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Characterization of *Pax3*-expressing cells from adult blood vessels

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Summary

We report expression of *Pax3*, an important regulator of skeletal muscle stem cell behaviour, in the brachial and femoral arteries of adult mice. In these contractile arteries of the limb, but not in the elastic arteries of the trunk, bands of GFP-positive cells were observed in $Pax3^{GFP/+}$ mice. Histological and biochemical examination of the vessels, together with clonal analysis after purification of Pax3–GFP-positive cells by flow cytometry, established their vascular smooth muscle identity. These blood-vessel-derived cells do not respond to inducers of other mesodermal cell types, such as bone, however, they can contribute to muscle fibre formation when co-cultured with skeletal muscle cells. This myogenic conversion depends on the expression of *Pax3,* but is rare and non-cell autonomous as it requires cell fusion. Myocardin, which promotes acquisition of a mature smooth muscle phenotype in these Pax3–GFP-positive cells, antagonises their potential for skeletal muscle differentiation. Genetic manipulation shows that *myocardin* is, however, positively regulated by Pax3, unlike genes for other myocardin-related factors, MRTFA, MRTFB or SRF. Expression of *Pax3* overlaps with that reported for *Msx2*, which is required for smooth muscle differentiation of blood vessel-derived multipotent mesoangioblasts. These observations are discussed with respect to the origin and function of *Pax3*-expressing cells in blood vessels, and more general questions of cell fate determination and adult cell plasticity and reprogramming.

Key words: Vascular smooth muscle, skeletal muscle, myocardin, Pax3

Introduction

The Pax family of transcription factors plays important roles in lineage specification during embryonic development (Buckingham and Relaix, 2007). Expression of *Pax* genes also persists in the adult, in stem and/or progenitor cell populations. A striking illustration of this scenario is provided by Pax3 and Pax7. These factors are required for the entry of progenitor cells into the myogenic programme in the embryo (Relaix et al., 2005). Their expression persists in skeletal muscle satellite cells after birth and in the adult. Pax7 marks these cells (Seale et al., 2000) and is co-expressed with Pax3 in many muscles (Relaix et al., 2006). Satellite cells are responsible for post-natal growth and regeneration of skeletal muscles (Buckingham and Montarras, 2008). $Pax3^{nlacZ/+}$ and $Pax3^{GFP/+}$ mouse lines have been instrumental in these studies and have permitted the direct isolation and characterization of muscle satellite cells (Montarras et al., 2005; Pallafacchina et al., 2010; Relaix et al., 2006).

Pax3 and *Pax7* are also expressed in domains of the central nervous system and Pax3 plays an important role in neural crest that migrates from the dorsal neural tube, including cardiac neural crest that invades the arterial pole of the developing heart (Conway et al., 1997). However, neither gene has been implicated directly in the formation of cardiac muscle. Smooth muscle in the blood vessels of the head and in the aortic arch arteries, in the anterior region of the embryo, derives from Pax3-positive cranial neural crest (Etchevers et al., 2001). In the body of the embryo, smooth muscle can be formed from a range of mesodermal sources, notably lateral mesoderm, but also from the paraxial mesoderm of the somites, which gives rise to smooth muscle of the dorsal aorta. In both chick (Ben-Yair and Kalcheim, 2008) and mouse (Esner et al., 2006), a single cell in the dorsal compartment of the somite, the dermomyotome, can give rise to both smooth and skeletal muscle. In $Pax3^{GFP/+}$ embryos, perduring GFP shows that smooth muscle cells that had expressed *Pax3* in the dermomyotome, are still GFP-positive, although no longer Pax3 positive, in the dorsal aorta (Esner et al., 2006). Mesoangioblast stem cells, isolated after culture of the wall of this vessel, can form a number of mesodermal derivatives including smooth muscle (Minasi et al., 2002). These cells express *Pax3*, which is required for their entry into the myogenic programme when co-cultured with skeletal muscle (Messina et al., 2009). In the adult, a sub-population of pericytes, smooth

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muscle-like cells located immediately adjacent to vascular endothelial cells, as well as cells described as myoendothelial cells, also show multipotency (Dellavalle et al., 2007; Kirillova et al., 2007; Zheng et al., 2007), with spontaneous differentiation into skeletal muscle. Myogenic reprogramming of blood-vessel-derived cells from the mouse retina was also observed but appeared to require fusion with differentiating skeletal muscle cells (Kirillova et al., 2007). Autonomous trans-differentiation of established smooth muscle cell lines into skeletal muscle cells has also been reported (Graves and Yablonka-Reuveni, 2000).

In all situations where skeletal muscle is formed previous activation of the myogenic determination genes, *MyoD* or *Myf5* (or *Mrf4* in the early embryo) is required, with subsequent expression of myogenic regulatory genes required for differentiation (myogenin, *Mrf4*, *MyoD*), leading to skeletal muscle fibre formation. Smooth muscle differentiation shares with skeletal muscle a requirement for transcription factors of the Mef2 family (Lin et al., 1998; Potthoff and Olson, 2007) and also SRF, which acts through CArG box motifs present in the regulatory regions of many striated and smooth muscle genes (Miano, 2003; Owens et al., 2004). SRF is a weak transcriptional activator and requires cofactors, of which myocardin is a major player in smooth muscle (Parmacek, 2007; Pipes et al., 2006). Myocardin also functions as a transcriptional repressor of the myogenin gene, thus potentially inhibiting skeletal muscle differentiation (Long et al., 2007). The homeodomain protein Msx2 is also implicated in smooth muscle differentiation, notably in the context of mesoangioblasts and other mesodermal progenitor cells where it acts with necdin in the absence of myocardin (Brunelli and Cossu, 2005; Brunelli et al., 2004), and indeed a subset of smooth muscle genes in blood vessels are not regulated by myocardin (Pipes et al., 2005; Yoshida et al., 2004; Yoshida and Owens, 2005). *Msx2* is expressed in a subpopulation of vascular smooth muscle cells, mainly in peripheral blood vessels, such as the brachial and femoral arteries (Goupille et al., 2008). These are contractile blood vessels, as distinct from the vessels in the trunk, such as the aorta, that have elastic properties (Gittenberger-de Groot et al., 1999; Megens et al., 2007; Patel et al., 2006). Vascular smooth muscle cells are highly plastic, changing from a well-differentiated contractile to a synthetic immature phenotype under different physiological and pathological conditions, and when cultured (Rensen et al., 2007). A number of markers, such as smoothelin or smooth muscle myosin heavy chain (SM-MHC; also known as myosin-11), are expressed at higher levels in mature differentiated smooth muscle cells. BMP signalling exerts a control on the phenotypic plasticity of smooth muscle cells acting through myocardin related (MRTFA, MRTFB) transcription factors (Lagna et al., 2007).

We report here unexpected expression of *Pax3* in a subset of smooth muscle cells of adult brachial and femoral arteries. Purification by flow cytometry from $Pax3^{GFP/+}$ mice led to their characterization in terms of smooth muscle phenotype, myocardin and *Msx2* expression and cell fate plasticity. We provide evidence that re-directing these cells to skeletal myogenesis is under the negative control of myocardin and the positive control of Pax3 and that this event is rare and non-cell autonomous as it occurs after fusion with differentiating muscle cells.

Results

Whole mount X-gal staining of adult $Pax3^{nlacZ/+}$ mice revealed unexpected expression of the reporter gene in brachial and femoral arteries (Fig. 1A,B) with staining increasing along the proximodistal axis of the limbs from where these vessels enter the limb muscle masses. No expression was detected in the aorta (Fig. 1C) or in other arteries of the trunk, such as the common or external iliac arteries (Fig. 1D). Expression in brachial and femoral arteries was discontinuous, with a banded pattern of reporter gene expression, also observed in $Pax3^{GFP/+}$ adult mice (Fig. 1E,F). Separation of these Pax3–GFP-positive cells by flow cytometry indicated that they made up ~8% of the total cells from brachial and femoral arteries. Closer examination on transverse sections showed that the reporter gene was expressed in a subset of mural cells that expressed smooth muscle actin (Fig. 2A); no GFP expression was found in endothelial cells (Fig. 2B). RT-PCR analysis (Fig. 2C) confirmed the pattern of expression seen with the reporters, with *Pax3* transcripts present in the femoral artery, but not in the aorta. As expected, transcripts for smooth muscle (SM22α and SM-MHC) and endothelial cell markers (von Willebrand factor; vWF) were found in both types of blood vessels (Fig. 2C). No transcripts for myogenic regulatory factors (Myf5, myogenin, MyoD), or for Pax7, were

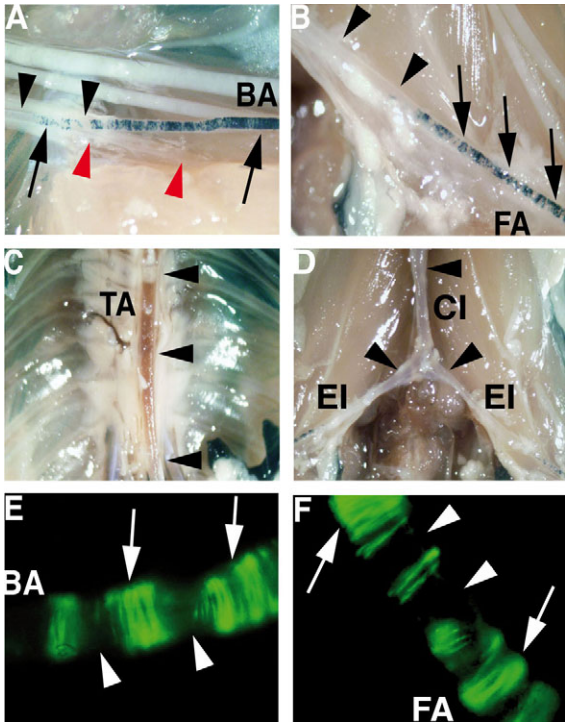


Fig. 1. *Pax3* is expressed in peripheral arteries of adult mice. The expression of *Pax3* was analyzed in adult $Pax3^{nlacZ/+}$ mice after X-gal staining and in $Pax3^{GFP/+}$ mice by direct observation of GFP fluorescence after dissection to expose the underlying tissues. (**A**,**B**) The X-gal staining patterns of $Pax3^{nlacZ/+}$ brachial (BA) and femoral (FA) arteries along the proximodistal axis of the limb. Higher levels of expression were found in the distal part of these contractile arteries (black arrows versus black arrowheads). No expression was found in the adjacent veins (red arrowheads). (**C**,**D**) $Pax3^{nlacZ/+}$ expression was not detected in elastic arteries of the trunk such as in the thoracic aorta (TA), the common iliac (CI) and the external iliac (EI). (**E**,**F**) The same expression pattern was observed in $Pax3^{GFP/+}$mice, with predominant expression of the reporter gene in the distal part of brachial (E) and femoral arteries (F). As with β-galactosidase (A,B) alternating bands of expression (white arrows) and non-expression (white arrowheads) are observed.

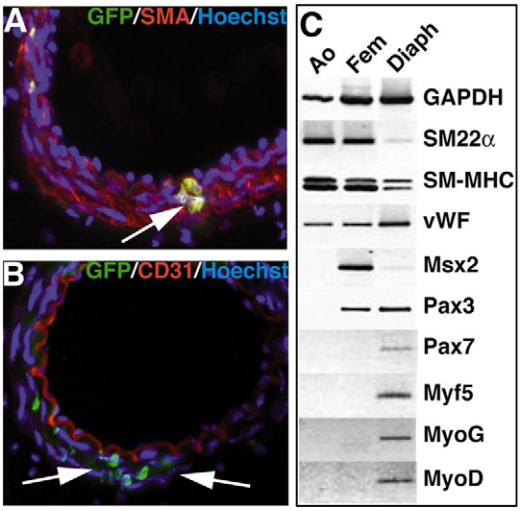


Fig. 2. *Pax3* is expressed in mural cells of peripheral arteries. (**A**,**B**) Immunohistochemical analysis on transverse sections of the femoral artery of $Pax3^{GFP/+}$ adult mice. GFP-positive cells were detected in the tunica media (white arrows) after staining with an anti-GFP antibody (green); anti-smooth muscle actin (SMA) and anti-CD31, both red staining, mark, respectively, the smooth muscle (A) and the endothelial layer (B). The co-expression of GFP and SMA, in A leads to a yellow staining. Nuclei were labelled with Hoechst dye. (**C**) RT-PCR analysis of RNA isolated from the aorta (Ao), the femoral artery (Fem) and the diaphragm (Diaph) of adult mice. Transcripts for smooth muscle transgelin (SM22α) and the smooth muscle myosin heavy chain (SM-MHC), the endothelial marker von Willebrand Factor (vWF), the homeobox transcription factor, Msx2, the paired box transcription factors (Pax3 and Pax7) and the myogenic regulatory factors, Myf5, MyoD and myogenin (MyoG) were analysed. GAPDH transcripts are shown as a control.

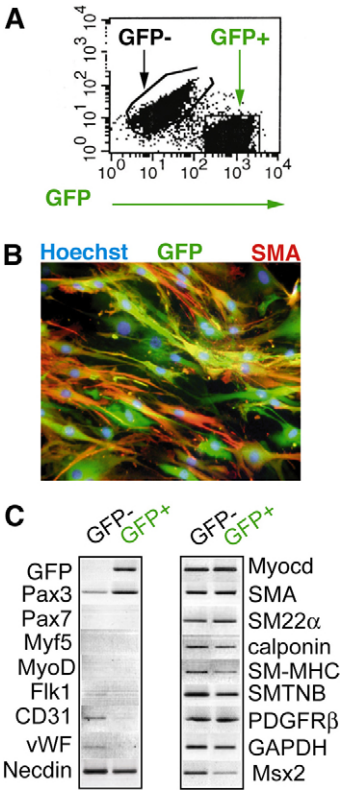


Fig. 3. Purified $Pax3^{GFP/+}$ cells have a smooth muscle phenotype in culture. (**A**) Flow cytometry separation of GFP-positive cells obtained from brachial and femoral arteries of $Pax3^{GFP/+}$ adult mice, after a phase of explantation–expansion in culture (see supplementary material Fig. S2). Cells were sorted according to their level of GFP fluorescence (green arrow). Y-axis, FL2 channel at 576 nm. (**B**) Immunohistochemical analysis of GFP-positive cells, isolated as in A and cultured. Anti-GFP (green) and anti-SMA (red) antibody staining. Nuclei were labelled with Hoechst. (**C**) RT-PCR analysis of cultured GFP-positive (GFP+) and GFP-negative (GFP–) cells obtained after flow cytometry purification as in A. Left lanes, expression of transcripts for GFP, Pax3, skeletal muscle markers (Pax7, Myf5, MyoD), endothelial markers (CD31, Flk1 and vWF) and necdin. Right lanes, expression of transcripts for smooth muscle markers, myocardin (Myocd), smooth muscle actin (SMA), transgelin (SM22α), calponin, smooth muscle myosin heavy chain (SM-MHC), smoothelin-B (SMTNB), platelet-derived growth factor receptor β (PDGFRβ), Msx2, and GAPDH used as an internal control.

detected in RNA preparations from the aorta or femoral arteries, although they were detected in RNA extracts from the diaphragm, a skeletal muscle, which like other skeletal muscles contained blood vessels and thus displayed expression of vascular markers. Expression of the *Msx2* gene was observed in the femoral artery but not the aorta, in agreement with previous observations (Goupille et al., 2008) indicating that *Msx2* is expressed in a subpopulation of mural cells in large peripheral arteries of adult mice. Similar to *Pax3*, *Msx2* is also expressed in bands of cells. Analysis of femoral arteries from $Pax3^{GFP/+}$:$Msx2^{nlacz/+}$ mice showed partial overlap of *Pax3* and *Msx2* reporters (supplementary material Fig. S1A,C,E), and furthermore, in a Pax3–GFP-positive population, obtained from the femoral artery of $Pax3^{GFP/+}$:$Msx2^{nlacz/+}$ mice, 22% of the cells were β-galactosidase positive (supplementary material Fig. S1B,D,F).

Pax3–GFP-positive cells, directly isolated by flow cytometry after enzymatic digestion of the femoral and brachial arteries of $Pax3^{GFP/+}$ mice, could not be expanded in culture (results not shown). However, when explants from these vessels were placed in culture, cells migrated out of the explant, spread over the dish and proliferated (supplementary material Fig. S2A,B). Pax3–GFP-positive cells constituted ~30% of this population. Most of these cells were smooth muscle actin (SMA)-positive (supplementary material Fig. S2C). When these Pax3–GFP-positive cells were isolated by flow cytometry (Fig. 3A) and compared with Pax3–GFP-positive cells directly obtained from brachial and femoral arteries, they were found to express similar surface markers characteristic of vascular smooth muscle cells, such as integrin β1, but not markers specific for endothelial or hematopoietic cells (supplementary material Fig. S2D).

This Pax3–GFP-positive cell population consisted of many cells displaying a smooth muscle phenotype as indicated by the presence of smooth muscle actin-positive cells (Fig. 3B). There was notable variation between cells in the relative levels of staining for this smooth muscle marker depending on the state of differentiation of the cells. Analysis of this GFP-positive population by RT-PCR showed the presence of transcripts typical of proliferating smooth muscle cells, with expression of higher levels of early markers (SMA, SM22α) characteristic of immature smooth muscle and lower levels of late markers characteristic of well-differentiated contractile smooth muscle, including calponin and SM-MHC (Rensen et al., 2007). Transcripts for myocardin, which is required for expression of these late markers (Huang et al., 2008) were also present

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(Fig. 3C). The population of GFP-negative cells (Fig. 3A,C) also expressed smooth muscle transcripts, however, the detection of transcripts for CD31-PECAM and vWF indicated the presence of endothelial cells (Fig. 3C). Expression of *Msx2* marks both populations, with a lower level in GFP-positive cells, 22% of which co-express both genes (supplementary material Fig. S1F). Transcripts for necdin, which acts with Msx2 (Brunelli et al., 2004), were present in both Pax3–GFP-positive and -negative populations (Fig. 3C).

Because Pax3 plays a crucial role upstream of the myogenic regulatory factors (MRFs) in the specification of muscle progenitor cells in the embryo, its expression in a subset of smooth muscle cells might be indicative of a capacity of these cells to adopt a skeletal muscle fate. Myocardin, by contrast, has been shown to act as a master regulator of the smooth muscle phenotype in the course of embryonic development and to induce the expression of a smooth muscle phenotype when overexpressed in skeletal muscle cell lines (Chen et al., 2002; Wang et al., 2003). In order to address the issue of the role of myocardin in Pax3–GFP-positive cells, we isolated clones expressing different levels of myocardin transcripts while continuing to express *Pax3* (Fig. 4A). These clones, which appeared spontaneously, did not differ substantially with respect to the expression of *SRF* or the myocardin-related genes, *MRTFA* and *MRTFB* (also known as *Mkl1* and *Mkl2*) (Fig. 4B). However, the capacity to express the late smooth muscle marker, SM-MHC, was markedly reduced in the clones expressing a lower level of myocardin, as seen for clone 5 (Fig. 4B). By contrast, transcripts for SMTNB (smoothelin-B), a late marker that does not depend on myocardin (Rensen et al., 2007; Yoshida and Owens, 2005), remained high.

When treated with BMP2, these clones showed increased levels of myocardin transcripts, with increased levels of transcripts for the late smooth muscle marker SM-MHC (supplementary material Fig. S3A), in keeping with the role of myocardin in activating such genes (Miano, 2003). No increase in markers of bone or cartilage was observed in these BMP2-treated clones (supplementary material Fig. S3B), in contrast to mesoangioblasts (Minasi et al., 2002). This might be due to absence of Runx2 upregulation (supplementary material Fig. S3B), which was shown to be crucial for vascular smooth muscle cells to express osteochondrogenic markers (Speer et al., 2010).

We investigated whether decreased myocardin expression had any influence on the capacity of the cells to adopt a skeletal muscle phenotype. None of these clones when cultured alone expressed transcripts for skeletal muscle markers such as Pax7 or MRFs, although they continued to transcribe *Pax3* (Fig. 4A; supplementary material Fig. S3C). We used a co-culture assay (Minasi et al., 2002) between BrdU-labelled clones 3, 4 and 5 and mitomycin-C-treated myoblasts from the C2 muscle cell line (Yaffe and Saxel, 1977). Mitomycin-C inhibits cell proliferation but does not affect myogenic cell differentiation, which could therefore be followed in the co-cultures (Fig. 4C; supplementary material Fig. S4). We observed that the lower the level of myocardin transcripts, the higher the number of MyoD-positive nuclei originating from the blood-vessel-derived clones, also marked by BrdU (Fig. 4D). Thus, there is an inverse correlation between the level of myocardin expression and the potential of these blood-vessel-derived cells to undergo skeletal myogenesis. This was further substantiated by stable transfection of clones 1 and 3, which expressed high levels of myocardin transcripts, as

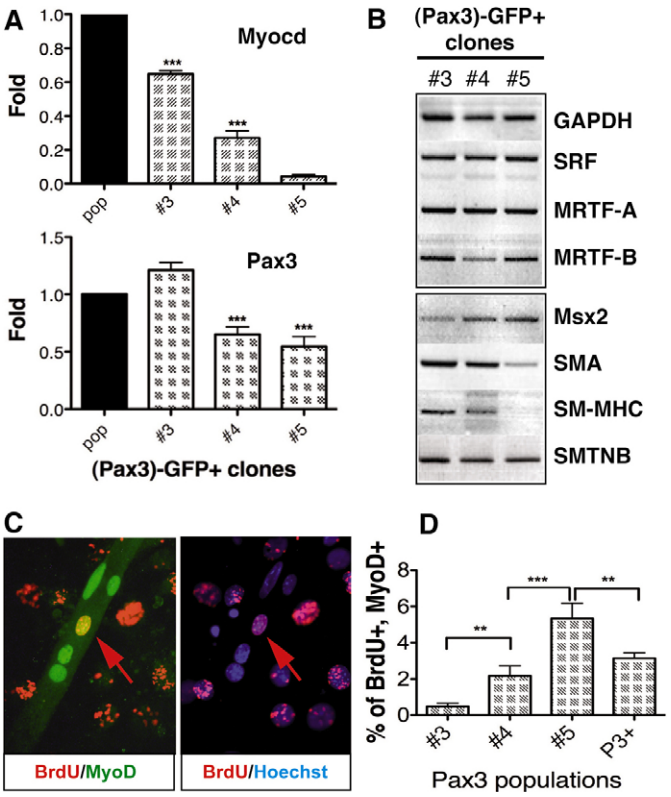


Fig. 4. Clonal analysis of GFP-positive cells isolated from arteries of *Pax3*$^{GFP/+}$ adult mice. (**A**) Quantitative RT-PCR analysis of *myocardin* (Myocd) and *Pax3* expression in the GFP-positive cell population (pop) and in three clones, (clones 3, 4 and 5) derived from this population. myocardin (top) and *Pax3* (bottom) mRNA levels were quantified using SYBR-QPCR and normalized to the level of *GAPDH* mRNA, and compared with the relative level of *Myocd* in the whole population that was arbitrarily set at 1. The results are expressed as the means ± s.d. ***$P<10^{-3}$. (**B**) Semi-quantitative RT-PCR analysis of the three clones shown in A. The expression of the *myocardin*-related genes, *MRTFA* and *MRTFB*, *SRF* and of transcripts for smooth muscle markers, *SMA*, *SM-MHC* and *SMTNB*, as well as *Msx2*, was analyzed using *GAPDH* as a control. (**C**) The myogenic potential of the clones and cell populations was tested in a co-culture assay with C2 myoblasts (see Materials and Methods). The clones were labelled with BrdU and then co-cultured with C2 myoblasts previously treated with mitomycin-C. After 14 days of co-culture, cells were immunolabelled with anti-BrdU (red) and anti-MyoD (green) antibodies. Nuclei were labelled with Hoechst. The red arrow points to a BrdU- and MyoD-positive nucleus (stained orange) within a myotube. (**D**) The percentage of nuclei positive for both BrdU and MyoD was quantified with respect to the total number of nuclei in 40–80 randomly selected fields of BrdU-positive cells (original magnification, ×40), corresponding to a total of at least 1200 cells. Results are expressed as the means ± s.d.. ***$P<10^{-3}$; **$10^{-2}<P<10^{-3}$. Results are shown for clones 3, 4, 5, which express different levels of myocardin, and for the GFP-positive population (P3+), which represent an average of such levels.

seen for clone 3 in Fig. 4A, with a plasmid encoding a dominant-negative form of myocardin [myocardin-DN (Wang et al., 2001; Wang et al., 2003)] carrying a deletion of the transactivation domain. This resulted in an increase in MyoD-positive cells derived from the BrdU-positive blood vessel cells (Fig. 5A). The reduction in expression of myocardin-dependent late smooth muscle markers, such as SM22α, calponin and SM-MHC (Fig. 5B), correlates with the reduction in myocardin activity in these cells. Despite their higher propensity to activate

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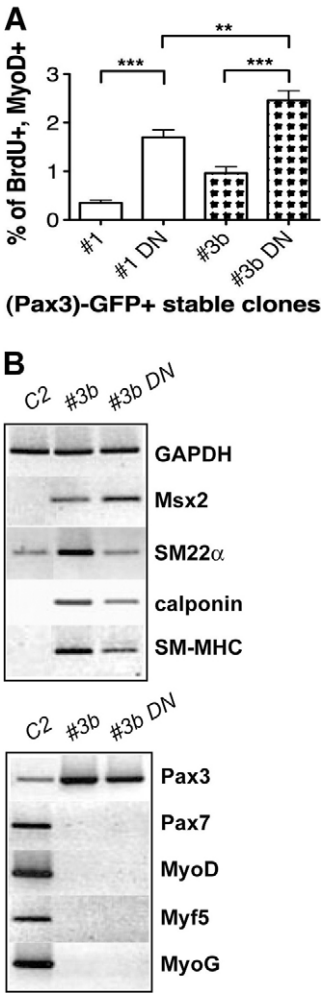


Fig. 5. Reduction in myocardin increases the myogenic potential of (Pax3)–GFP-positive mural cells. Two *Pax3*$^{GFP/+}$ representative clones (1 and 3b), which expressed a high level of myocardin, were stably transfected with an expression vector encoding a dominant-negative version of myocardin (DN) or an empty expression vector. (**A**) The capacity of these stably transfected clones to give rise to MyoD-positive nuclei was analyzed using a co-culture assay as in Fig. 4. The results are expressed as the means ± s.d. and correspond to the percentage of BrdU-positive (BrdU+), MyoD-positive (MyoD+) nuclei in the total BrdU-positive population. ***$P<10^{-3}$; **$10^{-2}<P<10^{-3}$. (**B**) RT-PCR analysis of transcripts for smooth muscle differentiation markers (Msx2, SM22α, calponin and SM-MHC) in the stably transfected clone 3b. The expression of *Pax3* and of the skeletal muscle markers *Pax7*, *MyoD*, *Myf5* and *myogenin* (MyoG) was also analyzed. Differentiated C2 cells were used as a positive control for skeletal muscle markers and as a negative control for most vascular smooth muscle markers.

myogenesis, these cells, when cultured alone, did not transcribe myogenic regulatory factor genes such as *MyoD* or *Pax7*; however, they continued to transcribe *Pax3* (Fig. 5B). Furthermore, when these experiments were performed in the presence of 1 mM EGTA to prevent cell fusion (Hu and Olson, 1990), mononucleated MyoD-positive cells of blood vessel origin were not detected (supplementary material Fig. S5). We therefore conclude that spontaneous fusion with differentiating muscle cells is required to reveal the myogenic potential of *Pax3*-expressing smooth muscle cells.

To address the role of Pax3 in these Pax3–GFP-positive cells, clones were isolated from blood vessels of *Pax3*$^{GFP/flox}$:*Rosa*$^{CreERT2/+}$ adult mice after tamoxifen treatment (Crist et al., 2009; Koushik et al., 2002). Invalidation of the *Pax3* allele was confirmed by PCR on genomic DNA (data not shown) and led to a 73% decrease in the expression of *Pax3* transcripts in the Pax3–GFP population after conditional ablation, with 25% of clones still expressing *Pax3* transcripts. Clones no longer expressing *Pax3* were further analyzed (Fig. 6A). These clones expressed very low levels of myocardin transcripts compared with *Pax3*-expressing cells, but similar levels of *SRF*; they also displayed a smooth muscle phenotype characterized, however,

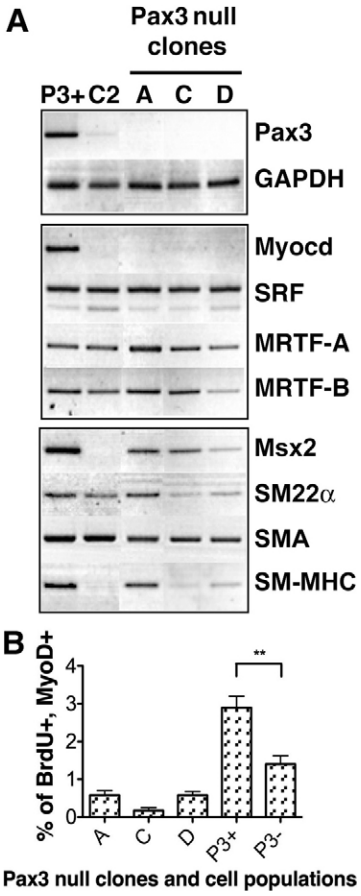


Fig. 6. Pax3 promotes the activation of myogenesis in blood vessel-derived cells. GFP-positive cells were isolated and cloned as previously described, from femoral arteries of *Pax3*$^{GFP/flox}$:*ROSA*$^{CreERT2/+}$ adult mice after tamoxifen injection. Three representative clones (A, C and D) are shown. (**A**) The expression of transcripts for the *Pax3* floxed allele (*Pax3* null), myocardin (Myocd), SRF, myocardin-related transcription factors (MRTFA and MRTFB), Msx2, early (SM22α, SMA) and late (SM-MHC) markers of vascular smooth muscle cell differentiation were examined. *GAPDH* was used as a control for transcript levels. C2 skeletal muscle cells and a population of Pax3–GFP-positive cells (P3+) were also used as controls. (**B**) The myogenic potential of *Pax3* null clones was tested in a co-culture assay as described previously. The results are expressed as the means ± s.d. of the percentage of BrdU+, MyoD+ cells in the total BrdU+ population for the three representative *Pax3* null clones. The GFP-positive populations from *Pax3*$^{GFP/+}$ mice (P3+) and *Pax3*$^{GFP/flox}$:*ROSA*$^{CreERT2/+}$ mice (P3−), in which there was 73% overall recombination after tamoxifen treatment, are also shown. **$10^{-2}<P<10^{-3}$.

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by lower levels of expression of both early and late smooth muscle markers, most notably in clones C and D. They also continued to express *Msx2* and *MRTFA* and *MRTFB* genes, although at variable levels, which might have contributed to the maintenance of their smooth-muscle-cell identity. In keeping with this notion, they also expressed the gene for the transcription factor, Gata6, which marks smooth muscle cells (data not shown). The myogenic potential of these clones was assessed using the co-culture assay with C2 cells. They all showed a very low capacity to contribute to myotube formation in this co-culture assay (Fig. 6B), despite a very low level of expression of myocardin transcripts. The percentage of nuclei found in myotubes that are positive for BrdU and MyoD – 0.6% at most (Fig. 6B) – was ten times lower than the percentages obtained with *Pax3*-expressing clones expressing low levels of myocardin, such as clone 5 (Fig. 4D). The GFP-positive cell population (P3–) obtained after tamoxifen treatment of $Pax3^{GFP/flox}$:$Rosa^{CreERT2/+}$ mice showed a higher capacity to fuse with C2 cells than those of clones A, C and D, reflecting incomplete recombination with 25% of the cells still expressing *Pax3*. Taken together, our results indicate that the capacity of adult blood-vessel-derived cells to differentiate into skeletal muscle depends on Pax3, and is negatively influenced by high levels of myocardin.

Discussion

We report here unexpected expression of *Pax3* in smooth muscle cells of femoral and brachial arteries that irrigate the limbs of adult mice. This was observed with *GFP* and *nlacZ* reporters introduced into an allele of *Pax3* and also by RT-PCR of purified Pax3–GFP-positive cells, which confirmed that *Pax3* transcripts were present.

A first question concerns the possible origin of these cells. Pax3-expressing neural crest does not invade the limb buds, whereas Pax3-positive cells migrate from the somites into the limbs. These will mainly contribute to skeletal muscle formation, but a sub-population of somite-derived cells also includes endothelial progenitors that can contribute to blood vessels (Kardon et al., 2002; Pouget et al., 2008). The somites also give rise to vascular smooth muscle, derived from *Pax3*-expressing cells, as shown for the dorsal aorta in the mouse embryo (Esner et al., 2006), although this has not been documented for vessels in the limb. Examination of angiogenesis in the developing mouse limb shows that vessels are organized before the invasion of Pax3-positive progenitors and that in *Pax3* mutant limbs where there is no migration of somitic myogenic progenitors, the vasculature is present (De Angelis et al., 1999; Tozer et al., 2007). This shows that the majority of vascular progenitors in the limb derive from Pax3-negative cells. This does not preclude that normally there is a contribution of such cells and that, as in the case of mesoangioblasts in the dorsal aorta (Minasi et al., 2002), occasional multipotent cell from the somite (Ben-Yair and Kalcheim, 2008) might be retained in the wall of the blood vessel. Indeed the striated distribution of Pax3-positive cells in the adult femoral and brachial arteries might suggest a clonal origin. It is notable that, similar to mesoangioblasts, Pax3–GFP-positive cells migrate out from explants of these blood vessels and that, as for mesoangioblasts, they grow and can be cloned in culture (Minasi et al., 2002). These cells have a range of positivity for smooth muscle markers, and when cloned they generate smooth muscle progeny. The Pax3–GFP-positive cells that we detected did not express the mesoangioblast endothelial markers CD31 and VE-cadherin. Unlike the mesoanglioblast (Minasi et al., 2002), or indeed a sub-group of pericytes of adult vessels (Dellavalle et al., 2007), the Pax3–GFP cells do not become osteogenic in response to BMP2, but rather upregulate contractile smooth muscle genes, as previously reported for vascular smooth muscle cells (Lagna et al., 2007). However, similar to mesoangioblasts, they can convert to skeletal myogenesis on co-culture with differentiated muscle cells but, as with blood-vessel-derived cells from mouse retina, this myogenic conversion requires fusion with differentiating muscle cells (Kirillova et al., 2007). Another possible origin, since muscle satellite cells leave the fibre when activated after injury, or can move into the interstitial space in normal muscle (Buckingham and Montarras, 2008; Hughes and Blau, 1990), is that these cells are recruited to blood vessels. Local recruitment of cells has been reported during neo-angiogenesis (Yoshida and Owens, 2005). However, it is arteries in the limb rather than the trunk that contain Pax3-expressing cells, whereas Pax3-positive satellite cells are mainly present in diaphragm and trunk muscles and in most forelimb, but not hindlimb muscles (Relaix et al., 2006). A factor arguing against a satellite cell origin, is the absence of Pax7, which is a marker of these cells in all adult muscles (Buckingham and Montarras, 2008).

A second question concerns the functional significance of such cells. The femoral and brachial arteries are contractile vessels, as distinct from the elastic arteries of the trunk (Gittenberger-de Groot et al., 1999; Megens et al., 2007; Patel et al., 2006) where Pax3 is not expressed. *Pax3* mutant mice die before birth, without apparent vascular problems. Conditional *Pax3* mutants are viable after birth (Lepper et al., 2009) (our unpublished results), however, this is under sedentary conditions in a cage and it is not yet clear how such mice respond to exercise.

Myocardin is a major co-activator of SRF in the smooth muscle context (Parmacek, 2007; Pipes et al., 2006). It is required for the activation of most genes specifically associated with a mature phenotype (Huang et al., 2008). In keeping with this, clones with low levels of myocardin expressed lower levels of mature smooth muscle genes such as that for SM-MHC, a finding confirmed by expression of dominant-negative myocardin. The Pax3–GFP-positive population also expresses genes for the myocardin-related co-factors MRTFA and MRTFB, at levels independent of myocardin expression. They also express *Msx2* at a higher level in clones where myocardin transcripts are low. Msx2 drives mesoangioblast smooth muscle differentiation, together with necdin (Brunelli and Cossu, 2005). Necdin transcripts are also detected in Pax3–GFP-positive cells. It is striking that *Msx2* transcripts are present in the contractile arteries that we have examined (Goupille et al., 2008). Given the role of Msx2 in the Pax3-positive mesoangioblast, it was tempting to consider that its expression would colocalise with that of *Pax3* in the femoral and brachial arteries. However, although *Msx2* is transcribed in similar bands of cells, these are not coincident with the Pax3–GFP population, showing only 22% overlap. It is possible that the Msx2–β-galactosidase-positive cells have derived from a Pax3-positive population, because the Pax3–GFP reporter is remarkably stable (Esner et al., 2006). The absence of reliable antibodies precludes colocalisation studies. At present we do not know how dynamic these populations are.

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The relationship between Pax3 and myocardin is intriguing. Clones that express lower levels of myocardin transcripts also tend to show a reduction in the level of *Pax3* transcripts, and in clones from mice in which *Pax3* has been conditionally mutated, myocardin transcripts are barely detectable. Under these conditions *Msx2* expression is also reduced, suggesting a possible link with Pax3. There is also evidence for a link between Pax3 and the myocardin gene in the somites. The myocardin gene is expressed in the Pax3-positive population of the dermomyotome (Long et al., 2007), and in a recent genetic screen that we have carried out for Pax3 targets in Pax3–GFP-positive cells of the somites, myocardin emerged as a potential target, notably in cells that migrate to the limb (Lagha et al., 2010). Myocardin antagonizes skeletal myogenesis (Long et al., 2007) and in keeping with this, in a clone expressing a dominant-negative myocardin construct, the proportion of Pax3–GFP cells that can participate in skeletal myogenesis on co-culture with muscle cells is increased. However, the percentage remains low (<10%), probably reflecting deficient levels of other upstream regulators of myogenesis. Myogenic conversion of the vascular cells depends on the presence of Pax3, because it is notably lower in Pax3–GFP cells where the level of Pax3 has been reduced by conditional mutagenesis, despite a major reduction in myocardin gene expression. This is reminiscent of mesoangioblasts that do not express myocardin and also require the maintenance of *Pax3* expression for realisation of their myogenic potential (Messina et al., 2009).

In conclusion, vascular smooth muscle cells display heterogeneity with respect to their origin and function. The presence of a subpopulation of VSMCs expressing *Pax3* in contractile arteries of the limbs constitutes further evidence for this heterogeneity. The persistence of *Pax3* expression in a subset of adult vascular mural cells does not constitute an unequivocal signature of skeletal muscle potential and indeed these cells primarily behave as smooth muscle cells. Because the vessel-derived cells that we describe do not have a myogenic phenotype before fusion, the effect of Pax3 and myocardin is seen against the transcriptional background of the muscle cell, which, as shown in classical heterokaryon experiments (Blau and Blakely, 1999; Pomerantz et al., 2009) can lead to nuclear reprogramming per se. It is not clear whether the *Pax3*-expressing subpopulation of cells that we have identified in limb contractile arteries contributes in any way to skeletal muscle regeneration under physiological conditions. Indeed injection of clone 5 Pax3–GFP-positive cells into *mdx* muscle did not result in substantial restoration of dystrophin in regenerating fibres, in contrast to the situation with Pax3–GFP-positive satellite cells (results not shown) (Montarras et al., 2005).

Myogenic conversion of mural cells (pericyte and VSMC) by forced (Pomerantz et al., 2009) or spontaneous fusion with myoblasts was previously reported (Kirillova et al., 2007) and was suggested to be an alternative physiological mechanism for muscle regeneration after injury (Lluis and Cosma, 2010). However, the use of a skeletal-muscle-specific genetic marker to track this event in a transplantation–regeneration assay (Washabaugh et al., 2004) did not support this proposition and instead indicated that recruitment of plastic cells from ectopic sources is unlikely to contribute to skeletal muscle regeneration under physiological conditions.

Conditional invalidation of *Pax3* in the smooth muscle cells that we describe here might throw light on their potential myogenic function and should elucidate the in vivo role of Pax3 under normal and pathological conditions (ischemia, hypertension, ageing) in these arteries.

Materials and Methods

Mouse lines

$Pax3^{nlacZ/+}$ (Relaix et al., 2004), $Pax3^{GFP/+}$ (Relaix et al., 2005), $ROSA26^{CreERT2}$ (Hameyer et al., 2007), $Pax3^{flox/flox}$ (Zhou et al., 2008) mouse lines have been described previously. They were used to generate $Pax3^{GFP/flox}$:$Rosa26^{CreERT2}$ mice. *Pax3* null mice were obtained after five to six intraperitoneal injections of tamoxifen (Sigma-Aldrich; 1 mg/injection) in $Pax3^{GFP/flox}$:$Rosa26^{CreERT2+}$ young adult mice (7–8 weeks old) over 8–10 days, followed by at least 1 week of recovery. Approximately 75% of the *Pax3 flox* alleles had been ablated, as estimated by PCR analysis of genomic DNA and by *Pax3* mRNA levels. Experiments on mice were carried out in accordance with the regulations of the French Ministry of Agriculture.

Explant culture and cell expansion

Brachial and femoral arteries were dissected from young adult (7–8 weeks old) $Pax3^{GFP/+}$ mice and $Pax3^{GFP/flox}$:$Rosa26^{CreERT2+}$ mice after tamoxifen induction. Fragments of arteries, free of nerves, muscle and fat, were plated in culture on collagen-coated dishes (50 μg/ml rat type I collagen; BD Biosciences) in a 1:1 mixture (v/v) of DMEM and F12 medium containing Glutamax, 1% penicillin–streptomycin, 10% (v/v) FCS, 1/100 dilution of insulin–transferrin–selenium (ITS; Sigma), 10 ng/ml human bFGF (Peprotech, Rocky Hill, NJ) and 100 ng/ml thymosin-β4 (AbCys, Paris, France) (Smart et al., 2007). This medium is referred to as expansion medium. After ~10 days, the cells spreading out from the explants were dissociated with trypsin (Roche) and grown under the same culture conditions for a few passages, before fluorescence-activated cell sorting purification of GFP-positive cells. Clones of GFP-positive cells from arteries of $Pax3^{GFP/+}$ and $Pax3^{GFP/flox}$ adult mice were obtained after automated plating of single GFP-positive cells sorted by flow cytometry. The clonal efficiency of both populations was between 10% and 28%.

Flow cytometry

Sorting of GFP-positive cells from crude cell populations was performed with a Moflo cell sorter (Cytomation, Trappes, France). Propidium iodide (1 μg/ml; Molecular Probes) uptake was used to exclude dead cells. Flow cytometry analysis was performed with an LSR analyzer (BD Biosciences) on cell populations freshly isolated from arteries, and cell populations obtained after a phase of explantation and expansion as described above, except that for this assay cells were dissociated from the plates with Accutase (Sigma-Aldrich) to minimize the alteration of surface antigens.

Antibodies

A monoclonal antibody against Thy1.2 (clone 30H12; BD Biosciences) coupled to PE (Phycoerythrin); antibodies against CD34 (clone RAM 34; BD Biosciences), CD29 (clone Ha2/5; BD Biosciences) and Sca1 (clone E13-161.7; BD Biosciences) were coupled to biotin and detected with streptavidin coupled to phycoerythrin (PE) or allophycocyanin (APC).

Antibodies against markers of the endothelial lineage were: anti-Flk1 (clone Avas12α1; BD Biosciences) coupled to PE, anti-VE-cadherin (Alexia, ALX-210-232B-T100) coupled to biotin, and anti-CD31 (clone MEC13.3; BD Biosciences) coupled to PE.

Antibodies against the hematopoietic lineage were: anti-CD45 (clone 30F11; BD Biosciences) coupled to biotin, anti-CD11b (clone M1/70; BD Biosciences) coupled to APC.

Co-culture assay for myogenic potential

C2 myoblasts [a myogenic cell line originally isolated by D. Yaffé (Yaffe and Saxel, 1977)] grown on 0.1% (w/v) gelatin-coated plates, in a 1:1 mixture (v/v) of F12 and DMEM medium containing 10% (v/v) FCS, were treated at low confluence (40%) with 5 μg/ml mitomycin-C for 4 hours to irreversibly inhibit their proliferation. Then cells were washed three times with PBS and fed fresh FCS-containing medium before plating of GFP-positive cells on the C2 cells.

GFP-positive cell populations or clones were cultured in the presence of 10^{-6} M bromodeoxyuridine (BrdU; Sigma) for 48 hours in expansion medium before plating onto mitomycin-C-treated myoblasts prepared as above. GFP-positive cells were washed three times in PBS, trypsinised, pelleted by centrifugation and plated at 10^5 cells per 35 mm plate on C2.4 mitomycin-C-treated myoblasts in a 1:1 mixture (v/v) of DMEM and F12 medium containing 5% FCS and 2% Ultroser SF (Biosepra, Cergy, France). After 3 days, cells were fed the same medium mix supplemented with 2% FCS and ITS to promote myoblast fusion; this medium was changed every 3 days. After 12 days, cultures were processed for immunodetection of BrdU and MyoD.

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The myogenic potential of blood-vessel-derived GFP-positive cells was measured by counting the number of cells that were positive for both BrdU and MyoD, expressed as the percentage of double positive nuclei with respect to total BrdU-positive and MyoD-positive nuclei present on 50–80 randomly chosen fields corresponding to at least 2000 nuclei from two independent experiments performed in duplicate. Statistical analysis was performed using a one-way nonparametric ANOVA test and Dunnett's multiple comparison post-test using GraphPad Prism 5. $P<10^{-2}$ was considered as significant.

Autonomous differentiation assay

In all cases cells were grown in a 1:1 mixture of F12 and DMEM medium and the medium was changed every 3 days.

Differentiation into smooth muscle cells was induced by culturing the cells for 2 weeks in medium supplemented with 5% FCS, ITS, 10 ng/ml bFGF (Peprotech), 100 ng/ml human BMP2 (Peprotech) and 100 ng/ml thymosin B4 (AbCys), and the cells were then grown on mouse type-4-collagen-coated plates.

For osteogenic differentiation, cells were maintained in medium supplemented with 5% FCS, ITS, 10 mM β-glycerophosphate (Sigma), 50 μg/ml L-ascorbic acid (Sigma), 10^{-8} M dexamethasone (Sigma) and 100 ng/ml BMP2 for 3 weeks on gelatin-coated plates.

For differentiation into skeletal muscle, cells were grown for two weeks in medium supplemented with 5% (v/v) FCS and 2% (v/v) Ultroser SF (Biosepra) on gelatin-coated plates. RT-PCR analyses for differentiation-specific genes were performed at the end of the differentiation phase.

Transfection

The plasmid expressing dominant-negative myocardin (Myocd DN, mutant 128–513) and the control plasmid, both permitting selection by neomycin resistance, were kindly provided by Eric Olson, University of Texas, Southwestern Medical Center, TX (Wang et al., 2001). pcDNA 3.1 empty vector (1 μg) or pcDNA 3.1-Myocd DN (1 μg) were linearised for transfection. Clones 3 and 1, expressing high levels of myocardin, were transfected, at 40% confluence, using the FuGENE 6 reagent (Roche) at a reagent:plasmid ratio of 3:1. Stably transfected cells were obtained after selection with 600 μg/ml G-418 for 14 days, and analyzed in the following passages.

RT-PCR and SYBR green qRT-PCR

Total RNA from cultured cells was purified using RNeasy (Qiagen) and RNA from freshly dissected tissues with TRIzol (Invitrogen). cDNAs were prepared from 250 ng of total RNA using Superscript III reverse transcriptase (Invitrogen) and random hexamers (Roche) in 20 μl according to the manufacturer's instructions.

Semi-quantitative RT-PCR analysis was performed with 1 μl cDNA in a total volume of 25 μl using primers located in different exons (supplementary material Table S1) and Taq polymerase (Bioline). GAPDH was used as a control. Amplification steps were as follows: 94℃ for 30 seconds, 56℃–60℃ for 30 seconds, 72℃ for 30 seconds, followed by a 10-minute incubation at 72℃, after the indicated number of cycles (supplementary material Table S1).

Quantitative PCR was performed using a StepOne Plus cycler (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems) with the indicated primers (supplementary material Table S1).

Expression levels of *Pax3* and *myocardin* transcripts were normalised to expression levels of *GAPDH* transcripts as a control gene, and are represented as fold change over values derived from control (Pax3)–GFP-positive cell populations. Results are from triplicate qRT-PCR analysis from two independent experiments. Statistical analysis was performed using a one-way nonparametric ANOVA test and Bonferroni's multiple comparison post-test (GraphPad Prism 5 software). $P<10^{-2}$ was considered significant.

β-galactosidase detection and immunostaining of sections

For in toto staining, adult tissues or organs were fixed for 30 minutes in 4% paraformaldehyde (PFA). The aorta and other large arteries were exposed by dissection. The activity of β-galactosidase was detected histochemically as described previously (Houzelstein et al., 1997). Blood vessels were photographed under a dissecting microscope, equipped with an Olympus DP70 camera.

Sections were prepared from dissected arteries that were fixed, embedded in OCT compound and sectioned at 10 μm. Sections were stained overnight at 37℃ with X-gal, for detection of β-galactosidase.

Before immunostaining, sections were treated as previously described (Goupille et al., 2008). Primary antibodies were anti-SMA-Cy3 (clone 1A4; Sigma; dilution 1:2000); anti-CD31 (clone MEC13.3; BD Biosciences; dilution 1:100); anti-GFP (598; MBL; dilution 1:1000). Fluorescent secondary antibodies, anti-rabbit or anti-rat IgG Alexa Fluor 488 or 594 (Molecular Probes) were used at 1:300 dilution. Nuclei, were visualized with Hoechst staining (Sigma). Sections were observed and photographed using a Zeiss Axiophot fluorescence microscope equipped with an Axiocam camera.

Cell immunostaining

Cells grown on 3.5 mm plates were fixed with 4% PFA for 5 minutes. Fixed cells were washed twice with PBS and incubated with 2 M HCl for 30 minutes. Acid treatment was neutralized by washing the cells five times with 0.1 M borate buffer (pH 8.5). Cells were then washed twice with PBS, permeabilized with 50 mM NH_4Cl, 0.2% Triton X-100 in PBS for 5 minutes, washed again twice with PBS and incubated in PBS containing 5% FCS, 1% BSA and 0.5% Triton X-100 for 1 hour at room temperature to prevent non-specific binding. The monoclonal anti-BrdU antibody (BD Biosciences; clone B44) and the polyclonal anti-MyoD antibody (Santa Cruz; C-20) were applied overnight at 4℃ at a 1:100 dilution. Secondary anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 594 antibodies were incubated for 2 hours at room temperature at a 1:300 dilution. Nuclei were stained with Hoechst (Sigma).

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