Stem Cell Reports
Article
ISSCR
OPEN ACCESS

PITX2 Enhances the Regenerative Potential of Dystrophic Skeletal Muscle Stem Cells

Daniel Vallejo,[1] Francisco Hernández-Torres,[1] Estefanía Lozano-Velasco,[1] Lara Rodriguez-Outeiriño,[1] Alejandra Carvajal,[2] Carlota Creus,[2] Diego Franco,[1] and Amelia Eva Aránega[1,*]
[1]Cardiac and Skeletal Myogenesis Group, Department of Experimental Biology, University of Jaén, CU Las Lagunillas B3-362, Jaén 23071, Spain
[2]Servicio de Neurología, Hospital Universitario Virgen de las Nieves, Granada 18014, Spain
*Correspondence: aaranega@ujaen.es
https://doi.org/10.1016/j.stemcr.2018.03.009

SUMMARY

Duchenne muscular dystrophy (DMD), one of the most lethal genetic disorders, involves progressive muscle degeneration resulting from the absence of DYSTROPHIN. Lack of DYSTROPHIN expression in DMD has critical consequences in muscle satellite stem cells including a reduced capacity to generate myogenic precursors. Here, we demonstrate that the c-isoform of PITX2 transcription factor modifies the myogenic potential of dystrophic-deficient satellite cells. We further show that PITX2c enhances the regenerative capability of mouse DYSTROPHIN-deficient satellite cells by increasing cell proliferation and the number of myogenic committed cells, but importantly also increasing dystrophin-positive (revertant) myofibers by regulating miR-31. These PITX2-mediated effects finally lead to improved muscle function in dystrophic (DMD/mdx) mice. Our studies reveal a critical role for PITX2 in skeletal muscle repair and may help to develop therapeutic strategies for muscular disorders.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a devastating genetic muscular disorder caused by mutations in the DYSTROPHIN gene, located on the short arm of the X chromosome. As a 427-kDa cytoskeletal protein, DYSTROPHIN is located underneath the sarcolemma and assembles with sarcolemmal proteins to form the DYSTROPHIN-associated protein complex (DAPC), which includes DYSTROGLYCANS, SARCOGLYCANS, SYNTROPHINS, and SARCOSPAN. The absence of or defects in DYSTROPHIN disrupt the DAPC, leading to chronic inflammation, progressive muscle degeneration, and replacement of muscle with fibroadipose tissue (Allen et al., 2010; Mercuri and Muntoni, 2013). DMD patients often lose independent ambulation by the time they reach 13 years of age, and generally die of respiratory failure in their late teens or early twenties. In DMD, one of the more severe features is progressive muscle wasting and weakness associated with exhaustion of muscle regeneration potential (Mah et al., 2014).

Skeletal muscle has the ability to repair and regenerate due to the presence of resident stem cells, termed muscle satellite cells. In mature muscle tissue, satellite cells occur as a small, dispersed population of mitotically and physiologically quiescent cells, marked by the expression of the transcription factor PAX7 (Kuang et al., 2007). Satellite stem cells in adult muscle represent a lineage continuum of the embryonic myogenic PAX3+/PAX7+ progenitor cells that remain in the adult muscle in a quiescent state. Upon injury, they become activated, proliferating, and entering a myogenic differentiation program by the upregulation of the myogenic determination genes *Myf5*, *MyoD*, and *Myogenin*, thus forming new myocytes that eventually fuse with each other to generate new muscle tissue (Yin et al., 2013). It bears highlighting that, in muscular dystrophies, the progressive muscle wasting and weakness is often associated with exhaustion of muscle regeneration potential. Therefore, the progressive loss of muscle mass has been attributed, at least partly, to the inability of muscle stem cells to efficiently regenerate tissue loss as the result of the disease (Berardi et al., 2014). Thus, critical for the development of effective strategies to treat muscle disorders is the optimization of approaches targeting muscle stem cells and capable of regenerating tissue loss as the result of the disease or as the result of normal muscle turnover (Bertoni, 2014). Notably, very recent reports have pointed out that muscle stem cells should be considered as a therapeutic target for restoring muscle function in individuals with DMD (Chal et al., 2015; Dumont et al., 2015). Chal et al. (2015) have shown that myofibers derived from embryonic stem cells of DMD/mdx mice exhibit an abnormally branched phenotype, suggesting that dystrophin is required for normal myogenesis. In addition, Dumont et al. (2015) have demonstrated that dystrophin has an essential role in regulating satellite cell polarity and asymmetric division. These intrinsic defects strongly reduce the generation of myogenic progenitors that are needed for proper muscle regeneration, indicating that muscle wasting in DMD is not only caused by myofiber fragility, but also is exacerbated by impaired regeneration owing to intrinsic satellite cell dysfunction (Dumont et al., 2015).

Pitx2 is a paired-related homeobox gene involved in the molecular process controlling embryonic and fetal

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

myogenesis (L'Honoré et al., 2007; Zacharias et al., 2010; L'Honoré et al., 2010; L'Honoré et al., 2014). Previous works from our laboratory showed that *Pitx2c* is the main *Pitx2*-isoform expressed in myoblasts playing a pivotal role modulating proliferation versus differentiation during myogenesis as well as balancing PAX3+/PAX7+ myogenic population *in vivo* (Martínez-Fernández et al., 2006; Lozano-Velasco et al., 2011). The role of PITX2 during adult myogenesis is beginning to be explored, thus several reports have shown that PITX2 is expressed in proliferating satellite cells promoting differentiation of satellite cell-derived myoblasts (Ono et al., 2010; Knopp et al., 2013). We have recently identified a PITX2c-microRNA (miRNA) pathway that regulates cell proliferation in early activated satellite cells and promotes their commitment to a myogenic cell fate (Lozano-Velasco et al., 2015). Despite the advances achieved in understanding the PITX2 involvement on satellite cell behavior and function, its role in adult regenerative myogenesis has not been yet determined.

In this study, we show that PITX2c is required for proper satellite cell differentiation since PITX2c gain and loss of function *in vitro* increase and decrease myogenic differentiation, respectively. In addition, we discovered that attenuated PITX2c expression is concomitant with defective myogenic differentiation of dystrophic satellite cells isolated from DMD/mdx mice (Bulfield et al., 1984) and PITX2c gain of function restores most of their differentiation potential. Importantly, cell transplantation of *Pitx2c*-overexpressing dystrophic satellite cells augments the number of myofibers, represses miR-31 reaching to increase revertant DYSTROPHIN protein, and finally improves muscle function in DMD/mdx mice. These results place PITX2 as a new player on skeletal muscle satellite cell biology and identify unknown functions of PITX2 during regenerative myogenesis.

RESULTS

PITX2c Promotes Satellite Cell Differentiation and Modifies the Myogenic Potential of Dystrophic-Deficient Satellite Cells

We have previously described a PITX2c-miRNA pathway regulating cell proliferation and promoting myogenic commitment in freshly isolated satellite cells (Lozano-Velasco et al., 2015). In addition, Knopp et al. (2013) demonstrated that PITX2 promotes satellite cell differentiation, increasing the percentage myoblast fusion index during the process of myogenic differentiation *in vitro*. Here, we have first evaluated the effects of PITX2c on satellite cell proliferation and myogenic potential by analyzing MYOD and Ki67 expression in isolated and culture satellite cells before they reach confluence. We observed that the number of Ki67+ and MYOD+ nuclei was higher in satellite cells overexpressing *Pitx2c* compared with control cells at 3 and 7 days of culture (Figures S1A–S1E), indicating that, in agreement with our previous reported results (Lozano-Velasco et al., 2015), *Pitx2c* overexpression increases satellite cell proliferation and myogenic commitment. Consequently, we also observed an enhanced differentiation capability as assessed by fusion index and proportion of MHC+ cells in differentiating satellite cells after myosin heavy chain (MF20) staining at 14 days of culture (Figures S1F and S1H). In contrast, *Pitx2c* loss of function (Figure S2A) resulted in fewer Ki67+ and MYOD+ nuclei with a less proportion of MHC+ cells and fusion index (Figures S2B–S2F). These results indicate that PITX2c function on satellite cell differentiation is due mainly to the PITX2c effect expanding satellite cell-derived myogenic committed population.

Since we detected that PITX2c regulates satellite cell differentiation, we investigated whether PITX2c expression would be altered during muscle regeneration as well as in a context where satellite cell differentiation and muscle regeneration is not successfully completed, such as in DMD (Shi et al., 2015; Partridge, 2013). To address this question, we first analyzed the expression profile of *Pitx2c* mRNA expression after induction of skeletal muscle damage by cardiotoxin injection in mice. As illustrated in Figure 1A, we found that *Pitx2c* mRNA increased 5-fold at day 1 after muscle damage induction. However, qRT-PCR analyses revealed that *Pitx2c* mRNA dramatically diminished in satellite cells isolated from DMD/mdx mice (Figure 1B). Next we used immunofluorescence staining to look for PITX2c+ cells in the muscle microenvironment. As observed in Figure 1C PITX2c is expressed in more than 50% of PAX7+ cells in uninjured tibialis anterioris (TA) muscles; PITX2c staining was also detectable in some myonuclei as reported previously (Hebert et al., 2013) (Figure 1C). Moreover, although the majority of PITX2c+ cells co-express CD34; we did not detect PITX2c staining in CD34+ interstitial muscle stem cells (Figure 1D). Consistently with qRT-PCR analyses, the number of PITX2c+ cells was clearly increased after muscle injury but decreased in dystrophic muscle (Figures 1E–1G).

In addition to PITX2c decreased expression (Figure 2A), and in agreement with previous recent reports (Chal et al., 2015; Dumont et al., 2015), we also observed that dystrophic satellite cells exhibited a clear defect to form myotubes (Figures 2B and 2C) together with a lower *in vitro* differentiation potential as observed by a decreased proportion of MHC+ cells and fusion index

Figure 1. PITX2c during Muscle Regeneration and DMD

(A) *Pitx2c* mRNA peak at day 1 after cardiotoxin injection in C57/BL3 mice.

(B) *Pitx2c* mRNA expression on muscles isolated from 4-month-old DMD/mdx mice compared with uninjured muscles isolated from 4-month-old C57/BL6 mice.

(C) Representative images of immunohistochemistry for PITX2C and PAX7 in uninjured tibialis anteriois (TA) muscles isolated from 4-month-old C57/BL3 mice. The yellow arrows point to PAX7+/PITX2c+ cells, the green arrows point to PAX7+ cells, and the red arrows point to PITX2c+ myonuclei.

(D) Representative images of immunohistochemistry for PITX2c and CD34 in uninjured TA muscles isolated from 4-month-old C57/BL3 mice. The yellow arrows point to CD34+/PITX2c+ cells, the red arrows point to CD34+ cells, and the green arrow point to PITX2c+ myonuclei.

(E) Representative images of immunohistochemistry for PITX2c and PAX7 in injured TA muscles isolated from 4-month-old C57/BL3 mice (3 days after injury). The yellow arrows point to PAX7+/PITX2c+ cells.

(F) Representative images of immunohistochemistry for PITX2c and PAX7 in uninjured TA muscles isolated from 4-month-old DMD/mdx mice. The yellow arrows point to PAX7+/PITX2c+ cells, the green arrows point to PAX7+ cells, and the red arrows point to PITX2c+ myonuclei.

(G) Percentage of PAX7+/PITX2c+ cells with respect to total nuclei on muscles isolated from 4-month-old C57/B/6 mice, injured muscle, and muscle isolated from DMD/mdx mice, respectively. The percentage of PAX7+/PITX2c+ cells was quantified based on results from at least four fields of view from each sample. All the experiments were repeated at least in three different biological samples to have a representative average.

Statistical significance was determined by Student's t test. $**p < 0.001$, $***p < 0.0001$.

(Figures 2D and 2E). Interestingly, *in vitro* experiments of *Pitx2c* gain of function on dystrophic-deficient satellite cells (Figure 3A) led to rescue their myogenic differentiation potential, as observed by a clear increase in the number of myotubes as well as in the proportion of MHC+ cells and fusion index (Figures 3B–3E). Moreover, *Pitx2c* overexpression in human satellite cells, isolated from a Becker muscular dystrophy patient, significantly increased the number of myogenin+ cells (Figure S3). These results reveal the importance of PITX2c for the dystrophic-deficient satellite cells to reach myogenic differentiation.

Figure 2. Dystrophic Satellite Cells Display Low Levels of *Pitx2c* Expression and Exhibit Defects on Myogenic Differentiation

(A) *Pitx2c* expression in satellite cells isolated from 4-month-old DMD/mdx mice versus control satellite cells isolated from C57/BL6 mice (n = 4).

(B) Ratio of number of myotubes through *in vitro* differentiation in satellite cells isolated from 4-month-old DMD/mdx mice versus control satellite cells isolated from C57/BL6 mice.

(C) Representative images of formed myotubes of satellite cells isolated from 4-month-old DMD/mdx mice versus control satellite cells isolated from C57/BL6 mice (days 3, 7, and 9 of culture).

(D) Representative images of immunohistochemistry for MF20 in differentiating satellite cells isolated from C57/BL6 mice versus satellite cells isolated from DMD/mdx mice (day 14 of culture).

(E) MHC+ cells and fusion index: five random fields of view from each group were scored for the presence of MHC+ cells. Values obtained in the control group (C57) were set at 100%. The fusion index was calculated as follows: (MF20-stained myocytes containing ≥ 2 nuclei/total number of nuclei) $\times$ 100. Values and error bars are means of SD of at least three independent experiments.

Statistical significance was determined by Student's t test. $^{***}p < 0.0001$, $^{****}p < 0.00001$.

Figure 3. *Pitx2c* Overexpression in Dystrophic-Deficient Satellite Cells Rescues Their Myogenic Differentiation Potential

(A) *Pitx2c* mRNA expression in satellite cells isolated from 4-month-old DMD/mdx mice transfected with LVX-*Pitx2c* versus LVX particles.

(B) Ratio of number of myotubes through *in vitro* differentiation in *Pitx2c*-overexpressing cells versus control.

(C) Representative images of formed myotubes of satellite cells isolated from C57/BL6 mice and dystrophic satellite cells overexpressing *Pitx2c* (LVX-Pitx2c) versus control cells (LVX) (days 3, 7, and 9 of culture).

(D) Representative images of immunohistochemistry for MF20 in differentiating satellite cells isolated from C57/BL6 mice and dystrophic satellite cells overexpressing *Pitx2c* (LVX-Pitx2c) versus control cells (LVX) (day 14 of culture).

(E) MHC+ cells and fusion index: five random fields of view from each group were scored for the presence of MHC+ cells. Values obtained in the control group (LVX) were set at 100%. All the experiments were repeated at least in three different biological samples to have a representative average.

Statistical significance was determined by Student's t test. $*p < 0.01$, $**p < 0.001$, $***p < 0.0001$, $****p < 0.00001$.

PITX2C Enhances Muscle Regeneration in DMD/mdx Mice

Based on the results presented above, we next posed the question as to whether we could alter the regenerative potential of dystrophic satellite cells by increasing PITX2c expression. To address this question, we developed a strategy by which freshly isolated satellite cells from the tibialis anterior muscle of DMD/mdx mice were transfected with the bicistronic lentiviral construction pLVX-*Pitx2c*-IRES-ZsGreen. Transfected satellite cells were then

Figure 4. Cell Transplantation of Dystrophic Satellite Cells Overexpressing *Pitx2c* into DMD/mdx Recipient Enhances the Number and Size of the Newly Formed Myofibers

(A) Schematic representation for cell transplant experiments.

(B) *Pitx2c* overexpression in TA muscles of DMD/mdx mice transplanted with dystrophic satellite cells overexpressing cells (LVX-Pitx2c-ZsGreen muscles) (n = 6) versus control cells transplanted with the empty lentiviral vector (LVX-ZsGreen muscles) (n = 6).

(C) Percentage of ZsGreen+ myofibers in LVX-Pitx2c-ZsGreen muscles (n = 6) versus control LVX-ZsGreen muscles (n = 6).

(legend continued on next page)

injected into the tibialis anterior muscle of a host immune-suppressed DMD/mx mice previously damaged by cardiotoxin injection. Satellite cells transfected with the empty lentiviral vector (LVX-IRES-ZsGreen) were injected in the contralateral TA in each experiment and used as control (Figure 4A). The use of pLVX-*Pitx2c*-IRES-ZsGreen lentiviral expression vector leads us to use ZsGreen to localize *Pitx2c*-overexpressing cells after transplantation. After 15 days of cell transplantation, quantification of ZsGreen+ myofibers indicate that the number of ZsGreen+ cells forming myofibers was significantly higher in muscles injected with satellite cells overexpressing *Pitx2c* with respect to that injected with satellite cells transfected with the empty lentiviral vector (Figures 4B–4D). Moreover, *Pitx2c* overexpression induced a shift in the distribution of the regenerating fiber size to the highest area classes (Figure 4E), indicating that regenerative potential was enhanced in DMD/mdx-satellite cells after *Pitx2c* overexpression.

Previously, we have reported in freshly isolated satellite cells that the c-isoform of the transcription factor PITX2 increases cell proliferation in early activated satellite cells by downregulating miR-15b, miR-23b, miR-106b, and miR-503, fortifying the MYF5+ satellite cells and thereby promoting their commitment to a myogenic cell fate (Lozano-Velasco et al., 2015). Therefore, to test whether this PITX2-miRNA pathway was also present in the muscles transplanted with cells overexpressing *Pitx2c*; the expression levels of these miRNAs were evaluated by qRT-PCR. As illustrated in Figures S4A and S4B, all four miRNAs were downregulated in transfected cells before transplantation as well as in transplanted muscles (15 days after cell transplantation). Also, in agreement with previous published results (Lozano-Velasco et al., 2015), we observed that *cyclin D1*, *cyclin D2,* and *Myf5* were upregulated in both transfected cells and the muscles transplanted with *Pitx2c*-overexpressing cells (Figures S4C and S4D), indicating that the *Pitx2*-miRNA pathway was also acting in donor satellite cells. We also analyzed the number of Ki67+ and MYF5+ cells in the transplanted muscles after 3 and 15 days of cell transplantation. This analysis showed that the number of Ki67+ cells as well as the number of MYF5+ cells was significantly greater in muscles transplanted with *Pitx2c*-overexpressing cells (Figures S4E and S4F).

Therefore, we conclude that *Pitx2c* overexpression in the transplanted dystrophic satellite cells boosts their regenerative capability by stimulating cell proliferation and raising the number of myogenic committed cells due to the activation of the previously described PITX2-miRNA pathway.

PITX2c Increases Full-Length Revertant Dystrophin Protein in DMD/mdx Mice by Regulating miR-31

Recently, Cacchiarelli et al. (2011) reported the identification of a miRNA—miR-31—which represses dystrophin expression by targeting its 3′ UTR (Cacchiarelli et al., 2011). In human DMD myoblasts treated with exon skipping, they also demonstrate that miR-31 inhibition increases DYSTROPHIN rescue. These results indicate that interfering with miR-31 activity can provide an ameliorating strategy for those DMD therapies that are aimed at efficiently recovering DYSTROPHIN synthesis (Cacchiarelli et al., 2011). Notably, miR-31 appears as downregulated by *Pitx2c* in a previously reported microarray analyses in Sol8 myoblasts (Lozano-Velasco et al., 2015). We have further validated PITX2c-mediated miR-31 downregulation by performing *in vitro* gain-of-function experiments in freshly isolated satellite cells. Therefore, qRT-PCR analyses in satellite cells overexpressing *Pitx2c* showed a clear miR-31 downregulation (Figure 5A). To reinforce the idea that PITX2c modulates the expression of miR-31, we screened for potential conserved PITX2 binding sites upstream of *miR-31* genomic loci. Two conserved PITX2 binding sites were identified ~6 kb upstream of *miR-31* gene locus (Figure 5B). To test the interaction of PITX2 with those putative binding sites, we performed chromatin immunoprecipitation (ChIP) assays in Sol8 cells. Exogenous PITX2 bound to the all-putative binding sites upstream of miR-31, genetic locus, as illustrated in Figures 5B and 5C.

According to those results, we found that miR-31 expression was significantly declined in muscles injected with *Pitx2c*-overexpressing cells compared with controls (Figure 6A). As expected, a sharp rise in *Dystrophin*-mRNA expression levels was also detected (Figure 6B). To further analyze how miR-31 regulates DYSTROPHIN expression in our system, we first performed *in vitro* experiments blocking transcription by using α-amanitin and, as illustrated in Figure 6C, we found that *Dystrophin mRNA* is clearly depressed when miR-31 is present, indicating that this miRNA acts by repressing *Dystrophin* mRNA stability.

A noteworthy phenomenon that occurs in DMD patients and DMD/mdx mice is the presence of revertant fibers that express DYSTROPHIN (Klein et al., 1992; Sicinski et al., 1989). The revertant fibers express DYSTROPHINS that

(D) Representative images for ZsGreen+ myofibers in LVX-Pitx2c-ZsGreen muscles versus LVX-ZsGreen muscles in negative control (uninjected muscle).
(E) Cross-sectional area in TA muscles of LVX-Pitx2c-ZsGreen muscles (n = 6) versus control LVX-ZsGreen muscles (n = 6).
Statistical significance was determined by Student’s t test. *p < 0.01, **p < 0.001, ***p < 0.0001, ****p < 0.00001.

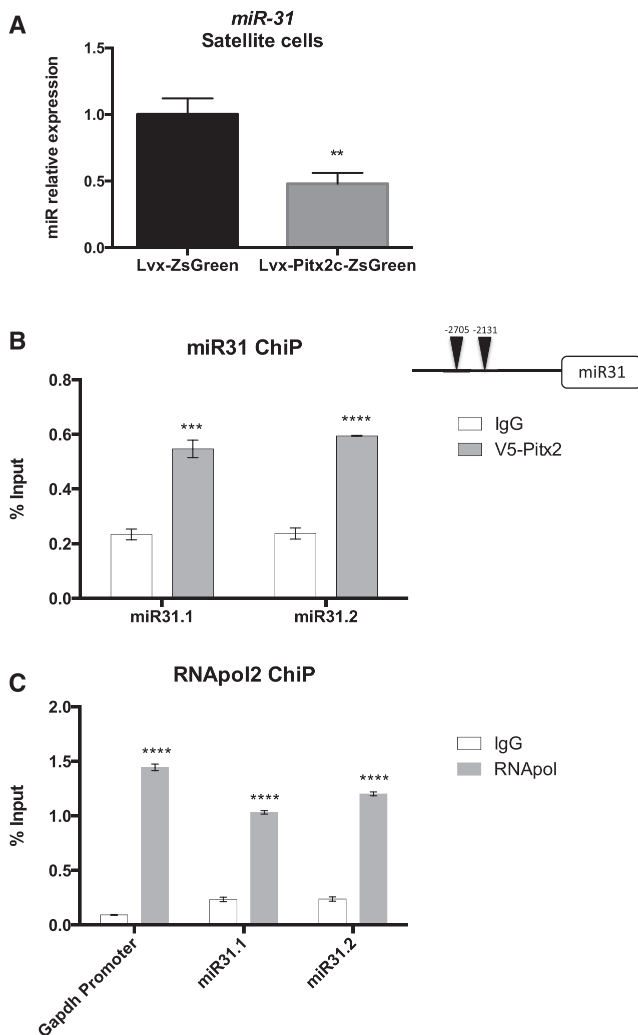

Figure 5. PITX2C Regulates miR-31 Expression

(A) miR-31 expression in isolated satellite cells overexpressing *Pitx2c* (LVX-Pitx2c-ZsGreen) versus control cells (LVX-ZsGreen). Data are presented as means ± SD for at least three independent experiments.

(B) PITX2 binds DNA regions upstream of miR-31 genetic locus.

(C) RNA polymerase II occupancy in tested DNA regions upstream of miR-31. For ChiP analysis, experiments were repeated at least twice with similar results. A representative example of a ChIP analysis is shown. Error bars display the SD of the mean.

Statistical significance was determined by Student's t test. $^{**}p < 0.001$, $^{***}p < 0.0001$, $^{****}p < 0.00001$.

arise for alternatively splicing transcripts lacking the mutant exon by exon skipping (Lu et al., 2000). Thus, using qRT-PCR, we tested the relative levels of *Dystrophin* mRNA lacking the mutant exon by analyzing the two alternative transcripts previously identified as directing the synthesis of the most commonly encountered revertant DYSTROPHIN isoforms in DMD/mdx mice (exon 18–35 splicing and exon 13–48 splicing *Dystrophin* mRNAs that direct 17% and 25% of the revertant DYSTROPHIN isoforms, respectively) (Lu et al., 2000). This analysis revealed that these alternative transcripts were significantly increased in the muscles injected with *Pitx2c*-overexpressing cells (Figures 6D and 6E). These findings indicate that, in our system, miR-31 downregulation mediated by PITX2c augments the amount of *Dystrophin* mRNA transcripts that lack the mutant exon.

Next, to check whether miR-31 increases the amount of reverted protein, we performed western blot analyses using an antibody that recognizes the C-terminal domain of DYSTROPHIN protein expressed in revertant fibers (Thanh et al., 1995). As shown in Figures 6F and 6G, DYSTROPHIN-deficient muscles transplanted with *Pitx2c*-overexpressing cells display a significant surge in the amount of most representative revertant DYSTROPHIN isoforms (exon 18–35 spliced and exon 13–48 spliced DYSTROPHINS) as assayed by western blot. In accordance with those findings, the number of DYSTROPHIN-positive fibers was significantly higher in the muscles injected with *Pitx2c*-overexpressing cells (Figures 6H and 6I). Moreover, *Pitx2c* overexpression in differentiated myotubes derived from DMD/mdx-satellite cells leads to increase *Dystrophin* mRNA expression (Figure 6J). Together these results indicate that truncated versions of DYSTROPHIN protein are detected in the recipient muscles due to upregulation of different *Dystrophin* mRNA variants, including the alternatively spliced mRNAs lacking the mutated exon, as a result of increased expression of *Pitx2c* in the fibers derived from the transplanted cells.

Finally, to assess functional performance, we performed treadmill tests in DMD/mdx mice transplanted with *Pitx2c*-overexpressing cells in both hind legs, until exhaustion 30 days after cell transplantation. As illustrated in Figure 6K, the running time and distance were 26%–37% higher, respectively, in transplanted mice with respect to control mice, indicating that transplantation of *Pitx2c*-overexpressing cells bolsters physical performance.

DISCUSSION

In this report we have identified PITX2c as an essential positive regulator of muscle regeneration in mice. Also, we found that *Pitx2c* is downregulated in DMD/mdx mice, which exhibit intrinsic defects on satellite cell differentiation. Based on these results, we have performed an *in vivo* experimental approach to demonstrate that greater *Pitx2c* expression in dystrophic satellite cells enhances their regenerative capacity. An important finding uncovered in this study is that PITX2C restores the expression of reverted DYSTROPHIN by regulating

A

miR-31
Cell transplanted -muscles
miR relative expression
0.0
0.5
1.0
1.5
**
Lvx-ZsGreen
Lvx-Pitx2c-ZsGreen

B

Dystrophin
mRNA expression level (a.u.)
0
2
4
6
***
Lvx-ZsGreen
Lvx-Pitx2c-ZsGreen

C

Dystrophin
mRNA expression level (a.u.)
0.0
0.5
1.0
1.5
***
**
***
CN
alpha amanitin
alpha amanitin pre-mir-31
alpha amanitin anti-mir-31

D

Exon 18-35 spliced
Dystrophin mRNAs
mRNA expression level (a.u.)
0
5
10
***
Lvx-ZsGreen
Lvx-Pitx2c-ZsGreen

E

Exon 13-48 splic
Dystrophin mRN
mRNA expression level (a.u.)
0
1
2
3
**
Lvx-ZsGreen
Lvx-Pitx2c-ZsGreen

F

C57/Bl6
Lvx-ZsGreen
Lvx-Pitx2c-ZsGreen
Dystrophin (full size)
Tubulin

G

Relative dystrophin intensity (fold increase over control)
0
1
2
3
***
***
DYSTROPHIN isoform -17%
DYSTROPHIN isoform -25%

H

LVX-ZsGreen
DYSTROPHIN
40 um
LVX-Pitx2c-ZsGreen
DYSTROPHIN
40 um

I

DYSTROPHIN + myofibers
% DYSTROPHYN +
0
10
20
30
40
**
Lvx-ZsGreen
Lvx-Pitx2c-ZsGreen

J

Dystrophin
(14 days)
mRNA expression level (a.u.)
0.0
0.5
1.0
1.5
2.0
*
LVX
Lvx-Pitx2c

K

Distance
metres
0
100
200
300
400
500
600
**
C57
Lvx-ZsGreen
Lvx-Pitx2c-ZsGreen

Time running
Minutes
0
10
20
30
40
***
C57
Lvx-ZsGreen
Lvx-Pitx2c-ZsGreen

(legend on next page)

miR-31 in DMD/mdx mice. Thus, these results provide *in vitro* and *in vivo* evidence for a role of PITX2 in skeletal muscle repair and in degenerative muscle diseases.

The knowledge of satellite cell molecular biology significantly contributes to the clarification of the molecular and cellular mechanisms of skeletal muscle regeneration (Lavasani et al., 2013). PITX2 is a transcription factor that has been shown to regulate satellite cell biology (Ono et al., 2010; Knopp et al., 2013). We have previously shown that overexpression of *Pitx2c* in Sol8 myoblasts maintain them with high proliferative capacity (Lozano-Velasco et al., 2011). More recently we have demonstrated that PITX2c increases proliferation in early activated satellite cells and promotes their commitment to a myogenic cell fate (Lozano-Velasco et al., 2015). However, Knopp et al. (2013) showed that PITX2c enhanced *in vitro* myogenic differentiation but reduced proliferation in satellite cell-derived myoblasts. In this study, we have observed that PITX2c promotes both satellite cell proliferation and their differentiation. The divergence of the PITX2c effects on satellite cell proliferation found by Knopp et al. (2013) could be explained by the different methods used for satellite cell isolation as well as the different times of *in vitro* cultures performed, since *in vitro* satellite cell proliferative behavior may be altered, depending of the method used for cell isolation (Montarras et al., 2005; Qu-Petersen et al., 2002). We have previously reported that *Pitx2c* overexpression in Sol8 myoblasts blocked terminal differentiation (Lozano-Velasco et al., 2011), while we demonstrate herein that PITX2c promotes satellite cell differentiation. The disparity between these results might be pointing out that PITX2c has different effects at distinct stages of muscle stem cell lineage commitment, promoting myogenic lineage progression upon early activation but blocking terminal differentiation in committed myoblasts.

Importantly, we found that *Pitx2c* expression peaked at day 1 after muscle damage, in line with our previous results that demonstrated an early *Pitx2c* upregulation during satellite cell activation *in vitro* (Lozano-Velasco et al., 2015). This *Pitx2c* upregulation was accompanied by an increase in the number of PITX2c+ cells during the early stages of muscle regeneration. However, *Pitx2c* expression levels were low in the dystrophic muscle, which display defects on muscle regeneration. Furthermore, few PITX2c+ cells were detected in the dystrophic muscle. These results reveal that PITX2c plays a critical role in skeletal muscle satellite cell biology and muscle regeneration.

The ability of satellite cells to effectively repair damaged skeletal muscle requires both coordinated proliferation as well as differentiation, and several previous reports have shown that myoblasts from DMD patients exhibit defects in cell proliferation (Blau et al., 1983) and dystrophin-deficient satellite cells display prolonged cell divisions, loss of apicobasal asymmetric division, and a higher proportion of abnormal division, leading to reduced generation of myogenic progenitors (Dumont et al., 2015). Here we found that, associated with defects in myogenic differentiation, satellite cells isolated from DMD/mdx mice clearly display lower PITX2c expression, and PITX2c gain of function rescued most of their myogenic potential, suggesting that this transcription factor also could act to modulate myogenic differentiation in dystrophic satellite cells. In addition, we show that *Pitx2c* overexpression in dystrophic satellite cells increase their regenerative potential *in vivo*.

Figure 6. *Pitx2c* Overexpression Downregulates miR-31, Leading to DYSTROPHIN Restoration in Transplanted Muscles

(A) miR-31 expression in muscle transplanted with *Pitx2c*-overexpressing cells (LVX-Pitx2c-ZsGreen) versus control muscles (LVX-ZsGreen).

(B) mRNA levels for *Dystrophin* in muscles transplanted with *Pitx2c*-overexpressing cells (LVX-Pitx2c-ZsGreen) versus control muscles (LVX-ZsGreen).

(C) *pre-miR-31* transfection clearly decreases mRNA levels for *Dystrophin* after ALPHA-AMANITIN treatment and, blocking endogenous miR-31 by *anti-miR-31* transfection, mRNA levels for *Dystrophin* are increased.

(D) qRT-PCR analysis for exon 18–35 spliced *Dystrophin* mRNAs.

(E) qRT-PCR analysis for exon 13–48 spliced *Dystrophin* mRNAs.

(F) A representative DYSTROPHIN western blot showing spliced DYSTROPHINS (arrows) at the expected size in muscle transplanted with *Pitx2c*-overexpressing cells (LVX-Pitx2c-ZsGreen) versus control muscles (LVX-ZsGreen).

(G) Quantification of western blot analysis.

(H) Representative images for Dystrophin+ myofibers in muscles transplanted with *Pitx2c*-overexpressing cells (LVX-Pitx2c-ZsGreen) versus control muscles (LVX-ZsGreen).

(I) Quantification of DYSTROPHIN+ myofibers in muscles transplanted with *Pitx2c*-overexpressing cells (LVX-Pitx2c-ZsGreen) versus control muscles (LVX-ZsGreen).

(J) qRT-PCR analyses for *Dystrophin* mRNA expression in differentiating satellite cells overexpressing *Pitx2c* (LVX-*Pitx2c*) versus control cells (LVX) (day 14 of culture).

(K) Treadmill test: running time and distance of C57/BL6 wild-type mice, DMD/mdx mice transplanted with *Pitx2c*-overexpressing dystrophic satellite cells (LVX-Pitx2c-ZsGreen), and DMD/mdx mice transplanted with control dystrophic satellite cells (LVX-ZsGreen) (n = 8). Values and error bars are means of SD of at least three independent experiments.

Statistical significance was determined by Student's t test. *p < 0.01, **p < 0.001, ***p < 0.0001.

A previous work in our laboratory has pointed to the existence of a PITX2-miRNA pathway controlling *in vitro* cell proliferation and myogenic cell fate in isolated satellite cells (Lozano-Velasco et al., 2015). Our analyses revealed that this PITX2-miRNA pathway is also present in cell-transplanted muscles, leading to greater cell proliferation and raising the number of myogenic committed cells in *Pitx2c*-overexpressing transplanted cells. Although PITX2 function regulating the redox state during fetal myogenesis has been described previously (L'Honoré et al., 2014), *Pitx2c* overexpression on dystrophic satellite cells has no impact on the expression of genes encoding antioxidant enzymes (Figure S5), suggesting differences between adult and fetal myogenesis. Therefore, we postulate that, in our system, activation of the PITX2-miRNA pathway in dystrophic transplanted cells promoted cell proliferation and myogenic cell fate during the process of muscle regeneration, thus finally enhancing their regenerative potential.

Muscles in Duchenne dystrophy patients characteristically lack DYSTROPHIN due to protein-truncating mutations that either disrupt the reading frame or cause premature termination of translation of the *Dystrophin*-encoding gene, which in turn results in the lack of functional protein (Pigozzo et al., 2013). However, these dystrophic muscles contain sporadic small clusters of DYSTROPHIN-expressing revertant fibers. These revertant fibers are also present in mdx mice, i.e., the dystrophic-deficient DMD/*mdx* mouse, and are believed to result from alternative splicing events that bypass mutation and restore an open reading frame (Lu et al., 2000). On other hand, recent studies have identified the post-transcriptional control of gene expression as a crucial level in the regulation of myogenesis (Guess et al., 2015; Zhang et al., 2015; McCarthy et al., 2007). It bears noting that a comprehensive analysis of the expression profiles for miRNAs has revealed that deregulation of miRNAs genes is common in muscle pathology, and several recent studies have revealed that miRNAs may be involved in the pathophysiology of muscular dystrophy (Cacchiarelli et al., 2011; Eisenberg et al., 2007; Greco et al., 2009). Moreover, the role of miR-31 has been reported in modulating the expression of DYSTROPHIN in a myoblast line obtained from dystrophic patients, indicating that miR-31 repression in the skeletal muscles could improve therapeutic treatments aimed at raising the levels of DYSTROPHIN synthesis (Kinoshita et al., 1994). Here, we present evidence that PITX2 negatively regulates miR-31 expression in myoblasts and satellite cells. In accordance with that finding, we also observed a clear miR-31 downregulation when dystrophic satellite cells overexpressing *Pitx2c* were transplanted into the muscles of DMD/mdx mice and, consequently, the levels of DYSTROPHIN-expressing revertant fibers were significantly higher. Our analyses indicate that miR-31 acts by promoting *Dystrophin* mRNA degradation, and that miR-31 downregulation mediated by PITX2 augments the total amount of *Dystrophin* mRNA, overall the revertant *Dystrophin* mRNA, and thus revertant DYSTROPHIN protein. Dumont et al. (2015) previously reported that DYSTROPHIN-deficient satellite cells exhibit a clear defect in asymmetric cell division (Dumont et al., 2015), we therefore cannot rule out that increased cell division observed in transplanted DYSTROPHIN-deficient *Pitx2c*-overexpressing cells can be linked to increased levels of revertant DYSTROPHIN protein. In addition, since it has been shown that manipulation of miR-31 levels affects satellite cell differentiation by regulation of MYF5 protein (Crist et al., 2012), the increase on myogenic differentiation observed by us in the transplanted muscles could be also due, at least in part, to PITX2c-mediated miR-31 downregulation. Finally, it bears highlighting that, as a consequence of the multiple functions mediated by PITX2 in dystrophic transplanted cells, DMD/mdx mice reach a major functional recovery, reinforcing the contention that PITX2 constitutes a crucial player modulating skeletal muscle repair.

Overall, our findings demonstrate that PITX2 orchestrates a number of molecular mechanisms that control muscle regeneration. Our *in vitro* and *in vivo* data demonstrate that Pitx2 enhances the regenerative capability of DYSTROPHIN-deficient satellite cells by increasing cell proliferation and enhancing the number of myogenic committed cells by activating the PITX2-miR-106b/miR-503/miR-23b/miR-15b pathway. Importantly we found that PITX2 represses miR-31, leading to an increase of revertant DYSTROPHIN protein and finally improving muscle regeneration (Figure 7). This study reveals the previously unknown function of PITX2 in skeletal muscle repair and may help to develop therapeutic strategies for muscular disorders.

EXPERIMENTAL PROCEDURES

Animals

C57BL/10ScSn and C57/Bl10ScSn-Dmdmdx/J (DMD/mdx) mice were supplied by The Jackson Laboratory. All mice were maintained inside a barrier facility, and experiments were performed in accordance with the University of Jaén regulations for animal care and handling.

Satellite Cell Isolation and Culture

Satellite cell isolation from 4-month-old mice (C57BL/10ScSn and C57/Bl10ScSn-Dmdmdx/J [DMD/mdx]), as well as from a human Becker muscle dystrophy patient (male, 35 years old), was performed by magnetic separations according to the manufacturer's protocol. Further details are provided in the Supplemental Information. After separation, freshly isolated satellite cells were cultured as described previously (Garriga et al., 2000).

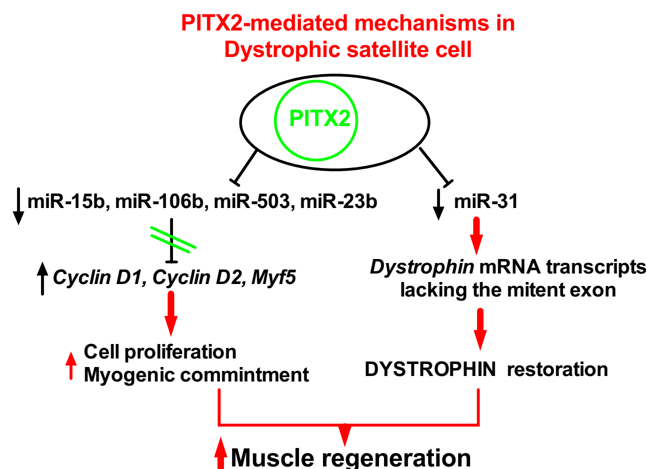

Figure 7. PITX2-Mediated Mechanisms in Dystrophic Satellite Cells during the Muscle-Regeneration Process

Pitx2c overexpression in dystrophic satellite cells increases cell proliferation and enhances the number of myogenic committed cells by activating the previously described PITX2-miR-106b/miR-503/miR-23b/miR-15b pathway but also repressing miR-31, leading to a higher amount of *Dystrophin* mRNA transcripts lacking the mutant exon and finally to an increase of DYSTROPHIN expressing revertant fibers. Together, PITX2c effects lead to improve muscle regeneration.

Pitx2c Overexpression and siRNA-Pitx2c

Pitx2c overexpression and *siRNA-Pitx2c* in satellite cells was performed as described previously (Lozano-Velasco et al., 2015). In brief, lentiviral particles containing the lentiviral vectors coding for PITX2c (pLVX-Pitx2c-IRES-ZsGreen) or the empty lentiviral vector (LVX-IRES-ZsGreen) were produced by using Lenti-X HTX Packaging Systems and following the manufacturer's procedure (Clontech). Detailed information is provided in the Supplemental Experimental Procedures.

qRT-PCR Analyses

RNA isolation and RT-PCR were performed as described previously (Lozano-Velasco et al., 2011), using standard procedures. Detailed information is provided in the Supplemental Experimental Procedures.

Cardiotoxin and Muscle Injury

Cardiotoxin was prepared by dissolving a freshly opened tube in PBS at 10 μM as described previously (Lepper et al., 2009). The TA muscles of 4-month-old C57BL/10ScSn (n = 4) mice were injected with 50 μL of cardiotoxin (Sigma). For immunohistochemistry and histological analysis, the animals were killed and TA muscles were collected at 7 and 15 days after cardiotoxin injection, frozen in liquid nitrogen-cooled isopentane for sectioning, or in liquid nitrogen for mRNA isolation, and preserved at −80°C.

Cell Transplantation Experiments

Four-month-old DMD/mdx mice (n = 12) pre-treated with the immunosuppressant FK506 (Kinoshita et al., 1994) were intramuscularly injected into the TA muscle with cardiotoxin (Lepper et al., 2009) to increase damage. Freshly isolated satellite cells were isolated from 4-month-old DMD/mdx donors and infected with lentiviral vectors as described previously (Benchaouir et al., 2007). For the histological, immunohistochemistry, and mRNA analyses, donor DMD/mdx cells infected with pLVX empty lentiviral vector were injected (5×10^4 in 50 μL F12 medium) into the right TA muscle and used as controls (n = 6), whereas donor DMD/mdx infected with pLVX-*Pitx2* lentiviral vector were injected (5×10^4 in 50 μL F12 medium) into the left TA muscle of the same recipient DMD/mdx (n = 6) (Torrente et al., 2004). Two weeks after cell transplantation, the animals were killed and TA muscles were frozen in liquid nitrogen-cooled isopentane for sectioning, or in liquid nitrogen for mRNA isolation, and preserved at −80°C.

For the treadmill test, both TA muscles of 4-month-old recipients DMD/mdx control mice (n = 8) were injected intramuscularly with 5×10^4 cells infected with pLVX empty lentiviral vector, whereas in the DMD/mdx experimental mice (n = 8) both TA muscles were injected with 5×10^4 cells infected with pLVX-*Pitx2* lentiviral vector. Four weeks after cell injection the animals were submitted to an exercise-tolerance test as described below.

miRNA and Anti-miRNA Transfection Assays

Satellite cells were cultured under growing conditions. Corresponding pre-miRNAs (Ambion) were transfected as described previously (Lozano-Velasco et al., 2011).

Immunocytochemistry and Immunohistochemistry

Immunocytochemistry experiments in satellite cells were performed as described previously (L'Honoré et al., 2007; Ono et al., 2010). Further details are provided in the Supplemental Experimental Procedures.

Cross-Section Area

To analyze the regenerating muscle transplanted with *Pitx2c*-overexpressing cells, we measured the regenerating fiber cross-sectional area after 2 weeks of the damage, as described by Moresi et al. (2009).

Western Blot

Western blot analyses were performed as described previously (Camici et al., 2007). Detailed information is provided in the Supplemental Experimental Procedures.

ChIP Assay

ChIP assays were performed as described previously in Sol8 cells (Lozano-Velasco et al., 2015). All PCR reactions were performed at an annealing temperature of 60°C. Different primers were used to amplify the DNA regions containing the PITX2 binding site 6 kb upstream of the coding sequence for miR-31. As controls, normal rabbit immunoglobulin G replaced the anti-V5 antibody to reveal nonspecific immunoprecipitation of chromatin.

α-Amanitin

RNA polymerase II satellite cells were inhibited by adding α-amanitin (Sigma-Aldrich, no. A2263) to the cell culture medium to a final concentration of 4 μg/mL, as described previously (Daimi et al., 2015).

Exercise-Tolerance Test

An exhaustion treadmill trial was performed to evaluate the endurance of the mice using a motorized treadmill (LE8708 single mouse, Panlab Treadmills, Harvard Apparatus) supplied with a shocker plate as described previously (Benchaouir et al., 2007).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.03.009.

AUTHOR CONTRIBUTIONS

D.V. conceived and carried out the experiments. F.H.-T. and E.L.-V. contributed to cell transplantation and qRT-PCR experiments. A.C. and C.C. performed collection of biopsies from dystrophic patients. L.R.-O. contributed to cell culture experiments. D.F. contributed to the project design and critical reading of the paper. A.E.A. carried out the experiments, provided overall project supervision, and produced the paper and figures.

ACKNOWLEDGMENTS

This work was partially supported by grants BFU2012-38111, BFU2015-67131 (Ministerio de Economía y Competitividad, Gobierno de España), CTS-1614, P08-CTS-03878, BIO-302 (Junta de Andalucia), and AFM2012-16074 (AFM).

Received: April 7, 2017
Revised: March 11, 2018
Accepted: March 12, 2018
Published: April 10, 2018

REFERENCES

Allen, D.G., Gervasio, O.L., Yeung, E.W., and Whitehead, N.P. (2010). Calcium and the damage pathways in muscular dystrophy. Can. J. Physiol. Pharmacol. *88*, 83–91.

Benchaouir, R., Meregalli, M., Farini, A., D'Antona, G., Belicchi, M., Goyenvalle, A., Battistelli, M., Bresolin, N., Bottinelli, R., García, L., et al. (2007). Restoration of human dystrophin following transplantation of exon-skipping-engineered DMD patient stem cells into dystrophic mice. Cell Stem Cell *13*, 646–657.

Berardi, E., Annibali, D., Cassano, M., Crippa, S., and Sampaolesi, M. (2014). Molecular and cell-based therapies for muscle degenerations: a road under construction. Front. Physiol. *5*, 119.

Bertoni, C. (2014). Emerging gene editing strategies for Duchenne muscular dystrophy targeting stem cells. Front. Physiol. *5*, 148.

Blau, H.M., Webster, C., and Pavlath, G.K. (1983). Defective mioblasts identified in Duchenne muscular dystrophy. Proc. Natl. Acad. Sci. USA *80*, 4856–4860.

Bulfield, G., Siller, W.G., Wight, P.A., and Moore, K.J. (1984). X chromosome-linked muscular dystrophy (DMD/mdx) in the mouse. Proc. Natl. Acad. Sci. USA *81*, 1189–1192.

Cacchiarelli, D., Incitti, T., Martone, J., Marcella, C., Cazzella, V., Santini, T., Sthandier, O., and Bozzoni, I. (2011). miR-31 modulates dystrophin expression: new implications for Duchenne muscular dystrophy therapy. EMBO Rep. *12*, 136–141.

Camici, G.G., Schiavoni, M., Francia, P., Bachschmid, M., Martin-Padura, I., Hersberger, M., Tanner, F.C., Pelicci, P., Volpe, M., Anversa, P., Lüscher, T.F., and Cosentino, F. (2007). Genetic deletion of p66(Shc) adaptor protein prevents hyperglycemia-induced endothelial dysfunction and oxidative stress. Proc. Natl. Acad. Sci. USA *104*, 5217–5222.

Chal, J., Oginuma, M., Al Tanoury, Z., Gobert, B., Sumara, O., Hick, A., Bousson, F., Zidouni, Y., Mursh, C., Moncuquet, P., et al. (2015). Differentiation of pluripotent stem cells to muscle fiber to model Duchenne muscular dystrophy. Nat. Biotechnol. *33*, 962–969.

Crist, C.G., Montarras, D., and Buckingham, M. (2012). Muscle satellite cells are primed for myogenesis but maintain quiescence with sequestration of Myf5 mRNA targeted by microRNA-31 in mRNP granules. Cell Stem Cell. *11*, 118–126.

Daimi, H., Lozano-Velasco, E., Haj Khelil, A., Chibani, J.B.E., Barana, A., Amorós, I., Gonzalez de la Fuente, M., Caballero, R., Aránega, A., and Franco, D. (2015). Regulation of SCN5A by microRNAs: miR-219 modulates SCN5A transcript expression and the effects of flecainide intoxication in mice. Heart Rhythm *12*, 1333–1342.

Dumont, N.A., Wang, Y.X., Von Maltzahn, J., Pasut, A., Bentzinger, C.F., Brun, C.E., and Rudnicki, M.A. (2015). Dystrophin expression in muscle stem cells regulates their polarity and asymmetric division. Nat. Med. *21*, 1455–1463.

Eisenberg, I., Eran, A., Nishino, I., Moggio, M., Lamperti, C., Amato, A.A., Lidov, H.G., Kang, P.B., North, K.N., Mitrani-Rosenbaum, S., et al. (2007). Distinctive patterns of microRNA expression in primary muscular disorders. Proc. Natl. Acad. Sci. USA *104*, 17016–17021.

Garriga, J., Adanero, E., Fernández-Solá, J., Urbano-Marquez, A., and Cussó, R. (2000). Ethanol inhibits skeletal muscle cell proliferation and delays its differentiation in cell culture. Alcohol Alcohol. *35*, 236–241.

Greco, S., De Simone, M., Colussi, C., Zaccagnini, G., Fasanaro, P., Pescatori, M., Cardani, R., Perbellini, R., Isaia, E., Sale, P., et al. (2009). Common micro-RNA signature in skeletal muscle damage and regeneration induced by Duchenne muscular dystrophy and acute ischemia. FASEB J. *23*, 3335–3346.

Guess, M.G., Barthel, K.B., Harrison, B.C., and Leinwand, L.A. (2015). miR-30 family microRNAs regulate myogenic differentiation and provide negative feedback on the microRNA pathway. PLoS One *10*, e0118229.

Hebert, S.L., Daniel, M.L., and McLoon, L.K. (2013). The role of Pitx2 in maintaining the phenotype of myogenic precursor cells in the extraocular muscles. PLoS One *8*, e58405.

Kinoshita, I., Vilguin, J.T., Guérette, B., Asselin, I., Roy, R., Lille, S., and Tremblay, J.P. (1994). Inmunosuppression with FK 506 insures good success of myoblast transplantation in MDX mice. Transplant. Proc. *26*, 3518.

Klein, C.J., Coovert, D.D., Bulman, D.E., Ray, P.N., Mendell, J.R., and Burghes, A.H. (1992). Somatic reversion/suppression in Duchenne muscular dystrophy (DMD): evidence supporting a frame-restoring mechanism in rare dystrophin-positive fibers. Am. J. Hum. Genet. *50*, 950–959.

Knopp, P., Figeac, N., Fortier, M., Moyle, L., and Zammit, P.S. (2013). Pitx genes are replayed in adult myogenesis where they can act to promote myogenic differentiation in muscle satellite cells. Dev. Biol. *377*, 293–304.

Kuang, S., Kuroda, K., Le Grand, F., and Rudnicki, M.A. (2007). Asymmetric self-renewal and commitment of satellite cells in muscle. Cell *129*, 999–1010.

L'Honoré, A., Coulon, V., Marcil, A., Lebel, M., Lafrance-Vanasse, J., Gage, P., and Drouin, J. (2007). Sequential expression and redundancy of Pitx2 and Pitx3 genes during muscle development. Dev. Biol. *307*, 421–433.

L'Honoré, A., Ouimette, J.F., Lavertu-Jolin, M., and Drouin, J. (2010). Pitx2 defines alternate pathways acting throught MyoD during limb and somatic myognesis. Development *137*, 3847–3856.

L'Honoré, A., Commère, P.H., Quimette, J.F., Montarras, D., Drouin, J., and Buckingham, M. (2014). Redox regulation by Pitx2 and Pitx3 is critical for fetal myogenesis. Dev. Cell. *29*, 392–405.

Lavasani, M., Lu, A., Thompson, S.D., Robbins, P.D., Huard, J., and Niedernhofer, L.J. (2013). Isolation of muscle-derived stem/progenitor cells based on adhesion characteristics to collagen-coated surfaces. Methods Mol. Biol. *976*, 53–65.

Lepper, C., Conway, S.J., and Fan, C.M. (2009). Adult satellite cells and embryonic muscle progenitors have distinct genetic requirements. Nature *460*, 627–631.

Lozano-Velasco, E., Contreras, A., Crist, C., Franco, D., and Aránega, A.E. (2011). Pitx2c modulates Pax3+/Pax7+ cell populations and regulates Pax3 expression by repressing miR27 expression during myogenesis. Dev. Biol. *357*, 165–178.

Lozano-Velasco, E., Vallejo, D., Esteban, F.J., Doherty, C., Hernández-Torres, F., Franco, D., and Aránega, A.E. (2015). Pitx2 modulates cell proliferation in myoblasts and skeletal-muscle satellite cells and promotes their commitment to a myogenic cell fate. Mol. Cell Biol. *35*, 2892–2909.

Lu, Q.L., Morris, G.E., Wilton, S.D., Ly, T., Artem'yeva, O.V., Strong, P., and Partridge, T.A. (2000). Massive idiosyncratic exon skipping corrects the nonsense mutation in dystrophic mouse muscle and produces functional revertant fibers by clonal expansion. J. Cell Biol. *148*, 985–996.

Mah, J.K., Korngut, L., Dykeman, J., Day, L., Pringsheim, T., and Jette, N. (2014). A systematic review and meta-analysis on the epidemiology of Duchenne and Becker muscular dystrophy. Neuromuscul. Disord. *26*, 428–491.

Martínez-Fernández, S., Hernández-Torres, F., Franco, D., Lyons, G.E., Navarro, F., and Aránega, A.E. (2006). Pitx2c overexpression promotes cell proliferation and arrests differentiation in myoblasts. Dev. Dyn. *235*, 2930–2939.

McCarthy, J.J., Esser, K.A., and Andrade, F.H. (2007). MicroRNA-206 is overexpressed in the diaphragm but not the hindlimbmuscle of mdx mouse. Am. J. Physiol. Cell Physiol. *293*, C451–C457.

Mercuri, E., and Muntoni, F. (2013). Muscular dystrophies. Lancet *881*, 841–860.

Montarras, D., Morgan, J., Collins, C., Relaix, F., Zaffran, S., Cumano, A., Partridge, T., and Buckingham, M. (2005). Direct isolation of satellite cells for skeletal muscle regeneration. Science *309*, 2064–2067.

Moresi, V., García-Alvarez, G., Pristerá, A., Rizzuto, E., Albertini, M.C., Marazzi, G., Sassoon, D., Adamo, S., and Coletti, D. (2009). Modulation of caspase activity regulates skeletal muscle regeneration and function in response to vasopressin and tumor necrosis factor. PLoS One *4*, e5570.

Ono, Y., Boldrin, L., Knopp, P., Morgan, J.E., and Zammit, P.S. (2010). Muscle satellite cells are a functionally heterogeneous population in both somite-derived and branchiomeric muscles. Dev. Biol. *337*, 29–41.

Partridge, T.A. (2013). The DMD/mdx mouse model as a surrogate for Duchenne muscular dystrophy. FEBS J. *280*, 4177–4186.

Pigozzo, S.R., Da Re, L., Romualdi, C., Mazzara, P.G., Galletta, E., Fletcher, S., Wilton, S.D., and Vitiello, L. (2013). Revertant fibers in the mdx murine model of Duchenne muscular dystrophy: an age and muscle-related reappraisal. PLoS One *8*, e72147.

Qu-Petersen, Z., Deasy, B., Jankowski, R., Ikezawa, M., Cummins, J., Pruchnic, R., Mytinger, R., Cao, B., Gates, C., Wernig, A., et al. (2002). Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. J. Cell Biol. *157*, 851–864.

Shi, H., Verma, M., Zhang, L., Dong, C., Flavell, R.A., and Bennett, A.M. (2015). Improved regenerative mogenesis and muscular dystrophy in mice lacking MKP5. J. Clin. Invest. *123*, 2064–2077.

Sicinski, P., Geng, Y., Ryder-Cook, A.S., Barnard, E.A., Darlison, M.G., and Barnard, P.J. (1989). The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. Science *244*, 1578–1580.

Thanh, L.T., Nguyen, T.M., Helliwell, T.R., and Morris, G.E. (1995). Characterization of revertant muscle fibers in Duchenne muscular dystrophy, using exon-specific monoclonal antibodies against dystrophin. Am. J. Hum. Genet. *56*, 725–731.

Torrente, Y., Belicchi, M., Sampaolesi, M., Pisati, F., Meregalli, M., D'Antona, G., Tonlorenzi, R., Porretti, L., Gavina, M., Mamchaoui, K., et al. (2004). Human circulating AC133(+) stem cells restore dystrophin expression and ameliorate function in dystrophic skeletal muscle. J. Clin. Invest. *114*, 182–195.

Yin, H., Price, F., and Rudnicki, M.A. (2013). Satellite cells and the muscle stem cells niche. Physiol. Rev. *93*, 23–67.

Zacharias, A.L., Lewandoski, M., Rudnicki, M.A., and Gage, P.J. (2010). Pitx2 is an upstream activator of extraocular myogenesis and survival. Dev. Biol. *349*, 395–405.

Zhang, W.W., Tong, H.L., Sun, X.F., Hu, Q., Yang, Y., Li, S.F., Yan, Y.Q., and Li, G.P. (2015). Identification of miR-2400 gene as a novel regulator in skeletal muscle satellite cells proliferation by targeting MYOG gene. Biochem. Biophys. Res. Commun. *463*, 624–631.