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Limb bud colonization by somite-derived angioblasts is a crucial step for myoblast emigration

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SUMMARY

We have combined the use of mouse genetic strains and the mouse-into-chicken chimera system to determine precisely the sequence of forelimb colonization by presomitic mesoderm (PSM)-derived myoblasts and angioblasts, and the possible role of this latter cell type in myoblast guidance. By creating a new Flk1/Pax3 double reporter mouse line, we have established the precise timetable for angioblast and myoblast delamination/migration from the somite to the limb bud. This timetable was conserved when mouse PSM was grafted into a chicken host, which further validates the experimental model. The use of *Pax3*$^{GFP/GFP}$ knockout mice showed that establishment of vascular endothelial and smooth muscle cells (SMCs) is not compromised by the absence of Pax3. Of note, *Pax3*$^{GFP/GFP}$ knockout mouse PSM-derived cells can contribute to aortic, but not to limb, SMCs that are derived from the somatopleure. Finally, using the *Flk1*$^{lacZ/lacZ}$ knockout mouse, we show that, in the absence of angioblast and vascular network formation, myoblasts are prevented from migrating into the limb. Taken together, our study establishes for the first time the time schedule for endothelial and skeletal muscle cell colonization in the mouse limb bud and establishes the absolute requirement of endothelial cells for myoblast delamination and migration to the limb. It also reveals that cells delaminating from the somites display marked differentiation traits, suggesting that if a common progenitor exists, its lifespan is extremely short and restricted to the somite.

KEY WORDS: Flk1, Pax3, Angioblast, Mouse-into-chicken chimera, Myoblast, Presomitic mesoderm

INTRODUCTION

Endothelial and myogenic precursors of the body wall and limbs are both derived from the somites, structures that segment along the anteroposterior axis following segregation of the presomitic mesoderm (PSM) and differentiate into distinct specialized compartments. Somites provide angioblasts and myoblasts that migrate into the limb to form differentiated tissues. One of the key issues regarding limb bud formation is the time schedule and the mechanisms by which these precursors enter the limb bud. This has been addressed in the avian embryo but remains largely unresolved in the mouse embryo, despite the existence of many genetic models.

The somitic origin of limb bud myoblasts was first shown in the avian model by grafts of quail somites into chicken hosts (Beresford, 1983; Chevallier et al., 1977; Christ et al., 1977; Hayashi and Ozawa, 1995; Jacob et al., 1979; Lance-Jones, 1988; Newman et al., 1981; Ordahl and Le Douarin, 1992; Schramm and Solursh, 1990). Graft analyses showed that myoblasts delaminate from the lateral region of the somite and migrate towards the limb bud between embryonic day (E) 2 (HH15 or the 24-somite stage) and E3 (HH20 or the 40-somite stage) (Chevallier et al., 1978; Christ et al., 1977; Solursh et al., 1987; Tozer et al., 2007).

The quail-chicken model also allowed the identification of two sources of angioblasts: (1) the splanchnic mesoderm, which gives rise to angioblasts vascularizing the viscerae and forming the double primitive aortic anlage (Noden, 1989); and (2) the somites, which contribute the entire endothelial network of the body wall and limbs (Noden, 1989; Pardanaud et al., 1996; Wilting et al., 1995). In addition to the trunk and limbs, somite-derived endothelial cells (ECs) have also been shown to contribute to the dorsoventral patterning of the aorta (Pardanaud et al., 1996; Pouget et al., 2006; Pouget et al., 2008). Angioblast colonization occurs at the 21-somite stage (Pardanaud et al., 1987) in forelimb facing somites 16-21 (Lance-Jones, 1988; Zhi et al., 1996), i.e. three somite-pair stages earlier than myoblasts. Finally, the somite has also been shown to provide smooth muscle cells (SMCs) of the aorta and trunk vessels that exhibit a major contribution to the formation of the vascular network (Pouget et al., 2008; Wiegreffe et al., 2007; Wiegreffe et al., 2009).

Using the mouse-into-chicken system, the onset of mouse myoblast delamination and migration to the forelimb was shown to take place around the 20-somite stage (Houzelstein et al., 1999; Sze et al., 1995), but the precise stage remains to be determined. Using the same experimental system, Ambler et al. (Ambler et al. , 2001) demonstrated that mouse PSM-derived angioblasts vascularize the body wall, limbs and kidney of the chicken embryo host without mapping their level of origin or their final destination. Finally, Tozer et al. (Tozer et al., 2007) briefly reported that the E9.5 mouse limb bud is already vascularized by the time myoblasts are initiating their delamination. Thus, neither the timetable for colonization nor the requirements for myoblasts emigration into the limb bud have been thoroughly addressed in the mouse model.

At the molecular level, transcription factor Pax3 controls the myogenic migration process in both avian and mouse species (Bober et al., 1994; Daston et al., 1996; Goulding et al., 1994; Relaix et al., 2003; Williams and Ordahl, 1994). Both delamination and migration appear to depend on the presence of the c-met receptor tyrosine kinase, a Pax3 target gene that associates with its ligand scatter factor/hepatocyte growth factor (SF/HGF) (Bladt et al., 1995; Brand-Saberi et al., 1996; Dietrich et al., 1999; Scaal et al., 1999; Schmidt et al., 1995). Once in the limb, myogenic cells start to express the regulatory myogenic factors Myf5 and MyoD, respectively (reviewed by Tajbakhsh, 2003). Angioblast

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determination was shown to be dependent on the tyrosine kinase receptor *Flk1* gene (Kdr – Mouse Genome Informatics), which encodes vascular endothelial growth factor receptor 2 (Vegfr2) (Dumont et al., 1998; Fong et al., 1995; Sato et al., 1995; Shalaby et al., 1995; Shivdasani et al., 1995; Visvader et al., 1998). Targeted inactivation of Flk1 leads to early embryonic death due to absence of ECs (Shalaby et al., 1995). Flk1 expression has been detected in the dorsolateral region of the somites precisely where the first angioblasts differentiate (Eichmann et al., 1993; Ema et al., 2006; Tozer et al., 2007).

The early colonization of the limb bud by ECs prompted investigators to propose that these cells could guide myoblast migration (Solursh et al., 1987). Huang et al. (Huang et al., 2003), however, demonstrated that ECs and myogenic cells derived from a unique quail somite grafted into a chicken host migrated independently and distributed into different locations, suggesting the absence of a relationship between the two cell type origins. A late role for ECs in splitting muscle masses was proposed by Tozer et al. (Tozer et al., 2007) but the migratory connections between the two cell lineages need further investigation.

Although the approaches described above indicate that angioblasts colonize the limb prior to myoblasts, no experimental analysis has determined the respective time schedules for these colonization processes. Furthermore, no studies have explicitly unravelled the development of the endothelial, smooth and skeletal muscle cells somitic derivatives or the role of Pax3 and Flk1 in the establishment of lineage patterning in the limb.

In addition, Pax3 and Flk1 are concomitantly expressed in the ventrolateral region of the somite, suggesting the existence of a bipotent progenitor (Eichmann et al., 1993; Ema et al., 2006; Kardon et al., 2002; Tozer et al., 2007). Whether the limb bud is colonized by progenitors endowed with multiple potentials or by single-lineage restricted cells remains to be established.

In the present study, we establish the precise timetable for mouse forelimb colonization by endothelial and myogenic precursors. This was achieved by using a novel Flk1/Pax3 double reporter mouse line, combined with the mouse-into-chicken PSM grafting technique. Results obtained with this latter approach are completely in accordance with the events occurring during normal mouse development, further validating the mouse-into-chicken technique for investigating the steps involved in endothelial, smooth and skeletal muscle lineage development in the trunk and limbs. More importantly, by using Pax3- or Flk1-deficient PSM, we demonstrate for the first time that acquisition of migratory competence of mouse myogenic progenitors requires Flk1 expression in Pax3-expressing hypaxial dermomyotomal cells. We show that $Pax3^{-/-}$ somitic cells give rise to vascular SMCs of the aorta. We also show that unlike ECs, SMCs of the limb blood vessels are not recruited from somites but from the somatopleure, and that their differentiation occurs independently of Flk1.

MATERIALS AND METHODS

Chicken embryos

Fertilized chicken eggs (*Gallus gallus*) from Couvoir Ferron (Le Lourroux Béconnais, France) were incubated at 37°C in a humidified atmosphere. The embryos were staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951). For mouse PSM grafting experiments, chicken eggs were incubated until they reached the 15-somite pair stage (HH11-12). For limb bud grafting onto the chorio-allantoic membrane (CAM), 5.5 day-old embryos were used (HH27).

Mouse embryos

For mutant analysis, $Flk1^{lacZ/+}$ (Shalaby et al., 1995) female mice were crossed with $Pax3^{GFP/+}$ (Relaix et al., 2005) male mice to obtain $Flk1^{lacZ/+}/Pax3^{GFP/+}$ double heterozygote embryos. Embryos were collected at 9 dpc (15 somites), 9.5 dpc (21 somites), 10.5 dpc (33 somites) and 11.5 dpc (45 somites) (Fig. 1). The day of the vaginal plug was considered to be 0.5 days post-coitum (dpc).

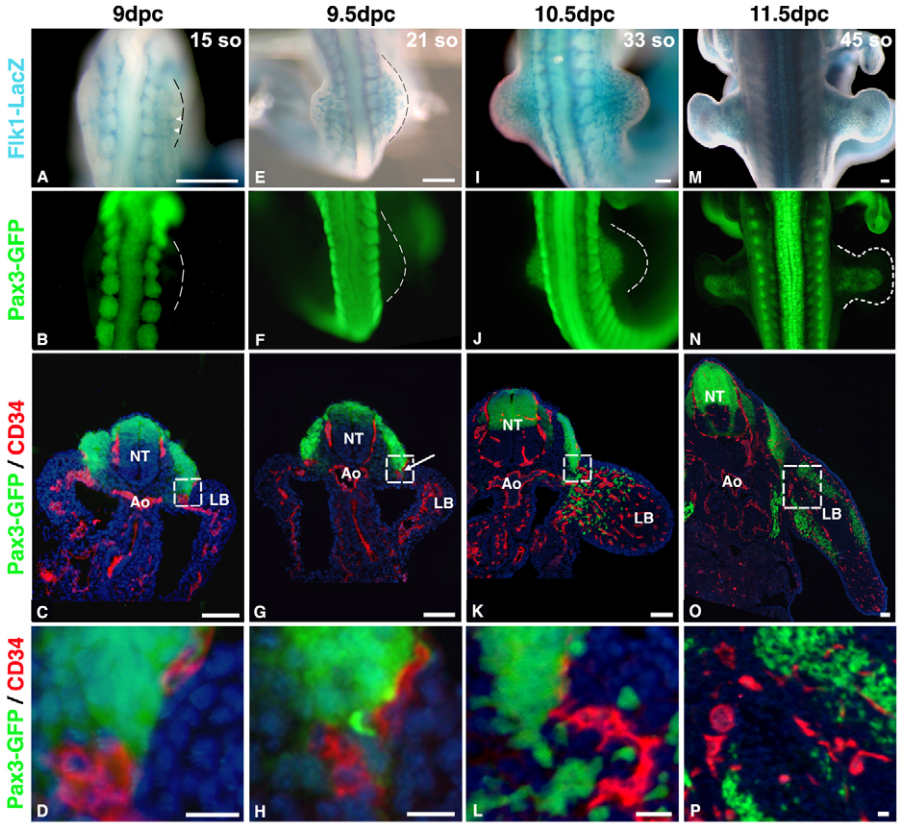

Fig. 1. Forelimb bud colonization studied in transgenic *Flk1-lacZ*/*Pax3-GFP* double reporter mouse embryos. (A,E,I,M) Whole-mount staining for *lacZ* activity detecting Flk1$^+$ ECs. (B,F,J,N) Identification of Pax3$^+$ myogenic progenitor cells through the GFP fluorescent reporter gene in live embryos. Stippled lines indicate the limb contour. (C,G,K,O) CD34$^+$ angioblasts and Pax3$^+$ myoblasts identified on transverse sections. DAPI-stained nuclei in blue. (D,H,L,P) Higher magnification of the framed areas in C,G,K,O. (**A-D**) ECs [9 dpc (15 somites)] are exiting the somites (arrowheads in A). Pax3 expression is restricted to the somites. (**E-H**) Flk1$^+$ ECs have colonized the limb 12 hours later (21 somites). A Pax3$^+$ cell (G, arrow in inset) is delaminating from the ventrolateral dermomyotome. (**I-L**) Flk1$^+$ (I) and CD34$^+$ (K) ECs [10.5 dpc (33 somites)] have invaded the whole limb, while myogenic cells (J,K) are restricted to the proximal zone. (**M-P**) The limb vascularization at 11.5 dpc (45 somites) has patterned into a network arranged around two muscular masses. Ao, dorsal aorta; LB, limb bud; NT, neural tube. Scale bars: 800 μm in A,B,E,F,I,J,M,N; 250 μm in C,G,K,O; 50 μm in D,H,L,P.

For mouse xenografts into chicken embryos, 8.5-9 dpc embryos were used from a cross between two GFP$^+$ transgenic parents (Okabe et al., 1997). *Flk1*$^{lacZ/+}$ male mice were crossed with *GFP* transgenic female mice, in which the GFP reporter gene is under the control of the ubiquitously expressed β-actin promoter. The *GFP/Flk1*$^{lacZ/+}$ mice were then mated to obtain *GFP/Flk1*$^{lacZ/lacZ}$ embryos. *Tie2-lacZ* transgenic mice (The Jackson Laboratory) were used according to a similar protocol to obtain *GFP/Tie2-lacZ* mice. Finally, transgenic *GFP* mice were crossed with *Des*$^{lacZ/+}$ mice (Li et al., 1996) to obtain *GFP/Des*$^{lacZ/+}$ embryos.

Mouse-into-chicken chimeras

GFP$^+$ PSM from a mouse embryo was grafted orthotopically and unilaterally into chicken hosts, as previously described (Fontaine-Perus et al., 1995). The PSM of 15-somite chicken embryos (HH11-12) was removed on the right-hand side. PSM from a 9- to 15-somite stage mouse was deposited according to its original dorsoventral and anteroposterior orientation (Fig. 2A). Chimeric embryos were incubated for an additional period of 12 to 120 hours.

Chorio-allantoic membrane grafting

Chicken chimeric forelimbs were isolated at different developmental stages between HH17 and HH19. They were transplanted onto the CAM of 5.5 day-old chicken embryos (Fig. 3A). These grafted limbs were fixed 3-4 days post-implantation.

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde (PFA), cryoprotected in 15% sucrose buffer and frozen in liquid nitrogen. Sections (10 μm) were collected on Superfrost slides (Thermo) and stored at –80°C. After rehydration in PBS, sections were blocked with PBS/10% FCS for 1 hour. The following primary antibodies were used: (1) anti-CD34 and anti-Flk1 (BD Pharmingen) to detect mouse ECs, and MEP-21 (McNagny et al., 1997) to detect chicken ECs; (2) anti-α-smooth muscle actin (α-SMA, Sigma-Aldrich) to detect SMCs; and (3) anti-Pax3 (Developmental Studies Hybridoma Bank), anti-myogenin (Myog) and anti-myosin heavy chain (My-HC) (Sigma-Aldrich, clone My-32) to detect myogenic cells. Anti-Pax3 allows the early detection of the

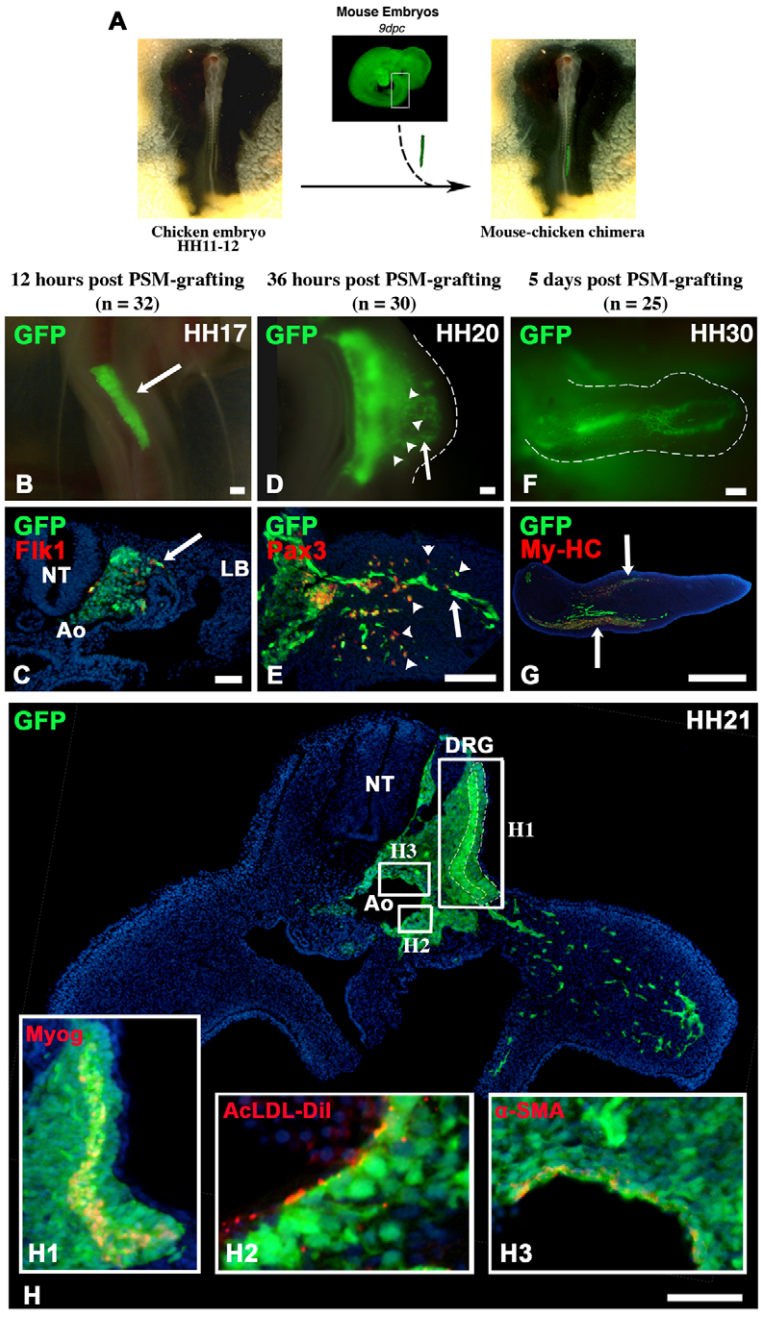

Fig. 2. Graft of mouse PSM into a chicken embryo. (**A**) Scheme of the graft. (B,D,F) UV illuminated live embryos, graft level. (C,E,G) Transverse sections stained with anti-Flk1-rhodamine (C), anti-Pax3 (E) and anti-My-HC (G). (**B**,**C**) The mouse GFP$^+$ PSM (arrow in B) has segmented into somites 12 hours post-grafting (HH17). Some pioneer GFP$^+$/Flk1$^+$ cells are leaving the grafted somites (C, arrow), whereas some others remain within the structure. (**D**,**E**) GFP$^+$ PSM-derived cells 36 hours post-grafting (HH20) have invaded the host limb bud. GFP$^+$ ECs have organized into tubules (arrow) whereas GFP$^+$/Pax3$^+$ myogenic cells are dispersed within the limb proximal zone (arrowheads). (**F**,**G**) Five days post-grafting (HH30). Mouse cells have organized into endothelial tubules and dorsal and ventral muscular My-HC$^+$ masses (arrows) (G). (**H**) Transverse section of a PSM graft 24 hours post-surgery (HH21). The myotome expresses myogenin (H1). At the dorsal aorta level, mouse ECs that line the vessel retain LDL-DiI (H2), whereas adjacent GFP$^+$ cells start to express smooth muscle actin (H3). Ao, dorsal aorta; DRG, dorsal root ganglia; LB, limb bud; NT, neural tube. *n*, number of experiments. Scale bars: 150 μm.

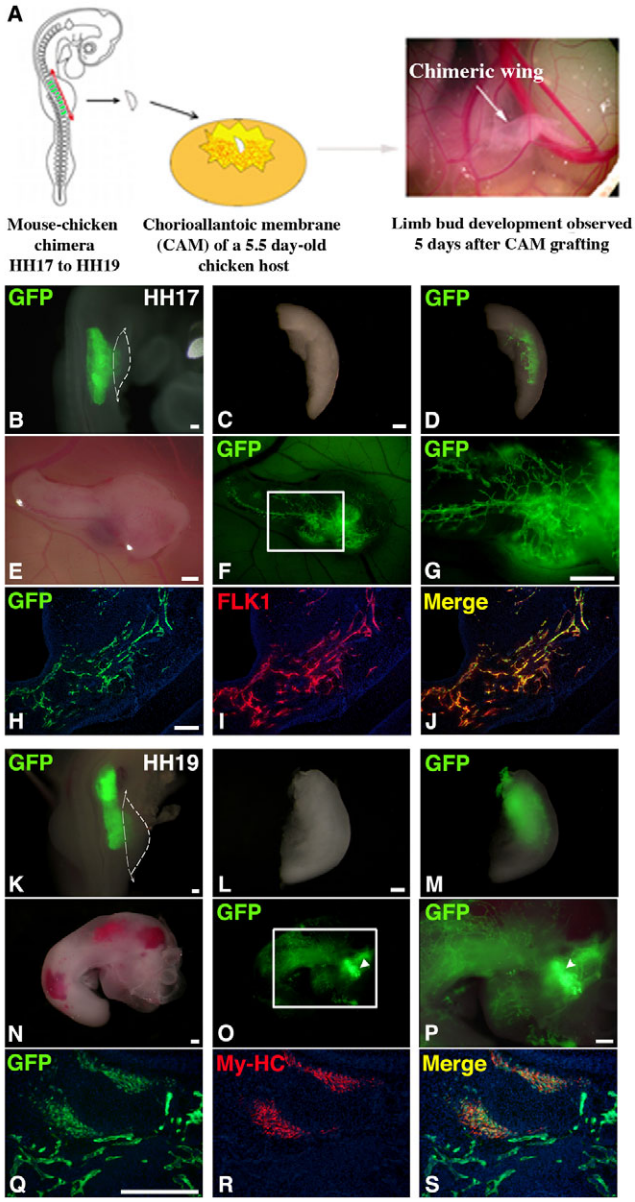

Fig. 3. Forelimb bud colonization by PSM-derived angioblasts and myoblasts analyzed through double-step grafting. (**A**) Scheme of the experiment. The limb has pursued its development on the CAM, acquiring the aspect of a wing (arrow in the picture on the right). (**B-D**) Twenty hours after PSM grafting (HH17), host limb bud in situ (B, UV light) and after dissection (C, visible light; D, UV light). Few GFP$^+$ cells have invaded the limb (D). (**E-G**) Three more days after grafting onto the CAM, the limb has developed and GFP$^+$ cells have organized into a complex superficial network. (**H-J**) Double GFP-Flk1 immunostaining of the chimeric limb showing that all GFP$^+$ cells are ECs. (**K-M**) Twenty-eight hours after PSM grafting (HH19), development and colonization of the limb has increased. (**N-P**) Four additional days after grafting onto the CAM, dorsal view of the re-transplanted limb shows numerous GFP$^+$ cells found at deeper levels (arrowheads). (**Q-S**) Two GFP$^{+/}$My-HC$^+$ muscular masses are identified on sections. Scale bars: 250 μm.

myoblast lineage until myogenin and myosin appear in differentiating muscle masses. Sections were incubated overnight at 4°C with primary antibodies diluted 1/100 in PBS/10% FCS. After several washes in PBS, sections were incubated for 90 minutes at room temperature with secondary antibodies diluted 1/100 in PBS. CD34 and Flk1 were revealed with rhodamine-conjugated goat anti-rat IgG2a (Southern Biotech). MEP21, Myog and My-HC were revealed with rhodamine-conjugated goat anti-mouse IgG1 and Pax3 with a rhodamine-conjugated goat anti-mouse IgG2a (Molecular Probes). Anti-α-SMA was visualized with a goat anti-mouse IgG2a coupled to either rhodamine or Alexa Fluor 350 (Molecular Probes). To visualize the endothelial network, human acetylated low density lipoprotein coupled to DiI (AcLDL-DiI, Molecular Probes) was injected into the heart of embryos that were analyzed 6 hours post-injection. Sections were finally counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize nuclei.

β-Galactosidase detection

Mouse embryos were fixed in 4% PFA, washed in PBS and whole-mount stained in PBS containing 2 mM 5-bromo-4-chloro-3-indolyl-β-D-galactosidase, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$ and 0.5% Triton X-100 (Sigma-Aldrich). Embryos were incubated at 37°C for 2-6 hours, then washed in PBS.

RESULTS

The vascular network of the limb bud is established prior to colonization by myogenic cells

During formation of the limb bud pattern, the precise timing at which EC and skeletal muscle precursors invade the bud has not been completely ascertained. In a previous study, Houzelstein et al. (Houzelstein et al., 1999) described the spatial and temporal contribution of mouse somites to the limb musculature with a Pax3 probe and determined that myoblasts delaminated from the dermomyotome when the embryo reached the 23-somite stage. However, this study used whole-mount approaches to establish the migration time, which may underestimate the onset of migration especially when a few dispersed single cells are concerned. Tozer et al. (Tozer et al., 2007) reported that mouse limb bud vascularization precedes myoblast colonization (at E9.5 dpc).

To address this issue, we created a new mouse reporter line for endothelial and muscle precursor by crossing the *Flk1*$^{lacZ/+}$ mouse strain to the *Pax3*$^{GFP/+}$ mouse strain. Double reporter embryos between 9 and 11.5 dpc were precisely analyzed to decipher how and when endothelial and myogenic precursors enter the limb bud. At 9 dpc (15-somite stage embryo, *n*=6), a few Flk1$^+$ ECs (Fig. 1A, arrowheads) were observed in the presumptive forelimb region, while Pax3$^+$ (probably myogenic) cells were still localized in the somites (Fig. 1B). This was confirmed on sections using CD34 immunostaining as an EC marker (Fig. 1C,D). At 9.5 dpc (21-somite stage embryo, *n*=10), ECs have colonized the whole limb except in its most distal part (Fig. 1E). No conspicuous Pax3$^+$ cells were identified leaving the somite (Fig. 1F). However, section analysis revealed that the first Pax3$^+$ cells delaminated from the ventrolateral lip of the somite (Fig. 1G, arrow; Fig. 1H). At 10.5 dpc (33-somite stage embryo, *n*=12), ECs have organized into a network (Fig. 1I) whereas Pax3$^+$ cells colonized the limb in a dispersed pattern (Fig. 1J-L). Furthermore, immunostaining indicates that both progenitors are committed as they leave the somite (see also supplementary material Fig. S1). At 11.5 dpc (45-somite stage embryo, *n*=12), vascularization became more complex and Pax3$^+$ cells have organized into dorsal and ventral muscular masses (Fig. 1M-P). Our results indicate that the onset of delamination of somite-derived Pax3$^+$ myogenic cells towards the forelimb takes place at precisely the 21-somite stage, when ECs have already invaded the limb bud and formed a vascular plexus.

Mouse-into-chicken chimeras as a model to study mouse PSM derivatives

Despite convincing reports in the chicken model, the origin(s) of angioblasts colonizing the mouse limb remains to be precisely determined. Because this crucial biological question cannot be directly addressed in the mouse embryo, we employed the mouse-into-chicken technique (Fontaine-Perus et al., 1995). We used transgenic embryos ubiquitously expressing GFP (Fig. 2A) as donors. This experimental model allows a single mouse cell emigrating from the grafted PSM to be visualized in the live embryo under a UV-equipped dissecting microscope.

For reliability of the results, it was crucial to use a stage at which no cell emigration has yet occurred in the avian embryo. The 15-somite stage was chosen because PSM faces the presumptive limb bud level at this stage. For the mouse, the eight-somite stage was chosen for the same reasons. However, because of high variability between littermate embryos, PSM from 8- to 15-somite stages were used, with no difference in the results.

In our experimental conditions, 2 hours were required for the mouse PSM to form one somite in the chicken host. Twelve hours after surgery (HH17), the first GFP$^+$ cells had detached from the newly formed somites facing the limb (Fig. 2B, arrow). Transverse sections revealed that the graft was perfectly integrated into the chicken host. Mouse-specific Flk1 EC expression was already visible within the graft (Fig. 2C, arrow; for a more detailed illustration, see also supplementary material Fig. S2). Thirty-six hours post-grafting (HH20), the limb had developed and colonization had intensified (Fig. 2D,E). GFP$^+$ cells were seen all over the limb with the exception of the most distal region; however, they were found concentrated in the proximal zone (Fig. 2D, arrowheads). A tubular vascular network encompassing the whole limb had organized (Fig. 2E, arrow), whereas dispersed Pax3$^+$ myogenic cells were found in the proximal region of the limb bud (Fig. 2E, arrowheads). The organized vascular network compared with the scattered Pax3 pattern suggests that ECs colonized the bud prior to myoblasts. Later on (HH30), a conspicuous superficial GFP$^+$ network formed in the limb, while a fluorescent mass was observed in depth (Fig. 2F). Sections showed that GFP$^+$ cells have contributed to the vascular network and also gave rise to the dorsal and ventral muscle masses, as identified by the expression of myosin heavy chain (Fig. 2G, arrows). Thus, mouse PSM first emitted ECs, followed by myogenic cells delaminating from the somite. Under our conditions, the mouse PSM appeared to be a major source of host limb ECs as demonstrated by specific CD34 immunostaining in a chimera 3 days after the graft (supplementary material Fig. S3).

Two days after grafting (HH21), mouse PSM had differentiated into somites that had segregated into dermomyotome and sclerotome (Fig. 2H). The GFP$^+$ myotome expressed the myogenic regulatory factor Myog (Fig. 2H1). Interestingly PSM provided both GFP$^+$/Ac-LDLDiI$^+$ ECs (Fig. 2H2) and a GFP$^+$/α-SMA$^+$ smooth muscular tunica around the dorsal aorta (Fig. 2H3). To gain additional insights into vascular and muscular contributions, we performed PSM xenografts using *Flk1*$^{lacZ/+}$ and *Tie2-lacZ* transgenic mice or *GFP/Des*$^{lacZ/+}$ transgenic mice, which allow labeling ECs or myogenic cells, respectively. The use of these mutant lines, in which embryonic development is not altered, enabled us to strengthen our previous observations on the colonization sequence of the limb by the different progenitors and their respective spatial organization (see supplementary material Figs S4, S5).

Thus, mouse PSM responded to chicken environmental cues and provided all the normal derivatives, i.e. endothelial, smooth and skeletal muscular cells that subsequently migrated to their specific targets. Our results in the chicken host limb bud suggested that mouse PSM sequentially provided endothelial and myogenic cells in a similar manner to that observed in the *Flk1*$^{lacZ/+}$/*Pax3*$^{GFP/+}$ double transgenic mouse embryo.

Establishing the calendar of limb bud colonization by PSM-derived endothelial and myogenic cells through double-step grafting

To further establish the precise time points at which endothelial and myogenic precursors entered the limb bud, we decided to use a combination of mouse-into-chicken chimera followed by chorio-allantoic membrane (CAM) grafts of the chimeric limbs. This experimental scheme allowed us to avoid further immigration of PSM-derived cells into the limb bud and to identify discrete cell populations that have colonized the limb because the structure has grown. Briefly, mouse PSM were grafted into chicken hosts and allowed to develop. The chimeric limb bud was retrieved at different time points and grafted on the CAM of a recipient avian embryo (Fig. 3A). Under these conditions, the isolated limb could pursue its development in the absence of further somitic cell colonization. Because a tiny limb bud had developed, the discrete cell populations colonizing the bud had grown and could now be easily identified and their contribution established.

When isolated 20 hours after PSM grafting (HH17), the limb contained a small population of GFP$^+$ cells (Fig. 3B-D). Three days after grafting on the CAM, the limb had grown and this small population of GFP$^+$ cells had formed a highly branched GFP$^+$ network (Fig. 3E-G) that, on sectioning, expressed Flk1 (Fig. 3H-J). Thus, 20 hours after delamination, mouse PSM had provided angioblasts to the limb, but no myogenic cell (supplementary material Fig. S6).

Twenty-eight hours after PSM grafting (HH19), the chimeric limb contained many GFP$^+$ cells (Fig. 3K-M). After 3-4 days of grafting onto the CAM, the branched GFP$^+$ network had extended. A GFP$^+$ cell mass (Fig. 3N-P, arrowheads) containing many My-HC$^+$ myogenic cells (Fig. 3Q-S) had formed deep into the limb. In order to establish precisely when myoblasts first entered the limb bud, chimeric limbs were obtained from CAM grafts 20 to 28 hours after the initial grafting period. We, thus, establish that myoblasts begin to colonize the limb bud 24 hours after mouse PSM transplantation (Table 1).

Table 1. Colonization timetable of the chicken forelimb by mouse PSM-derived angioblasts and myoblasts, determined in the mouse-into-chicken model followed by CAM grafting

	Chimeric limb bud grafted into the CAM after:					
	20 hours	22 hours	23 hours	24 hours	25 hours	28 hours
Angioblasts	+	+	+	+	+	+
Myoblasts	–	–	–	+	+	+
Number of cases	16	19	23	26	24	18

For time points 20 hours and 28 hours, experiments lasted 3 days, for the other time points they lasted 4 days.

ECs and SMCs can differentiate in the absence of Pax3

Pax3 was shown to be specifically required for skeletal muscle cell migration and specification, but its role in EC and SMC commitment has yet to be determined. This is a crucial issue because, although a common precursor for endothelial, smooth and striated muscle cells has been postulated (Ben-Yair and Kalcheim, 2008; Ema et al., 2006; Kardon et al., 2002), previous studies have demonstrated a normal developmental pattern of vascular smooth muscles in the dorsal aorta and limb vessels in the absence of Pax3 activity (Pax3-null embryos) (Esner et al., 2006; Tozer et al., 2007). Furthermore, as the lateral plate mesoderm has been reported to harbor ECs and SMCs (Pardanaud et al., 1996; Wasteson et al., 2008; Wiegreffe et al., 2007), the origin of limb ECs and smooth musculature of the dorsal aorta in the *Pax3*$^{GFP/GFP}$ embryos remains to be determined. Our mouse-into-chicken PSM grafting procedure thus appears as an appropriate model to address these questions in vivo, especially because Pax3 invalidation is lethal at 13.5 dpc. In the 11.5 dpc *Pax3*$^{GFP/GFP}$ embryos, ECs and SMCs displayed a normal vascular pattern, and a total absence of myogenic cells in the forelimb (Fig. 4A,B) was observed as described previously (Esner et al., 2006; Tozer et al., 2007). Vascular SMCs were present in the limb when analyzed at 13.5 dpc (Fig. 4C), suggesting that they were not dependent on Pax3^{+} somite-derived cells. *Pax3*$^{GFP/GFP}$ PSM were grafted into a chicken host. Two days post-surgery (HH22), GFP^{+} cells were restricted to the trunk, in keeping with the Pax3 knockout phenotype (Fig. 4D). Transverse sections indicated that the grafted PSM gave rise to CD34^{+} ECs that contributed to form the peri-neural vascular plexus (Fig. 4E). This latter structure appeared slightly less developed in the Pax3 mutant than in the wild type (compare with supplementary material Fig. S5). Moreover, mouse *Pax3*$^{GFP/GFP}$ PSM-derived ECs were also detected in the limb (Fig. 4F). In addition, no GFP^{+} cell was found in the vicinity of the aorta 2.5 days after the graft (HH25), the signal being restricted to the dermomyotome (Fig. 4G). However, mouse α-SMA^{+} cells, identified through their chromatin condensation visualized by bis-benzimide staining, were found surrounding the dorsal aorta in a peri-endothelial position (Fig. 4H, arrows). Thus, these experiments point to a crucial role for the somite in the generation of endothelial and SMC lineages, and demonstrate that Pax3 is required neither for their specification nor their migration. Nevertheless, we cannot exclude an early proliferation role for Pax3 on ECs.

Flk1 is not necessary for myogenic differentiation, but is required for the migration of skeletal myogenic progenitors

Extending the mouse-into-chicken findings to the normal mouse embryo prompted us to investigate the role of somite-derived ECs in myoblast migration. We took advantage of the Flk1 mutant that lacks EC differentiation and vessel formation (Shalaby et al., 1995). Because this mutant dies at 9 dpc, the role of somite-derived ECs in myoblast migration cannot be addressed directly in the embryo. We thus grafted Flk1 mutant PSM into the chicken embryo and analyzed myoblast migration in the absence of ECs. In order to trace all the mouse cells in vivo, the *Flk1*$^{lacZ/+}$ mutant was crossed with the transgenic *GFP* reporter mouse. At 9 dpc, *GFP/Flk1*$^{lacZ/lacZ}$ embryos were less developed than their littermates (Fig. 5A). These embryos displayed a normal Pax3 pattern (Fig. 5B).

To characterize myoblast differentiation in the absence of Flk1, we grafted a single *GFP/Flk1*$^{lacZ/lacZ}$ somite in a chicken embryo host (Fig. 5C). Two days after surgery (HH22), the somite had integrated

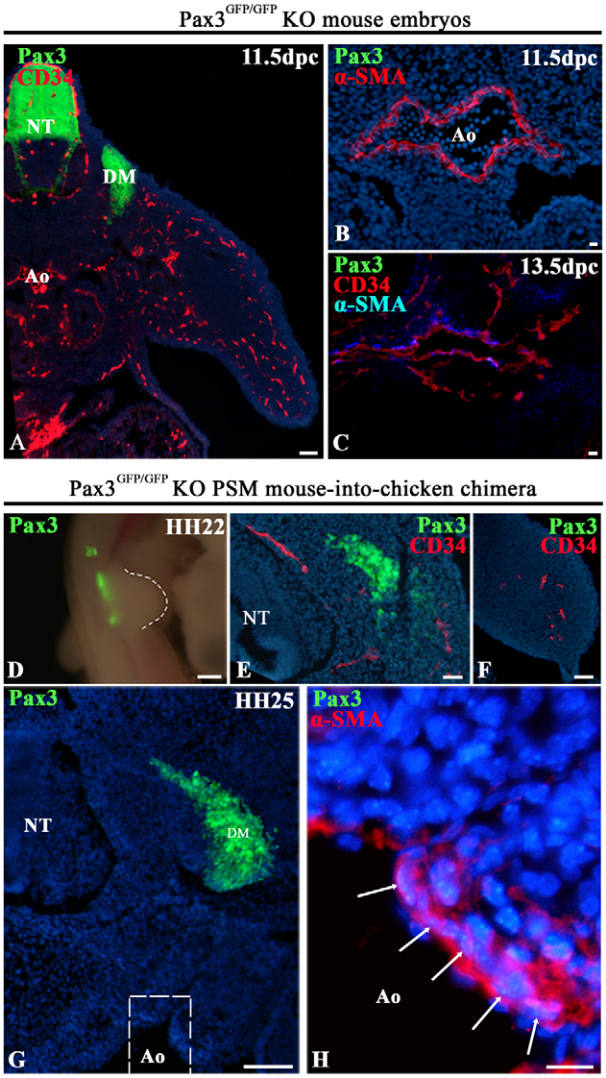

Fig. 4. Differentiation of smooth muscle and endothelial lineages analyzed in Pax3 mutants. (**A**) Section of a 11.5 dpc *Pax3*$^{GFP/GFP}$ mouse embryo at the limb bud level. GFP^{+} cells are present in the dorsal neural tube and the dermomyotome, which is drastically reduced as previously described (Relaix et al., 2003). CD34^{+} ECs display a normal pattern in the trunk and limb. (**B**) Immunostaining for α-SMA. Aortic smooth muscles differentiate in the absence of Pax3. (**C**) Limb level at 13.5 dpc. SMCs are detected. (**D-F**) *Pax3*$^{GFP/GFP}$ PSM graft 2 days post-surgery (HH22). (D) GFP^{+} cells are restricted to the grafted area. (E,F) The grafted PSM provided trunk (E) and limb (F) ECs, as shown by CD34 staining. (**G**) Three days post-grafting (HH25), a reduced dermomyotome has developed in the chicken host. (**H**) Anti-α-SMA staining shows that *Pax3*$^{GFP/GFP}$ PSM has provided the smooth musculature of the dorsal aorta. Note that mouse nuclei can be identified by their chromatin condensation (arrows). Ao, dorsal aorta; DM, dermomyotome; NT, neural tube. Scale bars: 500 μm in A,D,G; 50 μm in B,C,E,F,H.

the host, and some GFP^{+} fibers displaying an anteroposterior orientation were observed (Fig. 5C,E), a pattern similar to that observed after the graft of a single GFP^{+} somite (supplementary material Fig. S7). Transverse sections revealed that the grafted somite has given rise to all the somite derivatives, i.e. dermatome, sclerotome and myotome, the last expressing Pax3 (Fig. 5D) and My-HC (Fig. 5E). Under these conditions, we never observed GFP^{+}

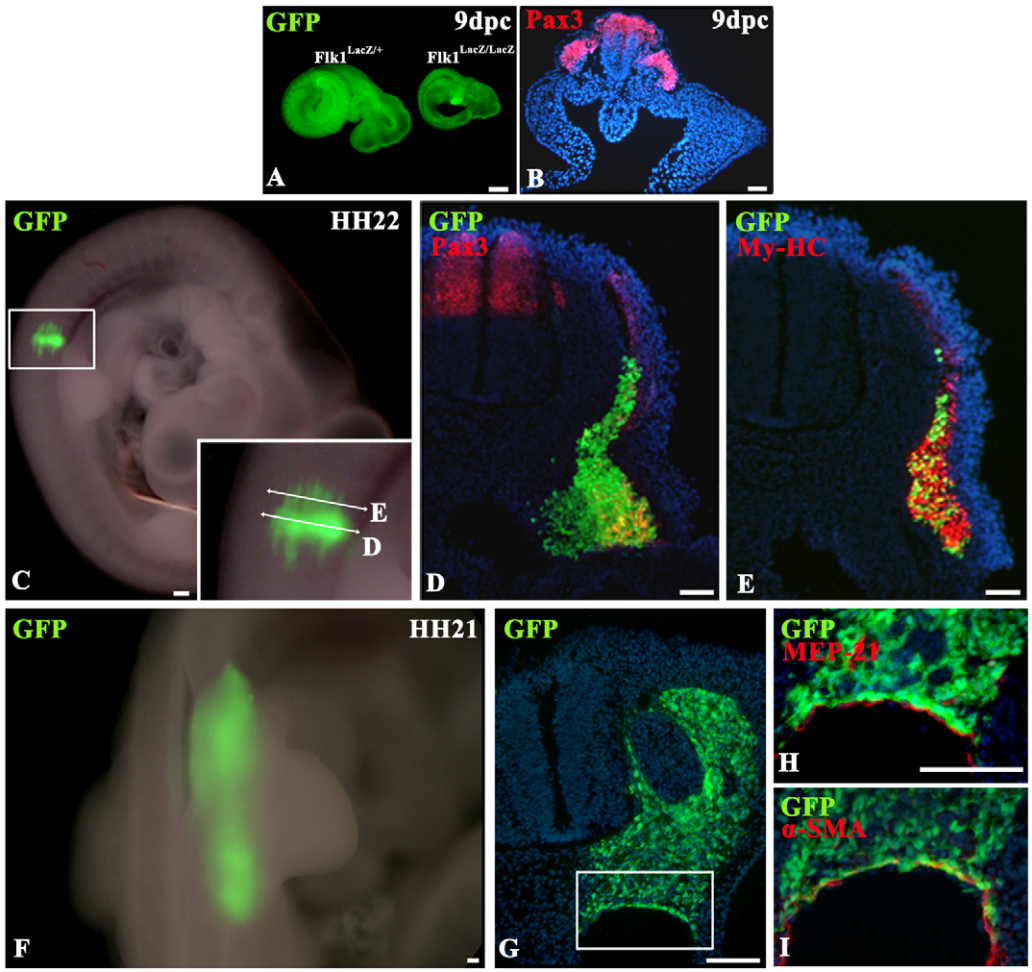

Fig. 5. Role of the Flk1 mutation on trunk and limb skeletal myogenesis. (**A**) *GFP/Flk1*$^{lacZ/+}$ and *GFP/Flk1*$^{lacZ/lacZ}$ mouse embryos at 9 dpc observed in toto. *Flk1* knockout embryos are smaller than their littermates. (**B**) Transverse section at the limb bud level of a *Flk1*$^{lacZ/lacZ}$ embryo. Pax3 immunostaining shows expression in the dermomyotome. (**C**) Graft of a single *GFP/Flk1* knockout somite 2 days post-surgery (HH22). Longitudinal fibers are observed aligned parallel to the neural tube. (**D**) Normal Pax3 expression in the dermomyotome. (**E**) GFP$^+$/My-HC$^+$ fibers are present in the myotome. (**F**) *GFP/Flk1*$^{lacZ/lacZ}$ PSM graft 2 days post-surgery (HH21). GFP$^+$ cells are restricted to the trunk. (**G-I**) Transverse sections show that the graft was correctly inserted in the chicken host. Vascularization is provided by the chicken host, as revealed the MEP-21 labeling (red in H), whereas SMCs of the dorsal aorta derive from the *Flk1* knockout PSM (red in I). Scale bars: 50 μm.

myogenic cells migrating into the limb. This result could not be attributed to species specificity between mouse myoblasts and chicken ECs. Indeed, when GFP$^+$ differentiated somites (i.e. with dermomyotome and sclerotome already formed) are grafted in the chicken embryo, few mouse ECs left the somites, whereas mouse myogenic cells colonized the limb and formed muscular masses, demonstrating that they are able to follow the routes opened by chicken ECs (supplementary material Fig. S8). In some cases, few GFP$^+$/Flk1$^{lacZ/lacZ}$/Pax3$^+$ cells were observed migrating ventrally to the body wall of the chicken embryo (supplementary material Fig. S9). This rules out a general effect of the lack of Flk1 on myogenic migration. Nevertheless, a more in depth analysis is required to analyze precisely this myogenic migration.

Having determined that the absence of ECs and Flk1 did not impair myogenic differentiation, we grafted a *GFP/Flk1*$^{lacZ/lacZ}$ PSM. Two days after the graft (HH21), the GFP signal was restricted to the trunk (Fig. 5F). The limb bud facing the graft had no mouse ECs and fewer chicken ECs (data not shown). More generally, no GFP$^+$ cells were seen in the limb bud, demonstrating that myoblasts migration was impaired in the absence of ECs. Transverse sections, however, showed that mouse PSM had developed in the host embryo trunk (Fig. 5G). The grafted side contained numerous GFP$^+$ cells, indicating that our experimental conditions allowed PSM development in keeping with single somite grafts. As a result of the Flk1 mutation, no GFP$^+$ cells were present in the luminal layer of the dorsal aorta, nor in trunk capillaries and cardinal veins. ECs were exclusively from the host (Fig. 5H). Nevertheless, cells surrounding the endothelial layer of the dorsal aorta were GFP$^+$ and expressed α-SMA, indicating the capacity of the Flk1 knockout PSM to provide vascular smooth muscles of the dorsal aorta (Fig. 5I).

DISCUSSION

Mouse PSM-derived angioblasts and myoblasts colonize the limb according to a two-step pattern

In the present study, we determined the sequence of limb colonization by angioblasts and myoblasts in the mouse embryo, using complementary approaches: (1) the *Flk1*$^{lacZ/+}$/*Pax3*$^{GFP/+}$

heterozygous embryos implemented here for the first time; and (2) the mouse-into-chicken chimera technique, which allowed to precisely determine the origin and the timing of limb colonization by both lineages. We used mouse embryos carrying an ubiquitously expressed GFP gene as donors, which allowed continuous monitoring of the cells emitted by the somites, and thus traced finely the whole developmental process of the mouse PSM. A similar timetable for PSM-derived cell migration at the forelimb level was observed using $Flk1^{lacZ/+}/Pax3^{GFP/+}$ double heterozygous embryos and mouse-into-chicken orthotopic transplantation. The two approaches converged towards the demonstration that mouse PSM-derived endothelial and myogenic progenitors colonize the forelimb in two distinct waves.

Mouse PSM was able to expand correctly in the chicken embryo, usually with the development of six somites in 12 hours, which respects the 'segmentation clock' defined by Dequeant et al. (Dequeant et al., 2006) using transcriptome analyses of the mouse PSM combined with in situ hybridization. In addition, our system of mouse PSM transplantation followed by grafting onto the CAM allowed us to determine more precisely the developmental calendar of the two somite-emitted lineages at the limb level and to propose a schematic model of mouse PSM development (Fig. 6). In a 9-somite stage mouse embryo (8.5 dpc), the PSM faces the future limb bud and takes 12 hours to form the somites that will provide angioblasts and myoblasts to the limb. At the 15-somite stage (9 dpc), the first cells escaping laterally the somites are ECs. Twelve hours later (9.5 dpc) mouse PSM provides myogenic progenitors to the limb, which are already colonized by endothelial progenitors. The chimeric model was unique, because it allowed the establishment of the PSM as the source of endothelial progenitors and the monitoring of its uninterrupted evolution.

In the chicken embryo, it has been described that angioblasts begin to colonize the forelimb at the 21-somite stage (Pardanaud et al., 1987), which corresponds to the completion of somite formation facing the forelimb, whereas myoblasts start to delaminate a few hours later at about the 24-somite stage (Solursh et al., 1987). These observations, combined with our results, suggest that chicken and mouse PSM follow a similar developmental pattern at the forelimb level, with a small delay for mouse myoblast delamination, reflecting species differences in developmental timing; as a matter of fact, 2 hours are needed for one somite to form in the mouse, versus 1.5 hours in the chicken (Palmeirim et al., 1997).

Mouse angioblasts and myoblasts are already committed when they leave the somites

Previous studies in the chicken embryo have shown that PSM is the only structure that provides ECs to the limb (Pardanaud et al., 1996; Pouget et al., 2006; Wilting et al., 1995). Here, we show that, in the mouse, the PSM not only provides ECs to the limb, but also is likely to be the unique source for these progenitors (supplementary material Fig. S3). Furthermore, in our grafting conditions, we never identified ECs leaving the somites as being $Pax3^{+}$, and, when re-grafted onto the CAM fewer than 24 hours

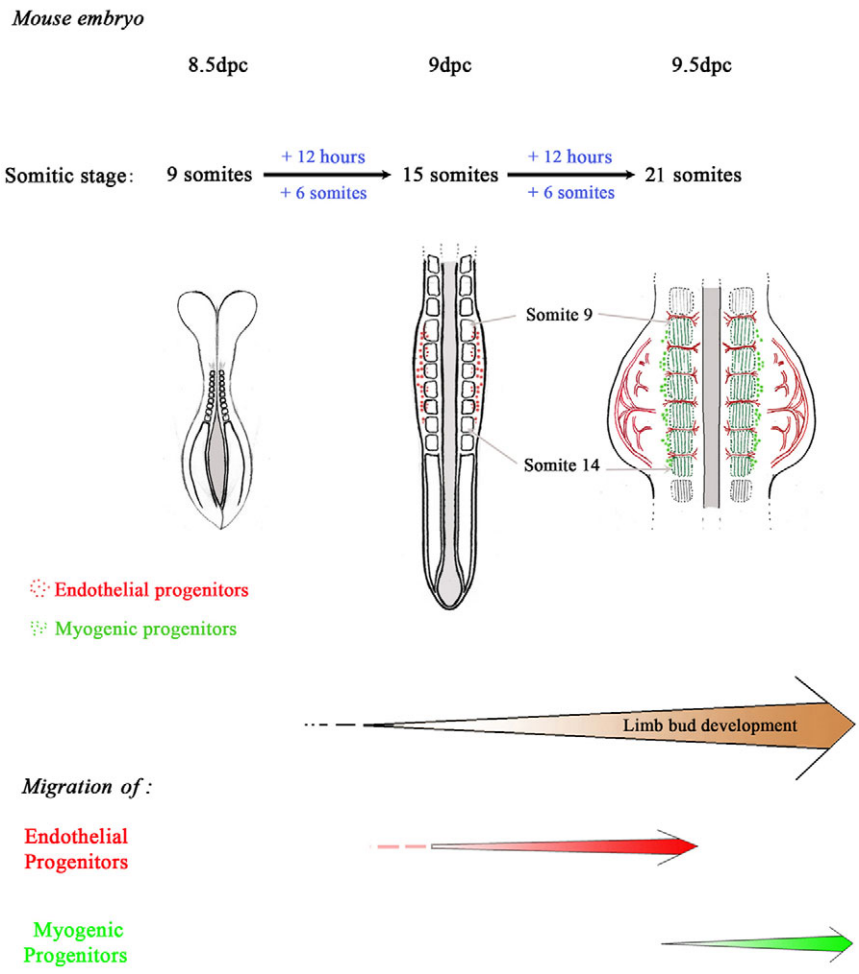

Fig. 6. Proposed model for mouse PSM development at the forelimb level. In the 9-somite stage embryo (8.5 dpc), the PSM faces the future limb bud. Twelve hours later (15-somite stage embryo, 9 dpc), the first cells that escape towards the limb are angioblasts (red dots), which subsequently colonize the bud. Twelve hours later (21-somite stage embryos, 9.5 dpc), the limb is vascularized by PSM-derived ECs while myoblasts start to delaminate from the somites (green dots).

after mouse PSM transplantation, the colonized limb bud displayed mouse ECs, but no mouse myoblasts. These results strongly support the interpretation that the cells leaving the somite are already committed to a specific lineage, and that a putative non-committed bipotential progenitor could exist within the somite only for a short period of time (Ema et al., 2006; Hutcheson et al., 2009; Kardon et al., 2002).

Pax3 is not involved in the settlement of PSM-derived ECs and SMCs

Few studies have evaluated the relationships between migrating endothelial and myogenic cells of the limb. Solursh et al. (Solursh et al., 1987) first suggested that ECs might have a guidance role for myogenic cells towards the limb, but this hypothesis was refuted by Huang et al. (Huang et al., 2003) who showed that myogenic cells and ECs from the same somite migrated to the limb through distinct routes. Nevertheless, Tozer et al. (Tozer et al., 2007) proposed that the endothelial network organization foreshadows muscle patterning and that vessels might delimit the future cleavage region of limb muscle masses. In order to bring some clues about the possible relationship between ECs and myogenic cells settlement, we used the *Pax3*$^{GFP/GFP}$ mouse embryo to test the PSM capacity to provide each derivative in the mouse-into-chicken model. Pax3 mutation leading to embryonic death, the transfer of *Pax3*$^{GFP/GFP}$ PSM into the chicken provided a unique tool with which to evaluate the implication of this gene in muscular and vascular lineages. We showed that *Pax3*$^{GFP/GFP}$ PSM was able to provide ECs to the limb in the chicken host, thus demonstrating that Pax3, even though co-expressed with Flk1 in some dermomyotome cells, is not required for migration and differentiation of these cells.

In addition, we found that *Pax3*$^{GFP/GFP}$ PSM provided the smooth musculature of the dorsal aorta with a normal developmental timing, which indicates that, although Pax3^{+} cells had been reported to contribute to ECs and SMCs of the dorsal aorta (Esner et al., 2006), SMCs can pursue their fully potency in vivo in the absence of this gene. It is now known that, in the avian and murine aortae, the primary population of SMCs originates from non-somitic mesoderm (Hungerford and Little, 1999; Takahashi et al., 1996; Wiegreffe et al., 2009), whereas the secondary population emanates from the somites (Pouget et al., 2006; Wasteson et al., 2008; Wiegreffe et al., 2007; Wiegreffe et al., 2009). Our observation also confirmed that mouse somites are the source of the second population of the dorsal aorta SMCs, and that their differentiation occurs independently of Pax3. This is in accordance with a recent study proposing that Pax3 and Foxc2 transcription factors repress each other, and that downregulation of Pax3 leads to specification of undifferentiated somitic cell into smooth muscular fate (Lagha et al., 2009).

Flk1 is essential for myoblast emigration to the limb but dispensable for myogenic and SMC differentiation

Our mouse-into-chicken chimera proved to be a powerful tool with which to study the consequence of Flk1 deletion in the establishment of smooth and skeletal muscle cells derived from the PSM. Indeed, Flk1 deletion leads to embryonic death at 9 dpc, before the beginning of trunk myogenesis and limb myogenic progenitor migration, with somites unable to produce ECs (Shalaby et al., 1995). We could thus demonstrate that PSM in Flk1 knockout embryos is able to activate the myogenic program, when grafted in the chicken embryo. We also found that Flk1 mutation did not impair the development of the smooth musculature of the dorsal aorta, which indicates that Flk1 is not required for the emergence of SMCs. Using the combination of Flk1 mutant and our chimeric procedure, we have unexpectedly demonstrated that SMCs coating the limb vessels originate from the limb mesenchyme; these cells are recruited to the vessels and then differentiate. This reveals a striking difference between the origin of the mural cells of the trunk vessels, which arise from somites, and those of the limb vessels, which arise from the somatopleura. We therefore demonstrated that the blood vessel defect did not prevent the initiation of axial myogenesis, viewed through the formation of the Pax3 dermomyotome and myotome, which can further differentiate into skeletal muscles. The mouse-into-chicken chimera technique also revealed that although Flk1 KO PSM-derived myoblasts are able to activate the myogenic program, they are unable to migrate and colonize the limb bud even if molecular cues are present. This new important finding reveals that myogenic cells require primary angioblast migration to emigrate into the limb, and that Flk1 is essential for limb hypaxial myogenesis, but dispensable for trunk myogenesis. Several hypotheses can be proposed to explain the role of Flk1 on myogenic progenitor migration: (1) hypaxial dermomyotomal cell *Flk1*$^{-/-}$ do not express c-met anymore, which is crucial for delamination and migration; (2) both c-met and Flk1 are required transiently in a cell autonomous way to initiate the genesis and later the delamination/migration process of hypaxial myogenic progenitors facing the limb; (3) the genesis of ECs in the hypaxial somite facing the limb bud instructs hypaxial dermomyotomal cells in the Pax3^{+}c-met^{+} myogenic migratory pathway. Therefore, more investigations are required to identify the mechanism involved in this process.

Molecular signals involved in limb myoblast emigration

Several putative molecular candidates have been identified as playing a role in myoblast migration. The complementary expression pattern of SDF1 in the limb mesenchyme and CXCR4 in myoblasts suggests a role for this molecular axis in the migration of both progenitors into the limb (Rehimi et al., 2008; Vasyutina et al., 2005; Yusuf et al., 2006). Another putative candidate is the ligand-receptor HGF/c-met couple shown as crucial for de-epithelialization of the lateral somite and subsequent myoblast emigration to the limb (Brand-Saberi et al., 1996; Dietrich et al., 1999; Scaal et al., 1999), and also for the patterning of the muscle masses in combination with Bmp2 and Bmp4 (Bonafede et al., 2006). Finally, based on its presence both dorsally and ventrally in the limb bud, the Tcf4-Wnt/β-catenin pathway has been proposed to establish a pre-pattern for limb muscles (Kardon et al., 2003). In all these studies, the endothelial compartment has been disregarded. Our results clearly suggest a crucial role for ECs in myoblasts emigration. The molecular nature of the signal provided by ECs remains to be elucidated. A crucial role for the non-myogenic cell-produced extracellular matrix for myoblasts migration has also been documented (Chiquet et al., 1981; Sanderson et al., 1986), together with a putative role of limb vessels in myoblast guidance (Venkatasubramanian and Solursh, 1984). Given the complexity of the process, a multistep control is likely and our results indicate that the EC compartment has to be integrated in this complex picture.

Concluding remarks

In conclusion, we have set up a powerful model that allows us to follow the different developmental steps of a unique embryonic structure, the PSM, something that cannot be achieved in vivo or in vitro in the mouse embryo. The mouse-into-chicken chimera

combined with our CAM grafting technique has enabled us to determine precisely that mouse PSM gives rise to angioblasts and myoblasts of the limb in two separate waves and that PSM-derived progenitors are already committed when they leave the somites. Furthermore, this chimeric model appears to be as a relevant tool with which to extend the lifespan of murine structures from embryos that bear a lethal mutation. This, in turn, allows us to study the functions of genes in different lineages without disturbing the 'local environmental cues'. Using this model, we demonstrate: (1) that *Pax3* and *Flk1* are not required for aortic SMC differentiation, with *Pax3* being also dispensable for angioblast migration and differentiation in the aorta and limb, but that, importantly, (2) angioblast emigration towards the limb is a necessary event for myoblast migration.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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