem cells that express some endothelial markers and give rise to a number of mesodermal derivatives, including skeletal muscle and smooth muscle (Minasi et al., 2002). This raises the possibility of a lineage relationship between these muscles and prompted us to investigate the origin of smooth muscle cells in the dorsal aorta.

At embryonic day (E) 8 in the mouse, the initial structure of the dorsal aorta is present as two tubes extending under the neural tube and notochord, along the anteroposterior axis of the embryo. Fusion of the tubes, in the central region of the trunk, takes place progressively towards the extremities, to give the single midline dorsal aorta. The nascent tube is formed from endothelial cells (Drake and Fleming, 2000). Mural cells, expressing smooth muscle markers, differentiate under the endothelial cell layer, which faces into the lumen. In the avian dorsal aorta, the first mural smooth muscle cells appear ventrally (Hungerford et al., 1996). Pericytes are a class of mural cell that characteristically lie within the basal lamina of the endothelium and constitute the smooth muscle component of capillaries, whereas thicker blood vessels, such as the dorsal aorta, accumulate additional outer layers of vascular smooth muscle cells (Gerhardt and Betsholtz, 2003).

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Smooth muscle is derived from mesoderm, except at certain sites, such as in the head and aortic arches, where it originates from the neural crest (Le Lievre and Le Douarin, 1975). It is thought that, during vascular development, smooth muscle cells are recruited from surrounding mesenchyme and induced to differentiate by factors produced by the endothelial cells of the vessel (Hirschi et al., 1998). However, endothelial and smooth muscle cells may derive from a common Flk1-expressing progenitor (Yamashita et al., 2000; Ema et al., 2003). Furthermore, labelling experiments have suggested that endothelial cells in the dorsal aorta can transdifferentiate into smooth muscle (DeRuiter et al., 1997). The mesoangioblast stem cells may correspond to an endothelial/pericyte intermediate cell type (Cossu and Bianco, 2003).

Endothelial cells in the dorsal aorta have been shown to derive from two distinct mesodermal sources. In both birds and mammals, it has been proposed that there may be a common progenitor cell, the hemangioblast, derived from splanchnic mesoderm, that gives rise to the endothelial cells of the vessel wall and to hematopoetic cells (Jaffredo et al., 1998; Nishikawa et al., 1998). Grafting experiments in avian embryos have shown that somites give rise to endothelial cells in the dorsal aorta; this paraxial mesoderm contribution was restricted to a dorsolateral location, whereas the ventral floor was colonized by cells derived from splanchnic mesoderm, at the stages examined (Pardanaud et al., 1996).

Somites form from paraxial mesoderm as segmented structures, following an anteroposterior developmental gradient, on either side of the neural tube, from about E8 in the mouse. Under the influence of signals from surrounding tissues, cells in the somite acquire different mesodermal identities (Tajbakhsh and Buckingham, 2000). Ventrally, mesenchymal cells of the sclerotome will give rise to the bone of the vertebral column and ribs. Dorsally the initial epithelial structure of the somite is retained as the dermomyotome, which gives rise to dorsal derm and to all the skeletal muscles of the body. The first skeletal muscle to form is the myotome. Muscle progenitor cells delaminate from the dermomyotome and migrate under this epithelium where they differentiate. Initially, this process takes place

from the epaxial dermomyotome, which is adjacent to the axial structures of the neural tube and notochord. Later, cells from the other, hypaxial, extremity of the dermomyotome also contribute to the myotome. Other muscle progenitor cells migrate out from the hypaxial dermomyotome to form, for example, the muscles of the limb. Dermomyotomal cells are characterized by the expression of Pax3, an important regulator of myogenesis. Endothelial cells are also associated with somites, from which they migrate into the body and limbs. Somite transplantation experiments in avian embryos have shown that angioblasts are derived from all somitic compartments (Noden, 1989; Wilting et al., 1995). More recently lineage tracking, using retrovirus vectors in the chick embryo, has shown that endothelial and skeletal muscle cells in the limb are derived from a common progenitor cell, labelled in the hypaxial dermomyotome (Kardon et al., 2002). It has been suggested that the mesenchymal cells of the avian sclerotome can be recruited to give rise to the smooth muscle of blood vessels formed in this region of the embryo (Christ et al., 2004).

In order to examine a possible lineage relationship between cells in the dorsal aorta and the skeletal muscle cells of the myotome in the mouse embryo, we adopted a genetic approach that permits retrospective clonal analysis (Bonnerot and Nicolas, 1993; Nicolas et al., 1996). This employs a *laacZ* reporter that contains a duplication of the *lacZ* coding sequence under the control of regulatory sequences directing expression to the tissues of interest. In the embryo, a rare intragenic recombination event will remove the duplication to give *lacZ*, which encodes a functional β-galactosidase (β-gal) protein when the gene is expressed. A common progenitor cell that has undergone such a recombination event will give rise to β-gal$^+$ cells that are clonally related. We have used mice in which we had targeted the α-cardiac actin gene with a *nlaacZ* reporter (Meilhac et al., 2003), in order to carry out such a clonal analysis in the myocardium. In addition to the heart, α-cardiac actin is also expressed in embryonic skeletal muscle and in the dorsal aorta (Sassoon et al., 1988).

The retrospective clonal analysis presented in this paper shows that cells in the dorsal aorta and in the myotome have a common clonal origin. The properties of the common progenitor cells are discussed. Based on the analysis of *Pax3$^{GFP/+}$* mice, we propose that GFP-labelled progenitor cells migrate from the somite to the dorsal aorta. We also document the spatiotemporal characteristics of clones in the dorsal aorta, in terms of cell type and position. Most of them are smooth muscle cells, but occasional labelled endothelial cells are present in the clones, in keeping with the existence of a common vascular progenitor.

## MATERIALS AND METHODS

### Mice

The *$\alpha_c$-actin$^{nlaacZ1.1/nlaacZ1.1}$* line, in which a *nlaacZ* sequence is targeted to the α-cardiac actin gene, used for clonal analysis, was as described by Meilhac et al. (Meilhac et al., 2003). The T4 transgenic line expresses *nlacZ* under the control of 9 kb of genomic sequence, upstream of α-cardiac actin (Biben et al., 1996). The *Pax3$^{GFP/+}$* mouse line was obtained by targeting a DNA sequence encoding GFP to an allele of *Pax3* (Relaix et al., 2005). *Pax3$^{GFP/+}$* mice carrying the T4 transgene were obtained by crossing males of the T4 line with *Pax3$^{GFP/+}$* mice. The *ROSA26R-lacZ* line was as described by Soriano (Soriano, 1999) and *Ht-PA-Cre* transgenic mice as by Pietri et al. (Pietri et al., 2003). Embryos in which the conditional *ROSA26* allele had been activated were obtained by crossing the two mouse lines.

### Production and description of clones

The *nlaacZ* sequence produces a truncated β-galactosidase (β-gal) protein, which is deprived of enzymatic activity. It will only give rise to a β-gal-positive cell if it undergoes an internal recombination event, which removes the duplication and the stop codons generated by it. This is a spontaneous event, which occurs during mitosis at a low frequency (Bonnerot and Nicolas, 1993). Descendants of a cell in which this has occurred will be detected provided that they express the α-cardiac actin gene. Clones that had been obtained by crossing heterozygous *$\alpha_c$-actin$^{nlaacZ1.1/+}$* males (Meilhac et al., 2003) were used to analyze labelling in the dorsal aorta at E10.5. Additional clones at E10.5 and E9.5 were produced by crossing superovulated wild-type females (C57/B6SJL) with homozygous *$\alpha_c$-actin$^{nlaacZ1.1/nlaacZ1.1}$* males, as described by Meilhac et al. (Meilhac et al., 2004b). At E10.5, a total of 729, and at E9.5, 449, *$\alpha_c$-actin$^{nlaacZ1.1/+}$* embryos were analyzed. Dissected embryos were fixed for 30 minutes in 4% paraformaldehyde (PFA) followed by staining in X-Gal solution at 37°C overnight [0.4 mg/ml X-Gal in 2 mM $MgCl_2$, 0.02% NP-40, 0.1 M PBS, 20 mM $K_4Fe(CN)_6$, 20 mM $K_3Fe(CN)_6$]. After X-Gal staining, the liver, gut and other tissues were removed to expose the dorsal aorta. Embryos were stored at 4°C in 4% PFA.

### Statistical establishment of clonality

The intragenic recombination that converts *nlaacZ* into *nlacZ* is a spontaneous, heritable and random event. The frequency of its occurrence can therefore be analyzed by the fluctuation test of Luria and Delbrück (Luria and Delbrück, 1943). This parameter has been estimated to be about $3.6\times10^{-6}$ per cell and per division in the myocardial lineage of the *$\alpha_c$-actin$^{nlaacZ1.1/+}$* line (Meilhac et al., 2004a). The fluctuation test of Luria and Delbrück also predicts that the number of independent recombinations follows a Poisson distribution. Based on the number of observations in the dorsal aorta (see Table 1), we can calculate that, at E10.5, a single case of two independent recombination events within the dorsal aorta is the maximum expected. No multiple independent recombination events are expected at E9.5. We can therefore conclude that β-gal$^+$ cells are clonally related.

The frequency of two independent events is equal to the product of the probability of each single event. Thus, the calculated number of observations of both independent events, A and B (Table 1), is $C=N_A*N_B/N_t$, where $N_A$ is the number of observations of event A, $N_B$ of event B, and $N_t$ the total number of embryos dissected at each stage.

Conformity of a frequency to a theoretical law was assessed by the classical $\chi^2$ test, calculating the parameter $\chi^2=(O-C^2)/C$, where O is the observed frequency and C the calculated frequency (1 degree of freedom).

In the case of the heart, which has a much larger number of α-cardiac actin-expressing cells at E10.5, the predicted frequency of double recombination events for this target tissue is much higher (Meilhac et al., 2003). We cannot therefore study clonality between the heart and dorsal aorta (23 embryos show staining in the heart and dorsal aorta, but with an expected number of 26 labelled embryos that correspond to a double recomination event) at this stage, as we can for the myotome and dorsal aorta, based on the statistical analysis.

### Histology and immunohistochemistry

Embryos were embedded in gelatin/sucrose and 10 μm thick sections were obtained using a cryostat. Before immunostaining, slides were washed three times in PBS and permeabilized with blocking solution (5% lamb serum, 1% BSA, 0.02% Tween). Incubation with the primary antibody was for 2 hours at room temperature, followed by washing with PBS, and a 1-hour incubation with the secondary antibody coupled to the fluorochrome.

To visualize nuclei, sections were incubated with Bisbenzimide H33342. Sections were observed and photographed using a Zeiss Axioplan microscope equipped with an Axiocam camera. Optical sections were performed using a Zeiss ApoTome system.

Antibodies used were as follows: anti-β-galactosidase, rabbit polyclonal, used at 1:200 dilution; anti-α-smooth muscle actin, mouse monoclonal, 1:400 (Sigma); anti-CD-31/PECAM, rat monoclonal, 1:200 (Pharmingen BD Bioscience); anti-laminin, rabbit polyclonal, 1:400 (Sigma); anti-GFP, rabbit polyclonal, 1:700 (Biovalley), anti-Pax3, mouse monoclonal, 1:50 (Developmental Studies Hybridoma Bank). Fluorochrome-conjugated goat secondary antibodies were used at 1:300 dilution (Alexa-Fluor 488 or 594 anti-mouse or anti-rabbit IgG, Molecular Probes).

### In situ hybridization

For in situ hybridization, embryos were fixed overnight in 4% PFA in PBS at 4°C. After fixation, embryos were washed in PBS, incubated in 15% sucrose and embedded into 7.5% gelatin. Sections (12 μm) were obtained using a cryostat. The in situ hybridization protocol, used with a digoxigenin-labelled riboprobe, was as described by Henrique et al. (Henrique et al., 1995), with modifications (http://www.hhmi.ucla.edu/derobertis/). In situ hybridization and immunodetection of GFP were performed on adjacent serial sections. Sections were observed and photographed using a Zeiss ApoTome system. The *Pax1* riboprobe was kindly provided by Dr Schughart (Institute of Mammalian Genetics, Neuherberg, Germany).

## RESULTS

### Expression of α-cardiac actin in the dorsal aorta

In order to visualize α-cardiac actin-expressing cells in the dorsal aorta, we used a transgenic mouse line, T4, that expresses *nlacZ* under the control of regulatory sequences of the α-cardiac actin gene (Biben et al., 1996). At embryonic day (E) 10.5, β-galactosidase-positive (β-gal$^+$) cells are observed in the heart, somites and dorsal aorta (Fig. 1A,B), where the endogenous gene is also expressed (Sassoon et al., 1988). In the somites, labelled cells are present in the skeletal muscle of the myotome (Fig. 1C,D). At this stage (40

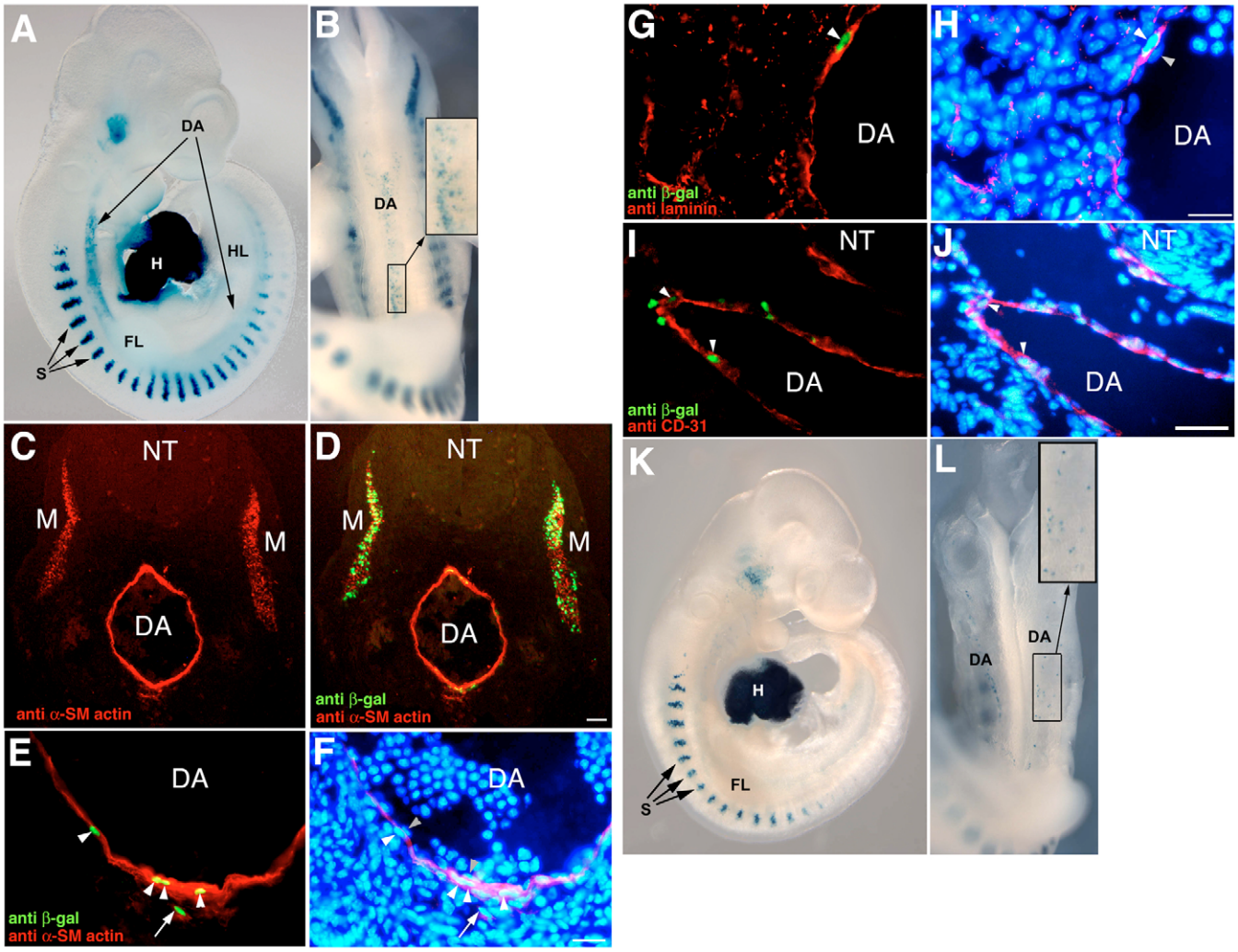



**Fig. 1. Expression of α-cardiac actin in cells of the dorsal aorta, visualized as β-gal activity with the T4 transgenic mouse line.** (**A**) Whole-mount X-gal staining of an E10.5 embryo, showing β-gal labelling (blue) in the heart (H), somites (S) and dorsal aorta (DA). FL, forelimb; HL, hindlimb. (**B**) A ventral view of A. (**C**) Immunohistological staining with an α-smooth muscle actin antibody (red) showing labelled cells in the dorsal aorta (DA) and myotomes (M), on either side of the neural tube (NT). (**D**) Co-immunohistology of the section shown in C, stained with antibodies to β-gal (green) and α-smooth muscle actin (red). (**E**) An enlargement of the dorsal aorta (DA), showing co-immunohistological staining with antibodies to α-smooth muscle actin (red) and β-gal (green). White arrowheads show α-smooth muscle actin-positive cells that have β-gal$^+$ nuclei and are in a periendothelial position. The white arrow points to a vascular smooth muscle cell that is also β-gal$^+$. (**F**) The same view with DAPI staining (blue) shows the nuclei of β-gal$^+$ smooth muscle cells (white arrowheads and white arrow) and adjacent endothelial cells (grey arrowheads). (**G**) Co-immunohistological staining with anti-laminin (red) and anti-β-gal (green) antibodies shows a cell (white arrowhead) with a β-gal$^+$ nucleus in the pericyte position within the basal lamina which underlies the endothelial cell layer of the dorsal aorta (DA) (**H**) The same section with DAPI staining shows the position of this cell (white arrowhead) immediately below an endothelial cell (grey arrowhead) which faces into the lumen of the dorsal aorta (DA). (**I**) Co-immunohistological staining with antibodies to the endothelial specific marker CD31/PECAM (red) and β-gal (green). CD31-positive endothelial cells that also express β-gal are marked by white arrowheads. Other cells with β-gal$^+$ nuclei (green) in the dorsal aorta (DA) are not endothelial. NT, neural tube. (**J**) The same view with DAPI staining showing the endothelial position of the nucleus adjacent to the lumen (white arrowheads) of the β-gal/CD31-positive cells. (**K**) Whole-mount X-gal staining of an E9.5 embryo, showing β-gal labelling in the heart (H) and somites (S). FL, forelimb bud. (**L**) A ventral view of K shows labelled cells in the dorsal aorta (DA). Scale bars: 20 μm.

**Table 1. Statistical analysis of clonality at E9.5 and E10.5 for β-gal⁺ cells in the dorsal aorta and myotome**

| | E9.5 | E10.5 |
|---|---|---|
| Total number *nlaacZ* embryos analyzed | 449 | 729 |
| β-gal+ cells in dorsal aorta (number of embryos) | 9 | 39 |
| β-gal+ cells in myotome (number of embryos) | 15 | 89 |
| β-gal+ cells in dorsal aorta and myotome | 6 | 32 |
| Expected frequency of double recombination (%) | 0.07 | 0.6 |
| $\chi^2$ | 108 | 145 |
| | *P*<0.001% | *P*<0.001% |

The total number of *α-cardiac actin*$^{nlaacZ/+}$ embryos analyzed is indicated together with the number of embryos with β-gal⁺ cells in the dorsal aorta and/or myotome. The expected percentage of embryos showing expression at both sites, as a result of two independent events of recombination, is shown, together with the $\chi^2$ test of statistical significance (see Materials and methods). The *P* value indicates the probability of two independent events. This figure is extremely low indicating that labelled cells originate from a common recombination event, and therefore are clonally related at both E9.5 and E10.5.

somites), the caudal limit of β-gal⁺ cells in the dorsal aorta is at the level of somite 30 (where somite 1 is the most anterior and therefore most mature), whereas labelled cells in the myotome are detectable up to about somite 34.

To characterize the α-cardiac actin-expressing cells of the dorsal aorta, we used double immunostaining with antibodies to β-gal, and to α-smooth muscle actin or CD31/PECAM, which mark smooth muscle or endothelial cells, respectively. In Fig. 1C,D, α-smooth muscle actin labelling of cells in the dorsal aorta and myotomes is shown. Not all α-smooth muscle actin-positive cells in the myotome are β-gal⁺ (Fig. 1D), which probably reflects the later onset of α-cardiac actin expression, as skeletal muscle cells differentiate. In the dorsal aorta, at E10.5, the β-gal⁺ cells represent a subpopulation (~10%) of the cells that express this smooth muscle marker. About 60% of cells are α-smooth muscle actin positive, of which about 45% are periendothelial cells in the pericyte position within the basal lamina of the endothelium (Fig. 1E-H) and 15% are vascular smooth muscle cells. A similar distribution of β-gal⁺ cells is seen between these two mural cell types. The remaining 40% of cells express the endothelial marker CD31. Very few (~0.2%) of these endothelial cells are β-gal⁺ (Fig. 1I,J). The cell composition and extent of β-gal labelling is similar in the anterior region where the dorsal aorta bifurcates, or in the central part of the trunk where it is a single tube.

At E9.5 (25 somite stage; Fig. 1K,L), β-gal⁺ cells are present in the anterior branches of the dorsal aorta and within the wall of the fused tube, extending caudally to the level of about somite 20. The dorsal aorta is smaller at this stage with fewer labelled cells, but the proportion of smooth muscle actin-positive cells that are β-gal⁺ remains at about 10%. Of the remaining 40% of endothelial cells, about 0.6% are β-gal⁺ at E9.5. Again the different cell types are similarly distributed on the anteroposterior axis. By E11.5, β-gal⁺ cells are no longer detectable in the dorsal aorta, indicating that the expression of α-cardiac actin is a transitory phenomenon in this structure.

## Clones of α-cardiac actin-expressing cells in the dorsal aorta also colonize the myotome

The introduction of a *nlaacZ* reporter into the α-cardiac actin gene made it possible to carry out a retrospective clonal analysis on cells that express this gene (Meilhac et al., 2003; Meilhac et al., 2004a). β-gal⁺ cells, in which a functional *nlacZ* sequence is present as a result of a rare intragenic recombination event, are observed in the myotomes and dorsal aorta of *α-cardiac actin*$^{nlaacZ/+}$ embryos. In order to examine a possible clonal relationship between labelled cells in these two structures, 729 embryos from the *α-cardiac actin*$^{nlaacZ/+}$ line were examined at E10.5, and 449 at E9.5. The numbers of embryos with β-gal⁺ cells in the myotome and dorsal aorta are shown in Table 1. The low frequency of embryos with labelled cells in the dorsal aorta, 5% at E10.5 and 2% at E9.5, indicates that these cells result from a rare recombination event and are clonally related (see Materials and methods). It is striking that many of these embryos also have β-gal⁺ cells in the myotome. The probability that labelled cells in the dorsal aorta and myotome arise from two independent recombination events in the same embryo is very low (Table 1, see statistical analysis). We therefore conclude that there are α-cardiac actin-expressing cells in the dorsal aorta that are clonally related to cells in the myotome.

## Classification of clones with β-gal⁺ cells in the dorsal aorta

Retrospective clonal analyses of cells in the myotome have already been described at E11.5 (Nicolas et al., 1996; Eloy-Trinquet and Nicolas, 2002a; Eloy-Trinquet and Nicolas, 2002b). We have used a similar classification to describe the characteristics of myotomal clones that also have labelled cells in the dorsal aorta. The main parameter taken into account, which reflects the age of the clone, was the extent to which β-gal⁺ cells are distributed along the anteroposterior axis, and whether they colonize somites on either side of the axis. An axial extension of seven somites was taken as the cut off point between long and short clones, based on previously observed clonal distributions at E11.5 (Eloy-Trinquet and Nicolas, 2002a). This extension probably reflects the number of somites prefigured in the presomitic mesoderm. The number of labelled cells in a clone is also an important indicator of age, as a more ancient recombination event will generate more cells that potentially express the *nlacZ* reporter when the α-cardiac actin gene is transcribed.

Four different categories of clones with labelled cells in the dorsal aorta were distinguished. (1) Long clones in which labelled cells are present in myotomes that extend over seven or more somites on the anteroposterior axis, with a mono- or bilateral distribution of β-gal⁺ cells. (2) Short clones, with a somite extension of less than seven, with a mono- or bilateral distribution of labelled cells. (3) Single somite clones in which β-gal⁺ cells are present in the myotome of a somite, on one side of the axis. (4) Clones of β-gal⁺ cells in the dorsal aorta, but not the myotomes. Examples of the different categories of clones are shown in Fig. 2 and the results are summarized in Fig. 3. At E10.5, all four categories of clones are observed.

Most long clones have a bilateral distribution. In this category, the progenitor cell in which the recombination event took place preceded the onset of segmentation and the introduction of bi-lateralization in the presomitic mesoderm. Recombination probably occurred in the self-renewing mesodermal population of the primitive streak. A clone such as E10.5-419, which has a very high number of labelled cells (as shown in Fig. 3A for the dorsal aorta), including extensive labelling in all compartments of the heart, probably reflects an early recombination event, probably predating the onset of gastrulation. Short clones are also necessarily derived from a *nlacZ* progenitor cell that arose before segmentation. The number of labelled cells in the dorsal aorta of these clones, like those of the long clones, tends to be higher when the labelled cells also have a longer extension on the axis of the aorta. Single somite clones, however, tend to have fewer labelled cells in the dorsal aorta, reflecting a more recent origin, probably after the onset of somitogenesis. In this case, and in clones that have no labelled cells in the somite, β-gal⁺ cells in the dorsal aorta are restricted to the level

of one or two somites. The average number of β-gal$^+$ cells per 'segment', at the level of each pair of somites, in the dorsal aorta (Fig. 3A, grey box) is remarkably constant in all categories of clones with labelled cells in the myotome. Both Student's *t*-test and the Snedecor test indicate that the distribution of values per segment in different classes of clones with expression in the somites is not significantly different (Fig. 3B). When the total number of myotomal clones was analysed, the same categories of clones with β-gal$^+$ cells in the myotome were found as those previously described (Eloy-Trinquet and Nicolas, 2002a). This suggests that myotomal clones that also colonise the dorsal aorta do not have distinct characteristics, but derive from a similar progenitor cell pool. At E9.5, fewer clones were analyzed but most of these are in the single somite or aorta only classes (Fig. 3C). The absence of long clones partly reflects the smaller number of somites and mature myotomes expressing α-cardiac actin at this stage; a clone such as E9.5-68 may correspond to another clonal category at later stages. Examples of the three categories found are shown in Fig. 2B.

## The nature of cells in the dorsal aorta that are derived from progenitor cells common to myotomal muscle

We have analyzed the location of clones of β-gal$^+$ cells in the dorsal aorta. Examples are shown in Fig. 4. Labelled smooth muscle cells are observed in dorsal (Fig. 4A,B), ventral (Fig. 4C,D,G,H), and lateral positions (data not shown), both in a periendothelial position (Fig. 4A-D), and in the outer layers of the wall of the aorta or in the blood vessels that derive from it (Fig. 4G,H). Rare β-gal$^+$ endothelial cells, protruding into the lumen, are detected (Fig. 4E,F). At E10.5 (Fig. 5A), the distribution of cell type varies with the age of the progenitor cell. Occasional β-gal$^+$ endothelial cells are observed in long and short clones. In these older clones, periendothelial smooth muscle cells predominate, whereas, in the other two categories of clone, vascular smooth muscle cells are more frequent, in the outer layers of the vessel. These observations are presented schematically in Fig. 5B. They provide evidence for the existence of a common progenitor for periendothelial and vascular smooth muscle cells, and suggest that endothelial cells may also derive from a common vascular progenitor cell. At E9.5 (Fig. 5C), fewer clones were available for analysis. Most β-gal$^+$ cells are in the periendothelial position, reflecting the immaturity of the aorta, which has relatively few outer vascular smooth muscle cells at this stage. However a few labelled endothelial cells are also detected at E9.5. In Table 2, the relative positions of different labelled cell types around the circumference of the dorsal aorta are summarized as a percentage for each category of clone. There is no notable difference in cell type distribution or the localization of aortic cells along the axis.

## The localization of β-gal$^+$ cells in the myotome

The localization of β-gal$^+$ cells in the myotome, and their spatial juxtaposition in relation to those in the dorsal aorta, are important parameters in determining how and when cells from the paraxial mesoderm colonize this vessel. Labelled cells tend to be located in the hypaxial part of the myotome at E10.5, when this somitic region can be distinguished (Fig. 2A, Fig. 6A-C), consistent with the presence of progenitor cells in the hypaxial dermomyotome. A quantitative analysis of the location of β–gal$^+$ cells in the myotome is shown in Table 3 and confirms this tendency. In clones with labelled cells only in the myotome, this hypaxial bias is not observed. As expected for clones derived from an earlier progenitor cell, most long clones also have cells in the dorsal aorta, with more extensive myotomal labelling.

## Labelling of progenitor cells by (Pax3)GFP

In order to explore further the origin of cells in the dorsal aorta, we employed *Pax3* expression as a potential marker. Pax3 is a transcription factor that is already expressed in presomitic paraxial mesoderm, and later in the dermomyotome, where it is required for the survival and delamination of muscle progenitor cells, as well as playing a role in their subsequent engagement in myogenesis (Tajbakhsh and Buckingham, 2000). We have generated a *Pax3* allele with a *GFP* reporter that marks *Pax3*-expressing cells as being GFP positive and also, because of GFP stability, marks cells that had previously expressed *Pax3* (Relaix et al., 2005). As shown in Fig. 6, GFP$^+$ cells are present in the somite, but are also detected in the region between the somite and the dorsal aorta (Fig. 6D,E), with which they are also associated (Fig. 6H). The Pax3 antibody, however, only marks cells in the somite, notably in the epithelial structure of the dermomyotome, and also migrating myogenic progenitor cells derived from the dermomyotome (Fig. 6G,J). The majority of periendothelial and vascular smooth muscle cells in the dorsal aorta are labelled with GFP (Fig. 6H), which co-localizes with α-smooth muscle actin (Fig. 6K). The *Pax3$^{GFP/+}$* line was crossed to the T4 transgenic line so that β-gal$^+$ cells expressing the α-cardiac actin-*nlacZ* transgene could be monitored. As seen in Fig. 6H,K, β-gal$^+$ cells in the dorsal aorta are GFP$^+$. Cells labelled in the clonal analysis, which is dependent on the expression of α-cardiac actin, are therefore mainly derived from *Pax3*-expressing cells.

*Pax3* is also expressed in the dorsal neural tube (Fig. 6E) and marks neural crest cells derived from this structure that will form, for example, the dorsal root ganglia (Fig. 6E). It is therefore possible that the GFP$^+$ cells observed outside of the somite are of neural crest origin. In the absence of Pax3, neural crest and its derivatives (Fig. 6F) are severely compromised. However, in *Pax3* mutant embryos, GFP$^+$ cells are still detected in the region between the somite and the dorsal aorta (Fig. 6F,I), and labelled cells, which co-localize with α-smooth muscle actin, are present in the dorsal aorta (Fig. 6L). This therefore strongly suggests that the GFP$^+$ cells in the dorsal aorta are not of neural crest origin and that, as expected, neural crest cells do not give rise to vascular smooth muscle in the trunk (Le Douarin and Kalcheim, 1999). Further confirmation was provided by an experiment in which the conditional *ROSA26R-lacZ* reporter (Soriano, 1999) was crossed with a mouse line carrying a Cre-recombinase transgene under the control of regulatory sequences for the human tissue plasminogen activator (*Ht-PA*) gene, which is a marker of neural crest cells (Pietri et al., 2003). Neural crest cells and their derivatives, such as the sympathetic ganglia in the vicinity of the dorsal aorta, expressed the *ROSA26-lacZ* reporter, but the dorsal aorta in the trunk was negative (see Fig. S1A,B in the supplementary material). Therefore, neural crest does not contribute to this structure. However, in the *Pax3* mutant, where the hypaxial dermomyotome is affected, the wall of the aorta is thinner (Fig. 6L) than in *Pax3$^{GFP/+}$* embryos (Fig. 6K), and the number of α-cardiac actin-*nlacZ*-expressing cells is reduced by a factor of three.

*Pax3* is expressed in presomitic mesoderm, just prior to somitogenesis, and throughout the early epithelial somite (Fig. 6O). Part of the GFP labelling seen ventrally in the more mature somites probably corresponds to sclerotomal cells derived from earlier progenitors in which *Pax3* was previously transcribed. Using *Pax1* expression as a marker of early sclerotome (Christ et al., 2004), we see co-localization with GFP (see Fig. S1C-E in the supplementary material). However, strongly labelled GFP$^+$ cells are located more ventrally, contiguous laterally with the hypaxial dermomyotome and extending towards the dorsal aorta.

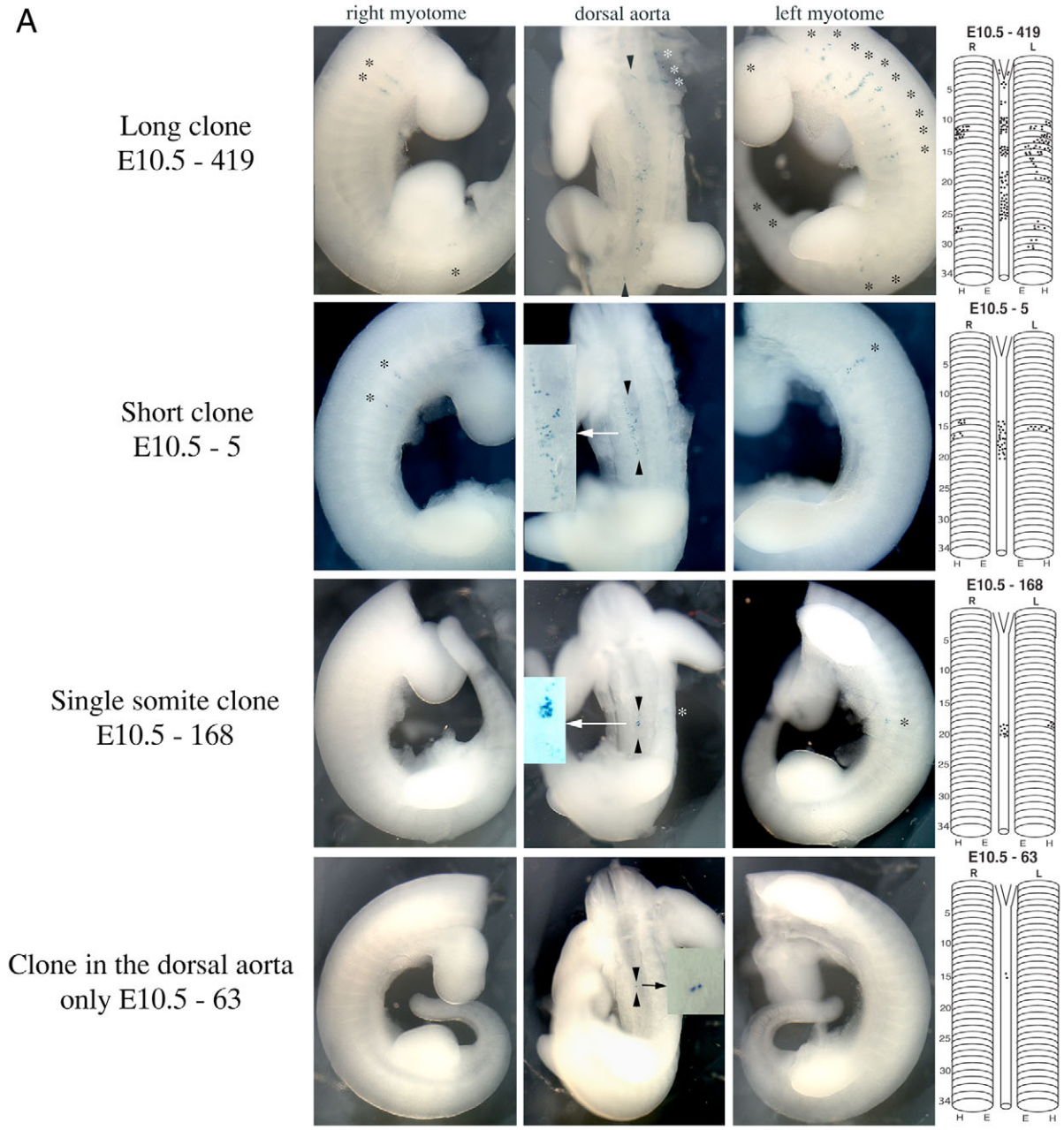



**Fig. 2. Examples of *α–cardiac actin*$^{nlaacZ1.1/+}$ embryos with β-gal$^+$ cells in the dorsal aorta.** (**A**) The different categories of clones at E10.5 are shown, with right and left lateral views to reveal cells in the myotomes (*) of the somites on either side of the neural tube and a ventral view (centre) to reveal cells (delimited by arrowheads) in the dorsal aorta. An enlargement of this region is shown as an inset (indicated by arrow). The position (not precise number) of labelled cells (black dots) in each clone is shown schematically on the right of the figure. Right (R) and left (L) somites (1-34) are presented on either side of the dorsal aorta. The hypaxial (H) and epaxial (E) extremities of the somites are indicated. (**B**) The different categories of clones at E9.5 are shown, with β-gal$^+$ cells in the dorsal aorta. Right and left lateral views reveal cells in the myotome (*) of somites on either side of the neural tube. A ventral view (centre) shows the dorsal aorta where β-gal$^+$ cells are delimited by black arrowheads. An enlargement of this region is shown as an inset (indicated by arrow). The position (not precise number) of labelled cells is shown schematically on the right of the figure. Right (R) and left (L) somites (1-25) are presented on either side of the dorsal aorta, which has not yet fused anteriorly.

Endothelial cells, at E10.5, identified by their position adjacent to the lumen, are not GFP$^+$ (Fig. 6H,K). Because they are the first cells to form the dorsal aorta, we also examined *Pax3*$^{GFP/+}$ embryos at E8.5. At this stage endothelial cells, marked by CD31/PECAM staining, are not labelled by GFP (Fig. 6M,N), even when they are adjacent to GFP$^+$ cells in the early somites (Fig. 6O). Immature epithelial somites have numerous GFP$^-$ cells (Fig. 6N), and, more posteriorly, where somites have not yet formed, *Pax3* is not expressed in the unsegmented paraxial mesoderm (Fig. 6M), and Pax3(GFP) does not therefore mark their derivatives, which may include endothelial cells of the dorsal aorta.

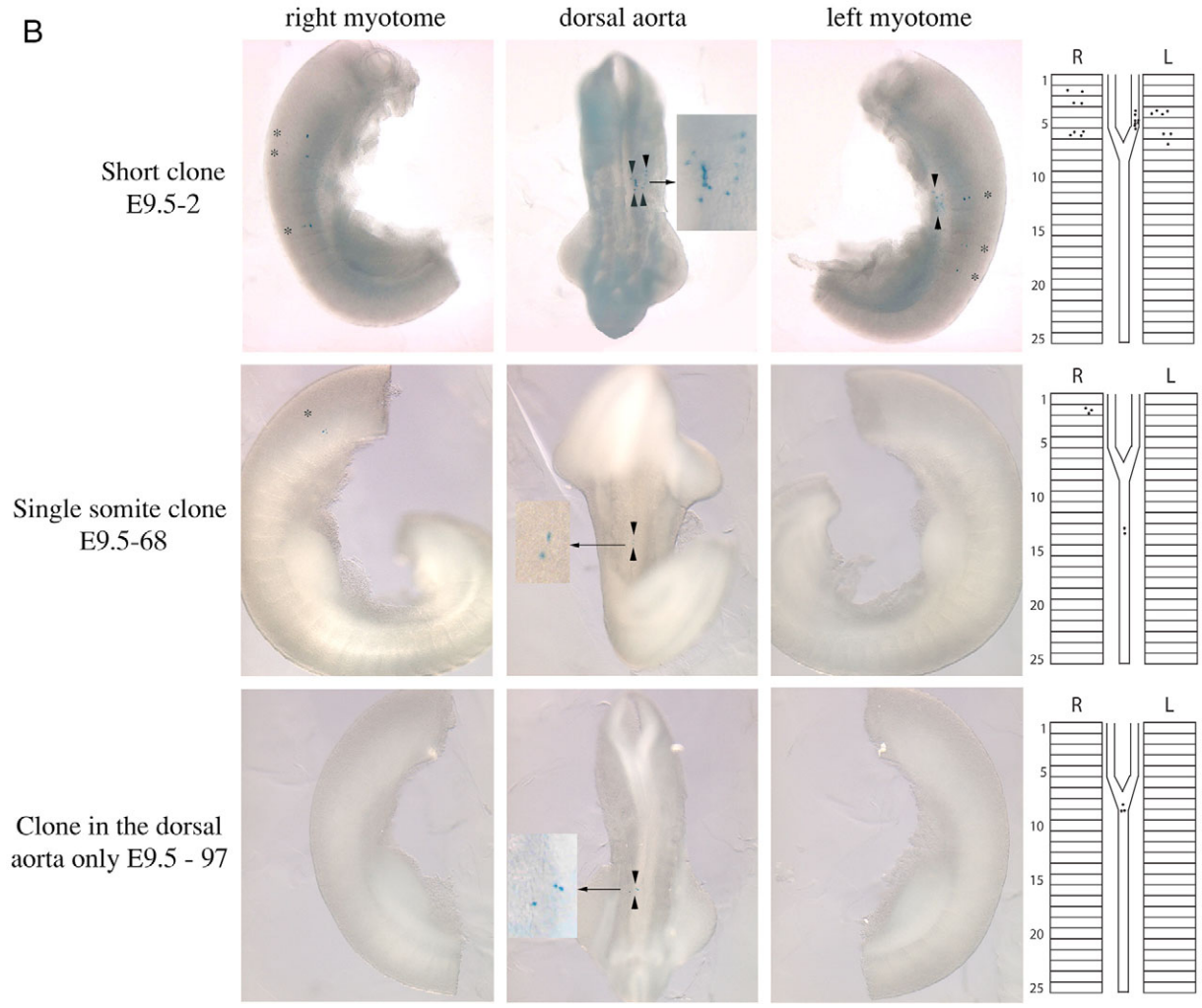


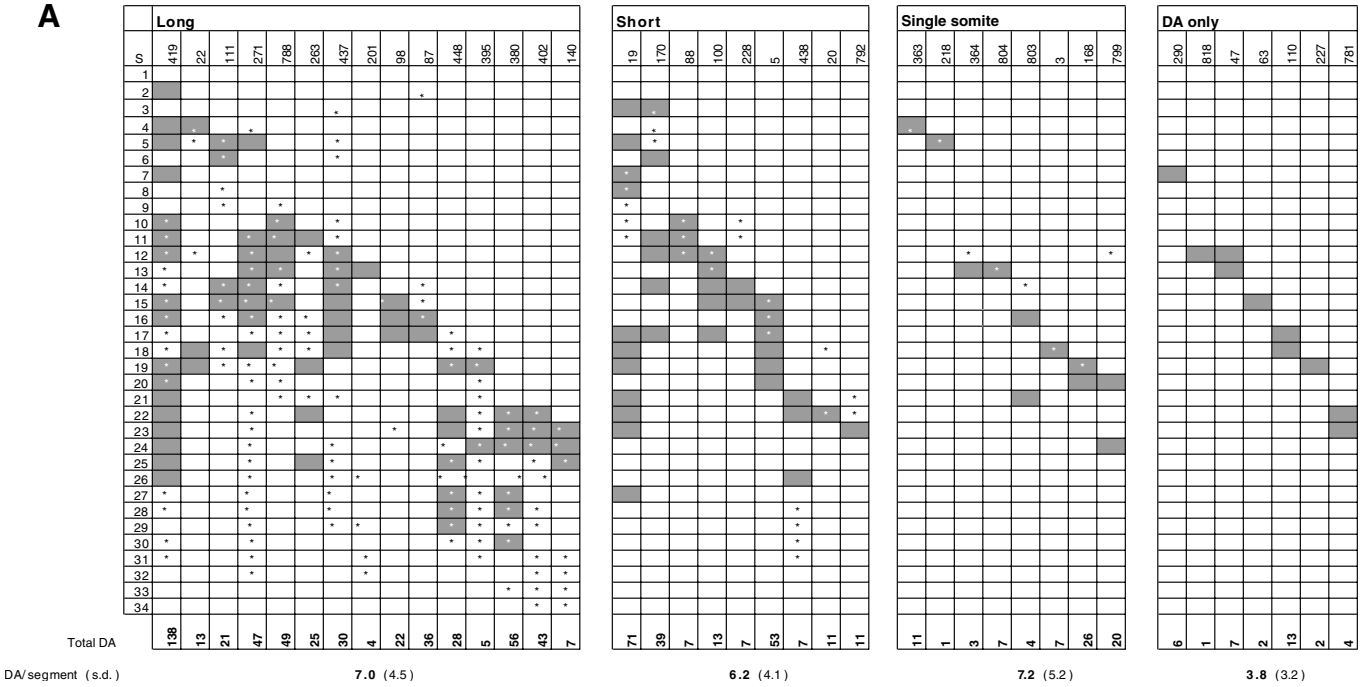

**Fig. 2B.** See previous page for legend.

## DISCUSSION

The retrospective clonal analysis presented here demonstrates that cells in the dorsal aorta and skeletal muscle cells in the myotomal compartment of the somite have a common clonal origin. The distribution of clonally related cells, together with observations on Pax3(GFP) labelling, suggest that progenitor cells are present in the hypaxial dermomyotome. Clones in the dorsal aorta include cells expressing smooth muscle markers, both in the periendothelial position and in the outer layers of the blood vessel wall, as well as rare endothelial cells, thus providing evidence for a common vascular progenitor cell.

### Clonal relationship between cell types in the dorsal aorta

The α-cardiac actin gene, which was targeted with the *nlaacZ* reporter for this clonal analysis, is expressed in a subpopulation of cells in the dorsal aorta, most of which express smooth muscle markers. We observe β-gal$^{+}$ cells in the dorsal aorta over a short time period. Association between smooth and cardiac actin isoforms is seen in other developing muscles. Overlapping expression with smooth muscle actin is observed in early skeletal muscle (Woodcock-Mitchell et al., 1988), and it is notable that in the hearts of α-cardiac actin mutant mice, smooth muscle actin is upregulated (Kumar et al., 1997). We have distinguished two types of smooth muscle-expressing mural cells in the dorsal aorta, based on their position. The lineage relationship between pericytes and vascular smooth muscle cells has not been clear (Gerhardt and Betsholtz, 2003); however, our clonal analysis demonstrates that the two types of mural cells in the dorsal aorta can derive from a common progenitor. The T4 line shows that α-cardiac actin is also expressed in some endothelial cells and we see rare labelled endothelial cells in some clones. The presence of endothelial cells in the same clone as periendothelial and vascular smooth muscle cells indicates that they share a common progenitor. At E10.5, only a few long and short clones show this phenomenon, indicating that the common progenitor is present before somitogenesis. At E9.5, labelled endothelial and smooth muscle cells are seen in a couple of 'single somite' clones. However, at this stage fewer somites have formed and such clones may have subsequently evolved into an older clonal category. Consistent with an early common progenitor, endothelial cells of the dorsal aorta appear to be laid down before Pax3(GFP) expression and the onset of somitogenesis. The observation that endothelial cells are Pax3(GFP) negative, whereas most mural smooth muscle cells are positive, does not support the proposal of a significant transdifferentiation of endothelial to smooth muscle cells in the




**B**

| | Student t-test, p=0.05 | | |
|---|---|---|---|
| | Long | Short | Single somite |
| Long | | **0.89** (1.99) | **0.07** (2) |
| Short | **1.17** (1.8) | | **0.37** (2.02) |
| Single somite | **0.75** (2.66) | **0.64** (2.7) | |
| | Snedecor F-test, p=0.05 | | |

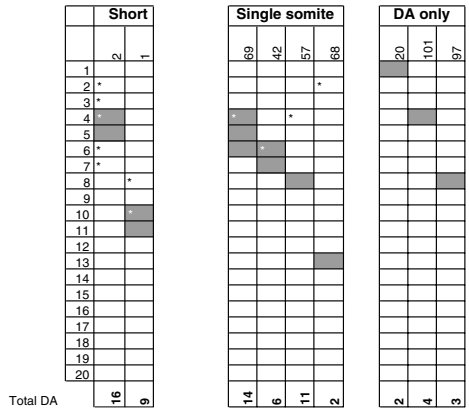



**Fig. 3. Distribution of β-gal⁺ cells in the dorsal aorta and myotomes, classified according to their myotomal labelling.** (**A**) The position of labelled cells in the dorsal aorta for clones within each class is represented by grey boxes, 'segments', at the corresponding somite (S) level, for somites 1-34 at E10.5. Asterisks (*) indicate the position of somites with β-gal⁺ cells in the myotome. The figure below each column indicates the total number of β-gal⁺ cells in the dorsal aorta (DA) for each clone (Total DA). The average number of β-gal⁺ cells in the dorsal aorta per 'segment' (DA/segment) is indicated at the bottom. s.d., standard deviation, is shown in parentheses. The number above each column identifies individual clones. The category of clone is indicated above each group. (**B**) Comparison between β-gal⁺ cells per 'segment' of the dorsal aorta in different categories of clone with labelled cells in the myotome. Student's *t*-test compares average values and the Snedecor F-test compares distributions. Numbers in brackets indicate the threshold figure for a significant statistical difference. In all cases, with a probability of $P$=0.05, there is no significant difference. (**C**) Representation of clones at E9.5, with a similar presentation to A.

dorsal aorta, previously made for avian embryos (DeRuiter et al., 1997). Previous evidence for a common vascular progenitor came from experiments with Flk1-positive mouse embryonic stem cells which were shown to differentiate into vascular smooth and endothelial cell types on injection into the chick embryo, or when analyzed under clonal conditions in vitro (Yamashita et al., 2000; Ema et al., 2003). We now show that such a common vascular cell progenitor can exist in vivo.

## Location of cells in the dorsal aorta in different categories of clone

In both long and short clones, most labelled cells are in the periendothelial position and are dispersed around the circumference of the dorsal aorta, as in the T4 line, whereas younger clones of the single somite and dorsal aorta only categories, have more labelled cells of the vascular smooth muscle type in the outer layer of the dorsal aorta. These tend to be located ventrally in all categories of

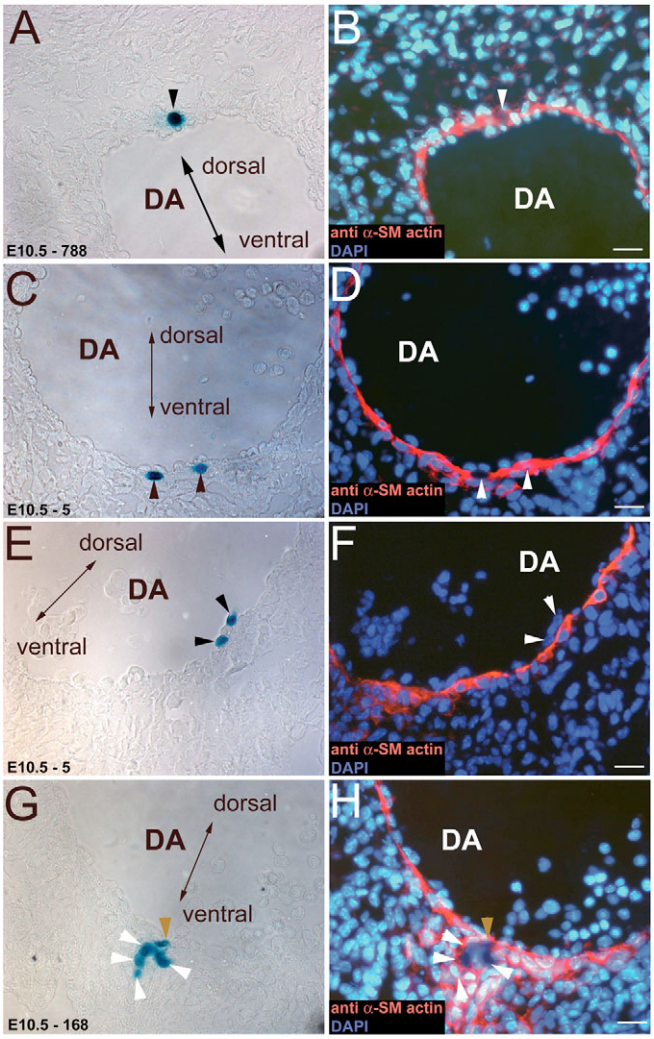



**Fig. 4. Localization and tissue identity of β-gal⁺ cells in the dorsal aorta in *α-cardiac actin*$^{nlaacZ/+}$ embryos.** (**A**) A section from the long clone, E10.5-788, stained with X-gal shows a β-gal⁺ cell (arrowhead) in the dorsal wall of the dorsal aorta in a periendothelial position. The dorsal/ventral orientation of the dorsal aorta (DA) is indicated. (**B**) Immunohistochemistry on the same section using an α-smooth muscle antibody (red) shows that the β-gal⁺ nucleus is in a cell expressing α-smooth muscle actin (white arrowhead). DAPI staining shows cell nuclei. (**C**) A section from the short clone E10.5–5, stained with X-gal, shows labelled cells in the ventral wall of the dorsal aorta in a periendothelial position (black arrowheads). (**D**) The same section treated with an α-smooth muscle actin antibody (red) shows that these cells are positive (white arrowheads). DAPI staining shows the nuclei. When nuclei are strongly β-gal⁺ they appear dark blue after DAPI staining. (**E**) Another section of the same clone as in C shows that β-gal⁺ cells (black arrowheads) are also present immediately adjacent to the lumen of the dorsal aorta in an endothelial cell position. (**F**) Immunohistochemistry on the same section as in E with an α-smooth muscle actin antibody shows that these cells (white arrowheads) are not smooth muscle positive, consistent with an endothelial identity. (**G**) A section from the single somite labelled clone E10.5–168, where a periendothelial cell (brown arrowhead), and vascular smooth muscle cells (white arrowheads) are stained by X-gal. (**H**) The same section as in G shows that all X-gal stained cells (brown and white arrowheads) are co-stained with an α-smooth muscle actin antibody (red). Scale bars: 20 μm.

clones. Periendothelial cells also are mainly found ventrolaterally in some but not all clones of more recent origin. This might suggest that both types of differentiated mural cell are first present in the ventrolateral wall, after which periendothelial cells colonize the circumference of the aorta, whereas vascular smooth muscle cells are still mainly located ventrally at E10.5. This would be consistent with previous work showing that cells expressing smooth muscle markers first appear in the ventral wall of the chick dorsal aorta (Hungerford et al., 1996).

Because α-cardiac actin is only expressed in a minority of endothelial cells, it is difficult to predict the importance of the paraxial mesoderm contribution to this cell type. However grafting experiments, in which quail somites are introduced into the chick embryo, have shown that endothelial cells in the dorsal aorta derive from paraxial mesoderm and that endothelial progenitor cells are still present in somites. In these experiments, quail endothelial cells were mainly located dorsally (Pardanaud et al., 1996), whereas the rare labelled endothelial cells identified in our clonal analysis were situated ventrolaterally, indicating that the paraxial mesoderm contribution is not limited to the dorsal wall of the aorta. Our Pax3(GFP) observations indicate that, in the mouse embryo, endothelial cells in the dorsal aorta are not derived from somitic cells that express *Pax3*. If they originate from paraxial mesoderm, as suggested by the clonal analysis, this must be prior to the expression of *Pax3*.

## Somitic origin of cells in the dorsal aorta

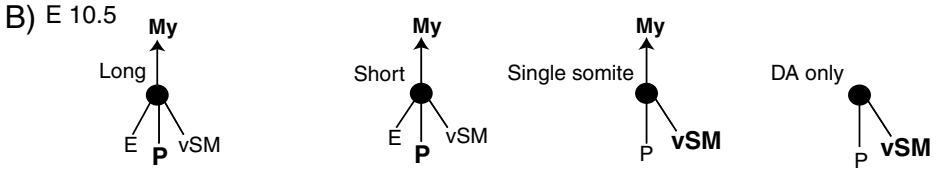
Clonally related cells in the dorsal aorta do not show a random distribution, but tend to be located adjacent to labelled somites. This segmental tendency is notable in more recent clones. Labelled cells in the dorsal aorta in single somite clones, or in clones where only the aorta is labelled, maintain a discrete distribution not extending beyond the limits of one, or at most two, somites. Furthermore, statistical analysis shows that there is no significant difference in the distribution of labelled cells per 'segment' in the dorsal aorta in different categories of clone. This supports the possibility of 'segmental' restriction in the clonal contribution, with no dispersion on the anteroposterior axis, as the number of labelled cells per 'segment' would be expected to decrease with the diminishing age and size of the clones if there was dispersion along the axis. This is consistent with a colonization of the smooth muscle compartment of the dorsal aorta by cells derived from adjacent somites.

The results of the clonal analysis, together with examination of (Pax3)GFP-positive cells at E10.5, suggests the presence of multipotent progenitor cells located in the hypaxial dermomyotome that can give rise to the skeletal muscle of the hypaxial myotome and to the mural smooth muscle cells of the dorsal aorta. More recent single somite clones support this model. In these clones, but also in short clones, there is a marked association between preferential labelling of the hypaxial myotome and the presence of labelled cells in the dorsal aorta, whereas in clones which do not colonize the dorsal aorta the epaxial and hypaxial myotome show similar labelling. Even in older long clones with cells in the dorsal aorta, a hypaxial bias is observed. This is consistent with the early segregation of progenitor cells for the medial (epaxial) and lateral (hypaxial) myotome, indicated by a clonal analysis in the mouse embryo at E11.5 (Eloy-Trinquet and Nicolas, 2002b). A minority of clones with labelled cells in the dorsal aorta show epaxial myotomal labelling. This is the case for some long clones, where the progenitor was present well before somitogenesis. The extensive Pax3(GFP) labelling of smooth muscle cells in the dorsal aorta

A)

| | E 10.5 | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Long | | | | Short | | | | Single somite | | | | DA only | | | |
| | E10.5 - 402 | E10.5 - 271 | E10.5 - 22 | E10.5 - 788 | E10.5 - 5 | E10.5 - 20 | E10.5 - 792 | E10.5 - 100 | E10.5 - 168 | E10.5-803 | E10.5 - 804 | | E10.5 - 110 | E10.5 - 47 | E10.5 - 227 | E10.5 - 290 |
| **E** | 1 | 1 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 |
| **P** | 24 | 31 | 2 | 47 | 31 | 10 | 7 | 7 | 3 | 0 | 7 | | 0 | 2 | 2 | 0 |
| **vSM** | 18 | 15 | 11 | 2 | 19 | 1 | 4 | 6 | 23 | 4 | 0 | | 13 | 5 | 0 | 6 |

B) E 10.5




C)

| | E 9.5 | | | | |
|---|---|---|---|---|---|
| | Short | Single somite | | | DA |
| | E9.5 - 2 | E9.5 - 42 | E9.5 - 69 | E9.5 - 57 | E9.5 - 97 |
| **E** | 0 | 2 | 2 | 0 | 0 |
| **P** | 13 | 4 | 11 | 10 | 3 |
| **vSM** | 3 | 0 | 2 | 1 | 0 |

D) E 9.5

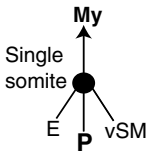



**Fig. 5. Distribution of different cell types in the dorsal aorta in different categories of clone.** (**A**) The numbers of labelled endothelial (E), periendothelial (P) and vascular smooth muscle (vSM) cells in different categories of clones, at E10.5. (**B**) Schematic diagram showing progenitors for the myotome (My) and dorsal aorta (summarizing all cell types observed) for each category of clone. The progenitors of long clones and short clones give rise to endothelial cells (E), as well as mural smooth muscle cells. The predominant cell type – periendothelial (P) or vascular smooth muscle (vSM) – in the dorsal aorta is indicated in heavy type. (**C**) The same as A, at E9.5. DA, dorsal aorta only. (**D**) At E9.5, a similar lineage tree to those in B, is shown for the category of single somite clones.

**Table 2. Distribution of labelled cells in the dorsal aorta in clones at E10.5 and E9.5**

| | | E10.5 | | | | E9.5 | | |
|---|---|---|---|---|---|---|---|---|
| | | Long clones | Short clones | Single somite | No myotome | Short clones | Single somite | No myotome |
| Endothelial | Dorsal | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Lateral | 0.7 | 3 | 0 | 0 | 0 | 19 | 0 |
| | Ventral | 0.7 | 0 | 0 | 0 | 0 | 0 | 0 |
| Periendothelial | Dorsal | 27 | 4 | 0 | 0 | 6 | 33 | 100 |
| | Lateral | 14.6 | 21 | 13.5 | 0 | 50 | 9.5 | 0 |
| | Ventral | 27 | 38 | 13.5 | 14 | 25 | 29 | 0 |
| Vascular smooth muscle | Dorsal | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Lateral | 5 | 1 | 3 | 12 | 19 | 9.5 | 0 |
| | Ventral | 21 | 33 | 70 | 74 | 0 | 0 | 0 |

β-gal$^+$ cells in the dorsal aorta, examined on sections, were counted and classified according to two parameters: their localization in the circumference of the tube, as ventral, lateral or dorsal, and their localization across the wall of the tube in the endothelial, periendothelial or vascular smooth muscle cell position, as demonstrated by antibody labelling. The figures represent the percentage of labelled cells within each category of clone.

**Table 3. Distribution of β-gal$^+$ cells in the myotome in different categories of clones**

| | Long clones | | Short clones | | Single somite | |
|---|---|---|---|---|---|---|
| | +DA | –DA | +DA | –DA | +DA | –DA |
| Hypaxial myotome | 17 | 2 | 12 | 7 | 5 | 7 |
| Epaxial myotome | 12 | 5 | 3 | 7 | 2 | 10 |
| All labelled | 12 | 0 | 0 | 1 | 0 | 0 |
| Somites analyzed | 41 | 7 | 15 | 15 | 7 | 17 |
| Clones analyzed | 4 | 1 | 7 | 7 | 7 | 17 |

In clones that were stained in both the dorsal aorta and the myotome (+DA), the hypaxial myotome is preferentially colonized, whereas in those clones labelled only in the myotome (–DA), β-gal$^+$ cells are more frequent in the epaxial myotome. The total number of somites and clones analyzed is indicated at the bottom of the table.

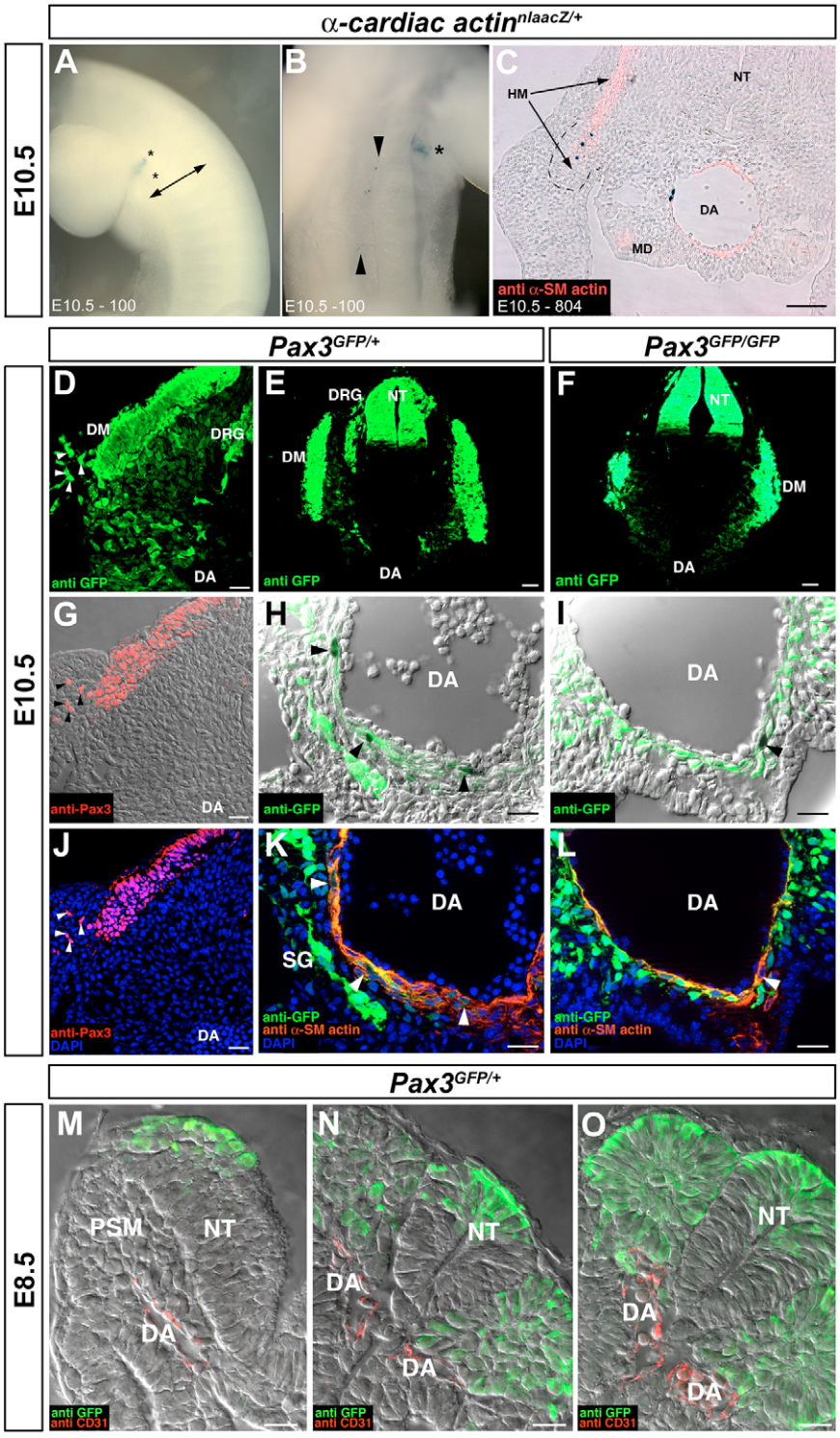



**Fig. 6. The hypaxial location of clones in the myotome and the distribution of (Pax3)GFP-labelled cells in the somite, dorsal aorta and intervening tissues.** (A-C) Examples of clones, at E10.5, with β-gal$^+$ cells in the dorsal aorta and hypaxial myotome. (**A**) Lateral view of an X-gal-stained embryo showing labelled cells in the hypaxial myotome (*). The full extension of an adjacent myotome is indicated by a double-headed arrow. (**B**) Ventral view of the same embryo showing β-gal$^+$ cells in the dorsal aorta. The extent of labelled cells is indicated by the black arrowheads. On the right side (*) β-gal$^+$ cells are visible in the hypaxial myotome. (**C**) A section of a single somite clone, E10.5-804, showing cells stained with X-gal in the hypaxial myotome (HM) lying under the epithelium of the dermomyotome (marked with a dotted line) and cells in the adjacent lateral wall of the dorsal aorta (DA). The section was stained with an α-smooth muscle actin antibody (red). Labelling is also seen in the mesonephric duct (MD). (**D-F**) Immunohistochemistry, using an anti-GFP antibody (green) on sections from *Pax3*$^{GFP/+}$ (D,E) and *Pax3*$^{GFP/GFP}$ (F) embryos at E10.5. Labelled cells in the dermomyotome (DM) and between this part of the somite and the dorsal aorta (DA), as well as in the latter, are seen in E, and in the enlargement of the more hypaxial region shown in D, where myogenic progenitor cells migrating to the hindlimb (white arrowheads) are indicated. The dorsal root ganglia (DRG) are also labelled in D and E, but not in F, where neural crest cells are compromised. (**G**) Immunohistochemistry with a Pax3 antibody (red) of a section from a *Pax3*$^{GFP/+}$ embryo at E10.5, showing staining in the dermomyotome and in the myogenic progenitor cells migrating to the hindlimb (black arrowheads). No staining is observed in the region between the somite and the dorsal aorta. (**H**) Immunohistochemistry with an anti-GFP antibody (green) of a section from a *Pax3*$^{GFP/+}$ embryo carrying the T4 transgene, at E10.5, shows staining of cells in the wall of the dorsal aorta, some of which (black arrowheads) are also stained with X-gal. (**I**) Immunohistochemistry using an anti-GFP antibody (green) of a section from a *Pax3*$^{GFP/GFP}$ embryo, carrying the T4 transgene, which has also been treated with X-gal. An X-gal positive cell (black arrowhead) is labelled. (**J**) The same section as is shown in G, with DAPI staining, together with immunohistochemistry with the Pax3 antibody (red). (**K**) Co-immunohistochemistry of the section shown in H, using an α-smooth muscle actin (red) antibody, as well as the antibody against GFP (green). White arrowheads indicate X-gal-stained nuclei in mural smooth muscle cells of the dorsal aorta. The highly fluorescent cells of an adjacent sympathetic ganglion (SG) are also observed. (**L**) Co-immunohistochemistry of the section shown in I, using an α-smooth muscle actin antibody (red), as well as the antibody to GFP (green). A white arrowhead indicates an X-gal-stained nucleus of an α-smooth muscle actin-positive periendothelial cell in the dorsal aorta. Note that in the absence of Pax3, the sympathetic ganglion is absent, although GFP-positive cells are still present outside the wall of the aorta. (**M-O**) Co-immunohistochemistry with an anti-GFP antibody (green) and a CD31/PECAM antibody (red) on sections from a *Pax3*$^{GFP/+}$ embryo at E8.5. (M) The dorsal extremity of the non-fused neural tube (NT) is already GFP positive, whereas presomitic mesoderm (PSM) at the posterior part of the embryo is not. The endothelial cells of the dorsal aorta, stained with the CD31/PECAM antibody (red), are GFP negative. (N) GFP-positive cells are detected within the first epithelial somite, but not all somitic cells are positive. The dorsal part of the fused neural tube (NT) is GFP positive. Endothelial cells of the dorsal aorta (DA), stained with a CD31/PECAM antibody (red), are GFP negative. (O) In this more mature epithelial somite, most cells are GFP positive. The endothelial cells of the dorsal aorta (DA), stained with a CD31/PECAM antibody (red) do not co-localize with GFP. Scale bars: in C, 100 μm; in E,F, 50 μm; in D,G-O, 20 μm.

indicates that the precursors of these cells, as well as those of the myotome, are in the somite. Epaxial labelling is also seen in some short and single somite clones, suggesting that the common progenitor, present in the epaxial somite, can also give rise to cells that migrate from this location to the dorsal aorta. GFP-positive cells are not labelled with the α-smooth muscle actin antibody (nor with that for PECAM) in the region between the somite and the wall of the aorta, indicating that differentiated cells do not migrate, and it is the precursors of mural smooth muscle cells that leave the somite. These cells no longer express Pax3, based on antibody staining, but did so previously, as they are GFP positive. Unlike skeletal muscle progenitor cells, which require Pax3 to delaminate and migrate to the limb, (Pax3)GFP-positive cells continue to contribute to the dorsal aorta in *Pax3* mutant embryos, although to a reduced extent. In *Pax3* mutants, the hypaxial dermomyotome undergoes apoptosis and this reduction of smooth muscle cells is therefore consistent with their derivation from this part of the somite (Tajbakhsh and Buckingham, 2000).

The possibility that sclerotomal cells contribute to the smooth muscle cells of the vasculature has been raised by experiments in the chick embryo (Christ et al., 2004). However, the clonal analysis presented here clearly shows that at least part of the smooth muscle component of the dorsal aorta, marked by α-cardiac actin, is clonally related to skeletal muscle cells of the myotome. Whereas in the case of older clones, the progenitor is present in presomitic mesoderm, in single somite clones the common progenitor is probably located in the somite, necessarily in the dermomyotome rather than in the sclerotomal compartment, which does not give rise to skeletal muscle (Tajbakhsh and Buckingham, 2000). Pax3(GFP) labelling of cells in the dermomyotome is intense, whereas only residual GFP is seen in cells of the sclerotome, reflecting previous expression throughout the epithelial somite. The relative intensity of the GFP staining and its location compared with *Pax1* transcripts, which mark sclerotomal cells, suggest a dermomyotomal origin for GFP-positive smooth muscle cells in the dorsal aorta. Neural crest cells represent another potential source of Pax3-GFP cells. However, GFP-positive cells are still present in the dorsal aorta in the *Pax3* mutant, where neural crest is compromised, and a lineage tracing experiment with a neural crest-specific Cre mouse line confirms that neural crest does not contribute to the smooth muscle of the dorsal aorta in the trunk.

## The dermomyotome as a source of multipotent progenitor cells

Experiments in the chick embryo have indicated that the dermomyotome is a source of multipotent cells, with a common progenitor for derm and skeletal muscle (Ben-Yair and Kalcheim, 2005), and for the migrating population that contributes to the skeletal muscle and endothelial cells of the limb (Kardon et al., 2002). Our results now suggest that it is also a source of progenitor cells that give rise to skeletal and smooth muscle. This is particularly evident in single somite clones with labelled cells in the dorsal aorta and hypaxial myotome. Clones with labelled cells only in the dorsal aorta, or only in the myotome, may derive from a precursor cell in the dermomyotome that has already segregated from the skeletal/smooth muscle lineage.

The cells that we have followed in the dorsal aorta present interesting parallels with the mesoangioblast stem cell isolated from this structure (De Angelis et al., 1999; Minasi et al., 2002). These cells express α-cardiac actin and *Pax3* (G.C., unpublished). In vivo transplantation experiments of mouse dorsal aorta into the chick embryo showed that mouse cells contributed both to blood vessels and to adjacent muscle fibres (Minasi et al., 2002). This has led to the suggestion that the vasculature may be a source of muscle progenitor cells during development (De Angelis et al., 1999; Cossu and Mavilio, 2000). One can speculate that the mesoangioblast may correspond to a progenitor cell from the hypaxial dermomyotome that has retained multipotent properties.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/4/737/DC1

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