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Skeletal Muscle Differentiation of Embryonic Mesoangioblasts Requires Pax3 Activity

GRAZIELLA MESSINA,[a] DARIO SIRABELLA,[b] STEFANIA MONTEVERDE,[a] BEATRIZ G. GALVEZ,[a]
ROSSANA TONLORENZI,[a] ESTHER SCHNAPP,[a] LUCIANA DE ANGELIS,[b] SILVIA BRUNELLI,[a,c] FREDERIC RELAIX,[d]
MARGARET BUCKINGHAM,[e] GIULIO COSSU[a,b,f]

[a]Stem Cell Research Institute, H. San Raffaele Scientific Institute, Milan, Italy; [b]San Raffaele Biomedical Science
Park of Rome, Rome, Italy; [c]Department of Experimental, Environmental Medicine and Medical Biotechnology,
University of Milano-Bicocca, Monza, Italy; [d]UMR S 787, INSERM–UPMC–Paris VI, Faculté de Médecine
Pitié-Salpétrière, Paris, France; [e]C.N.R.S. URA 2578, Department of Developmental Biology, Pasteur Institute,
Paris, France; [f]Department of Biology, University of Milano, Milan, Italy

ABSTRACT

Mesoangioblasts have been characterized as a population of vessel-associated stem cells able to differentiate into several mesodermal cell types, including skeletal muscle. Here, we report that the paired box transcription factor Pax3 plays a crucial role in directing mouse mesoangioblasts toward skeletal myogenesis in vitro and in vivo. Mesoangioblasts isolated from the aorta of *Pax3* null embryos are severely impaired in skeletal muscle differentiation, whereas most other differentiation programs are not affected by the absence of Pax3. Moreover, *Pax3*$^{-/-}$ null mesoangioblasts failed to rescue the myopathic phenotype of the α-sarcoglycan mutant mouse. In contrast, mesoangioblasts from Pax3 gain of function, *Pax3*$^{PAX3-FKHR/+}$, mice display enhanced myogenesis in vitro and are more efficient in regenerating new muscle fibers in this model of muscular dystrophy. These data demonstrate that Pax3 is required for the differentiation of mesoangioblast stem cells into skeletal muscle, in keeping with its role in orchestrating entry into the myogenic program. STEM CELLS 2009;27: 157–164

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Mesoangioblasts are vessel-associated stem cells that can self-renew and are able to differentiate into several types of mesodermal tissues such as smooth, cardiac, and skeletal muscle, bone, and fat, when treated with the appropriate signaling molecules or cocultured with differentiating cells of the same tissue [1]. When delivered through the arterial circulation, mesoangioblasts cause a significant structural and functional improvement in different animal models of muscular dystrophy [2, 3]. More recently, cells with similar properties have been isolated from biopsies of postnatal human skeletal muscle. These cells, which express markers of pericytes, were also shown to differentiate into skeletal muscle in vitro and to ameliorate the dystrophic phenotype when delivered intra-arterially into the muscle of *mdx-SCID* dystrophic mice [4]. Although the myogenic potential of mesoangioblasts has been demonstrated in vitro and in vivo, the molecules regulating this process are still unknown. Genome-wide RNA expression analysis, performed on mesoan-gioblasts, revealed a characteristic pattern of expression that correlates with the biological features and the developmental potency of these cells [5]. Mesoangioblasts were found to express *Pax3* at a high level. The paired box/homeodomain transcription factors of the Pax family play important roles in the formation of tissues and organs during embryonic development [6]. In particular, Pax3 is implicated in neurogenesis in regions of the central nervous system, regulates neural crest and its derivatives, and plays a key role in skeletal myogenesis. In the latter context, Pax3 is expressed in paraxial mesoderm and marks multipotent cells of the dorsal somite, the dermomyotome, from which skeletal muscle is derived. Endothelial and smooth muscle cells are also derived from paraxial mesoderm, and the same Pax$^+$ progenitor in the dermomyotome can give rise to both the smooth muscle cells of blood vessels and skeletal muscle [7, 8]. During the first wave of early myogenesis, Pax3 is required for the survival of cells in the hypaxial dermomyotome and their migration to sites outside the somite. Subsequently, in the limb buds, for example, it directly activates the myogenic determination gene *Myf5* [9]. Transcriptional ac-

tivity of Pax3 itself, which also impacts *MyoD*, is regulated in the somite by noncanonical Wnt signaling from the dorsal ectoderm [10]. In addition to *Myf5*, Pax3 directly activates myogenic expression of *Fgfr4*, and also lies genetically upstream of *Sprouty1* [11], which encodes an intracellular inhibitor of this signaling pathway, thus modulating the balance [12] between progenitor cells and their entry into the myogenic program that is promoted by fibroblast growth factor signaling [11]. Subsequent myogenesis depends on a population of progenitor cells that originates from the central domain of the dermomyotome where Pax7 as well as Pax3 are present. These cells provide a reserve of resident stem cells, within the developing muscle masses, where they constitute a self-renewing population that also differentiates into muscle fibers [13–15]. Lack of both Pax3 and Pax7 causes a major muscle deficit during late embryonic development; many progenitor cells die or change fate, in the absence of *Myf5*, *Mrf4*, and *MyoD* activation [15]. These three myogenic factor genes are essential at this stage for determining muscle cell fate. A fourth member of this family of basic helix-loop-helix transcription factors, myogenin, acts as a differentiation factor, controlling downstream muscle genes, such as those encoding myosin heavy chains (MHCs). The population of Pax3$^+$/Pax7$^+$ cells from the central dermomyotome later gives rise to satellite cells, the progenitor cells of postnatal muscle. Pax7 plays a key role in these cells [16], ensuring their survival [17], whereas both Pax7 and Pax3, expressed in a subset of satellite cells, control *MyoD* activation and satellite cell myogenesis.

In this report, we studied the role of Pax3 in the myogenic differentiation of mesoangioblasts. We generated mesoangioblasts from *Pax3* null embryos as well as from mice expressing a constitutively active form of Pax3, Pax3-FKHR [18]. Our analysis shows that Pax3, but not Pax7, regulates the differentiation of mesoangioblasts into skeletal muscle. This is consistent with the paraxial mesoderm origin of these cells in the embryonic dorsal aorta [8] and underlines the key role of Pax3/Pax7 in controlling the entry of cells into the myogenic program.

MATERIALS AND METHODS

Isolation and Culture of *Pax3* Null and *PAX3-FKHR* Mesoangioblasts

Pax3 null and *PAX3-FKHR* mesoangioblasts were generated from the dorsal aorta of *Pax3^GFP/GFP* and *Pax3^PAX3FKHR-IRESnLacZ/+* mouse embryos (embryonic day, 9.5), respectively [15, 18]. Cells were isolated, cloned, and expanded as described previously [19]. The well-characterized D16, D15, and D53 clones of mouse mesoangioblasts were cultured as described previously [1, 20]. Human satellite cells, used in the cocultures, were cultured as described [21].

Differentiation Assays

Differentiation into smooth muscle cells and osteoblasts was induced by treatment with transforming growth factor (TGF)β1 and bone morphogenic protein (BMP)2, respectively, and analyzed as previously described [1, 5]. For mineralized nodule assays, mesoangioblasts were maintained in growth medium supplemented with 50 μg/ml ascorbic acid, 10 mM μ-glycerol phosphate, dexamethasone 10 – 8 M, and 100 ng/ml BMP2 for 25 days. At the end of the culture period, cultures were stained for alkaline phosphatase, and mineral deposition was assessed using the von Kossa method, according to the standard procedure.

Differentiation into skeletal muscle was induced by coculturing murine mesoangioblasts with human myoblasts at a 1:10 ratio. To induce muscle fiber formation, cultures were shifted to differentiation medium (Dulbecco's modified Eagle's medium supplemented with 2% horse serum). After 7 days, cultures were stained with antibodies against striated MHC (MF20) and lamin A/C, which stains the human nuclei. The percentage of mesoangioblast myogenic differentiation was calculated by counting the number of murine 4′,6-diamidino-2-phenylindole (DAPI)$^+$ (lamin A/C$^-$) nuclei in myosin-positive cells as a percentage of total DAPI$^+$ (lamin A/C$^-$) nuclei. Biochemical differentiation was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) using mouse specific oligonucleotides for *MyoD* and *myogenin* transcripts. Skeletal myogenesis of *PAX3-FKHR* expressing mesoangioblasts was induced by plating cells onto Matrigel-coated dishes in differentiation medium. After 7 days, cultures were fixed and stained with antibodies against striated myosin (MF20) and MyoD.

Immunoblotting

Western blotting analysis of cells or tissues was performed as described [22]. The antibodies used were: anti-Pax3 (Hybridoma Bank, Iowa City, IA, http://dshb.biology.uiowa.edu), mouse anti-α-sarcoglycan (α-SG) (Novocastra Ltd., Newcastle upon Tyne, U.K., http://www.novocastra.co.uk), mouse anti-β-tubulin (Covance, Princeton, NJ, http://www.covance.com), and anti-MHC (MF20, Hybridoma Bank).

Immunofluorescence

Cells were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde for 10 minutes. Muscle samples from mesoangioblast-transplanted α-SG null mice were frozen in liquid nitrogen cooled isopentane, and serial 8-μm thick sections were cut with a Leica cryostat (Leica Microsystems GmbH, Wetzlar, Germany, http://www.leica-microsystems.com). Cells and tissue sections were processed for immunofluorescence as previously described [2]. The antibodies used were: mouse anti-smooth muscle actin (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), rabbit anti-green fluorescent protein (GFP) (Molecular Probes Inc., Eugene, OR, http://probes.invitrogen.com), anti-MHC (MF20; Hybridoma Bank), mouse anti-laminin (Sigma), mouse anti-lamin A/C (Novocastra), and anti-α-SG (Novocastra).

RT-PCR

RNA (1 μg) collected from different mesoangioblast cultures was converted into double-stranded cDNA by reverse transcription using the cDNA synthesis Thermoscript RT-PCR System kit (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). cDNA was then amplified using the following primers:

VE-cadherin (VE-Cad, 227 bp; forward [Fw], 5′ gga tgc aga ggc tca cag ag 3′; reverse [Rev], 5′ ctg gcg gtt cac gtt gga ct 3′), Flk1 (270 bp; Fw, 5′ tct gtg gtt ctg cgt gga ga 3′; Rev, 5′gta tca ttt cca acc acc ct 3′), CD34 (300 bp; Fw, 5′ ttg act tct gca acc acg ga 3′; Rev, 5′ tag atg gca ggc tgg act tc 3′), c-Kit (400 bp; Fw, 5′ ggc tca taa atg gca tgc tc 3′; Rev, 5′ ctt cca ttg tac ttc ata cat g 3′), Cbfa1 (387 bp; Fw, 5′ ccg cac gac aac cgc acc at 3′; Rev, 5′ cgt ccc ggc cca caa atc tc 3′), bone sialoprotein (BSP, 563 bp; Fw, 5′ gaa acg gtt tcc agt cca g 3′; Rev, 5′ tga aac ccg ttc aga agg 3′); collagen 1A2 (Coll1A2, 484 bp; Fw, 5′gca atc ggg atc agt acg aa 3′; Rev, 5′ ctt tca cgc ctt tga agc ca 3′); osteocalcin (292 bp; Fw, 5′ aag cag gag ggc aat aag gt 3′; Rev, 5′ agc tgc tgt gac atc cat ac 3′); osteopontin (437 bp; Fw, 5′ tca cca ttc gga tga gtc tg 3′; Rev, 5′ act tgt ggc tct gat gtt cc 3′); osteonectin (499 bp; Fw, 5′ cac ata act ctg agg cca ttg 3′; Rev, 5′ gcc caa ttg cag ttg agt gat 3′); MyoD (455 bp; Fw, 5′ctg ctt ttg gca gat gca cc 3′; Rev, 5′ cag tga ggt gca gcc aga gt 3′), and myogenin (272 bp; Fw, 5′ctg ggg acc cct gag cat tg 3′; Rev, 5′ atc gcg ctc ctc ctg gtt ga 3′).

In Vivo Injection

Two-month-old α-SG [23] null mice (injected slowly through the entire anterior surface of the tibialis muscle with a 27-gauge needle containing 100 μl of 5 μM cardiotoxin [Sigma] 12 hours before treatment) were injected through the right femoral artery or by intramuscular injection with 5 × 10^5 mouse mesoangioblasts (pretreated with stromal cell-derived factor 1 to increase cell migration), as described in [24]. To be sure that the lack of Pax3 did not affect cell migration, 5 × 10^5 *Pax3* wild-type (wt) or *Pax3* null mesoangioblasts were also injected into the tibialis anterior of the α-SG null

mice. A set of animals was sacrificed after 1 week to follow mesoangioblast migration into the muscles. A real-time PCR for GFP was performed on all the samples as described below (see Real-time PCR). After 1 month, different muscles (quadriceps, gastrocnemius, and tibialis) were collected and α-SG expression was checked by Western blot analysis.

Real-Time PCR

Total RNA from muscles (gastrocnemius) and cells was isolated using the TRIzol protocol (Invitrogen) and reverse transcribed with the Taqman kit (Platinum Taq DNA polymerase; Invitrogen). Real-time quantitative PCR was carried out with a real-time PCR system (Mx3000P; Stratagene, La Jolla, CA, http://www.stratagene.com). Each cDNA sample was amplified in duplicate using SYBR Green Supermix (Bio-Rad, Hercules, CA, http://www.bio-rad.com) for GFP (Fw, aagttcatctgcaccaccg; Rev, tccttgaagaagatggtgcg), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Fw, 5′-gatgatgac-ccgtttggctcc-3′; Rev, 5′-gatgatgacccgtttggctcc-3′), and LacZ (Fw, 5′-actatcccgaccgccttact-3′; Rev, 5′-tagcggctgatgttgaactg-3′). Delta Ct values were normalized to those obtained from the amplification of GAPDH transcripts.

For the migration assay, data are expressed as the percentage of migrated cells, which is calculated by comparing the expression level of the *GFP* reporter gene in the experimental sample with that measured in the total input of injected cells.

Cell Transfection and Infection

The D16 mesoangioblast cell line was transfected by the liposome Fugene (Roche Diagnostics, Basel, Switzerland, http://www.roche-applied-science.com). In order to obtain stable transfected clones, cells were cotransfected with the Pax3-Engrailed (*Pax3-Engr*) gene plasmid and the plasmid delivering puromycin as a transfection selection marker (ratio 5:1). The Pax3 dominant negative form was obtained by fusing in-frame sequences encoding the *Drosophila melanogaster* Engrailed repression domain (298 amino acids [24]) to the first 374 amino acids of Pax3 [16]. After 48 hours, 2 μg/ml of puromycin was added. Forty-eight hours after selection, single clones were expanded and assayed for plasmid expression. In the *myogenin-lacZ* reporter construct (kindly provided by Dr. Libera Berghella) *LacZ* is under the transcriptional control of the 1.1-kb upstream region of the *myogenin* promoter.

The cDNA coding for FOXO1A (*FKHR*) was subcloned into the pcDNA3 expression vector (Invitrogen). Mesoangioblasts were transfected with pcDNA3-FOXO1A or pcDNA3 alone as a control. After 48 hours, 0.5 mg/ml of G-418 (Sigma) was added and the selected populations were tested for muscle differentiation as described above.

RESULTS

Generation of *Pax3* Null Mesoangioblasts: Morphology, Proliferation, and Gene Expression

To confirm data obtained by the RNA-microarray analysis [5], we analyzed the expression of Pax3 in three well-characterized clones of mouse mesoangioblasts (D16, D15, and D63) by Western blot. As shown in Figure 1A, all the clones expressed Pax3, but not Pax7. To investigate the possible role of Pax3 in mesoangioblast commitment to the skeletal muscle fate, we isolated these cells from the dorsal aorta of E10.5 $Pax3^{GFP/+}$ heterozygote and $Pax3^{GFP/GFP}$ null as well as wt embryos [14]. Both the wt and the *Pax3* null cells showed good clonogenic efficiency. Under normal culture conditions, *Pax3* wt and *Pax3* null mesoangioblasts displayed the same phenotype, morphology (Fig. 1D), and proliferation rate (not shown). Analysis by RT-PCR (Fig. 1C) and examination of surface markers by flow cytometry (not shown) showed no significant differences in gene expression between *Pax3* null and wt mesoangioblasts. Like previously described mesoangioblast clones [1], both wt
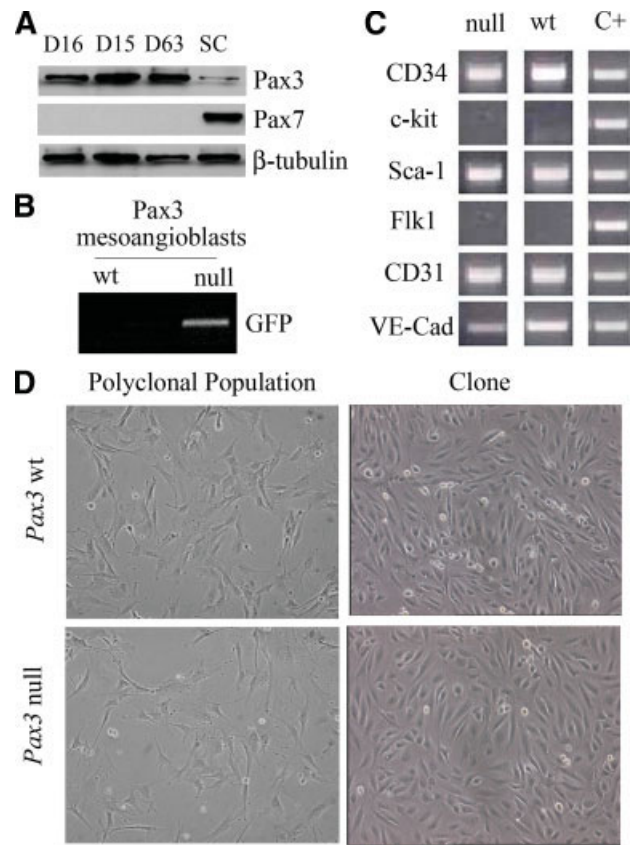

Figure 1. Morphology, proliferation, and gene expression profile of *Pax3* null mesoangioblasts. **(A):** *Pax3* and *Pax7* expression in three different well-characterized clones of mouse mesoangioblasts, D16, D15, and D63. β-tubulin was used to normalize the amount of proteins loaded. **(B):** Expression of the reporter gene *GFP* in the *Pax3* null mesoangioblasts by RT-PCR. **(C):** RT-PCR of the markers normally expressed in wt mesoangioblasts in comparison with *Pax3* null cells. C+ (a total embryo extract) was used as a positive control. **(D):** Phase contrast images of *Pax3* null mesoangioblasts (*Pax3* null) in comparison with wt cells (*Pax3*wt). Abbreviations: GFP, green fluorescent protein; RT-PCR, reverse transcription-polymerase chain reaction; SC, satellite cells; VE-Cad, VE-cadherin; wt, wild-type.

and *Pax3* null mesoangioblasts were found to express CD34, CD31, VE-Cad, and Sca-1. In addition, although GFP expression was progressively extinguished with passage in culture, the presence of the reporter gene was still detectable by RT-PCR in the *Pax3* null cells (Fig. 1B).

Pax3 Null Mesoangioblasts Fail to Differentiate into Skeletal Muscle

We tested the ability of *Pax3* null mesoangioblasts to differentiate into different mesodermal cell types using assays previously described [1].

Following TGFβ treatment, both wt and *Pax3* null mesoangioblasts (selected clones and the polyclonal populations) differentiated at a high frequency into smooth muscle (Fig. 2A). Treatment of *Pax3* null mesoangioblasts with BMP2, normally used to induce the osteogenic program, led to bone differentiation similar to that in wt cells, as revealed by the analysis of osteogenic markers, such as BSP, osteopontin, osteonectin, and osteocalcin (Fig. 2B), by RT-PCR. Moreover, when cultured in the presence of dexamethasone, ascorbic acid, and β-glycerophosphate, *Pax3* null mesoangioblast clones gave rise to von Kossa-positive bone-like nodules in vitro with the same efficiency (8%) as their wt counterparts (10%) (Fig. 2C), although
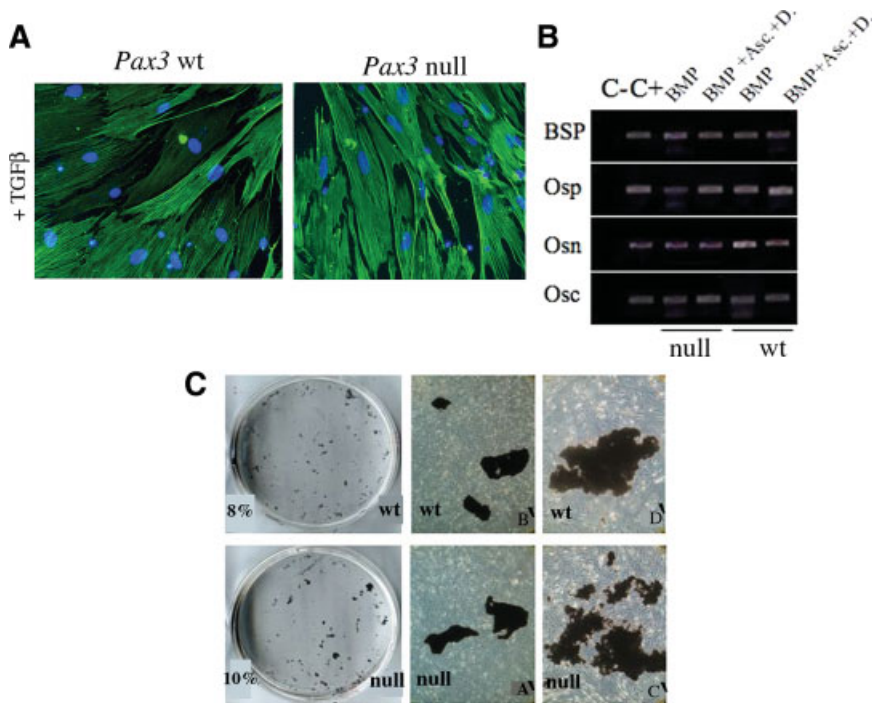
Figure 2. *Pax3* null mesoangioblast differentiation into smooth muscle and bone. **(A):** Immunofluorescence analysis of *Pax3* wt and null mesoangioblasts treated with TGFβ (5 ng/ml) and stained with antibody against α-smooth muscle actin (green). The nuclei are stained with DAPI. **B**. RT-PCR of osteogenic markers expressed in *Pax3* wt (wt) and null mesoangioblasts (null) following osteogenic-promoting treatment. C⁻, H₂O; C⁺, total E10.5 embryo. **(C):** Von Kossa staining of mesoangioblasts treated with BMP2 under osteogenic-promoting conditions. The percentage of Von Kossa staining is the expression of the ratio between the number of positive osteogenic nodules and the total cell number viewed by contrast phase. In A′–D′ higher magnification shows the calcified nodules (A′, C′, wt; B′, D′, null). Abbreviations: BMP, bone morphogenic protein; BSP, bone sialoprotein; DAPI, 4′,6-diamidino-2-phenylindole; Osc, osteocalcin; Osn, osteonectin; Osp, osteopontin; TGFβ, transforming growth factor; wt, wild-type.

alkaline phosphatase activity was greatly reduced in the mutant cells, as shown by alkaline phosphatase staining (purple cells, Fig. 3A). When exposed to an adipogenic medium, *Pax3* null cells were not able to differentiate into adipocytes, in comparison with wt cells (Fig. 3B), thus suggesting an unexpected role of *Pax3* in adipogenic differentiation. Finally, we tested the ability of the *Pax3* null mesoangioblasts to differentiate into skeletal muscle. Similar to wt mesoangioblasts, *Pax3* null cells did not differentiate spontaneously in vitro. To induce skeletal muscle in vitro, we cocultured wt or *Pax3* null mesoangioblasts with human muscle satellite cells. The contribution of mouse versus human cells to myotubes was analyzed using an antibody that is specific for human nuclear laminin and by the analysis of mouse or human muscle-specific gene expression by RT-PCR. As shown in Figure 4A and B, the *Pax3* null mesoangioblasts, unlike wt cells, failed to fuse into human myotubes and to activate skeletal muscle-specific genes. RT-PCR, performed with mouse-specific primers for transcripts of the myogenic factors MyoD and myogenin, amplified these messenger RNAs in wt but not in *Pax3* null cells cocultured with human myogenic cells (Fig. 4C). To further confirm this, we transfected the D16 mesoangioblast cell line [2] with a construct expressing *Pax3Engr*, where the Pax3 DNA-binding domain is fused to the Engrailed transcriptional repression domain [25]. Previous work demonstrated that the Pax3-Engrailed fusion protein negatively affects myogenesis in cultured satellite cells [17]. Stable clones expressing Pax3-Engrailed or a control vector were subsequently transiently transfected with a *myogenin-lacZ* reporter construct, where the *lacZ* reporter is regulated by the *myogenin* promoter, activated at the onset of myogenic differentiation. Following 3-day culture in differentiation medium, *myogenin-lacZ* expression was measured by quantitative real-time PCR. As shown in Figure 4D, *myogenin* activation was reduced more than threefold in the D16 *Pax3Engr* cells in comparison with control cells. Moreover, to demonstrate that the difference in myogenic differentiation was directly dependent upon Pax3 expression, we transiently cotransfected *Pax3* null mesoangioblasts with Pax3-GFP and GFP expression vectors. GFP⁺ and GFP⁻ cells were separated by fluorescence-activated cell sort-
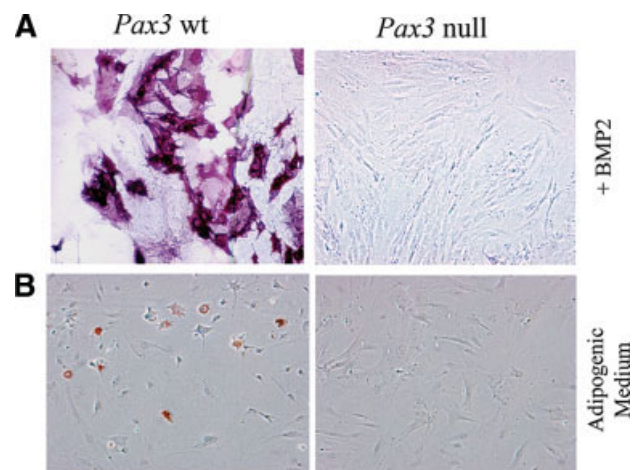

Figure 3. Alkaline phosphatase activity and adipogenic differentiation of *Pax3* null mesoangioblasts. **(A):** Staining for alkaline phosphatase activity (purple cells) in *Pax3* wt and *Pax3* null mesoangioblasts following BMP2 treatment. **(B):** Red oil staining of *Pax3* wt and null mesoangioblasts treated with adipogenic-promoting medium. Abbreviations: BMP, bone morphogenic protein; wt, wild-type.

ing and cocultured with human myoblasts as described above. Results (not shown) indicated that GFP⁺ cells differentiated in coculture as efficiently as wt mesoangioblasts, whereas GFP⁻ cells did not. Notably, no spontaneous myogenic differentiation (i.e., without coculture) was observed under any of these conditions. Overall, these data strongly suggest a crucial role of Pax3 in the commitment of embryonic mouse mesoangioblasts to an inducible myogenic fate.

PAX3-FKHR Mesoangioblasts Show Enhanced Myogenic Differentiation

To further investigate the possible mechanism through which Pax3 controls the entry of mesoangioblasts into the myogenic program, we isolated mesoangioblasts from $PAX3^{PAX3-FKHR\text{-}IRESnLacZ/+}$ mouse embryos [18]. It was shown
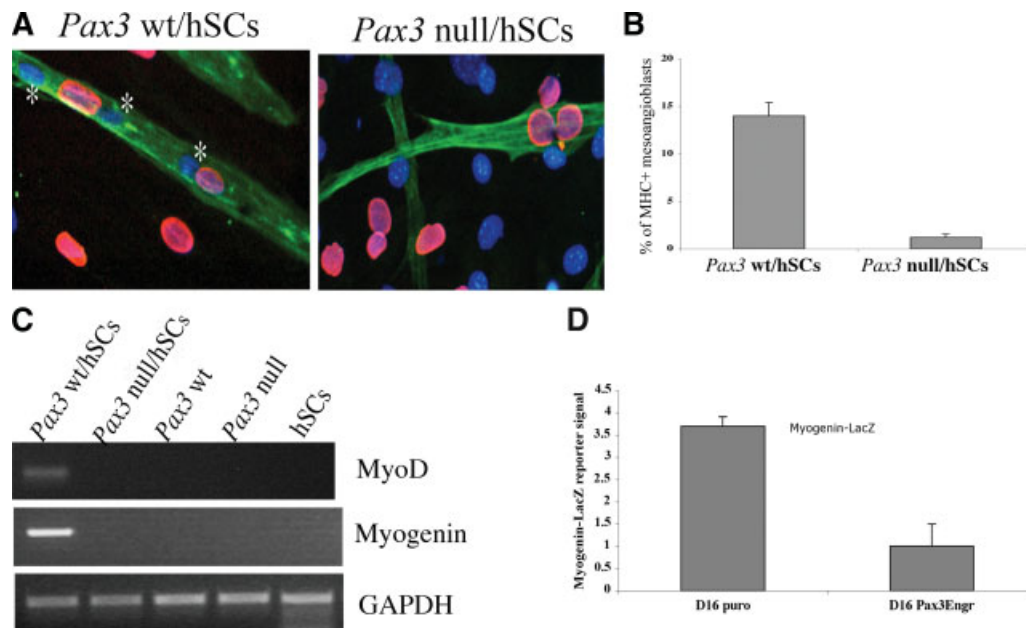
Figure 4. *Pax3* null mesoangioblasts fail to differentiate into skeletal muscle. **(A):** Skeletal muscle differentiation of *Pax3* wt or null mesoangioblasts is detected after co-cultivation with hSCs. The human nuclei are identified using an anti-human nuclei-specific antibody (lamin A/C, red) and the expression of MHC is identified using an anti-MHC antibody (MF20, green). All the nuclei are stained with DAPI and the human nuclei appear pink in the merged image. The white asterisks identify mouse nuclei in the myofibers. **(B):** The differentiated mesoangioblasts were quantified as the number of MHC$^+$ mesoangioblasts compared with the total number of mesoangioblasts in the coculture. **(C):** RT-PCR carried out using mouse-specific primers for *MyoD* and *myogenin* transcripts. **(D):** Activity of a *myogenin-lacZ* reporter gene in D16 mesoangioblasts (cocultured with C2C12 myoblasts), coexpressing either a Pax3Engr or a control (puro) vector. LacZ mRNA was analyzed by real-time RT-PCR. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hSCs, human satellite cells; MHC, myosin heavy chain; RT-PCR, reverse transcription-polymerase chain reaction; wt, wild-type.

previously that the oncogenic fusion protein Pax3-FKHR rescues the early *Pax3* mutant phenotype, with overactivation of Pax3 targets [18]. We generated both a polyclonal population and clones from these embryos. Figure 5A shows the similar morphology of *PAX3-FKHR* and *Pax3* wt mesoangioblasts. Analysis of surface markers by RT-PCR (Fig. 5B) showed no significant differences between *PAX3-FKHR* and wt mesoangioblasts, with the exception of the endothelial markers VE-Cad and Tie2, which were more highly expressed in *PAX3-FKHR* mesoangioblasts. We also looked for activation of *MyoD* and *Myf5* in the *PAX3-FKHR* cells, but did not observe expression at different time points, either by Western blot or by RT-PCR (data not shown). However, when *PAX3-FKHR* mesoangioblasts were cultured in a differentiation-promoting medium, a few cells ($\cong$2%), not detected under our RT-PCR conditions, spontaneously differentiated as indicated by MHC expression (Fig. 5C), a phenomenon never observed with wt embryonic mesoangioblasts nor with *Pax3* null mesoangioblasts rescued by wt Pax3 expression. Moreover, when either clones or polyclonal populations of *PAX3-FKHR* mesoangioblasts were cocultured with human satellite cells, they differentiated into multinucleated myotubes with higher efficiency (20%–30% versus 5%–10% in different experiments) than wt mesoangioblasts (Fig. 5D, 5E). RT-PCR using mouse-specific primers for *MyoD* and *myogenin* transcripts confirmed this result (Fig. 5F). Differentiation to other mesodermal cell types (bone, fat, smooth and cardiac muscle) did not show differences between *PAX3-FKHR* and wt cells (not shown). To discriminate between the effects due to Pax3 and possible effects due to the *FKHR* moiety, the C-terminal transcription activation domain of *FKHR* (FOX01A) was also expressed in *Pax3* null as well as in *Pax3* wt mesoangioblasts. Despite a decrease in cell proliferation, we did not observe spontaneous muscle differentiation in either wt or *Pax3* null cells (data not shown).

Therefore, these results further demonstrate the importance of Pax3 function in mesoangioblast muscle differentiation.

Pax3 Is Required for Mesoangioblasts to Restore α-SG Synthesis in Dystrophic Mice

Finally, we examined whether the failure of *Pax3* null mesoangioblasts to differentiate into skeletal muscle in culture might also prevent their ability to ameliorate the dystrophic phenotype [2, 3] and if expression of *PAX3-FKHR* in these cells could rescue this defect. Therefore, *Pax3* wt, *Pax3* null, and *PAX3-FKHR* mesoangioblasts, previously transduced with a lentiviral vector expressing GFP, were injected through the right femoral artery or directly into the tibialis anterior muscle of α-SG null mice: their ability to migrate to colonize the muscle and to give rise to α-SG expressing fibers was analyzed. As shown in Figure 6A, the *Pax3* null mesoangioblasts (GFP$^+$) were able to migrate, cross the blood vessels, and colonize the muscles, like *Pax3* wt and *PAX3-FKHR* cells. However, despite their ability to migrate to the muscle fibers (Fig. 6A), the *Pax3* null mesoangioblasts failed to express α-SG, in contrast to wt cells (Fig. 6B–6D). More interestingly, the *PAX3-FKHR* cells contributed to muscle fiber repair more efficiently than the wt cells, thus demonstrating the role of Pax3 in mesoangioblast muscle differentiation and in amelioration of the dystrophic phenotype.

DISCUSSION

We demonstrate that skeletal muscle differentiation of mesoangioblasts in vitro and in vivo depends on Pax3. The presence of Pax3 in multipotent mesoangioblasts does not appear to be required for their proliferation or survival, in contrast to the situation in myogenic progenitor cells in the embryo and adult.
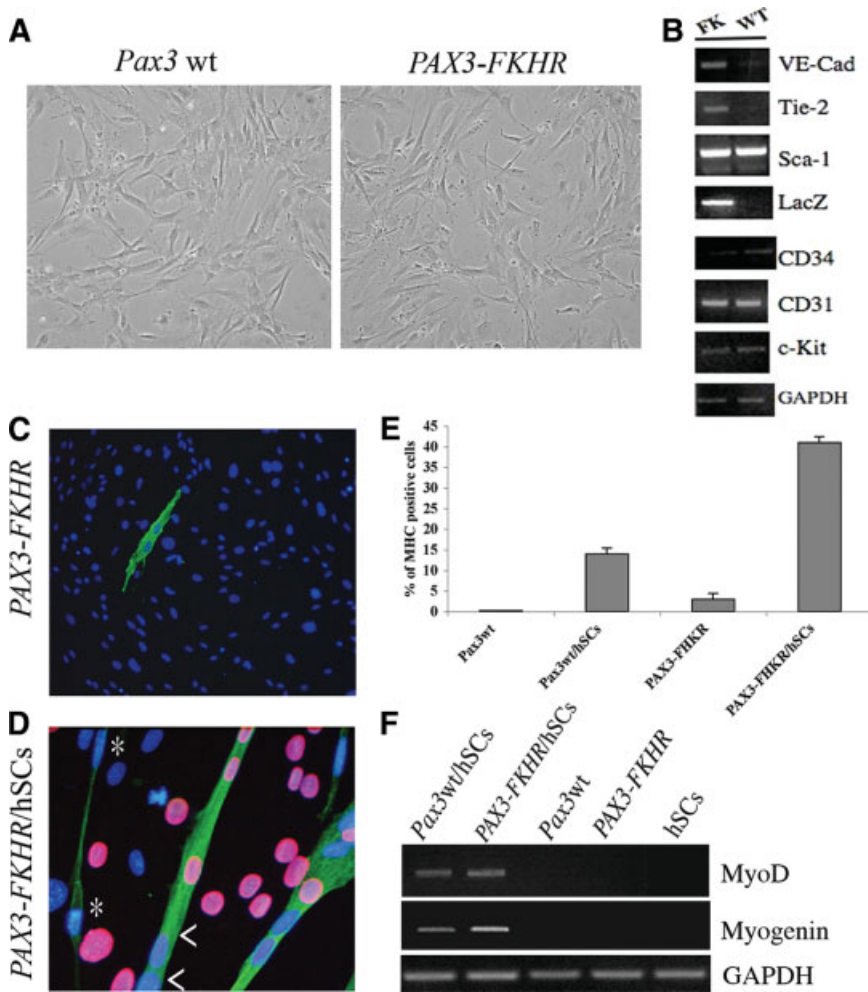
Figure 5. *PAX3-FKHR* mesoangioblasts show robust myogenic differentiation. **(A):** Phase contrast of *Pax3*^{*Pax3-FKHR*-IRESnlacZ/+} mesoangioblasts in comparison with wt cells. **(B):** RT-PCR of the markers normally expressed in wt mesoangioblasts (WT) in comparison with *PAX3-FKHR* expressing cells (FK). **(C, D):** Skeletal muscle differentiation of the *PAX3-FKHR* cells alone **(C)** or in coculture with hSCs **(D)**. The human nuclei are identified using an anti-human nuclei-specific antibody (lamin A/C, red) and the expression of MHC is identified using the anti-MHC antibody MF20 (green). All the nuclei are stained with DAPI and the human nuclei appear pink in the merged image. The arrowheads indicate mouse nuclei inside mouse only myotubes; the asterisks identify mouse nuclei inside chimeric myotubes also containing human nuclei from satellite cells. **(E):** The differentiated mesoangioblasts were quantified as the number of MHC$^+$ mesoangioblasts compared with the total number of mesoangioblasts in the coculture. **(F):** RT-PCR was carried out using mouse-specific primers for *MyoD* and *myogenin* transcripts. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hSCs, human satellite cells; MHC, myosin heavy chain; RT-PCR, reverse transcription-polymerase chain reaction; wt, wild-type.

Differentiation of mesoangioblasts into most mesodermal derivatives is also not affected by a lack of Pax3. An exception to this are adipocytes, suggesting that this mesoangioblast derivative may be closely linked to myogenic progenitor cells. There is some precedent for this; Pax3$^+$ satellite cells, for example, can assume an adipocytic phenotype [26], and in mutant embryos in the absence of myogenic determination factors, excess adipose tissue is observed, potentially derived from myogenic progenitors [27]. In *Pax3* null mesoangioblasts, cartilage/bone differentiation occurs, but does not lead to alkaline phosphatase expression, suggesting that the absence of Pax3 may have some impact on the maturation of this phenotype. An effect on this tissue differentiation pathway may be related to the initial expression of *Pax3* in paraxial mesoderm cells that subsequently downregulate transcription of this gene as they enter the sclerotome compartment and differentiate into cartilage and bone of the vertebral column and ribs. It is also interesting to note that, besides its expression in osteoprogenitor cells, alkaline phosphatase is also expressed in postnatal pericytes of striated muscle [28]. This led us to identify pericytes as the postnatal counterpart of embryonic mesoangioblasts [4]. Lack of alkaline phosphatase expression in *Pax3* null mesoangioblasts may thus suggest that alkaline phosphatase-positive pericytes are derived postnatally from embryonic mesoangioblasts. Unfortunately this possibility cannot be tested in *Pax3* null embryos that do not survive the fetal stage and awaits mutant mice engineered for time-dependent Pax3 ablation in the mesoderm.

It is striking that Pax7 is not expressed in mesoangioblasts and that the skeletal muscle fate of these cells is Pax3, not Pax7, dependent. Again, the analogy with what happens in the embryo is informative. Retrospective clonal analysis and the persistence of the Pax3-GFP reporter indicate that pericytes and smooth muscle cells of the dorsal aorta are derived from Pax3$^+$ cells in paraxial mesoderm that also give rise to skeletal muscle [8]. These cells that locate to the hypaxial dermomyotome, unlike those in the central compartment of this structure, do not express Pax7. In the chick embryo, labeling of cells in the dermomyotome also clearly demonstrates that a single Pax$^+$ progenitor can give rise to both skeletal muscle and smooth muscle of the blood vessels [7]. It is therefore quite possible that some multipotent mesoangioblast cells in the wall of the dorsal aorta are derived from the Pax3$^+$ multipotent cells of the paraxial mesoderm. Interestingly, it has been reported that Pax3 is also required for myogenic differentiation of P19 embryonal carcinoma cells, which have a spectrum of differentiation options that is larger than that of mesoangioblasts [29]. Very recently, Gang et al. [30] reported that murine mesenchymal stem cells can also undergo myogenic differentiation when Pax3 is overexpressed, whereas other murine cell types could not. These data are in general agreement with our report. However, it should be noted that in the case of mesoangioblasts, spontaneous myogenic differentiation does not occur and Pax3 is required to make cells competent to respond to inductive signals from neighboring differentiating myoblasts. Thus, these data underline a pivotal role of Pax3 for entry into skeletal myogenesis. It is not only the dorsal aorta that gives rise to mesoangioblasts, which can also be isolated
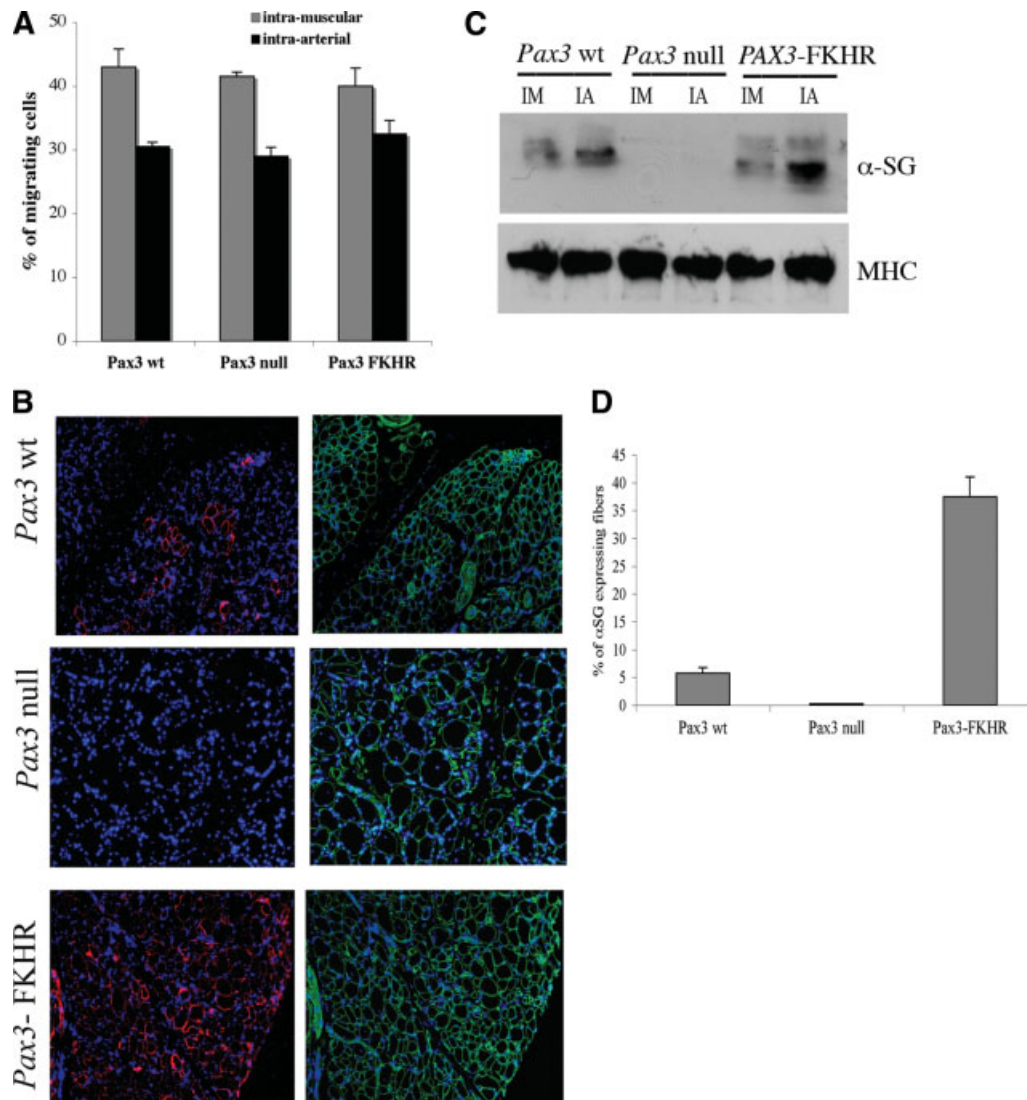
Figure 6. Pax3 is necessary for mesoangioblast rescue of the dystrophic phenotype. **(A):** In vivo mesoangioblast cell migration. *Pax3* wt, *Pax3* null, and *PAX3-FKHR* mesoangioblasts labeled with a GFP-expressing vector were injected into the right femoral artery or by intramuscular injection of 2-month-old (preinjected with cardiotoxin) α-SG null mice. After 1 week, the muscles (the gastrocnemius for the intra-arterial and the tibialis for the intramuscular injection) were collected and the number of GFP+ cells accumulated inside the muscle was calculated by real-time PCR for GFP and expressed as a percentage of the total number of injected cells (% of migrated cells). **(B):** Immunofluorescence analysis of muscle sections for α-SG null mice, 1 month after injection with wt, *Pax3* null, or *PAX3-FKHR* mesoangioblasts, stained with antibodies against laminin (green) and α-SG (red). Nuclei are stained with DAPI (blue). **(C):** Western blot for α-SG protein in lysates from muscles (quadriceps) from α-SG null mice collected 1 month after injection with wt, *Pax3* null, or *PAX3-FKHR* mesoangioblasts. MHC is shown as a control. A representative experiment of four independent experiments is shown. **(D):** Graph showing the percentage of α-SG-expressing fibers following *Pax3* wt, *Pax3* null, or *PAX3-FKHR* mesoangioblast engraftment into the tibialis anterior. Abbreviations: α-SG, α-sarcoglycan; DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein; IA, intra-arterial; IM, intramuscular; MHC, myosin heavy chain; wt, wild-type; PCR, polymerase chain reaction.

from postnatal blood vessels. $Pax3^{GFP/+}$ expression is also observed sporadically in postnatal blood vessels, suggesting that Pax3+ progenitors are still present, potentially the descendants of multipotent Pax3+ cells that had migrated from the somites to their sites of angiogenesis during embryogenesis.

The potential therapeutic applications of mesoangioblasts for cell therapy of muscle disease make it particularly important to understand how their acquisition of a muscle fate is regulated. Pax3/Pax7 alone is not sufficient to convert cells to myogenesis, and indeed they require cofactors to function as efficient transcriptional activators [6], as well as cooperation with other upstream regulatory factors such as the homeodomain proteins Six1/4 [31]. The signaling factors that come from differentiating muscle cells, human satellite cells in the experiments described

here, and their regulatory targets within the mesoangioblasts remain to be defined. However, the results reported here represent an important step forward in understanding how the mesoangioblast cell acts as a myogenic progenitor and underlines the essential role of Pax3/7 in directing cells into the skeletal muscle differentiation program.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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