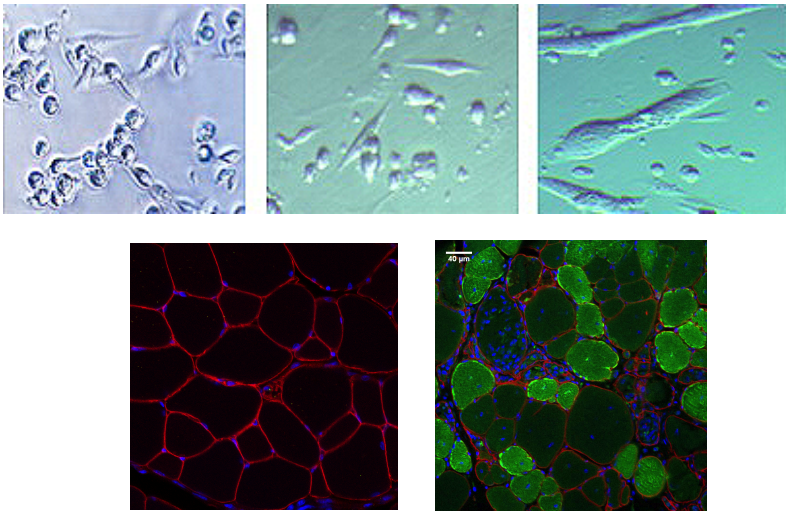
UNIVERSIDAD DE JAÉN

FACULTAD DE CIENCIAS EXPERIMETALES
DEPARTAMENTO DE BIOLOGÍA EXPERIMENTAL

DOCTORAL THESIS

Role and functions of Pitx2 regulating satellite cell biology



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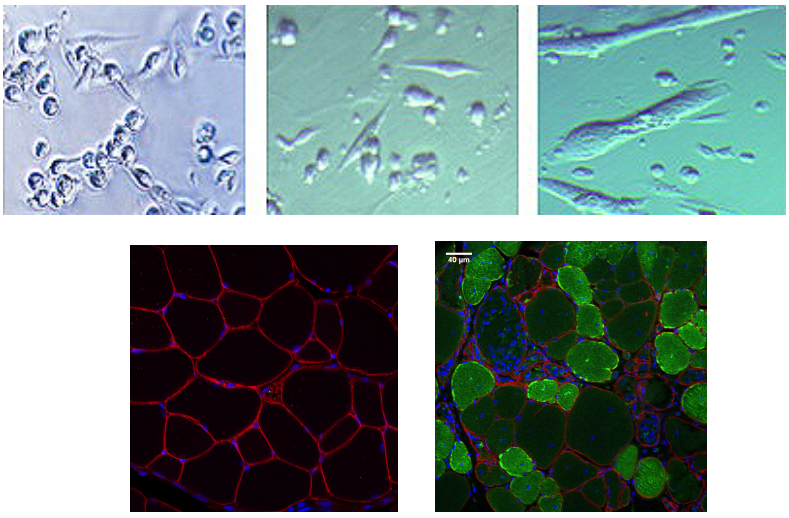
TESIS DOCTORAL

Papel de Pitx2 en la regulación de la biología de las células madre satélite musculares



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INDICE

INTRODUCCIÓN

El músculo esquelético tiene la capacidad de reparar y regenerar debido a la presencia de células madre residentes, denominados células satélite de músculo. Las células satélite (SC) son una población de células musculares mononucleadas que representan 2-10% de los mionúcleos totales (2×10^5 a 1 × 106 células/g Músculo) [30]. Las células satélite (SC) se encuentran en un nicho especializado localizado entre la sarcolemma de la miofibrilla y la matriz extracelular circundante (ECM), que se denomina lámina basal. En los músculos adultos en reposo, las SC se encuentran en estado quiescente pero, tras la lesión o estímulo de crecimiento, las SC se activan y dan lugar a la progenitores miogénicos necesarios para formar nuevas miofibras, pero también necesitan renovarse para mantener el pool de células satélite. Experimentos de lesiones musculares repetidas han demostrado que el número de células satélite permanece constante incluso después de múltiples traumas. La capacidad de las SC para equilibrar adecuadamente la quiescencia, auto-renovación y compromiso miogénico es crucial para garantizar el mantenimiento del músculo esquelético. La adquisición de un estado de proliferativo desde la quiescencia, así como el cambio entre la proliferación y la diferenciación de las células madre del músculo esquelético (células satélite) son pasos clave para la regeneración muscular adecuada. Sin embargo, cómo se produce la regulación de la proliferación de estas células satélite no esta completamente entendido.

Por otro lado, en el marco de los trastornos del músculo esquelético, las distrofias musculares representan un grupo de enfermedades genéticas heterogéneas que se caracterizan por degeneración y debilitamiento progresivo del músculo esquelético. La distrofia muscular de Duchenne (DMD), uno de los trastornos genéticos más letales, implica la degeneración muscular progresiva resultante de la ausencia de la proteína distrofina. La falta de expresión de distrofina en la DMD tiene consecuencias críticas en las células madre satélite musculares, incluida una capacidad reducida para generar precursores miogénicos y regenerar el músculo. Así, la pérdida progresiva de masa muscular se ha atribuido, al menos en parte, a la incapacidad de las células madre musculares para regenerar eficazmente la pérdida de tejido como resultado de la enfermedad. Por lo tanto, crítico para el desarrollo de estrategias eficaces para tratar los trastornos musculares es la optimización de los enfoques dirigidos a las células madre musculares.

RESUMEN

En este trabajo demostramos que la isoforma-c del factor de transcripción Pitx2 incrementa la proliferación celular en una línea celular de mioblastos, regulando negativamente los miRNA 15b (miR-15b), miR-23b, miR-106b y miR-503. Esta cascada Pitx2c-microRNA (miRNA) también regula la proliferación celular en las células satélite en las etapas tempranas de su activación, incrementando el número de células con un destino celular miogénico. Este estudio revela funciones desconocidas de varios miRNAs en el comportamiento de mioblastos y células satélite y, por lo tanto, puede tener aplicaciones futuras en medicina regenerativa.

Así mismo, en esta Tesis Doctoral demostramos que la isoforma-c del factor de transcripción Pitx2 modifica el potencial miogénico de las células satélite deficientes en distrofina (distróficas). Además, encontramos que Pitx2c potencia la capacidad regenerativa de las células satélites distroficas de ratón, aumentando la proliferación celular y el número de células comprometidas a diferenciación miogénica. Así mismo, Pitx2c restaura la expresión de distrofina mediante regulación de la expresión del miR-31. Estos efectos mediados por Pitx2 finalmente conducen a mejorar la función muscular en ratones distróficos (DMD/mdx). Nuestros estudios revelan un papel fundamental para Pitx2 en la reparación del músculo esquelético y pueden ayudar a desarrollar estrategias terapéuticas para los trastornos musculares.

CONCLUSIONES

1.- Hemos identificado un subconjunto de microRNAs regulados por Pitx2, con funciones previas desconocidas en células miogénicas y que tienen profundos efectos sobre la proliferación de mioblastos. Notablemente, encontramos que la cascada molecular Pitx2-miRNA que regula la proliferación celular se conserva en células satélite recién aisladas, proporcionando señales que potencian el compromiso de las células satélite con la diferenciación hacia linaje miogénico mediante la regulación negativa de la expresión de miR-106b. En general, el presente estudio describe una vía de Pitx2-miRNA desconocida previa que controla la proliferación celular en células miogénicas, proporcionando nuevas para mejorar la capacidad regenerativa de las células precursoras miogénicas del músculo esquelético de las extremidades para el tratamiento de enfermedades del músculo esquelético.

2.- Nuestros hallazgos demuestran que Pitx2 orquesta varios mecanismos moleculares que controlan la regeneración muscular. Nuestros datos *in vitro* e *in vivo* demuestran que Pitx2 mejora la capacidad regenerativa de las células satélites deficientes en distrofina al aumentar la proliferación celular y mejorar el número de células comprometidas miogénicas activando la ruta molecular Pitx2-miR-106b/miR-503/miR-23b/miR-15b. Es importante destacar que hemos demostrado que Pitx2 reprime el miR-31, lo que lleva a la restauración de la distrofina y finalmente a una mejora en la regeneración muscular. Este estudio revela la función previamente desconocida de Pitx2 en la reparación del músculo esquelético.

CHAPTER I: GENERAL INTRODUCTION

CHAPTER I: GENERAL INTRODUCTION

THE REGENERATIVE POTENTIAL OF SKELETAL MUSCLE: ADULT MUSCLE SATELLITE CELLS

The striated muscles represent approximately 40% of the body mass in humans and are responsible for posture and movements. The skeletal muscles are made up of numerous multinucleated myofibers that possess the contractile machinery to generate movement.

The basic unit of skeletal muscles is the muscle fibre (myofibre), multinucleated syncytium deriving from the fusion of mononucleated muscle cells (myoblasts) [1]. Myofibres are surrounded by a connective tissue structure composed by *epimysium*, *perimysium* and *endomysium* (or basal lamina), important to define functional units where contraction is transformed into movement [2]. Adult skeletal muscles are composed by myofibres with different physiological properties (slow and fast contracting type). Their proportion within a muscle determines the overall contractile property [2]. However, the basic mechanism of muscle contraction is common and results from a “sliding mechanism” of the thick filaments (myosin-rich) over the thin filaments (actin-rich) after neuronal activation [2]. The arrangement of myosin and actin into filaments give rise to repeating units defined as sarcomeres.

The maintenance of a working skeletal musculature is conferred by its remarkable ability to regenerate. Indeed, upon muscle injury a finely orchestrated set of celular responses is activated, resulting in the regeneration of the contractile muscle apparatus [2]. That regenerative capability is due to the presence of resident stem cells, termed muscle satellite cells. Therefore, muscle regeneration is characterized by different myogenic stages of satellite cells, namely: activation, proliferation, differentiation, and self-renewal/return to quiescence. The following sections describe the origin and characteritics of the skeletal muscles and their morphological changes during muscle regeneration.

-Muscle satellite cells in muscle repair

The satellite cells (SC) are a population of mononuclear muscle cells representing 2-10% of the total myonuclei (2 × 10^5 to 1 × 10^6 cells/g Muscle tissue)[3]. The satellite cells (SC) are located in a specialized niche located between the sarcolemma of the myofibril and the surrounding extracellular matrix (ECM), which is called the basal lamina (Fig. 1). In resting adult muscles, SCs are quiescent and are characterized by the expression of the Pax7 transcription factor. After the lesion or growth stimulus, the SCs are activated and express the myogenic regulation factors Myf5 and / or MyoD (also known as Myod1).

Activated CS, called myoblasts, proliferate massively and generate the myogenic progenitors necessary for muscle regeneration [4,5]. Subsequently, myoblasts negatively regulate Pax7 expression and activate the expression of factors such as myogenin (Myog) and myogenic regulatory factor 4 (MRF4, also known as Myf6) to exit the cell cycle, differentiate and fuse to form myofibers (Fig. 1). The satellite cells are responsible for the tremendous regenerative capacity of the skeletal muscles; Several studies have shown a complete lack of regeneration in the skeletal muscles with a reduced number of satellite cells expressing Pax7

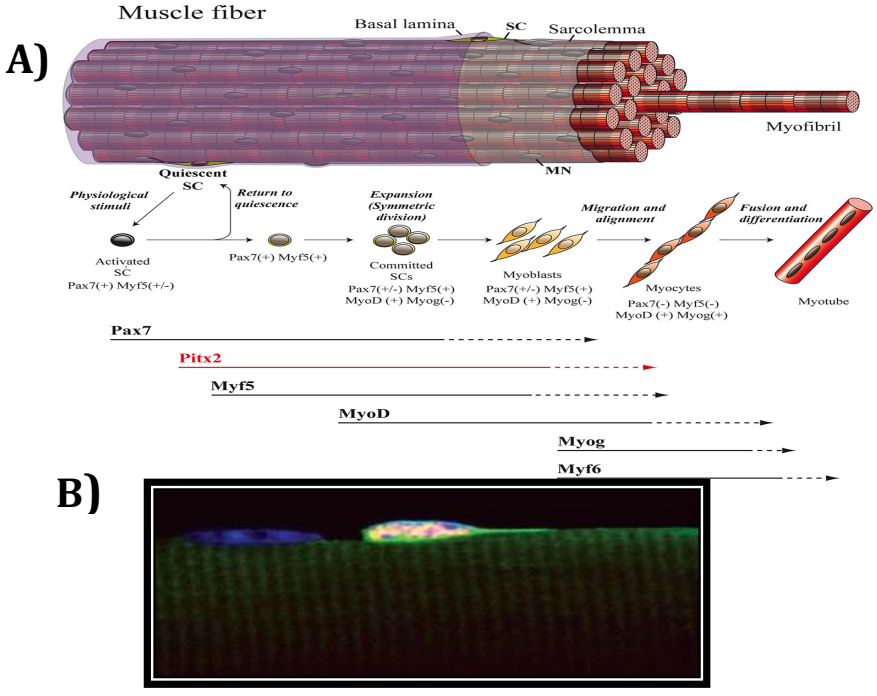

Figure 1: A) Localization, activation and progression along myogenic differentiation of the SC (Image taken from Hernández-Torres et al, 2017) [8]. B) Marking of Pax7 (red); nuclei (blue) and laminin (green) in a myofibril (Image taken from Bentzinger, Wang and Rudnicki, 2012) [9].

After muscle injury, the activated satellite cells give rise to the necessary myogenic progenitors to form new myofibers, but they also need to be renewed to maintain the satellite cell pool. Experiments of repeated muscle injuries have shown that the number of satellite cells remains constant even after multiple traumas [10]. The ability of SCs to adequately balance quiescence, self-renewal and myogenic compromise is crucial to ensure the maintenance of skeletal muscle. Importantly, many studies support the notion that satellite cells are heterogeneous and comprise a subpopulation of compromised cells that are more likely to progress in myogenic differentiation, and a subpopulation of satellite stem cells that are predisposed to self-renewal [11–13]

-Embryonic origin of muscle satellite cells

During embryonic development, the myogenic progenitors that generate the skeletal muscles come from the dorsal portion of the somites, known as the dermomyotome [9]. The embryonic myogenic progenitors are characterized by the expression of the transcription factors Pax3 and Pax7 [14–16]. Embryonic myogenesis in these precursor cells takes place by the sequential expression of the Myf5 and/or MyoD followed by Myog and MRF4 transcription factors, resulting in the formation of the different compartments of the embryonic muscle [17–20]. Parallel to the formation of myofibers, a subpopulation of myogenic precursor cells that do not express myogenic regulatory factors and maintain Pax3/Pax7 expression, is observed adjacent to late myofibers during the fetal mouse, around E16.5-18.5 [21]. It is hypothesized that these cells give rise to the population of satellite cells found in the adult muscle (Figure 2). Although adult satellite cells do not express MyoD under resting conditions, the use of a MyoD-iCre Mouse with a reporter lineage tracking allele suggests that essentially all adult satellite cells transcribed MyoD in the prenatal stage [22].

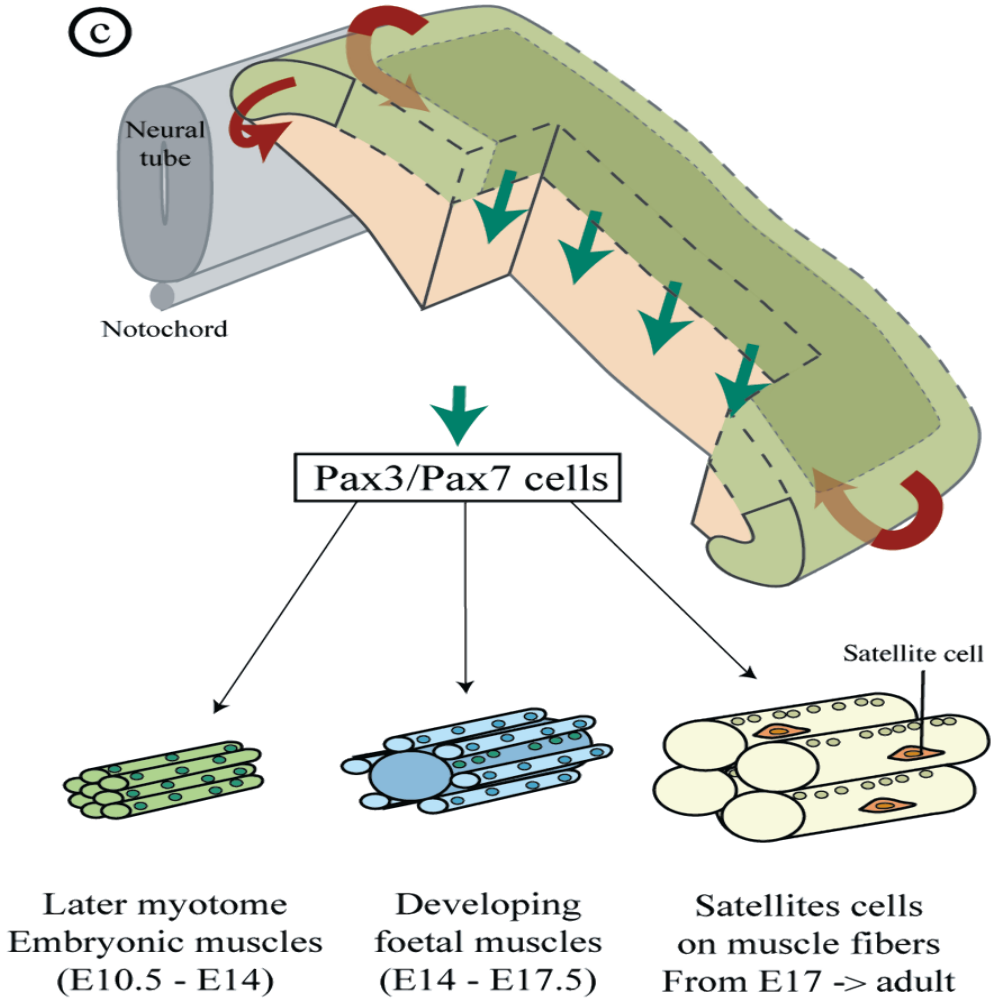

Figure 2: Embryonic origin of SC (Image taken from Lagha et al, 2017) [23].

Contrary to the expression of MyoD, different populations of Myf5-positive and Myf5-negative satellite cells are present in adult muscles, as observed in both Myf5-nlacZ reporter mice and by direct detection of Myf5 protein levels [24,25]. Thus, in Myf5-Cre / ROSA26-YFP mice it has been shown that a subpopulation of ~ 10% of total satellite cells have never expressed Myf5 during development [12]. This heterogeneity in the origins of the development of satellite cells raises the possibility of that a subset of satellite cells have a greater capacity for self-renewal and act as muscle stem cells. In this sense, it is interesting to note that in the Myf5-Cre/ROSA26-YFP mice, the YFP-negative satellite cells possess

greater self-renewal than the YFP-positive cells, which are more prone to commit to myogenic progenitors [11]. Likewise, using Pax7-NGFP, it was demonstrated that, under regeneration conditions, activated satellite cells expressing higher levels of Pax7 are less prone to compromise than those that express lower levels of Pax7 [11].

Taken together, these studies demonstrate that satellite cells are a heterogeneous population that can be divided into subpopulations of compromised satellite cells (ie, cells prompted to progress through the myogenic lineage once activated), as well as a subpopulation of satellite stem cells (ie, cells capable of self-renewal and maintaining the pool of satellite stem cells). However, if the populations of satellite stem cells identified with the different animal models represent the same population or different subsets of satellite stem cells has not yet been determined.

- **Muscle satellite cell quiescence and activation**

In resting adult muscles, the satellite cells remain dormant as quiescent or reversible G0 states (Figure 3). The ability of satellite cells to maintain the quiescent state is essential for the long-term conservation of the total satellite cell pool [26,27]. This quiescent state is different from the exit of the cell cycle observed before the differentiation. The most notable difference is its reversibility, which allows cells to return to a proliferative state in response to injury. The rapid re-entry into the cell cycle of satellite cells after injury suggests that the quiescent state is highly regulated and represents a 'ready' state for activation. Analysis by microarrays revealed that more than 500 genes are highly upregulated in quiescent cells compared to myoblast in division [28,29]. Within this quiescent molecular signature are the negative regulators of the cell cycle, including cyclin 1B-dependent kinase inhibitors (Cdkn1b, also known as p27 or p27Kip1) and 1C (Cdkn1c, also known as p57 or p57Kip2), the protein tumor suppressor of retinoblastoma (Rb, also known as Rb1), regulators of protein G 2 and 5 (Rgs2, Rgs5), peripheral myelin protein 22 (Pmp22) and the negative regulator of fibroblast growth factor (FGF), Sprouty1 (Spry1) (Figure

3). It is important to note that anomalies in the ability of satellite cells to maintain quiescence are associated with a reduced capacity for self-renewal and muscle regeneration.

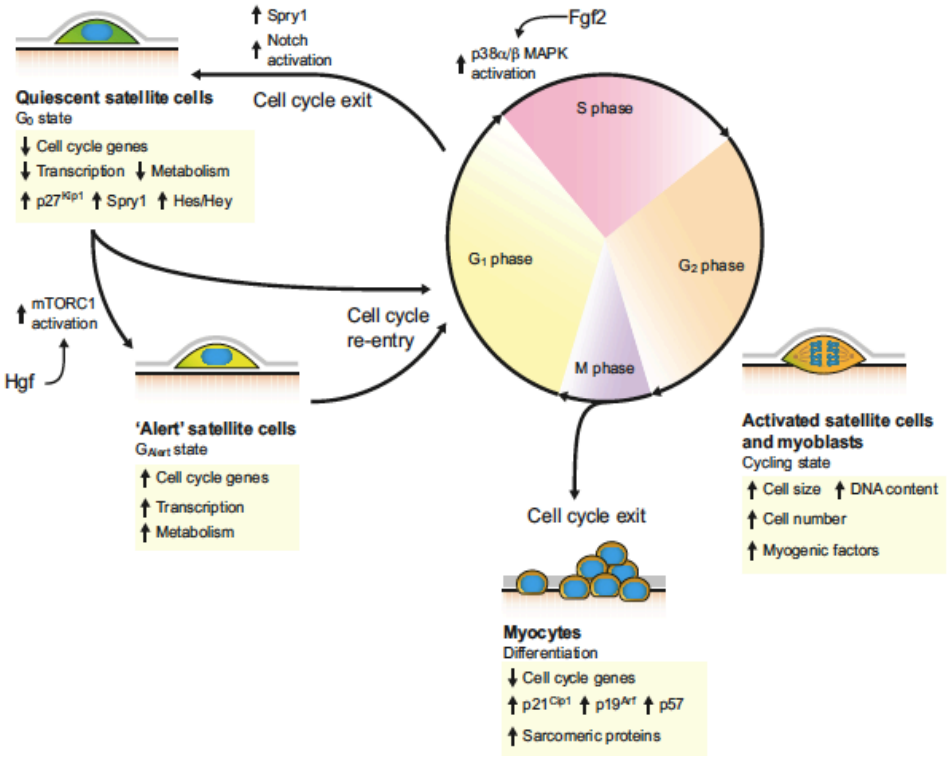

Figure 3: Regulation of the cell cycle in satellite cells. Under resting conditions, the intrinsic regulators of the cell cycle maintain the satellite cells reversibly in an inactive G0 state. Activated satellite cells re-enter the cell cycle, either directly or through an intermediate state called GAlert. After activation, satellite cells can exit the cell cycle and return to stillness by up-regulating Spry1 or by increasing Notch signaling. The proliferating myoblasts also leave the cell cycle to differentiate into myocytes and progress towards the myogenic lineage. (Image taken from Dumont et al, 2015) [13].

In recent years, the Notch pathway has emerged as the main regulator of quiescence. Notch signalling is higher in quiescent satellite cells than in activated myogenic cells [26] (Figure 3). This increase in activity is mediated by the interaction between the Notch Delta1 ligand (delta-like 1 in mouse), which is expressed in myofibers, and the Notch receptor (and its syndecan 3 co-receptor), which is present in the satellite cells [30,31]. Upon binding of Notch to its receptor, the intracellular Notch domain (NICD) is released and translocated to the nucleus where it interacts with the Rbpj protein and triggers the transcription of several genes, including those of the Hes and Hey families. Consequently,

the deletion of Rbpj in satellite cells or the double knockout of Hey1 and HeyL results in the spontaneous activation of quiescent satellite cells, deterioration in self-renewal and depletion of the satellite cell pool [26,27,32]. Interestingly, the genetic models of the inactivation of Notch also lead to the spontaneous differentiation of satellite cells, suggesting that Notch has a double role in maintaining the myogenic progenitor state. During the regeneration process, the negative regulation of Notch is mandatory for the progress towards a lineage [33]. In addition to re-entering the cell cycle, activated satellite cells must also determine the cell fate of their daughter cells, if they auto-renew or generate myogenic progenitors. The analyses of the MRF expression of proliferating satellite cells revealed the asymmetric expression of determining factors, including Myf5, MyoD and Myog in a subpopulation of daughter cells of satellite cells [12,34,35]. It is believed that the subpopulations of stem cells are capable of undergoing both symmetric and asymmetric modes of self-renewal in order to maintain the population of satellite cells through repetitive rounds of regeneration. The analyses of the activation of Myf5 in Myf5-Cre/ROSA26-YFP mice revealed that the YFP-negative satellite stem cells are capable to perform symmetric divisions, which give rise two identical daughter cells that auto-renew the pool of satellite stem cells [12]. Alternatively, satellite stem cells can perform asymmetric divisions, which generate a stem cell and a compromised cell that will progress through myogenic differentiation (Figure 4) [12].

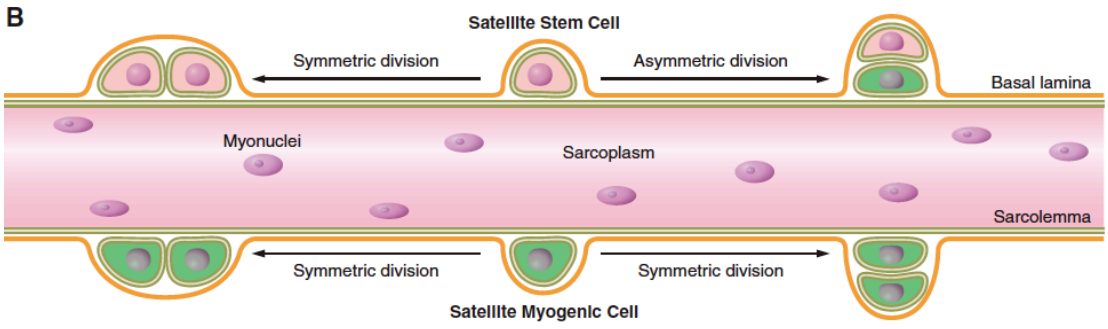

Figure 4: Symmetric and asymmetric divisions in satellite stem cells (Myf5- in pink) and myogenic satellite cells (Myf5 + in green). (Image taken from Yin et al, 2013)[36].

It is interesting to note that in Myf5-Cre/ROSA26-YFP mice ~ 30-40% of the first divisions of YFP-negative cells are symmetric, while the remaining divisions are asymmetric (Figure 4) [37]. Similar percentages were observed in Pax7-nGFPHi satellite cells [38]. The symmetric division is promoted by the activation of the

PCP cell polarity pathway, which leads to the symmetric distribution of polarity effectors such as Vangl2 in daughter cells [37]. The deletion of Vangl2 in isolated myofibers gives rise to a greater number of Myog-positive progenitors, but the satellite cell pool decreases, indicating a role of the symmetric division in the self-renewal and expansion of the satellite cell pool [37].

Asymmetric divisions are characterized by the segregation of different determinants of cell fate in daughter cells. During the asymmetric division of the Myf5-negative satellite stem cells, the Notch3 receptor is enriched in daughter cells that remain as stem-cell (Myf5-negative), while the daughter cell committed to the myogenic lineage (Myf5-positive) inherits the ligand Notch Delta1 [12]. As a result, it has been shown that the Notch Numb antagonist was asymmetrically located and remained in Miog-positive cells [31,39]. These results are consistent with the role of Notch in promoting he return to quiescence and self-renewal [30,40,41]. After asymmetric division, the ability of the two daughter cells to activate the myogenic program is also controlled by Pax7 [42]. For example, it has been demonstrated that MyoD could be distributed asymmetrically in the two daughter cells, resulting in a Pax7+/MyoD- cell "Reserve" and a myogenic committed cell Pax7-/MyoD+ [38,43]. The segregation of the DNA template has also been proposed as a model for measuring asymmetric divisions in activated satellite cells. In fact, by using the Pax7-nGFP transgenic model, it has been reported that while activated satellite cells expressing low levels of Pax7 (Pax7Lo) perform random DNA segregation, those expressing higher levels of Pax7 (Pax7Hi) mainly perform asymmetric DNA segregation during cell division [11]. The daughter cell that inherits the old DNA template retains Pax7 expression, while the one that receives the new template DNA expresses Myog [38].

The ability of satellite cells to choose between symmetric or asymmetric division allows them to coordinate their activity with the needs of the regenerating muscle. For example, a greater proportion of symmetric divisions would promote the expansion of the set of satellite stem cells, while an increase on the asymmetric divisions would favour the generation of myogenic progenitors and the maintenance of the stock of stem cells (Figure 4) [44]. Thus, A dynamic

equilibrium must exist between symmetrical and asymmetric divisions that fluctuate during the different stages of muscle regeneration. On the contrary, it could be hypothesized that an imbalance towards symmetrical divisions will lead to an alteration of the regenerating muscle. A better understanding of the decision-making mechanisms of satellite cell fate is necessary to determine satellite cells behaviour in pathological conditions.

MUSCULAR DYSTROPHIES IN THE CONTEXT OF MUSCLE REPAIR (DUCHENNE MUSCULAR DYSTROPHY)

Within the framework of skeletal muscle disorders, muscular dystrophies represent a group of heterogeneous genetic diseases that are characterized by progressive degeneration and weakening of skeletal muscle. The most commonly affected genes encode structural proteins that are important for the maintenance of the integrity of the muscle fibber. Duchenne muscular dystrophy (DMD) is a severely debilitating and fatal disease that affects approximately 1 of 3,500 males [45]. The first signs of motor deterioration appear between 2-5 years of age. Rapid progression of the disease and proximal muscle weakness results in patients needing wheelchairs at 12 years of age. Additionally, around 18 years of age, patients begin to suffer from cardiomyopathy and respiratory and cardiac insufficiency are the main cause of death around the second or third decade of life [46]. Although the use of corticosteroids has helped to prolong the survival of the patient, there is still no effective cure for DMD.

Duchenne muscular dystrophy (DMD) is caused by mutations in the gene located on the X chromosome that encodes the 427-kDa cytoskeletal protein dystrophin [47] (Figure 5). The gene reaches about 2.5 megabases and is composed of 79 exons, is the largest known human gene and, consequently, is prone to 91 mutations. DMD is caused by deletions in the frame of displacement, duplications and points of nonsense mutations resulting in complete loss of the protein or a non-functional protein [48]. Becker muscular dystrophy (BMD), which is less common than DMD, is caused by mutations in

the reading frame that generate a semi-functional form of dystrophin, leading to delayed muscle weakness and a milder disease phenotype.

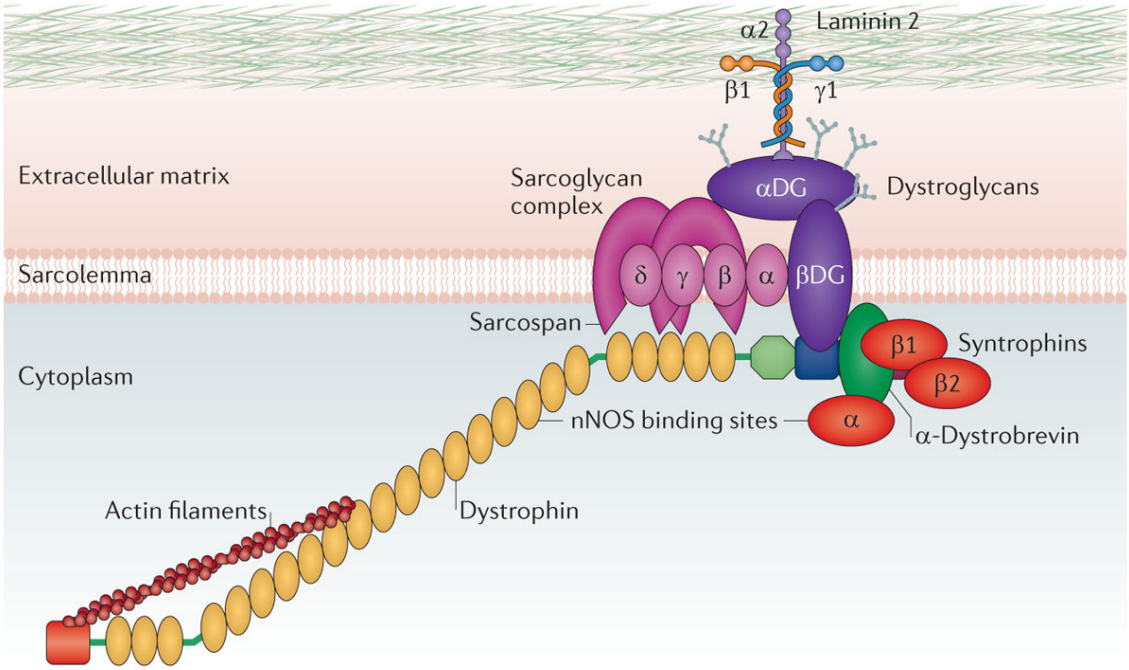

Figure 5: Intracellular localization of the dystrophin protein and its connection to the DGC complex) (Taken from Fairclough et al, 2013) [49]

Dystrophin is expressed mainly in skeletal and cardiac muscle and to a lesser extent in smooth muscle, as well as the brain [50]. Dystrophin functions as an essential component of the large oligomeric complex dystrophin-glycoprotein (DGC) (Figure 5) [51,52]. The DGC acts connecting the actin cytoskeleton of the myofibra to the surrounding extracellular matrix through the sarcolemma. In the absence of dystrophin, the DGC complex is altered, which weakens the muscle fibers making them very susceptible to injury. Thus, the stress induced by muscle contraction produces constant cycles of degeneration and regeneration, inflammation and fibrosis; which leads to progressive muscle weakness and loss of muscle mass and function [53,54]. It is interesting to note that, in muscular dystrophies, progressive muscular atrophy and weakness are often associated with depletion of the muscle regeneration potential.

The precise contribution of satellite cells to the mechanism of DMD disease progression remains elusive. Since dystrophin expression was not detected in

myoblast primary cultures [55,56], it was assumed that satellite cells also lacked dystrophin expression. Therefore, any change on satellite cell behaviour was assumed as an indirect effect due to the dystrophic environment. A widely accepted issue has been the concept of "muscle stem cells depletion" caused by repetitive cycles of muscle degeneration and regeneration [57,58]. This model suggests that satellite cells are ultimately unable to keep up with the high demand for regeneration in a context of dystrophic muscle, resulting thus in an eventual loss of regenerative capacity.

In contrast to stem cell depletion model, multiple studies have reported an increase in the number of satellite cells in the dystrophic muscle. The analysis of muscle biopsies of DMD patients aged 2 to 7 years revealed that the number of satellite cells is elevated in the dystrophic muscle compared to controls for all age groups [59]. Another study also showed that the content of satellite cells was spectacularly and specifically increased in type I muscle fibers from DMD patients in an advanced stage of the disease [60]. Recent studies examining myofibers isolated from mdx mice - a mouse model commonly used for DMD harbouring a naturally occurring null mutation in the Dmd gene [61] - also found a high number of satellite cells in the muscle fibers of young mdx mice comparing with wild-type controls [62–64]. These results collectively suggest that the impaired regenerative capacity of the dystrophic muscle cannot simply be due to depletion of the muscle stem cells.

It should be noted that the first studies that examined mdx mice lacking MyoD, a critical myogenic determinant expressed by compromised satellite cells, established that the mdx: MyoD-/- double mutant mice showed severe myopathy, thus demonstrating that the loss of MyoD exacerbated the degenerative mdx-phenotype. More recently, Sacco and colleagues introduced a null mutation of telomerase in mdx mice to generate double knock-out mice that concomitantly lacked dystrophin expression and telomerase activity [57]. Surprisingly, these mice exhibit severe muscular dystrophy that resembles more markedly the pathology of human DMD. In these mice, analyses of dystrophic satellite cells showed an impared potential for proliferation and graft, suggesting that the greater severity of the dystrophic phenotype was due largely to the

impaired regenerative function of the satellite cells. In contrast to previous results reported for DMD in humans and mdx, these mice showed a reduced number of satellite cells compared to wild-type control animals. Although none of these approaches directly address the contribution of satellite cells functions into the mechanism of DMD, these results lead to authors to conclude that the severity of the disease correlates with the regenerative capacity of the endogenous satellite cells.

A very recent RNA-Seq and microarrays analyses performed in isolated satellite cell has revealed that dystrophin is highly transcribed in satellite cells [64]. Moreover, immunofluorescence analyses with anti-dystrophin antibodies in satellite cells isolated by FACS and in cultured cells have also confirmed that dystrophin protein is expressed in activated satellite cells [64]. Interestingly, dystrophin protein displays a polarized expression in satellite cells, reaching its maximum levels when the cells are near to undergo cell division [64].

It has been documented that in mammalian cells, dystrophin interacts with the cell polarity regulatory kinase Mark2 in the muscle fiber membrane [65]. In addition, cellular polarity proteins have previously been implicated in the determination of asymmetric cell divisions in satellite cells [35,66]. The analysis of satellite cell divisions in myofibers isolated from mice has revealed asymmetric localization of PARD3 in dividing satellite cells leads to asymmetric activation of p38α/β and the expression of the myogenic determination factor MyoD in committed daughter cells (Figure 6). The absence of Pard3 expression and the lack of activation of p38α/β have also been found to lead to self-renewal and the return the satellite stem cell to quiescence [35].

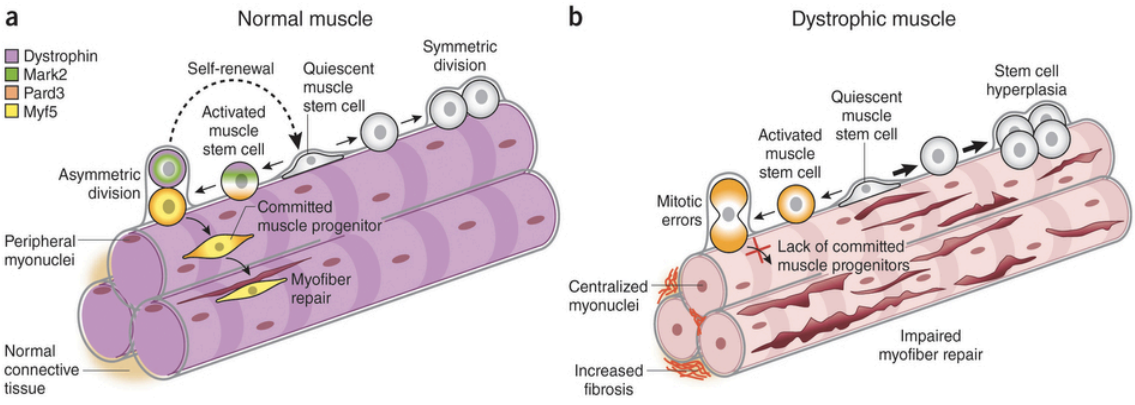

Figure 6: Role of dystrophin in DMD. (Image taken from Dumont et al, 2015) [13].

In activated satellite cells, localized expression of dystrophin promotes the polarization of Mark2 and Pard3 to opposite sides of the dividing cell. This polarization promotes asymmetric cell division, which leads to the generation of satellite stem cells lacking Myf5 and committed muscle progenitors. Dumont et al (2015) [64] demonstrated that satellite cells null for dystrophin loosed the cellular polarity mediated by Pard3, which lead to cell division errors and a decrease in asymmetric cell divisions. This scenario finally resulted in a decrease in differentiated myocytes leading to impaired regeneration (Figure 6) [64]. Thus, for the first time, a direct role of dystrophin in the regulation of satellite cell function have been described, providing a clear link between the cells dystrophic satellite and the loss of muscle regeneration capacity in DMD.

In summary, the progressive loss of muscle mass has been attributed, at least in part, to the inability of muscle stem cells to effectively regenerate tissue loss as a result of the disease. Therefore, critical to the development of effective strategies to treat muscle disorders is the optimization of approaches targeting muscle stem cells.

THE TRANSCRIPTIONAL AND POSTTRANSCIPTIONAL CONTROL OF THE SKELETAL MUSCLE DEVELOPMENT AND REGENERATION

In vertebrates, all the myogenic cells that form the striated skeletal muscles of the limb and trunk originate from the somites (Figure 10-A). Somites are bilaterally paired blocks of paraxial mesoderm that form along the head-to-tail axis of the developing embryo in segmented animals. The somite is initially a spherical accumulation of cells that progressively subdivides into two compartments, the ventral mesenchymal sclerotome and the dorsal epithelial dermomyotome. Shortly afterwards, The medial halves of the somites give rise to back and intercostal muscles -the epaxial musculature-, while cells derived from lateral somites will produce the muscles of the limbs and of the body wall, forming the hypaxial musculature. The cells from the hypaxial dermomyotome adjacent to limb regions leave the epithelial structure after EMT and begin migrating towards the fore and hind limbs to form dorsal and ventral muscle masses in the limb-bud mesenchyme, where they begin to differentiate and express muscle-specific genes [67].

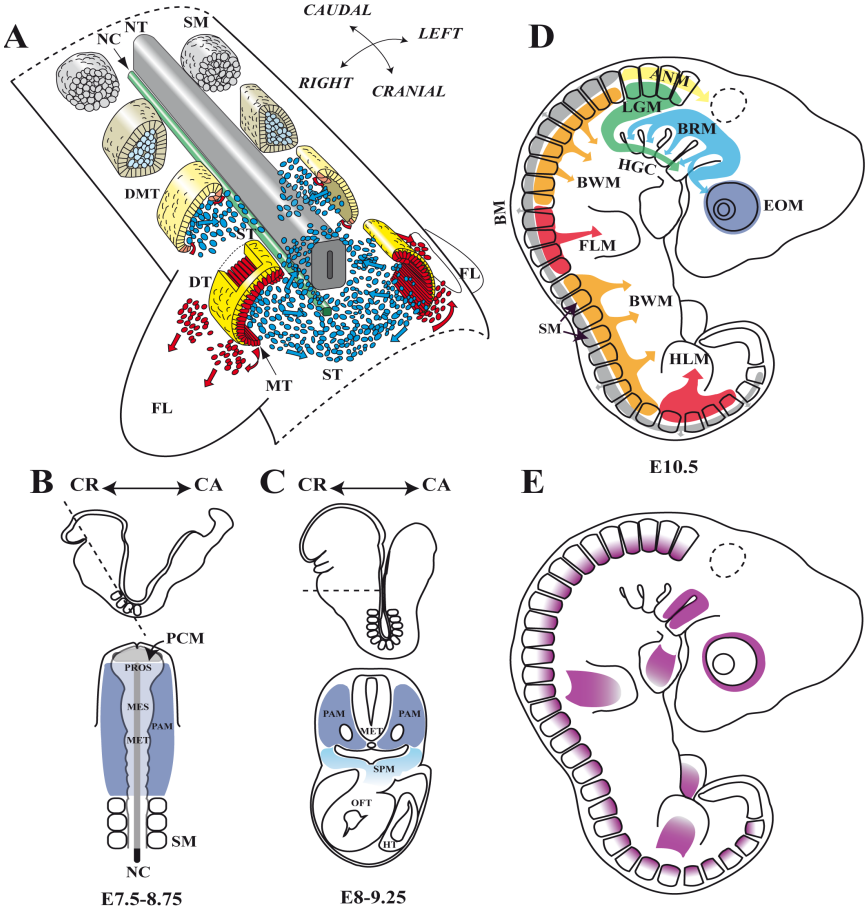

Figure 7: Embryonic myogenesis **A)** Schematic representation of somite maturation. Somites mature following an anterior to posterior developmental gradient. Modified from *Gray's Anatomy*. The Anatomical Basis of Clinical Practice, 40th Edition (Standring, 2008). **NT**, Neural Tube; **NC**, Notochord; **SM**, Somites; **DMT**, Dermomyotome; **ST**, Sclerotome; **DT**, Dermotome; **MT**, Myotome; **FL**, Forelimb. **B)** Head frontal plane of a mouse embryo between stages of development E7.5-8.75 in mouse . Dashed line illustrates the cutting plane. **CR**, Cranial; **CA** Caudal **NC**, Notochord; **SM**, Somites; **PAM**, Head Paraxial Mesoderm; **PCM**, Prechordal Mesoderm; **PROS**, Prosencephalon; **MES**, Mesencephalon; **MET**, Metencephalon. **C)** Transverse plane of a mouse embryo between stages of development E8-9.25 in mouse. Dashed line represents the cutting plane. **PAM**, Paraxial Mesoderm of the head; **MET**, Metencephalon; **SPM**, Splanchnic mesoderm; **OFT**, Outflow Tract of heart; **HT** Heart Tube. **D)** Origins of skeletal muscles. **EOM**, Extra-Ocular Muscles; **BRM**, Branchial Muscles; **LGM**, Laryngoglossal Muscles; **HGC**, Hypoglossal Cord; **ANM**, Axial Neck Muscles; **BM**, Back Muscles; **BWM**, Body Wall Muscles; **FLM** Forelimbs Muscles; **HLM** Hind limbs Muscles **SM**, Somites. **E)** *Pitx2* expression domains at the E10.5 stage of development in mouse. (Image taken from Hernández-Torres et al, 2017) [8]

Cell commitment in the somite is highly dependent on a number of transcription factors acting in a hierarchical molecular cascade to orchestrate specification, determination, and differentiation of myogenic precursors. The cells in the dermomyotome are first specified to the myogenic lineage by the transcription factor Pax3 (Figure 8). Subsequently, *Pax7* is activated in the *Pax3*-expressing

myogenic precursors, which give rise the myogenic progenitor of the embryonic and fetal muscles in the trunk and limbs [68,69]. *Pax* genes directly control the activation of the myogenic program by binding to the myogenic regulatory factors *Myf5*, followed by *Myod* [68,69]. *Myf5* is the first MRF expressed during embryonic development, being transiently upregulated in the paraxial mesoderm and later during the formation of the myotome. Subsequently, the *Myod* gene is activated after the onset of *Myf5* expression in the dermomyotome [70] (Figure 8). Both factors, *Myf5* and *MyoD*, are considered as muscle-determination genes. Finally, the transcription factors *Myog* and *Myf6* are required for the onset of the expression of terminal differentiation genes needed for myoblasts fusion and myotube formation [9].

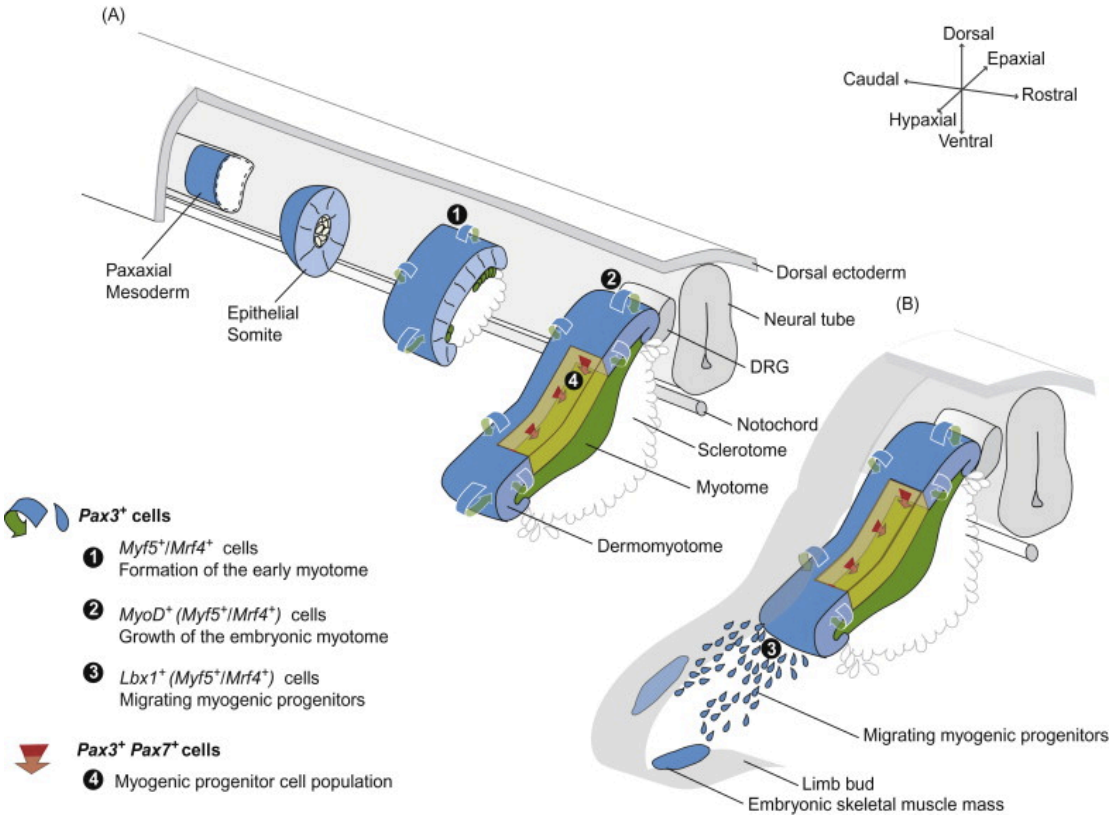

Figure 8: Cell commintment in the somite. (Image taken from Buckingham and Rigby, 2014) [71]

Although all skeletal muscle of the body originates within paraxial mesoderm, in the head, identifiable compartments such as somites, myotomes or sclerotomes are not histologically evident. The unsegmented head mesoderm is remodeled at early stages of embryonic development. Initially, the head mesoderm

includes the prechordal mesoderm and the paraxial mesoderm, but when the neural tube closes dorsally and the endoderm ventrally; the prechordal mesoderm is integrated within the remaining paraxial mesoderm located anterior to the somites (Figure 7-B-C). The unsegmented head mesoderm gives rise all craniofacial skeletal muscles, which can be catalogued as four distinct populations: extra-ocular (EOMs), branchial, laryngoglossal, and axial neck muscles [72]. EOMs are formed by cells from the cranial paraxial mesoderm that migrates through the first branchial arch (FBA) as well as from the prechordal mesoderm [73,74] (Figure 7-D). Branchial arch muscles are formed mainly by migrating cells from the cranial paraxial mesoderm and the lateral splanchnic mesoderm [75,76]. Finally, the axial neck muscles in the transition zone between the head and the trunk arise from medio-dorsal and latero-ventral domains of occipital and cervical somites [77–79].

The genetic hierarchy governing myogenesis in the trunk does not appear to operate for head-muscle formation. Activation of the myogenic program in the head therefore depends on different upstream factors and also displays site-dependent regulation. Branchial-arch-derived muscles depend on *Myf5/Myf6/Myod*, whereas extraocular muscle formation is initiated by *Myf5/Myf6* and in their absence cannot be restored by *Myod* [80].

In addition, during the adult life the skeletal muscle has the ability to renew developing mechanisms that compensates for the physilogical turnover and damage to maintain tissue homeostasis. [81,82]. This adult myogenesis depends on the activation of satellite cells that have the potential to differentiate into new fibers [2]. It has been very well establish that satellite cells are closely related to progenitors of embryonic origin [83–87]. Thus, many similarities, such as common transcription factors and signaling molecules, between embryonic myogenesis and regeneration in the mature skeletal musculature have been discovered [88].
During the last decade the homeobox transcription factor *Pitx2* has emerged as another key element implicated in the fine-tuning mechanism that regulates skeletal muscle development. Concurrently, several recent experimental evidences pointed out the role of Pitx2 in satellite cell biology are emerging.

-The homeobox transcription factor PITX2 in the transcriptional control of myogenesis

-Description and identification of Pitx2 gene

The homeotic transcription factor PITX2 is encoded by the Pitx2 gene, which is located on chromosome 3 in the mouse and consists of six exonic sequences separated by five introns [8] (**Figure 7**). Mutations in PITX2 were first identified as the molecular cause of the Rieger syndrome congenital malformations [8]. Pitx2 is a member of the bicoid family of homeodomain transcription factors that plays an important role in morphogenesis. Pitx2 is expressed in many tissues during development, including the left lateral plate mesoderm, derivatives of the first brachial arch, the eye, brain, pituitary gland, mandible, heart, and limbs [8] . Several laboratories have shown that Pitx2 is a mediator of left–right signaling in vertebrates acting downstream of the Nodal/Lefty cascade [8]. Pitx2-deficient mice are characterized by failure of body-wall closure, arrest in embryo turning, ocular defects, right pulmonary isomerism and defects in cardiac, tooth, and pituitary development [8].

Molecular studies have demonstrated that three different Pitx2 isoforms (Pitx2a, Pitx2b, and Pitx2c) are expressed during development, and a fourth Pitx2 isoform (Pitx2D) has only been described in humans [8]. Pitx2a and Pitx2b are generated by alternative splicing mechanisms, and Pitx2c uses an alternative promoter upstream of exon 4 (Figure 9). Thus, each Pitx2 isoform has identical C termini but different N termini. All *Pitx2* isoforms share a K50 DNA binding homeodomain through which join the consensus sequence TAATCC [89,90], thus being able to induce a transcriptional activation of *Prl* [89] or *Anf* [91] promoters.

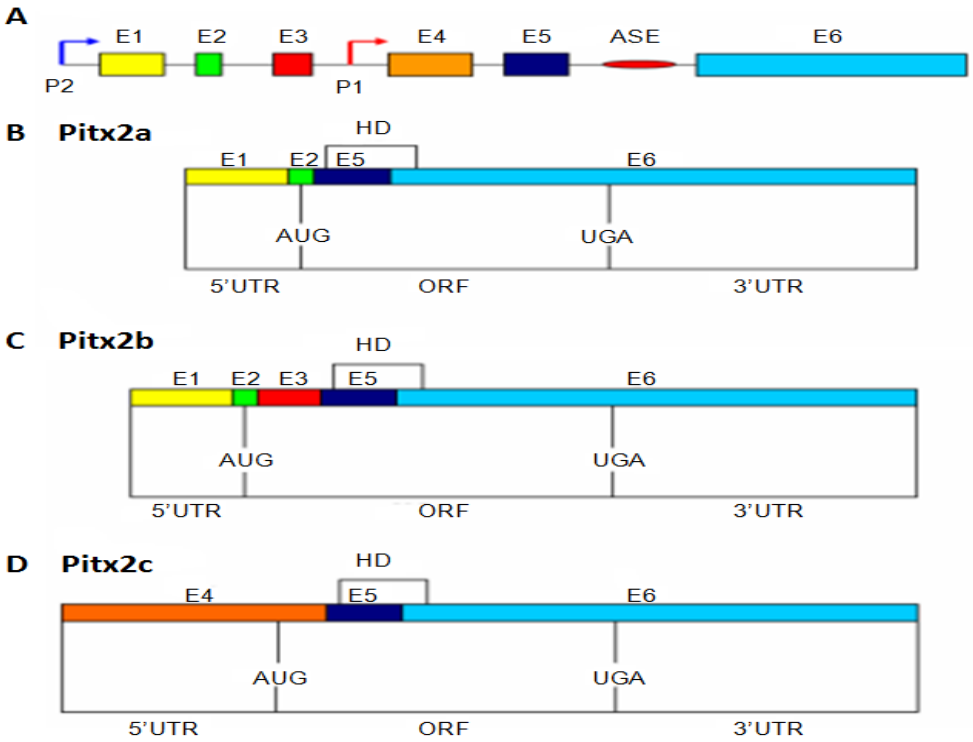

Figure 9: The Pitx2 gene: Genomic structure and splice variants in the mouse. (A) Genomic structure. (B) Pitx2a isoform. (C) Pitx2b isoform. (D) Pitx2c isoform. The arrows in (A) indicate the promoters used by the Pitx2a and Pitx2b isoforms (P2, blue arrow) and Pitx2c (P1, red arrow). ASE: Asymmetric enhancer. Exons: E followed by the corresponding number. The coding region (ORF) and the region of the Homeodomain (HD) as well as the start (AUG) and end (UGA) codons of the translation are indicated.

-Pitx2 and muscle development

During the last two decades, several reports have pointed out that the role of Pitx2 in the molecular cascade controlling myogenesis strongly depends on the myogenic precursors position.

The first experimental evidence involving *Pitx2* in the molecular process controlling myogenesis was reported by Kitamura *et al.* (1999) [92]. These authors found *Pitx2* expression in myotomes co-localizing with *Pax3*, a muscle specification marker that plays a key role in delamination and migration of myogenic progenitors to the limb buds [93]. Subsequent, Marcil *et al.* demonstrated the presence of *PITX2* protein in myoblasts of the limb bud, displaying a similar expression pattern to *Pax3* and *Myod* [94]. Together, all these data suggested a putative *Pitx2* involvement on muscle-pattern during

development. A more detailed temporal and spatial analysis during muscle anlagen formation by using *lacZ* expression from a *Pitx2* gene insertion revealed that *Pitx2* was expressed specifically in most of the muscles of head and trunk in late embryos and adults [95]. Therefore, Shih et al. observed the presence of a cluster of *Pitx2*-expressing cells lateral to the dermomyotome. This cluster was detected first in the forelimbs at E10.25. *Pitx2(+/LacZ*)-expressing cells were also detected on appendicular sections after E10.5. Curiously, only subsets of *Pitx2*(+) cells within these areas were also positive for *Pax3* and/or others muscle-regulatory factors (MRFs), and virtually all *Pitx2*(+) cells within these areas expressed at least one of these known myogenic markers [95]. These observations led the authors to conclude that *Pitx2* could represent one of the most effective molecular markers for myogenic lineage. In agreement with that interpretation, L'Honore *et al.* (2007) found extensive co-labeling of *Pitx2*, *Pax3*, and *Pax7* in proliferating cells of the myotome and dermomyotome [96]. Notably, they also observed that *Pax3*-positive cells that completed their migration at the proximal limb bud also expressed *Pitx2* while not all *Pitx2*-positive cells expressed *Pax3*. These results suggested that *Pitx2* might be a key player within the molecular pathways controlling muscle progenitor fate.

More recently, L'honore *et al.* (2010) reported additional information concerning the hierarchical position of *Pitx2* within the genetic patnway controlling myogenesis in the trunk and limbs [97]. Therefore, they delineated differential requirements for *Pitx2* in migratory vs. non-migratory somite-derived myogenic cells by using *Pitx2*$^{-/-}$ and *Pitx2*$^{-/-}$;*Myf5*$^{nlacZ/nlacZ}$ double-mutant mouse models. Thus, the authors showed a delay on *Myod* expression in limb buds of *Pitx2*$^{-/-}$ embryos, and chromatin immunoprecipitation (ChIP) analyses led them to assert that delay on *Myod* expression could be due to failure on *Pitx2*-mediated activation over the *Myod* core enhancer. Moreover, *Pitx2* and *Myf5*(*LacZ/LacZ*) double mutants displayed a deficient *Myod* activation while the formation or migration of myogenic precursors to the limb buds was unaltered. However, the presence of one active *Myf5* allele in a *Pitx2*-deficient background prevented MyoD loss in about 60% of cells [97], suggesting that *Myf5* somehow compensated for *Pitx2* deficiency in those mice. These results suggested that

Myf5 acts in parallel with *Pitx2* controlling *Myod* expression during early limb-bud myogenesis (Figure 10.2). In contrast to limb-muscle cells, *Myod* expression on the myotome was not delayed in Pitx2$^{-/-}$ embryos, but the absence of Pitx2 and Myf5 in *Pitx2*$^{-/-}$;*Myf5*$^{nlacZ/lacZ}$ embryos led to an almost complete loss of *Myod* expression [97]. Therefore, the onset of *Myod* expression in myotome seems not to be depended on *Pitx2,* but on the predominant role of *Myf5* and *Myf6* (Figure 10.1).

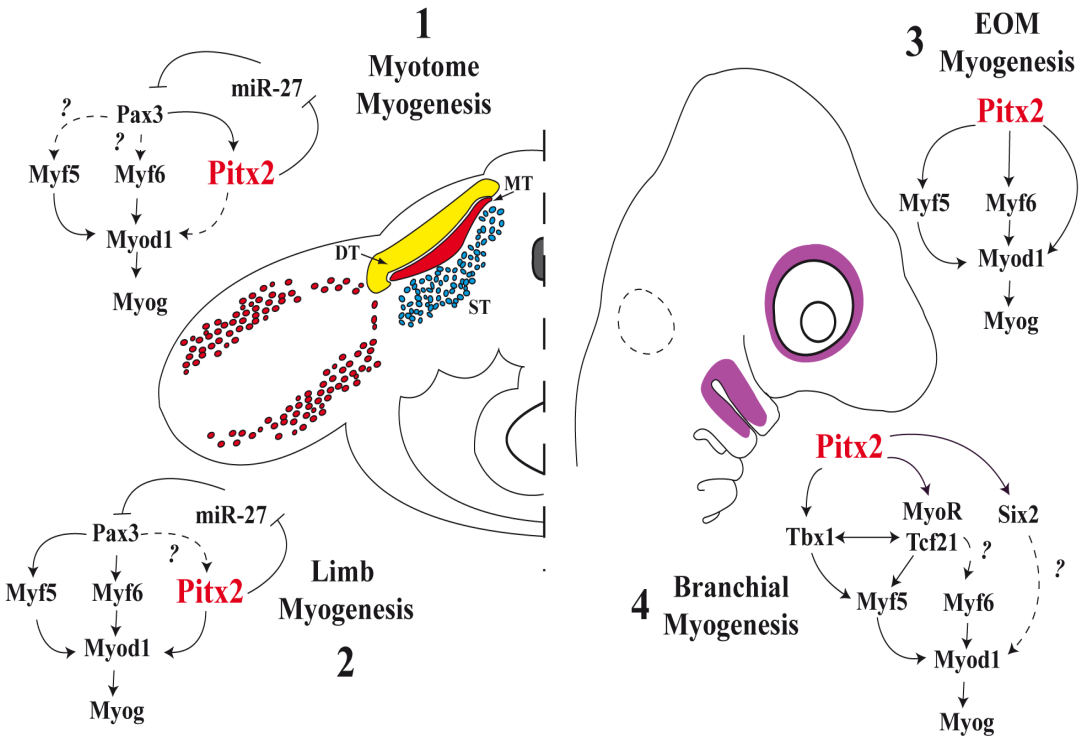

Figure 10: *Pitx2* functions on Myogenesis. During embryonic stages, *Pitx2* contribution is depending on the muscle anlagen (Myotome Myogenesis (1), Limb Myogenesis (2), EOM Myogenesis (3) or Branchial Myogenesis (4). (Image taken from Hernández-Torres et al. 2017) [8]

Additionally, the analysis of *Pax3 Splotch* mutant mice revealed a complete deficit of *Pitx2* expression restricted to the myotome, indicating that *Pitx2* could acts downstream of *Pax3* during myotome myogenesis. This notion has been also supported by the transcriptome analyses of *Pax3*$^{GFP/+}$ *and Pax3*$^{GFP/PAX3-FKHR}$ transgenic mice [98]. Therefore, in gain-of-function screens for *Pax3* targets,

Lagha et al found a *Pitx2* up-regulation in somites but not in limb buds. Although all of these seminal works suggest that *Pitx2* could be acting downstream of *Pax3*, at least in the myotome and in parallel to *Myf5*, not all *Pitx2*-expressing cells were positive for *Pax3* (as note above), and *Pitx2* expression precedes to *Myf5* exprossion in the limb buds [97]. Therefore, additional studies using *Pitx2* conditional in specific myogenic cell populations would be help to elucidate the function of Pitx2 in embryonic myogenesis.

Concurrently, recent evidences have emerged supporting the role of *Pitx2* on cell proliferation in myogenic cells. Thus, it has been reported that *Pitx2* is a target gene of the *Wnt/Dvl2*/*beta-catenin* pathway and operates to control proliferation in specific cell types by regulating expression of the growth-control genes *Ccnd1*, *Ccnd2*, and *c-Myc;* pointing out that the *Pitx2* N-terminal domain is required for its effects on cell proliferation in a myoblast cell line [99,100]. We have previously demonstrated that *Pitx2c* is the main *Pitx2*-isoform expressed in Sol8 myoblasts and that overexpression of *Pitx2c* in Sol8 cells displayed high proliferative capacity and completely blocked terminal differentiation of this myoblast cell line mainly because high levels of *Pax3* expression were maintained [101]. Additional *in vivo* data have also supported *Pitx2* function on cell proliferation during myogenesis. In this sense, Abu-Elmagd *et al.* [102] have showed that *Pitx2* loss of function decreased the number of myogenic cells in the somites of the chick embryo, whereas *Pitx2* gain of function increased the myocyte number, particularly in the epaxial region of the myotome. In agreement with these results, and by using *Pitx2c*$^{-/-}$ mutant embryos, we have previously reported that *Pitx2c* plays a pivotal role in the control of the subtle equilibrium between proliferation and differentiation during myogenesis in myotomes and limb-buds balancing *Pax3+/Pax7+* myogenic population *in vivo.* Interestengly we also demonstrated that Pitx2 regulates key myogenic transcription factors such as *Pax3* through the repression of *miR-27* [103] (Figure 10-1-2). This new function of *Pitx2c* mediated by miRNAs introduces a new level of complexity in the intricate regulatory network that governs myogenesis in the embryo.

Throughout development the networks of transcription factors enabling the phases of determination, specification and differentiation during myogenesis can be re-used spatially and temporarily. For instance, *Pax3* which activates MRF expression in the somites, is not expressed in the developing craniofacial muscles [68,104] and it has been proposed that *Pitx2*, rather than *Pax3*, plays a major role as an upstream regulator of craniofacial myogenesis [71,105] This is supported by the fact that *Pitx2* null mice displays Extraocular muscle (EOM) dysmorhogenesis [92,106]. However, the mechanisms by which EOM development was impaired in *Pitx2* null mice remained elusive. In this sense, other authors have subsequently suggested that this phenotype could be due to *Pitx2*-mediated effect on proliferation rate of myogenic precursors [72], in agreement with previously reported data for others myogenic cells [100,101]. In this context, the hypothesis that *Pitx2* is a player controlling cell proliferation on myogenic cells is also supported by the fact that conditional inactivation of *Pitx2* in neural-crest-derived cells did not affect the early differentiation of extraocular muscles [107], but conditional *Pitx2* deletion in the mesoderm lead to *Myf5*, *Myf6*, *Myod1* and *Myog* down-regulation delaying thus the onset of myogenesis in developing EOMs [105]. In this regard, Sambasivan *et al. (2009),* by analyzing double defective *Myf5*(*Myf6)* (*Myf5*$^{nlacZ/+}$, *Myf5*$^{nlacZ/nlacZ}$) mutant mice embryos as well as *Myf4*$^{-/-}$ mutants, demosntrated that, in the absence of both *Myf5* and *Myf6, Pitx2* cannot ensure survival and activation of *Myod* expression in EOMs [76]. Subsequently, Zacharias *et al.* (2011) by using a tamoxifen inducible *UBC-CreER*^{T2} promoter to inactivate the expression of *Pitx2* in mesodermal EOM precursors, showed that *Pitx2* is required for specification and survival of EOM precursors, acting as an anti-apoptotic factor in pre-myogenic mesoderm and subsequently activating the myogenic program in these cells through direct binding to *Myf5* and *Myod* promoters [105]. Taken together, all these data clearly suggest that *Pitx2* is an upstream regulator of *Myf5*, *Myf6,* and *Myod* in EOM embryonic myogenesis (Figure 10.3).

Pitx2 is also expressed in the myogenic precursors of the first branchial arch (FBA). Tbx1 expression is required for specification of FBA premyoblasts leading to *Myf5* and *Myod1* activation in those cells [108]. Strikingly, *Pitx2* systemic mutants, whether *Pitx2*$^{-/-}$ [109] or *Pitx2*$^{LacZ/LacZ}$ [110], displayed *Tbx1*

downregulation in FBA, although *Pitx2* expression is unaffected in *Tbx1* null mutants [109]. These data, together with the fact that *Pitx2* directly interacts with *Tbx1* regulatory elements [110] suggest that *Pitx2* is an upstream activator of *Tbx1* in FBA. A more detailed analysis of both *Pitx2* mutants revealed that of *Pitx2* inactivation in FBA resulted in an increased cell death in the mesodermal core and a loss of early specification markers of premyoblasts such as *Six2, Tcf21*, and *MyoR.* Although the role that *Six2* in FBA-myogenesis remains elusive*, Tcf21* and *MyoR* are known to be upstream effectors of *Myf5*, *Myod*, and *Myog* in this muscle anlagen [111]*.* All together, these results indicate that *Pitx2* controls *Myod1* expression and the onset of myogenesis in FBA through *Tbx1, Tcf231*, and *MyoR* (Figure 10-4).

Finally, a new role for *Pitx2* has recently been unraveled during fetal myogenesis. L'honore *et al.,* by using *Pitx2:Pitx3* double conditional mutants, have demonstrated that *Pitx2* and *Pitx3* control the expression of the redox system through regulation of *Nrf1* and antioxidant enzymes during muscle differentiation [112]. Thus, *Pitx2/3 depletion* at the onset of differentiation induces an abnormal increase of reactive oxygen species (*ROS)* levels in differentiating myoblasts and leads to impaired myogenesis due to apoptosis of these cells. These results emphasize the role of *Pitx2* controlling redox conditions during fetal myogenesis.

-Pitx2 in adult myogenesis

The role of *Pitx2* during adult myogenesis and satellite stem cell biology is beginning to be explored. The first evidence regarding *Pitx2* expression in satellite cells (SCs) was reported by Ono et al. (2010) [113]. These authors showed that all *Pitx2* isoforms are expressed in proliferating SC-derived myoblasts. These authors compared SCs from the extensor digitorum longus (EDL) of the limb with SCs from the masseter of the head (MAS). They showed that *Pitx2b* and *Pitx2c* levels were higher in cells from the EDL than SCs isolated from MAS. They also found that *Pitx2c* was the main *Pitx2* isoform expressed in proliferating SCs in the limbs [113]. Based on these analyses, the authors suggested that, even after activation and entry into the cell cycle, SCs

retain their identity consistently with their ontogeny underlying the heterogeneity of SCs populations.

Subsequent studies have pointed out that *Pitx2a*, *Pitx2b,* and *Pitx2c* were expressed at very low levels in proliferating SCs, but increased during the early stages of myogenic differentiation. However, the constitutive expression of any *Pitx2* isoform suppressed SC proliferation, but promoted myogenic differentiation [114]. However, additional evidence pointing out the functional relevance of *Pitx2* on SC proliferation has been reported. Therefore, Herbet et al. (2013) demonstrated a key role of *Pitx2* maintaining the phenotype of myogenic precursor cells in the extraocular muscles (EOM) [115]. These authors found higher levels of *Pitx2* expression in EOM in comparison with limb muscles. These Pitx2 higher levels were concomitant with a longer proliferative state in EOM-derived SCs as compared with limb cells. Moreover, *Pitx2* knockdown experiments in SCs isolated from EOM diminished their proliferation rate, and a similar trend was also seen for SCs isolated from tibialis anterioris muscle. These data suggested that *Pitx2* play a role maintaining a pool of proliferating myogenic precursor cells. Finally, these authors highlight that this high proliferative capacity may facilitate muscle repair on EOM tissue, thereby contributing to the sparing of EOM in muscular dystrophies [115]. Despite the advances achieved in understanding the Pitx2 involvement on satellite-cell behaviour and function, its role in satellite cell behaviour and adult regenerative myogenesis has not been yet determined.

POSTTRANSCRIPTIONAL CONTROL: microRNAS IN THE SKELETAL MUSCLE

Recent studies have identified post-transcriptional control of gene expression as a crucial level of regulation of myogenesis. During the last decade, our understanding of the mechanisms involved in gene regulation has increased enormously. In particular, a class of small ncRNAs, known as microRNAs (miRNAs), has emerged as having a central role in the post-transcriptional regulation of gene expression for a broad range of biological processes. Muscle-specific miRNAs or myomirs have been shown to control various processes in skeletal muscles, from myogenesis and muscle homeostasis to different responses to environmental stimuli. Importantly, myomirs are also involved in the development of several muscular and neuromuscular disorders.

-Biogenesis and functional mechanisims of microRNAs

The biogenesis of a miRNA starts on DNAs in our genome; miRNA genes are transcribed by RNA polymerase II (pol II) to generate long pri- mary transcripts (pri-miRNAs), which can be several kilobases long. The pri-miRNAs are capped,spliced and polyadenylated. They may encode a single miRNA, clusters of distinct miRNAs, or a protein and can therefore also act as mRNA precursors [116]. The next step also takes place in the nucleus and is orchestrated by the microprocessor complex. The principal components of this complex are the RNase III enzyme known as Drosha and its binding partner DiGeorge síndrome critical región gene 8 (DGCR8), a double-stranded RNA-binding protein [117] (Figure 11).

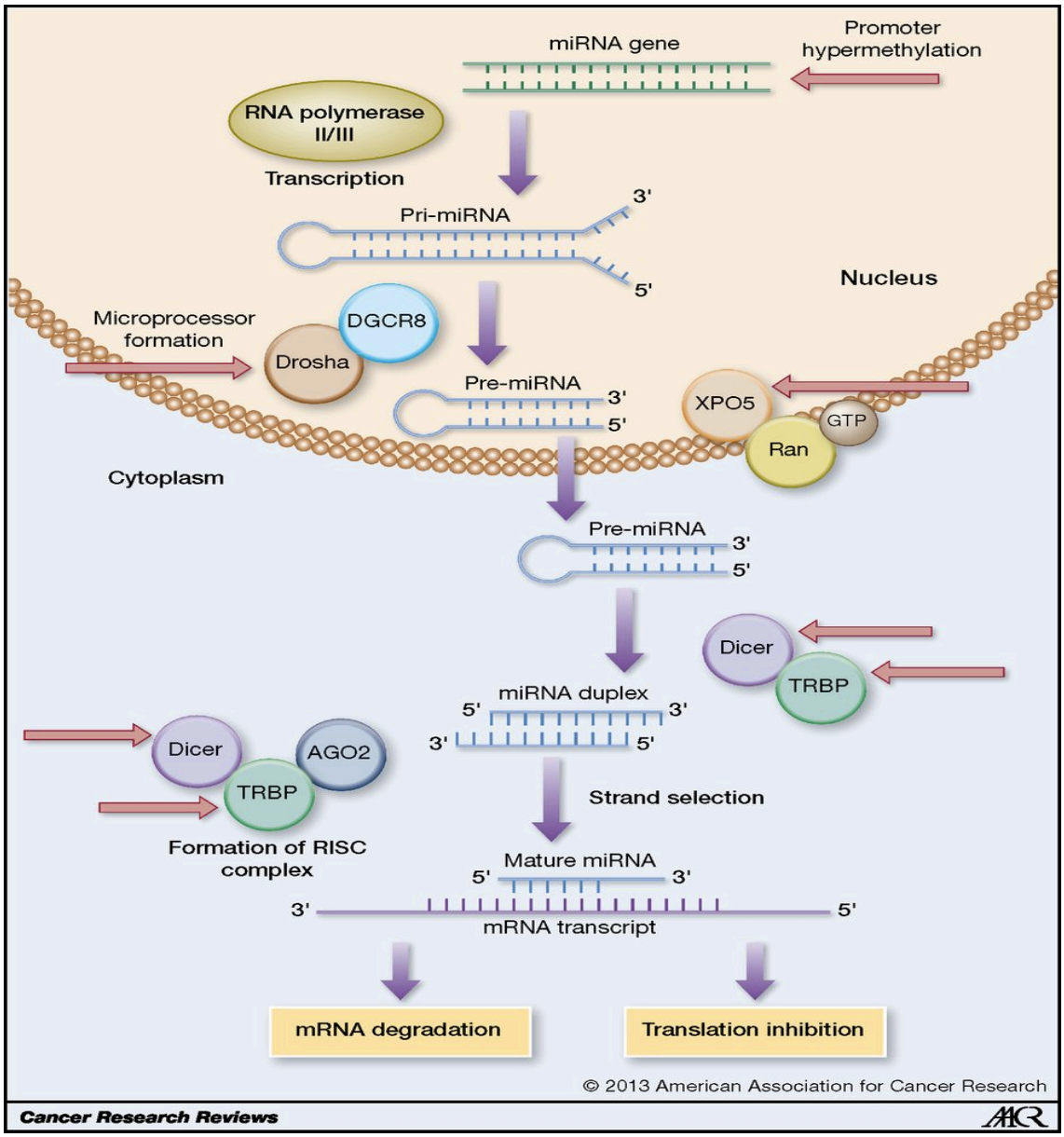

Figure 11: miRNA biogenic process. (Image taken from Mulrane et al, Cancer Research, 2013) [118].

After nuclear processing, the pre-miRNA is exported into the cytoplasm by Exportin-5 (XPO5) in a RanGTPdependent manner. In the cytoplasm, the pre-miRNA is processed by RNAse III Dicer, into miRNA:miRNA∗ duplex (dsRNA) of ~ 20 to 25 nt. Then, the dsRNA is separated by action of helicases, such as argonaute 2, RISC catalytic component (AGO2). One miRNA strand of the miRNA:miRNA∗ duplex is loaded onto an AGOprotein to form an effector complex known as the RNA-induced silencing complex (RISC) (Figure 11). One strand of the two different mature miRNAs originated from dsRNA is preferentially incorporated into the RISC, depending on its thermodynamic

stability, whereas the other strand usually is degraded [119]. The RISC functions as a guide by base pairing of miRNA with its target mRNAs, resulting in mRNA cleavage, mRNA deadenylation, and translation repression (Figure 11). The miRNA-binding sites are usually found in the 3′ -UTR of target mRNAs [120–122]. However, recent works support the existence of new sites of binding for miRNA outside the 3′ -UTRs of mRNA. In this context, it was recently showed that 5′ -UTRs are important for miRNA-induced translational repression [123]. Interestingly, the domain at the 5′ end of miRNAs, which extends from nucleotide 2 to 7, which has been denominated as “miRNA seed,” is critical for target recognition. However, the downstream nucleotides of miRNA, including nucleotide 8 and nucleotides 13 to 16, have also been shown to contribute to base pairing with some mRNA targets [124]. Depending on the degree of homology to the 3′ UTR target sequence, miRNAs can induce the translational repression or degradation of mRNAs. Given that each miRNA is capable of regulating the expression of many genes, each miRNA can simultaneously regulate multiple cellular signaling pathways.

miRNA sequences are located in diverse regions of the genome. The majority of canonical miRNAs in humans are encoded within introns of coding or noncoding genes. However, some miRNAs may be encoded by exons or may be associated in the same loci, organized as a polycistronic transcription unit. Indeed, some miRNAs have a promoter region, which allows autonomously expressed miRNA similar to those found on protein-coding genes. Surprisingly, some miRNAs genes have multiple transcription sites, including in some cases promoters distinct from those of their host genes. Additionally, it has been revealed that miRNAs located in the same cluster can be transcribed and regulated independently [124–126], demonstrating the complexity of miRNA biogenesis and function. Recent reports evidenced that miRNA biogenesis is regulated at multiple levels, which have emerged as additional mechanisms involved in defining the pattern of miRNA expression and its effect on phenotype.

Besides its regulation at the transcriptional level, and its processing by Drosha and Dicer in the nucleus and cytoplasm respectively, miRNA may also be

modified by RNA editing, RNA methylation, uridylation and adenylation, AGO loading, and RNA decay. Additionally, noncanonical pathways, independent of Drosha and Dicer, have also emerged as alternative pathways for miRNA biogenesis [124]. In addition, differential processing of pre-miRNAs has been shown and might be an alternative mechanism to control miRNA function in different tissues [127]. Furthermore, several mechanisms downstream of miRNA biogenesis have been reported to affect miRNA function, including regulation of AGO and GW182 levels, which are core miRISC components [125].

In addition to their intracellular biogenesis and action, emerging studies have identified the presence of miRNAs in exosomes, which are membrane-bound vesicles released from most cell types into the extracellular space after fused with the plasma membrane. Exosomal miRNAs may interact with neighboring or distant cells, representing a new process of cell-to-cell communication. Furthermore, the incorporation of exosomal miRNAs may exert function in recipient cells, influencing their phenotype [122,128]. In addition to being packed into microvesicles and exosomes, extracellular miRNAs can be associated with HDL [129] or with AGO2 protein [130], which maintains their stability and protects them from degradation. Interestingly, several reports have revealed that miRNAs-containing vesicles play an important role in disease progression, stimulating angiogenesis, and consequently facilitating metastasis in cancers [128].

- **MicroRNAs AS NEW PLAYERS REGULATING SKELETAL MYOGENESIS AND REGENERATION**

Recent studies have identified the post-transcriptional control of gene expression as a crucial level of regulation of myogenesis. Among the critical mediators of such control, an important role is played by *microRNAs* (*miRNAs*), small non coding *RNAs* that specifically bind the 3'untranslated regions (3'UTRs) of *mRNAs* and control their stability and translational efficiency. Several miRNAs have been identified, some of which, *miR-1*, *miR-133a* and miR-206, are expressed specifically in muscle tissue [131]. The binding of muscle regulatory factors (such as MyoD and Mef2c) to the presumptive promoters of

muscle-restricted *miRNAs*, together with the over-expression and knock-down of those *miRNAs* in muscle tissues and in myogenic cell lines, have provided experimental support for their role in muscle differentiation [132,133]. Interestingly, *miR-1* and *miR-206* promote myogenesis by targeting transcriptional repressors of muscle gene differentiation, whereas *miR-133* inhibits myogenesis by enhancing myoblast proliferation [131,134] and it has been reported that *miR-27* is induced during *in vivo* muscle differentiation and represses Pax3 [135] (Figure 12). More recently, it has been reported that overexpression of *miR-30* family promotes differentiation, while inhibition restricts differentiation of myoblasts *in vitro* [136].

Besides miR-206, miR-486 has been shown to be upregulated during myogenesis, which leads to cell cycle quiescence. Interestingly, both miRNAs accelerated the myogenic differentiation program by suppressing Pax7, and the use of miR-206 and miR-486 inhibitors blocked the myogenesis (Figure 12) [137]. Skeletal myogenesis is also affected by miR-125b, which is downregulated during myoblast differentiation and overexpression of miR-125b was shown to negatively modulate myoblast differentiation *in vitro*, by targeting IGF-II levels [138].

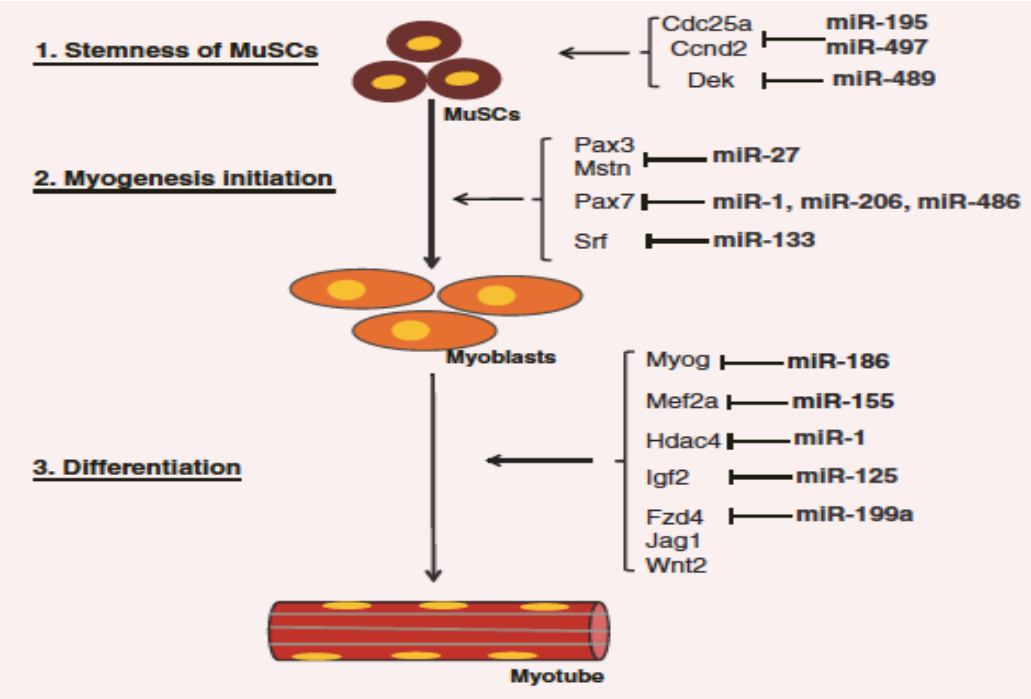

Figure 12: miRNAs in myogenesis. (Image taken from Diniz and Wang, Comprehensive Biology, 2016).[139]

The involvement of miR-199 in myoblast differentiation has also been demonstrated. Thus, miR199a-5p was shown to affect myoblast differentiation by targeting several myogenic cell proliferation and differentiation regulatory factors of the WNT signalling pathway, such as FZD4, JAG1, and WNT2. However, overexpression of miR-199-5p did not affect the ability of myoblast to differentiate into myotube but miR-199-5p overexpression induced myofiber disruption [140]. Moreover, miR-199a-3p was found highly expressed in skeletal muscle and was induced during C2C12 myoblast differentiation. miR-199a-3p mimics treatment inhibited C2C12 myogenic differentiation, and reduced the expression of MyoG and MyHC, which are markers of myogenic differentiation. Gain- and loss-of-function ofmiR-199a-3p affected the expression of IGF-1, mTOR and RPS6KA6, indicating that the suppression of the IGF-1/AKT/mTOR signalling pathway is one of the potential mechanisms by which miR-199a-3p regulates myogenesis [141].

It have also been reported that miR-351 is implicated in the maintenance of myogenic progenitor cell proliferation, as well as in the transition into myotubes. Experiments of miR-351 knockdown inhibited differentiation on myogenic progenitor cells and induced apoptosis. By contrast, miR-351 overexpression rescued myogenic progenitor cell from apoptosis during the differentiation process by inhibiting E2f3 expression, which is a factor involved in cell cycle progression and proliferation [142]. Several evidences have pointed out the involvement of miR-124 in the myogenic process, since its overexpression inhibited the myogenic program and myotube formation, its inhibition increased myogenic marker expression and induced myotube formation [143]. Moreover, miR-146b was recently characterized as a novel regulator of skeletal myogenesis, since higher levels of miR-146b were found during myoblast differentiation. Indeed, miR-146b overexpression increased myoblast differentiation, while miR-146b inhibition reduced myoblast differentiation and prevented the downregulation of Smad4, Notch1 and Hmga2 [144].
miR-322/424 and miR-503 are also upregulated during myogenesis, targeting a phosphatase responsible for removing inhibitory phosphorylation of cdk2 (Cdc25A) suggesting that Cdc25A repression is an important step in the myogenic differentiation of C2C12 cells [145]. Equivalently, the miR-24 expression

was increased in differentiated C2C12 myoblasts, whereas miR-24 was reduced in response to the TGF-β via Smad3, which inhibits the differentiation of C2C12 myoblasts. Furthermore, these authors showed that of miR-24 overexpression partially restored the levels of myogenic markers and muscle transcriptional factors inhibited by TGF-β, suggesting that miR-24 reduction may be one of the mechanisms by which TGF-β represses myogenesis [146]. miR-128a is highly expressed during myoblast differentiation in the skeletal. Overexpression of miR-128a attenuated myoblast proliferation by suppressing insulin receptor substrate 1 and phosphorylated Akt levels, whereas miR-128a inhibition induced myoblast proliferation and myotube hypertrophy [147]. Together, these findings suggest that miR-128a exerts an important role both in cell proliferation and growth of skeletal muscle.

In the context of adult satellite cells, recent reports have pointed out the role of *miRNAs* modulating cell proliferation in adult satellite cells. Diverse miRNAs have been demonstrated to control satellite cell activity, including miR-489, which is highly expressed in quiescent satellite cells, maintaining muscle stem cell quiescence by suppressing Dek expression [148]. On other hand, miR-31, which targets Myf5, affects satellite cell differentiation. Therefore, in quiescent satellite cells, miR-31 is sequestered in mRNP granules., These mRNP granules are dissociated during the activation of satellite cells leading to a reduction of miR-31 expression and an increase of Myf5 levels [149]. In the zebrafish embryo, skeletal muscle progenitor cells also express miR-214, which to modulate the Hedgehog signaling to specify muscle cell fate [150]. Others miRNAs have been reported to regulate skeletal muscle stem cells. miR-195 and miR-497 expression are increased in adult muscle stem cells, resulting in cell cycle arrest, by targeting Cdc25, and quiescence, by suppressing Ccnd. Furthermore, overexpression of miR-195 and miR-497 decreased the expression of Cdc25a, Cdc25b1/2, and Ccnd2. Moreover, the use of siRNA for Ccnd2 induced quiescence of juvenile muscle stem cells, which was also observed after miR-195 and miR-497 overexpression in these cells. Underlying the potential regenerative role of these miRNAs, muscle stem cells transfected with miR-195/497 showed more efficient regenerative myogenesis [151]. Together, these studies indicate that *miRNAs* could be previously unrecognized

regulators of muscle progenitor cell proliferation, fate specification, and differentiation. Therefore, these data suggest that miRNAs can play important role in the regulation of satellite cell activation during muscle regeneration.

Finally, it is interesting to note that a comprehensive analysis of the expression profiles for *miRNAs* has revealed that deregulation of *miRNAs* genes is a common feature in the pathology of muscle. Thus, the analyses of the expression profile of *miRNAs* in muscle samples obtained from various muscle disorders in humans lead to identify a number of *miRNAs* that are deregulated in myopathies. Indeed, expression of *miR-206* was significantly increased in the diaphragm and in the regenerating myofibres of mdx mice [152,153] as well as in biopsies from patients with DMD and Spinal muscular atrophy [154,155]. Moreover, it has been reported the role of *miR-31* modulating the expression of dystrophin in a myoblasts line obtained from dystrophic patients [156]. Therefore, these studies indicate that miRNAs may be involved in the pathophysiology of muscular dystrophy.

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AIMS OF THE THESIS

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**Chapter II:*

To elucidate the function of Pitx2 in the transcriptional regulation of miRNAs in myoblasts and satellite cells.

**Chapter III:*

To dissect the role of Pitx2 on the regenerative potential of satellite cells.

CHAPTER II: RESULTS (I)

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A *Pitx2*-MicroRNA Pathway Modulates Cell Proliferation in Myoblasts and Skeletal-Muscle Satellite Cells and Promotes Their Commitment to a Myogenic Cell Fate

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The acquisition of a proliferating-cell status from a quiescent state as well as the shift between proliferation and differentiation are key developmental steps in skeletal-muscle stem cells (satellite cells) to provide proper muscle regeneration. However, how satellite cell proliferation is regulated is not fully understood. Here, we report that the c-isoform of the transcription factor Pitx2 increases cell proliferation in myoblasts by downregulating microRNA 15b (miR-15b), miR-23b, miR-106b, and miR-503. This *Pitx2c*-microRNA (miRNA) pathway also regulates cell proliferation in early-activated satellite cells, enhancing Myf5$^+$ satellite cells and thereby promoting their commitment to a myogenic cell fate. This study reveals unknown functions of several miRNAs in myoblast and satellite cell behavior and thus may have future applications in regenerative medicine.

The maintenance and repair of adult muscle tissue are directed by satellite cells. Quiescent satellite cells are activated by exercise or injury and enter the cell cycle to produce progeny myogenic precursor cells that undergo multiple rounds of division before entering terminal differentiation and fusing to multinucleated myofibers (1). Together with skeletal muscles, satellite cells originate from cells of the segmented paraxial mesoderm known as somites. Somite formation starts at around embryonic day 7.75 (E7.75) in the mouse embryo and continues until the species-specific number of somites is reached (2). As the somite matures, myogenic progenitor cells become confined to the dorsolateral part of the somite, the dermomyotome. The dermomyotome contains multipotent progenitor cells of different cell types, including the skeletal-muscle progenitors. These cells in the dermomyotome are specified to the myogenic lineage by *Pax3*. Later, *Pax7* is activated within these *Pax3*-expressing myogenic precursors, which produce progenitor cells of the embryonic and fetal body muscles (3, 4). *Pax* genes directly control the activation of the myogenic program in the limb by binding and activating the myogenic regulatory factors Myf5 and Mrf4, followed by MyoD (5–8). *Pax7* is maintained in fetal myogenic precursors and satellite cells in adults, whereas *Pax3* is downregulated during the fetal period (9), although the *Pax3* locus remains active in a subset of satellite cells of particular muscles in adults (10, 11). In adults, satellite cells can be recruited to supply myoblasts for routine muscle fiber homeostasis or for the more sporadic demands of myofiber hypertrophy or repair (12). In addition to producing progeny destined for differentiation, satellite cells also maintain their own population by self-renewal, thus fulfilling the defining criteria of a stem cell (13).

Pitx2, a member of the bicoid family of homeodomain transcription factors, plays a major role in developmental myogenesis. Pitx2 expression occurs in muscle progenitors during musculature development, colabeling with Pax3$^+$ and Pax7$^+$ myotomal cells (14). Moreover, previous works have demonstrated that *Pitx2* can act as an upstream activator of myogenesis in the extraocular muscles, whereas it cooperates with the *Myf5*/*Myf4* pathway to control somite-derived myogenesis (15, 16), and recently, an essential role of Pitx2 and Pitx3 in redox regulation during fetal myogenesis was also reported (17). Previously, we have shown that *Pitx2c* is the main *Pitx2* isoform expressed in Sol8 myoblasts and that overexpression of *Pitx2c* in Sol8 cells displays a high proliferative capacity and completely blocked terminal differentiation of this skeletal-muscle cell line, mainly because high levels of *Pax3* expression were maintained (18). Recent results from our laboratory have revealed that these roles of *Pitx2c* in balancing proliferation versus differentiation as well as signaling through *Pax3* also occur during embryonic myogenesis (19). In addition, the role of Pitx2 during adult myogenesis is beginning to be explored. Recent findings indicate that *Pitx2* is expressed in proliferating satellite cells and can act to promote differentiation of satellite cell-derived myoblasts (20, 21), yet the role of Pitx2 in satellite cell function remains poorly understood. Recent studies have identified the posttranscriptional control mediated by microRNAs (miRNAs) as a crucial level in the regulation of myogenesis. Also, miRNAs have been shown to play crucial roles in muscle development and in the regulation of muscle cell proliferation and differentiation (22, 23). In this context, it has been reported that miRNA 206 (miR-206) and miR-486 induce myoblast differentiation by downregulating *Pax7* (24). More recently, Gagan et al. (25) identified a feed-forward loop where *MyoD* indirectly downregulates its inhibitor

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TABLE 1 Primers for site-directed mutagenesis[a]

Primer	Sequence
MD15abc_503_1Ccnd1_F	CTCCTCTCATGG*CGC**AAA**TA*CCGATGACTCCCA
MD15abc_503_1Ccnd1_R	TGGGAGTCATCGG*TA**TTT**GC*GCCATGAGAGGAG
MD15abc_503_2Ccnd1_F	TCACGTTGTTTTC*GC**AAA**TA*TTGGAGGGTCAGT
MD15abc_503_2Ccnd1_R	ACTGACCCTCCAA*TA**TTT**GC*GAAAACAACGTGA
MD106ab_Ccnd1_F	ATTCCATTTCAAAGC*AAA**TT*TGGTCAGCTAGCT
MD106ab_Ccnd1_R	AGCTAGCTGACCAA*A**TTT**GC*TTTGAAATGGAAT
MD23abc_Ccnd1_F	TCTATTTTGGCTTA*AAAAA*GATTACCGCTGTATT
MD23abc_Ccnd1_R	AATACAGCGGTAA*TC**TTT**TT*AAGCCAAAATAGA
MD15abc_503_1Ccnd2_F	GCTGACTAAAGTAG*C**AAA**TA*CCTAAGGGATATG
MD15abc_503_1Ccnd2_R	CATATCCCTTAGG*TA**TTT**GC*TACTTTAGTCAGC
MD15abc_503_2Ccnd2_F	ATTATTATTTTTC*GC**AAA**TA*AGAAGCTAAGATC
MD15abc_503_2Ccnd2_R	GATCTTAGCTTCTT*A**TTT**GC*GAAAAATAATAAT
MD15abc_503_3Ccnd2_F	TGTTTCACGGTGTG*C**AAA**TA*TTTTAGAAACATT
MD15abc_503_3Ccnd2_R	AATGTTTCTAAAA*TA**TTT**GC*ACACCGTGAAACA
MD106ab_Ccnd2_F	TTCCATTAGAAAAGC*AAA**TT*GAAAATTTTGGGG
MD106ab_Ccnd2_R	CCCCAAAATTTTC*AA**TTT**GC*TTTTCTAATGGAA
MD106ab_Myf5_F	AATACTGTCTTGCC*A**AAA**TA*TGAGAAAATAGAT
MD106ab_Myf5_R	ATCTATTTTCTCA*TA**TTT**T*GGCAAGACAGTATT

[a] Italics indicate the miRNA seed sequences in the 3′ UTRs of the Ccnd1, Ccdn2, and Myf5 genes. Boldface indicates the directed mutagenesis performed for each seed sequence.

MyoR via miR-378 during myoblast differentiation. In addition, miR-27 has also been implicated in the myogenic process, inducing *in vivo* muscle differentiation and repression of *Pax3* during myogenic differentiation (26). We have recently shown that *Pitx2c* plays an important role during myogenic development, controlling miR-27 and *Pax3* expression and thus maintaining the cells in a predifferentiated state. Furthermore, miRNAs modulate stem cell fate decisions, and some miRNAs involved in satellite cell quiescence and activation are starting to be identified (27–29). In the present study, we have further elaborated the transcriptional regulation of miRNAs by *Pitx2* in myoblasts and satellite cells, aiming to unravel whether impaired microRNA expression mediated by *Pitx2* might contribute to the cellular and molecular phenotypes previously reported, i.e., increased cell proliferation.

MATERIALS AND METHODS

Microarrays and statistical and bioinformatics analyses. In the present study, mirVana microarrays (Ambion) were used to profile the microRNA signature under different *Pitx2c* overexpression conditions, namely, two different doses (400 and 800 ng/ml of the cytomegalovirus [CMV]-Pitx2c plasmid) after 24 h of transfection. Thirty micrograms of total RNA was used to hybridize the distinct microRNA microarrays under each condition analyzed. MicroRNA-Cy5 labeling, microarray hybridization, and washing steps were performed according to the manufacturer's guidelines. The obtained original raw data files included quadruplicates of any given microRNA probe (662 unique mouse/human/rat mature microRNAs). Thus, the raw intensity value for each replicate of the same probe was considered an independent sample under each condition and then normalized by using the "justvsn" function implemented in the "vsn" Bioconductor library (http://bioconductor.org/) run in R software (http://r-project.org/). The normalized data were then uploaded and analyzed with the TM4 microarray software suite (http://tm4.org/), where a one-way analysis of variance (ANOVA) was carried out; those microRNAs showing a false-significant proportion of <0.05 were selected as significant, and their expression levels were used to obtain gene hierarchical clustering (using Pearson absolute correlation and complete-linkage algorithms).

DNA plasmid/siRNA transfection experiments and luciferase assays. DNA plasmid and small interfering RNA (siRNA) transfections were performed in Sol8 myoblasts, as previously described (19). The DNA plasmids used were the CMV-Pitx2c and CMV-enhanced green fluorescent protein (EGFP) plasmids. For RNA interference, siRNA against Pitx2c (Sigma) was used (19). For luciferase assays, the *cyclin D1*, *cyclin D2*, and *Myf5* 3′ untranslated regions (UTRs) were amplified from mouse genomic DNA and cloned into the pGLuc-Basic vector (New England BioLabs). Cyclin D1, cyclin D2, and Myf5 gene 3′ UTRs were amplified from mouse genomic DNA with specific primers bearing HindIII/BamHI restriction sites and cloned into the pGLuc-Basic vector (New England BioLabs). PCR-based site-directed mutagenesis was performed by using the Stratagene QuikChange site-directed mutagenesis kit but with the enzymes and buffers from the Bio-Rad iPROOF PCR kit. Primers used for site-directed mutagenesis (Table 1) introduced mutations into miR-15b, miR-106b, miR-23b, and miR-503 seed sequences present in the *cyclin D1* 3′ UTR, *cyclin D2* 3′ UTR, and *Myf5* 3′ UTR. Independent cotransfection experiments with pre-miRNAs were carried out simultaneously in Sol8 cells with 20 μl of culture medium; luciferase activity was measured 24 h after transfection by using a BioLux Gaussia luciferase assay kit or a BioLux Cypridina luciferase assay kit (New England BioLabs). In all cases, transfections were carried out in triplicate.

Generation of conditional tissue-specific null mutant mice. *Pax3*-Cre transgenic mice, purchased from the Jackson Laboratory, were crossed into homozygous *Pitx2* floxed mice, and *Pax3-cre*$^{+/-}$ *Pitx2*$^{fl+/-}$ heterozygotes were backcrossed. The littermates were PCR screened with *Pitx2*- and Cre-specific primers (30). Cre-positive heterozygote mice were selected as wild-type controls (*Pax3-cre*$^{+/-}$). All mice were maintained inside a barrier facility, and experiments were performed in accordance with University of Jaén regulations for animal care and handling.

Satellite cell isolation. Satellite cells were isolated as described previously by Qu-Petersen et al. (31). Two populations of early-preplate (EP) cells were used: EP cells after 3 days of culturing, which were previously described as phenotypically EPq, relatively quiescent early-activated cells, and EP cells after 5 days of culturing, which were previously described as phenotypically EPa, activated cells (31). Primary muscle cultures were prepared from young (3- to 4-month-old) normal mice by using a modified version of a previously described preplate technique (32–36). The hind limb muscles of young mice were removed, and the bones were dissected. The muscle was then minced into a coarse slurry by using scalpels. The muscle tissue was enzymatically dissociated at 37°C in 0.2% collagenase type XI (Sigma-Aldrich) for 1 h and then centrifuged at 3,500 rpm for 5 min. The cells were collected, incubated in dispase (Gibco BRL), prepared as 2.4 U/1 ml Hanks balanced salt solution (HBSS) (Gibco BRL)

TABLE 2 Specific primers for each gene, annealing temperatures, and amplicon sizes

Gene	Direction	Primer	Annealing temp (°C)	Amplicon size (bp)
β-*actin*	Sense	5′-CCA GAG GCA TAC AGG GAC-3′	60	143
	Antisense	5′-TGA GGA GCA CCC TGT GCT-3′		
Gapdh	Sense	5′-TCT TGC TCA GTG TCC TTG CTG G-3′	60	180
	Antisense	5′- TCC TGG TAT GAC AAT GAA TAC GC-3′		
Pitx2c	Sense	5′-CCT CAC CCT TCT GTC ACC AT-3′	60	175
	Antisense	5′-GCC CAC ATC CTC ATT CTT TC-3		
ccnd1	Sense	5′-TTG ACT GCC GAG AAG TTG TG-3′	60	154
	Antisense	5′-CTG GCA TTT TGG AGA GGA AG-3′		
ccnd2	Sense	5′-ATG CTG CTC TTG ACG GAA CT-3′	60	201
	Antisense	5′-ATG CTG CTC TTG ACG GAA CT-3′		
Pax7	Sense	5′-ACC ACT TGG CTA GGA TTT TCA AG-3′	60	240
	Antisense	5′- AGT AGG CTT GTC CCG TTT CC-3′		
Myf5	Sense	5′-TGA GGG AAC AGG TGG AGA AC-3′	60	187
	Antisense	5′-AGC TGG ACA CGG AGC TTT TA-3′		

TABLE 3 Specific primers for each miRNA analyzed

MicroRNA	Primer
hsa-miR-1	UGGAAUGUAAAGAAGUAUGUAU
hsa-miR-15a	UAGCAGCACAUAAUGGUUUGUG
hsa-miR-15b	UAGCAGCACAUCAUGGUUUACA
hsa-miR-21	UAGCUUAUCAGACUGAUGUUGA
hsa-miR-23a	AUCACAUUGCCAGGGAUUUCC
hsa-miR-23b	AUCACAUUGCCAGGGAUUACC
hsa-miR-106b	UAAAGUGCUGACAGUGCAGAU
hsa-miR-130a	CAGUGCAAUGUUAAAAGGGCAU
hsa-miR-133a	UUUGGUCCCCUUCAACCAGCUG
hsa-miR-206	UGGAAUGUAAGGAAGUGUGUGG
hsa-miR-503	UAGCAGCGGGAACAGUUCUGCAG

for 45 min, and then incubated for 30 min in 0.1% trypsin-EDTA (Gibco BRL) diluted in HBSS. After enzymatic dissociation, the muscle cells were centrifuged and resuspended in proliferation medium (PM). PM consists of Dulbecco's modified Eagle's medium containing 10% horse serum, 10% fetal bovine serum (FBS), 0.5% chicken embryo extract, and 1% penicillin-streptomycin (all reagents were purchased from Gibco BRL). Different populations of muscle-derived cells were isolated based on their adhesion characteristics. The muscle cells were plated into collagen-coated flasks (collagen type I; Sigma-Aldrich) for 2 h (preplate 1 [pp1]). The nonadherent cells were then transferred to other flasks (pp2), and the adherent cells in pp1 were discarded. It has been reported that the cells that rapidly attach are highly fibroblastic in nature (32–35). After 24 h, the floating cells in pp2 were collected, centrifuged, and plated into new flasks (pp3). These procedures were repeated at 24-h intervals until serial preplates (pp5) were obtained. All cell populations (pp3 to pp5) were maintained in PM with daily changes. Based on data from a previous report (31), 30 to 40% of the cells in pp2 and pp3 are known to be nonmyogenic, whereas up to 95% desmin-negative cells can be found in pp4 and pp5 (35). To further purify the myogenic cell population, pp2 and pp3 were discarded, and only pp4 and pp5 were combined and termed the EP cell population, as previously described (36).

Lentiviral vector production and satellite cell transduction. For the construction of the *Pitx2c* expression cassettes, the coding region of mouse *Pitx2c* was PCR amplified and cloned into pGEM-T. After sequence verification, the DNA was subcloned into the lentiviral vector pLVX-IRES-ZsGreen1 (Clontech), which allows the simultaneous expression of Pitx2c protein and a green fluorescent protein (ZsGreen1) and sequencing for verification. The recombinant DNA was cotransfected with a mixture of plasmids that express the respective viral proteins needed for producing viral particles in packing cells (Lenti-X 293T), by using Lenti-X HTX packaging systems according to the manufacturer's instructions (Clontech). The titer of lentivirus was determined by transducing Lenti-X 293T cells and by using the Lenti-X reverse transcriptase quantitative PCR (qRT-PCR) titration kit (Clontech). Freshly isolated satellite cells (EPq and EPa) were transduced with lentiviral vectors coding for Pitx2c (pLVX-Pitx2c-ZsGreen) or empty lentiviral vector cDNA (pLVX-IRES-ZsGreen1), at a multiplicity of 50 to 100 genome units, determined by quantitative PCR (qPCR), and adsorption of the lentiviral vectors was done for 8 to 24 h on cultures after viral particles were added. Transduction was monitored in all experiments by flow cytometry and by fluorescence microscopy.

Flow cytometry and fluorescence microscopy. Cells were fixed with 4% paraformaldehyde. ZsGreen1-positive (ZsGreen1$^+$) cells were analyzed by flow cytometry using an LSR-Fortessa cytometer (Beckman Coulter, Brea, CA). DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Fluorescence microscope images were acquired with a Leica TCS SL confocal microscope (Leica LCS version 2.0).

MicroRNA and anti-microRNA transfection assays. Sol8 cells as well as EPq and EPa satellite cells were cultured under growth conditions. The corresponding pre-miRNAs (Ambion) were transfected as described previously (19).

qRT-PCR analyses. RNA isolation and RT-PCR were performed as described previously (19), using standard procedures. Total RNA was extracted from mouse muscle tissue, Sol8 cells, EPq cells, EPa cells, and differentiating myoblasts by using the TriPure isolation reagent (Roche) according to the supplier's guidelines. To minimize genomic DNA contamination, total RNA was treated with 20 U of RNase-free DNase (Roche) for 1 h at 37°C and then purified by using a standard phenol-chloroform protocol. One microgram of total RNA was reverse transcribed by using Superscript RNase H$^-$ reverse transcriptase (Invitrogen) or an Exiqon microRNA qRT-PCR detection system, according to the manufacturer's protocol. As a reverse transcription control, each sample was subjected to the same process without reverse transcriptase. Real-time PCR was performed by using an MxPro Mx3005p PCR thermal cycler (Stratagene, Spain) and a SYBR green detection system (DyNamo HS SYBR green qPCR kit; Finnzymes). PCRs were performed in 0.2-ml optical tubes (Cultek) with a 20-μl total volume containing SYBR green mix (Finnzymes) and 2 μl of the reverse-transcribed RNA. The β-*actin* and *Gapdh* genes were used in parallel for each run as an internal control. Amplification conditions were 95°C for 5 min and 40 cycles of 95°C for 30 s, the annealing temperature for 30 s, and 72°C for 30 s. The final cycle was performed at 72°C for 7 min. Specific primers for each gene analyzed, annealing temperatures, and amplicon sizes are shown in Table 2. The

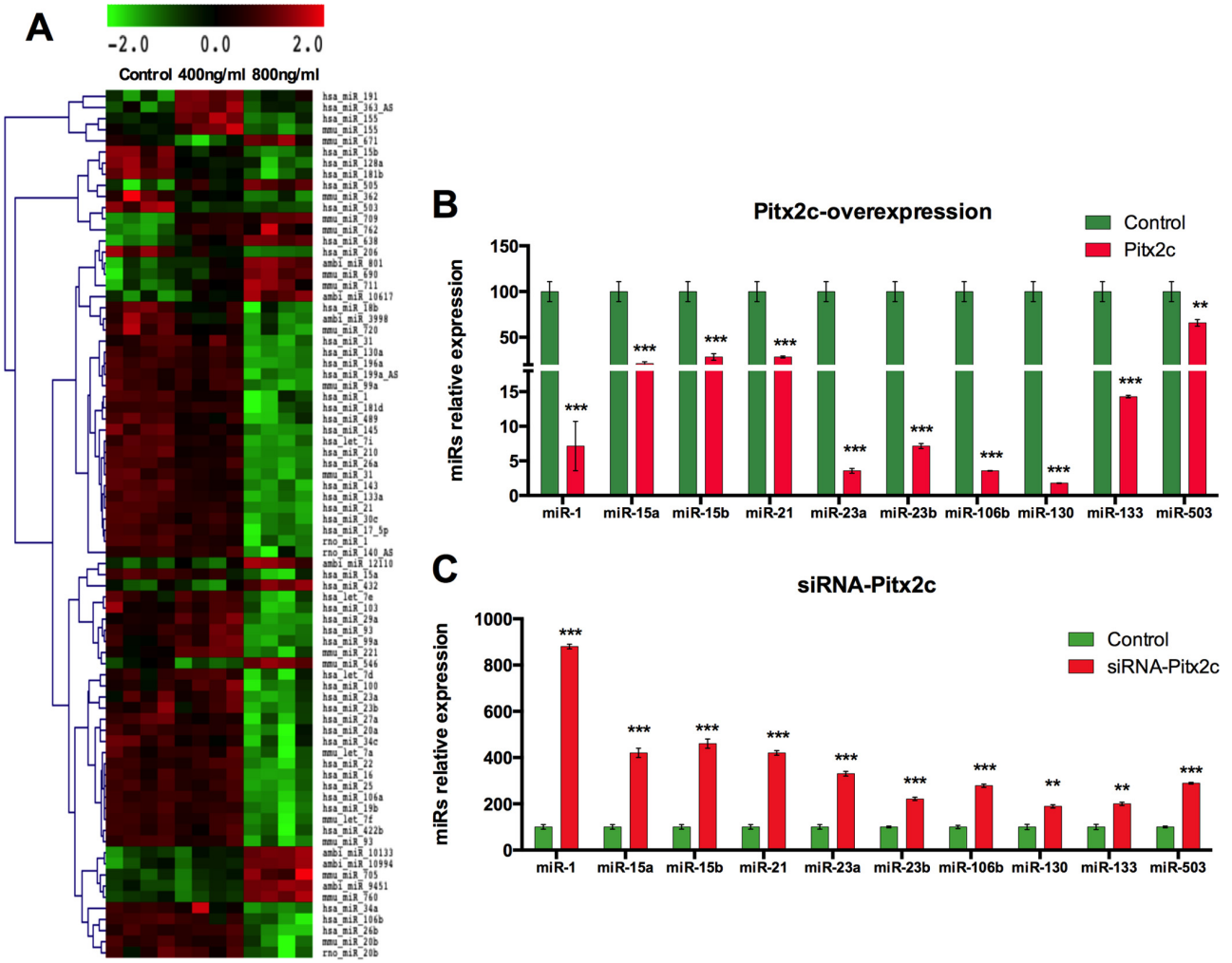


FIG 1 (A) Hierarchical clustering of statistically significant microRNA microarray expression profiles with the Sol8 cell line transfected with 400 ng/ml and 800 ng/ml of the CMV-*Pitx2c* plasmid 24 h after transfection. The color range (−2 to 2) is related to Z-scored expression values. (B) Expression profiles of statistically significant miRNAs by qRT-PCR in Sol8 *Pitx2c*-transfected cells at 400 and 800 ng/ml of the Pitx2c plasmid compared to controls. (C) Expression profiles of the statistically significant miRNAs by qRT-PCR in *Pitx2c*-silenced Sol8 cells (siRNA against Pitx2c) compared to controls.

relative level of expression of each gene was calculated as the ratio of the extrapolated levels of expression of each gene to the *Gapdh* level. PCR product sizes were verified by 2% agarose gel electrophoresis. For each pool of cDNA used, Pitx2c expression was confirmed. Each PCR was performed in triplicate and repeated with at least three different biological samples to obtain a representative average.

For microRNAs, qRT-PCRs were performed by using the Exiqon LNA microRNA primers and detection kit according to the manufacturer's guidelines. All reactions were always run in triplicates by using 5S as a normalizing control, as recommended by the manufacturer. SYBR green was used as a quantification system on a Stratagene Q-Max 2005P qRT-PCR thermocycler. Relative measurements were calculated as described previously by Livak and Schmittgen (37), and control measurements were normalized to represent 100%, as previously described (38). Specific primers for each miRNA analyzed are shown in Table 3.

***In situ* hybridization.** *In situ* hybridization was performed on serial cryosections of limb muscles obtained from C57BL/10 mice by using double-digoxigenin (DIG)-labeled LNA oligonucleotides (Exiqon) or antisense RNA probes, as previously described (39). Combined immunohistochemical detection was performed as previously described (40), by using anti-Pax7 antibody (Developmental Hybridoma Bank).

Immunocytochemistry. Immunocytochemistry experiments with Sol8 cell cultures and EPq and EPa cultured satellite cells were performed as described previously (14, 19). The antibodies used for immunostaining included anti-Ki67 (Abcam) anti-Myf5 (Santa Cruz Biotechnology), and anti-PHH3 (Millipore). For immunofluorescence, fluorochrome (Alexa Fluor 546)-conjugated secondary anti-rabbit antibodies (Invitrogen) were used for visualization. Nuclear staining was performed by using DRAQ-5 (red fluorescent cell-permeable DNA probe; Biostatus Limited). Immunofluorescence detection was performed by confocal analyses using a Leica TCS SL confocal microscope (Leica LCS version 2.0). Quantification was performed at a ×10 magnification for PHH3-positive cells and at a ×20 magnification for Ki67- and Myf5-positive cells; each experimental point is represented by the average of data from analyses of five different images for each independent experiment, and experiments were repeated with at least three different biological samples to obtain a representative average.

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation (ChIP) assays were performed as described previously (41),

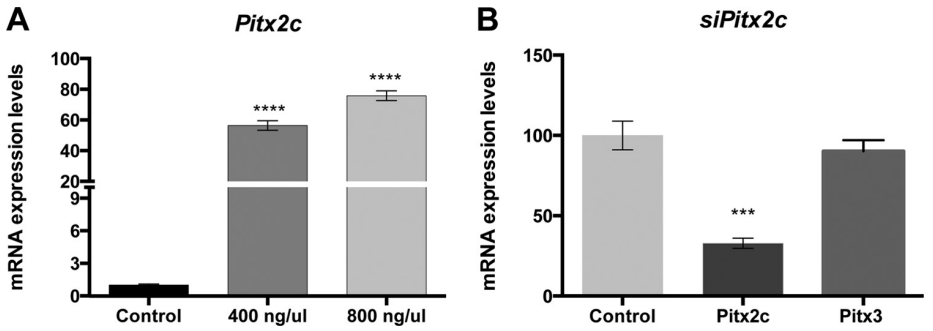


FIG 2 (A) qRT-PCR analyses showing *Pitx2c* overexpression after transfection with two different doses of the CMV-*Pitx2c* plasmid (400 and 800 ng/ml). (B) mRNA expression levels of Pitx2c and Pitx3 after treatment with siRNA against *Pitx2* in Sol8 myoblasts.

with modifications. Sol8 cells were transfected with 8 μg of the pcDNA-V5-Pitx2c plasmid in 100-mm dishes. After 24 h of Pitx2c transfection, the cells were cross-linked with 1% formaldehyde for 10 min at 37°C. For chromatin immunoprecipitation, the antibodies used were anti-V5 (clone V5-10; Sigma) or anti-polymerase II (anti-Pol II) (8WG16) (Santa Cruz); antibody against mouse dystrophin was used as a mouse IgG control. All PCRs 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 sequences for miR-15b, miR-106b, miR-503, and miR-23b (Table 3). As controls, normal rabbit IgG replaced the anti-V5 antibody to reveal nonspecific immunoprecipitation of chromatin. Three parallel real-time PCRs were also performed in triplicate with dilutions of input DNA to determine the linear range of amplification. Enrichment of RNA polymerase II served as an internal positive control for the ChIP assays, which was observed in all DNA regions analyzed.

Statistics. Data are presented as means, with error bars representing standard deviations. Data were analyzed for significance by Student's *t* test and considered significantly different if the *P* value was <0.01.

Microarray data accession number. The data discussed here have been deposited in the NCBI Gene Expression Omnibus (GEO) (42) and are accessible through GEO series accession number GSE53943 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE53943).

RESULTS

Pitx2-mediated microRNA expression in skeletal myoblasts. Pitx2 is expressed in developing myoblasts very early in development, and it was recently demonstrated to play a pivotal role in regulating key myogenic steps. We previously documented that *Pitx2c* is key in modulating proliferation versus differentiation and balancing different progenitor cell populations during myo-

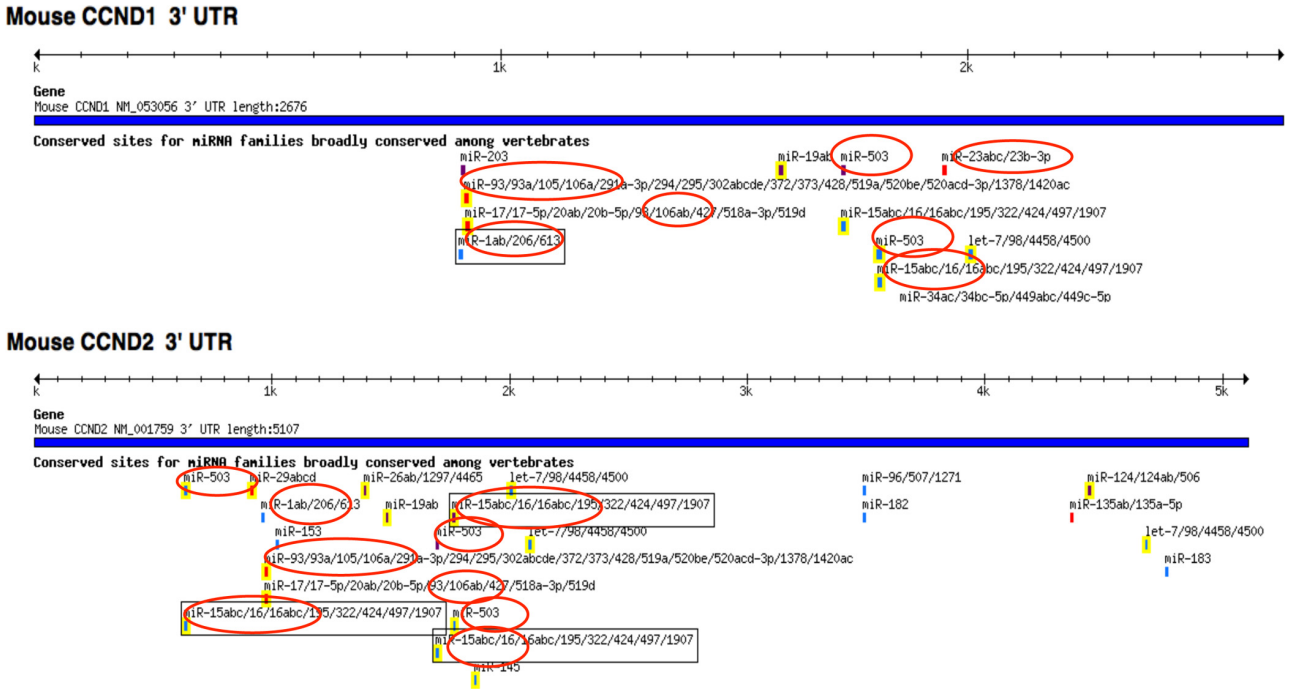


FIG 3 Schematic representation of the putative microRNA binding sites in the cyclin D1 (*ccnd1*) and cyclin D2 (*ccnd2*) genes as revealed by the TargetScan algorithm (http://www.targetscan.org/).

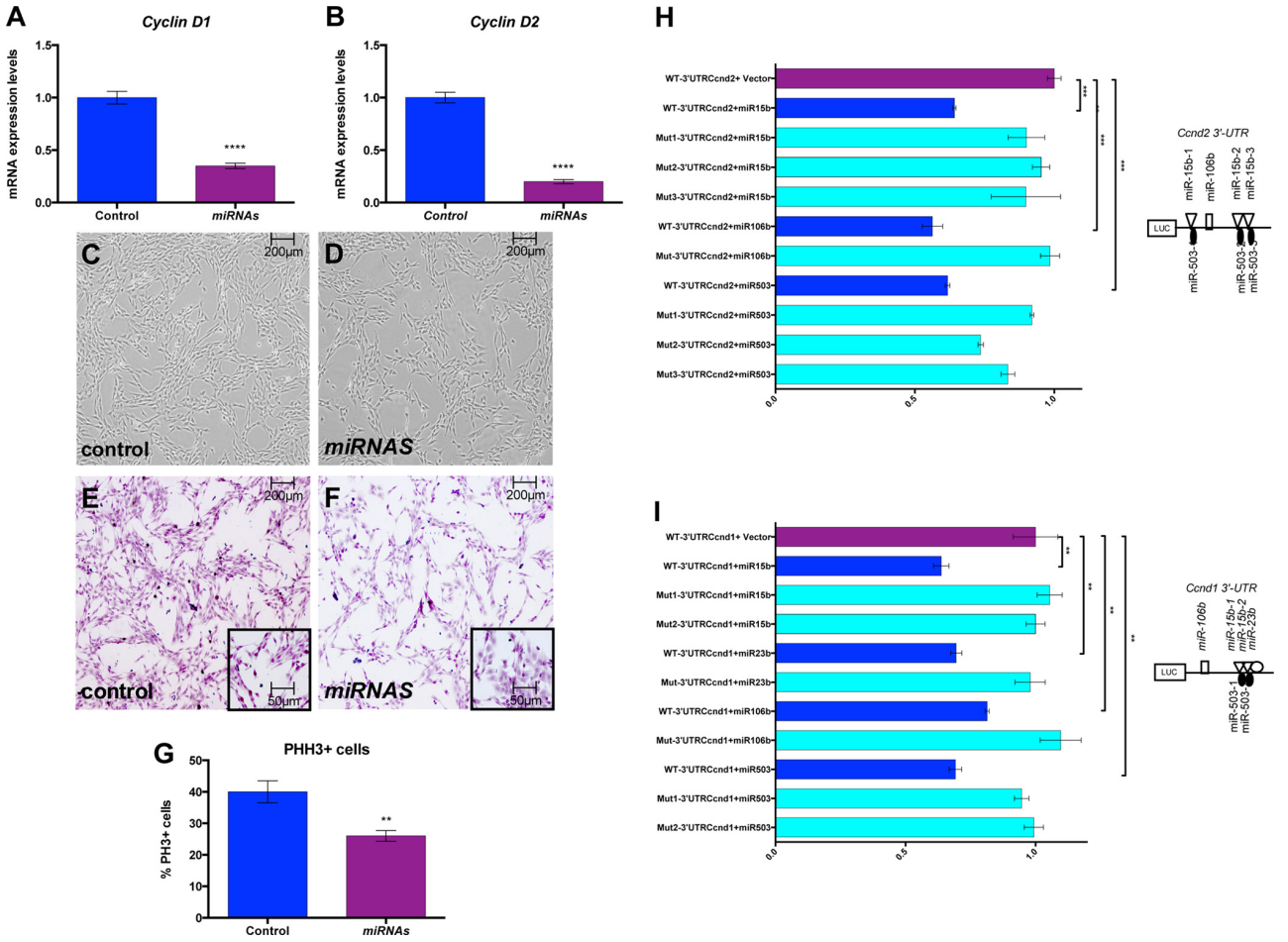


FIG 4 (A and B) Expression levels of *ccnd1* and *ccnd2* in Sol8 cells overexpressing a cocktail of miR-15b, miR-23b, miR-106b, and miR-503 (miRNAs) compared to control cells. (C and D) Representative images of Sol8 cells transfected with the miRNA cocktail and control cell cultures. (E and F) Representative images of immunohistochemical PHH3 staining in the Sol8 cell line overexpressing miRNA compared to controls. The inset represents closeup views of the corresponding immunostained cells. (G) Quantification of $PHH3^+$/total cells in transfected Sol8 cells compared to controls after 24 h of miRNA transfection. (H and I) Normalized luciferase activity of the 3′-UTR cyclin D1 and cyclin D2 gene luciferase reporter (wild type [WT] *cyclin D1* and wild-type *cyclin D2* 3′ UTRs) with an empty plasmid or pre-miRNAs shows a loss of luciferase activity with expression of miR-15b, miR-23b, miR-106b, and miR-503. There was no loss of luciferase activity when the miRNA seed sequences were mutated.

genesis. Furthermore, we have shown that *Pitx2c* posttranscriptionally modulates key myogenic transcription factors such as Pax3 by repressing miR-27 expression (18, 19). To further analyze the role of *Pitx2c* in the posttranscriptional control of myogenesis, we performed microRNA microarray analyses using *Pitx2c*-overexpressing Sol8 myoblasts. Because we previously demonstrated that *Pitx2c*-mediated effects on myoblasts are dose dependent, we used two different doses of the CMV-*Pitx2c* plasmid (400 and 800 ng/ml of the CMV-*Pitx2c* plasmid), which previously affected myoblast proliferation and differentiation (19), thereby providing insights into the dose dependency of *Pitx2*-regulated miRNAs (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE53943). Figure 1A illustrates the hierarchical clustering of differentially expressed and statistically significant miRNAs after 24 h of cell transfection. Of 497 miRNAs, 60 (~10%) displayed statistically significant differences, thus suggesting that they are regulated by *Pitx2*. Detailed analyses demonstrated at least four distinct expression patterns. Small proportions of miRNAs display higher levels (7/60; ~11%) or lower levels in transfections with both doses of the CMV-*Pitx2c* plasmid (Fig. 1A). Most miRNAs (40/60; ~67%) display significantly lower expression levels only at *Pitx2c* doses previously shown to have more profound effects on the myoblast phenotype (transfections with 800 ng/ml of the CMV-*Pitx2c* plasmid) (Fig. 1A), while only a minority (1/60; ~1%) showed increased expression levels at this dosage. The remaining miRNAs displayed a transient increase (2/60; ~3%) or decrease (3/60; ~5%) of expression levels at low doses (transfections with 400 ng/ml of the CMV-*Pitx2c* plasmid). Overall, these data illustrate that *Pitx2* regulates the expression of different subsets of miRNAs. Importantly, most miRNAs regulated by *Pitx2* in skeletal-muscle myoblasts displayed lower levels (50/60; ~83%), and only a small proportion (10/60; ~17%) showed higher expression levels. We validated our microarray expression data by using qRT-PCR. As illustrated in Fig. 1B and 2A, miR-1, miR-15a, miR-15b, miR-21, miR-23a, miR-23b, miR-106b, miR-130, miR-133, and miR-503 displayed significantly lower levels after *Pitx2c* overexpression,

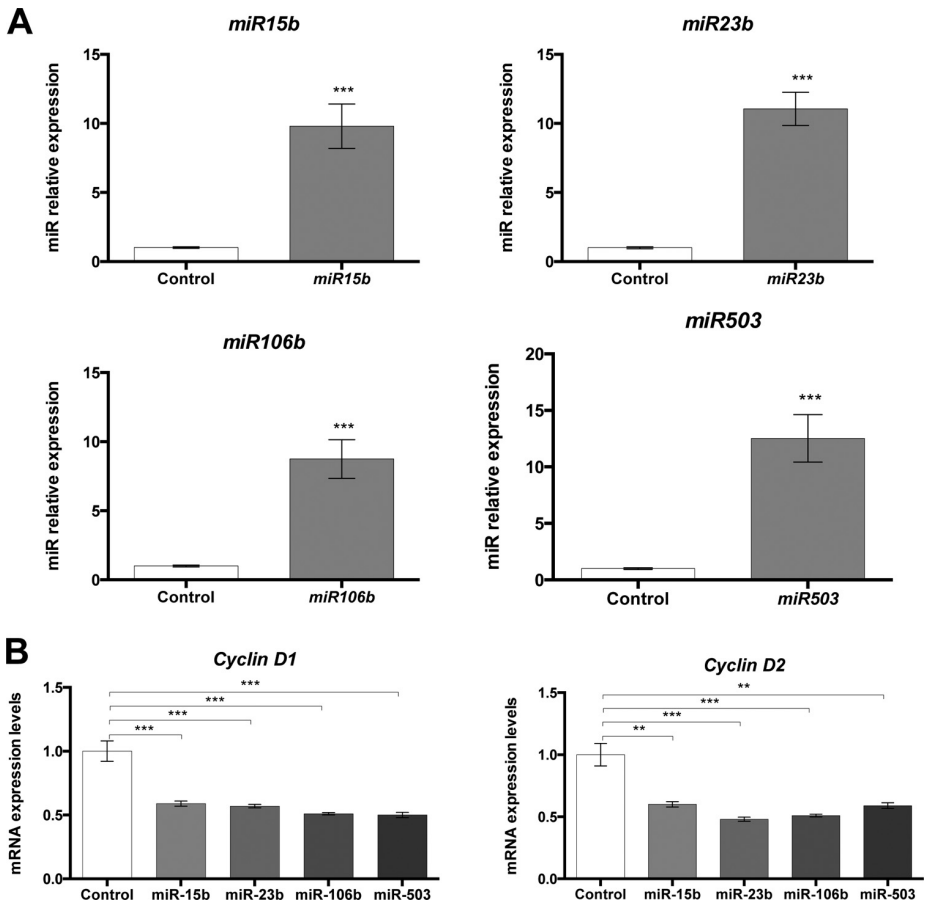


FIG 5 (A) qRT-PCR analyses showing miRNA overexpression after pre-miRNA-independent transfections. (B) mRNA expression levels for cyclin D1 and cyclin D2 genes were 50 to 60% lower when miRNAs were transfected separately in Sol8 cells.

whereas siRNA against *Pitx2* in Sol8 myoblasts resulted in the upregulation of miR-1, miR-15a, miR-15b, miR-21, miR-23a, miR-23b, miR-106b, miR-130, miR-133, and miR-503 (Fig. 1C and 2B), supporting our microarray observations. Individual miRNAs can target a large number of transcripts, and thus, their functional roles can vary greatly according to the biological context. To provide an initial insight into the putative mechanisms by which *Pitx2*-regulated miRNAs can exert their function in skeletal-muscle myoblasts, we undertook gene ontology analyses using DAVID software (http://david.abcc.ncifcrf.gov/). These gene ontology analyses revealed that the *Pitx2*-upregulated and *Pitx2*-downregulated miRNAs might modulate highly similar pathways: transcription, regulation of transcription, cell morphogenesis, and cell proliferation (see Table S1 in the supplemental material). In addition, cell projection, cell organization, cell motility, cell proliferation, and cell maturation were also revealed for *Pitx2*-downregulated miRNAs (see Table S1 in the supplemental material). Overall, these data support the notion that cell behavior and proliferation pathways induced by *Pitx2* could be driven by microRNA expression in myoblasts.

Role for *Pitx2*-mediated microRNAs in regulating myoblast cell proliferation. Previously, we demonstrated that overexpression of *Pitx2c* in mouse myoblasts leads to greater cell proliferation and loss of the ability to fuse and thus to form myotubes and terminally differentiate (18). *Pitx2* overexpression raises levels of *Pax3* expression, which in turn inhibits *MyoD* and myogenin gene expression and terminal differentiation, a process mediated by *Pitx2* regulation of miR-27 (19). However, cell proliferation in *Pitx2c*-overexpressing myoblasts is independent of *Pax3* and/or miR-27 regulation (19). Additionally, a role of Pitx2 in controlling myocyte numbers on the chick myotome has been reported (43), suggesting that the effect of Pitx2 on cell proliferation in myogenic cells is conserved in different vertebrate species. Thus, we focused our attention on whether deregulation of other miRNAs, mediated by Pitx2, can affect cell cycle regulation. We bioinformatically analyzed the putative microRNAs targeting key genes of cell cycle regulation, such as the cyclin D1 (*ccnd1*) and cyclin D2 (*ccnd2*) genes, and we found that >65% of the predicted target sites for microRNA in the *ccnd1* (8/12) and *ccnd2* (11/16) 3′ UTRs correspond to four downregulated miRNAs in Sol8 after *Pitx2c* overexpression (Fig. 3). Thus, these data suggest that Pitx2 controls transcriptional inhibition of a large subset of miRNAs, which in turn can modulate cell proliferation in Sol8 myoblasts. Notably, except for miR-1 and miR-206 (22), the role of these miRNAs in the regulation of cell proliferation on skeletal-muscle cells has not been reported previously. To investigate the function of miRNAs regulated by Pitx2 with previously unknown functions in myoblast cell proliferation, such as miR-15b, miR-23b, miR-106b, and

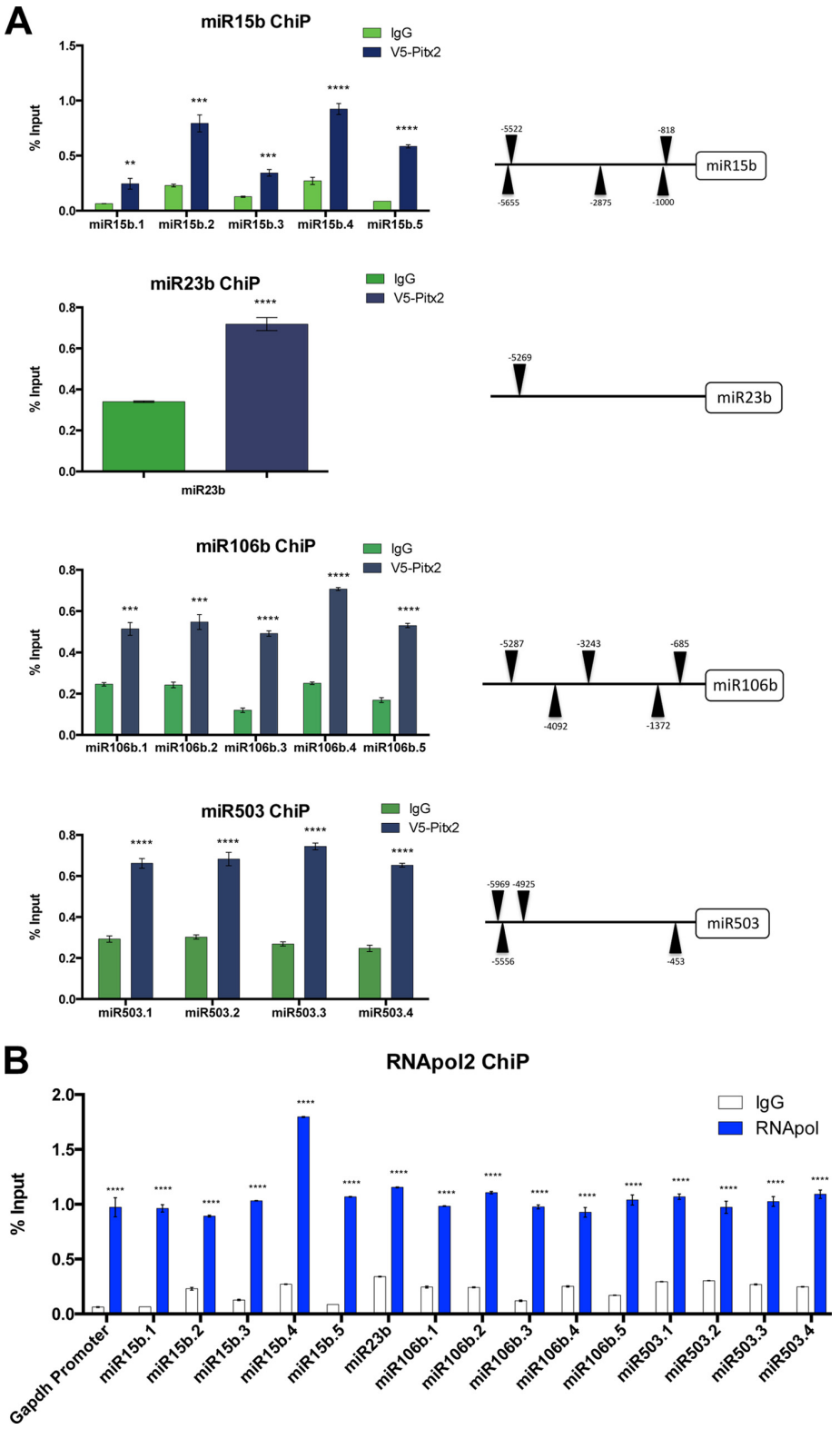
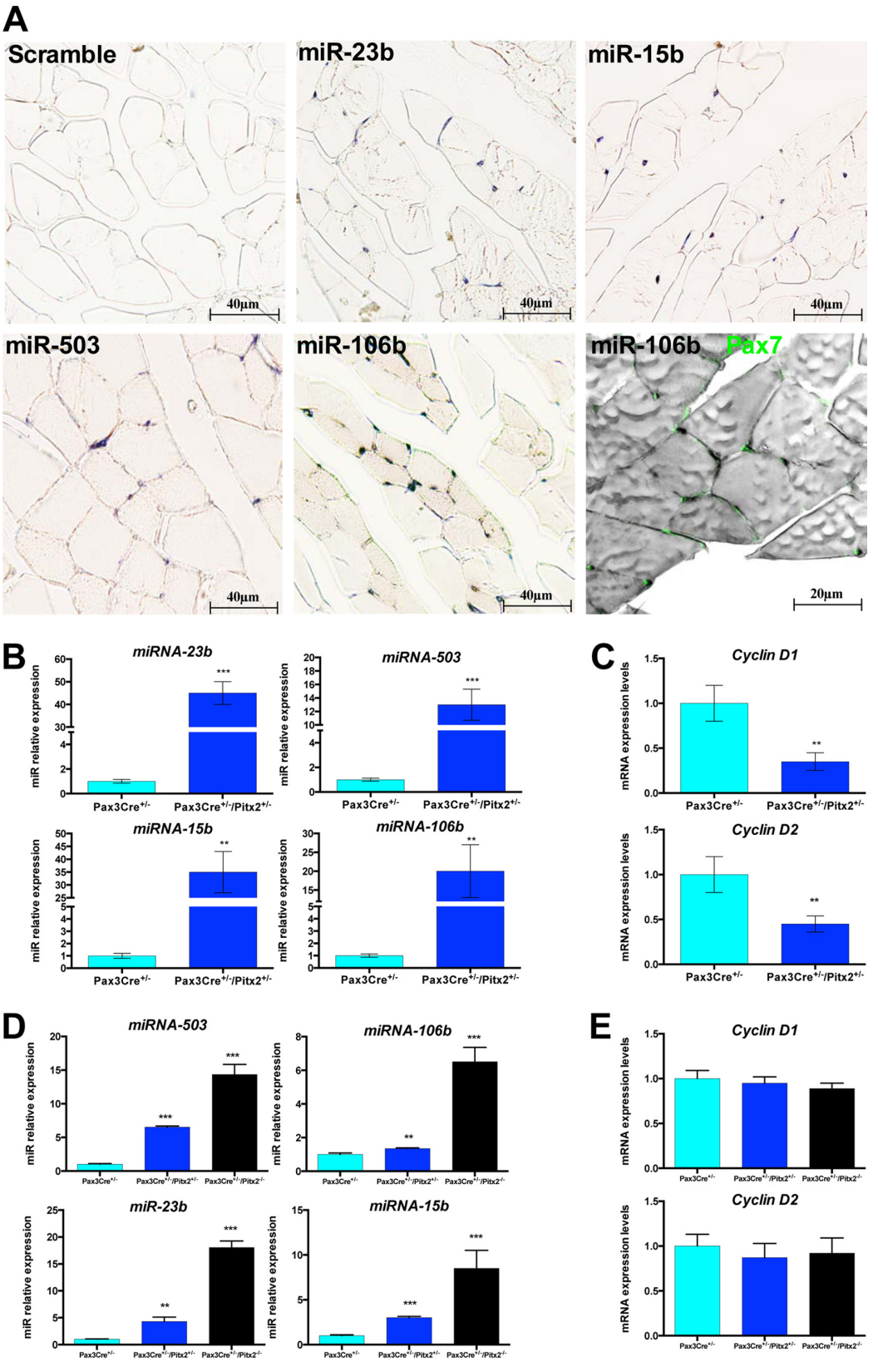


FIG 6 (A) ChIP assays. Pitx2 binds DNA regions upstream of the miR-15b, miR-23b, miR-106b, and miR-503 genetic loci. There was an observed enrichment in Pitx2 binding in all miRNAs analyzed. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. These experiments were performed in Sol8 cells. (B) RNA polymerase II occupancy in the tested DNA regions upstream of miR-15b, miR-23b, miR-106b, and miR-503. Notably, all of these DNA regions have RNA polymerase II occupancy levels similar to those of the *Gapdh* promoter used as a control.


A
Scramble
miR-23b
miR-15b
miR-503
miR-106b
miR-106b Pax7
40μm
20μm
B
miRNA-23b
miRNA-503
miRNA-15b
miRNA-106b
miR relative expression
Pax3Cre+/-
Pax3Cre+/-/Pitx2+/-
C
Cyclin D1
Cyclin D2
mRNA expression levels
D
miRNA-503
miRNA-106b
miR-23b
miRNA-15b
Pax3Cre+/-/Pitx2-/-
E
Cyclin D1
Cyclin D2
mRNA expression levels

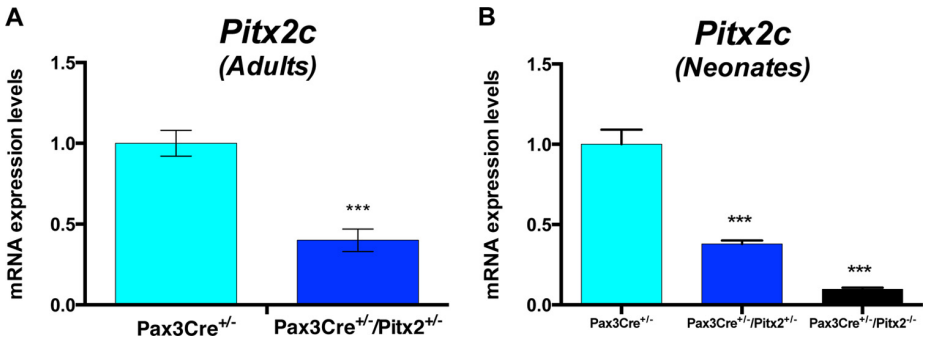


FIG 8 (A) qRT-PCR for *Pitx2c* expression in *Pax3-cre*$^{+/-}$/*Pitx2*$^{+/-}$ heterozygote adult mice. (B) qRT-PCR for *Pitx2c* expression in *Pax3-cre*$^{+/-}$/*Pitx2*$^{+/-}$ heterozygote and *Pax3-cre*$^{+/-}$/*Pitx2*$^{-/-}$ homozygote neonates.

miR-503, we performed transfection experiments with these miRNAs on Sol8 myoblasts at low confluence (10^5 myoblasts/well) and assessed cell proliferation by phospho-histone 3 immunolabeling after 24 h of transfection. In addition, cyclin D1 and cyclin D2 gene expression levels were measured by qRT-PCR. As reflected in Fig. 4A and B, transfection experiments with a cocktail of these miRNAs resulted in 80% lower expression levels of the cyclin D1 and cyclin D2 genes. In line with these findings, cells displayed a lower phospho-histone 3-immunolabeled index and thus were clearly proliferating at a slower pace, as illustrated in Fig. 4C to G. To test whether the predicted miR-15b, miR-23b, miR-106b, and miR-503 elements in the 3′ UTRs of the cyclin D1 and cyclin D2 genes were functional, we ligated these sequences downstream of the luciferase gene in the pGLuc-Basic vector and cotransfected them independently with pre-miR-15b, pre-miR-23b, pre-miR-106b, and pre-miR-503 into Sol8 cells (Fig. 4H and I). Luciferase activity for the cyclin D1 and cyclin D2 gene 3′ UTRs was approximately halved with cotransfection with pre-miR-15b, pre-miR-23b, pre-miR-106b, and pre-miR-503 compared with the empty vector, and importantly, site-directed mutagenesis of the predicted pre-miR-15b, pre-miR-23b, pre-miR-106b, and pre-miR-503 binding sites in the cyclin D1 and cyclin D2 gene 3′ UTRs eliminated such repression (Fig. 4H and I). Transfection with pre-miR-15b, pre-miR-23b, pre-miR-106b, and pre-miR-503a separately decreased the expression levels of the cyclin D1 and cyclin D2 genes only 50 to 60% (Fig. 5), suggesting synergism or additive effects among them.

In order to reinforce the notion that Pitx2 directly modulates the expression of these four miRNAs, we screened for potential conserved *Pitx2* binding sites upstream of the miR-15b, miR-23b, miR-106b, and miR-503 genetic loci. Five conserved Pitx2 binding sites were identified ~6 kb upstream of the miR-15b and miR-106b genetic loci, four sites were identified ~6 kb upstream of the miR-503 genetic locus, and one site was located ~6 kb upstream of the miR-23b gene locus (Fig. 6A). To test the interaction of Pitx2 with these putative binding sites, we performed chromatin immunoprecipitation (ChIP) assays in Sol8 cells. Exogenous Pitx2 bound to the all-putative binding sites upstream of the miR-15b, miR-23b, miR-106b, and miR-503 genetic loci, as illustrated in Fig. 6A. RNA polymerase II occupancy suggests that all DNA regions tested are transcriptionally active (Fig. 6B). Taken together, these data point out a *Pitx2*-miRNA pathway controlling the expression of key regulatory cell cycle genes, which in turn modulate cell proliferation in myoblasts.

***Pitx2*-mediated miRNAs and regulatory cell cycle gene expression are deregulated in conditional tissue-specific *Pitx2* mutant mice.** First, we checked the expression pattern for miRNAs regulated by *Pitx2c* in serial sections obtained from wild-type mouse limb muscles by LNA *in situ* hybridization. As illustrated in Fig. 7A, miR-15b, miR-23b, miR-106b, and miR-503 displayed a tissue expression pattern compatible with marked expression on satellite cells. Second, to determine whether *Pitx2c*-mediated miRNA regulation on myogenic cells is maintained *in vivo*, we used qRT-PCR to analyze the expression levels of miRNA regulated by *Pitx2c* in conditional tissue-specific *Pitx2* mutant mice by intercrossing a *Pitx2* floxed mouse line with a *Cre* deleter mouse line, which rendered muscle lineage-specific *Pax3-Cre* recombination. This conditional mutant mouse line is currently being analyzed and characterized (E. Lozano-Velasco, F. Ramirez, P. Hernández-Torres, D. Vallejo, D. Franco, and A. E. Aránega, unpublished data). Because *Pax3-cre*$^{+/-}$/*Pitx2*$^{-/-}$ homozygote mutant neonates were born alive but died soon after birth, in the present work, we analyzed adult heterozygote and neonatal mutants. Notably, limb muscles from *Pax3-cre*$^{+/-}$/*Pitx2*$^{+/-}$ heterozygote mice, in which *Pitx2* expression is reduced by >50% (Fig. 8A), displayed higher expression levels of miRNAs regulated by *Pitx2c* (miR-15b, miR-23b, miR-106b, and miR-503) than did *Pax3-cre*$^{+/-}$/*Pitx2*$^{+/+}$ control limb muscles (Fig. 7B). Moreover, given that even in the absence of overt damage, the rate of myonuclear turnover in rodents is 1 to 2% per week (44), we also analyzed the expression levels of the cyclin D1 and cyclin D2 genes, and we found that they were clearly downregulated in

FIG 7 (A) Tissue distribution of miR-15b, miR-23b, miR-106b, and miR-503 in limb muscles of C57BL/10 mice. miRNAs were expressed mostly in cells that presented a tissue distribution very similar to that of satellite cells, as illustrated by Pax7 costaining for miR-106b. An LNA probe with a scrambled sequence was used to test the specificity of the probes. (B) Expression profiles for miR-15b, miR-23b, miR-106b, and miR-503 in *Pax3-cre*$^{+/-}$/*Pitx2*$^{+/-}$ heterozygote mice (n = 9) compared to *Pax3-cre*$^{+/-}$ control mice (n = 8). (C) Cyclin D1 and cyclin D2 gene expression profiles in *Pax3-cre*$^{+/-}$/*Pitx2*$^{+/-}$ heterozygote versus *Pax3-cre*$^{+/-}$ control mice. (D) Expression profiles for miR-15b, miR-23b, miR-106b, and miR-503 in *Pax3-cre*$^{+/-}$/*Pitx2*$^{+/-}$ heterozygote (n = 6) and *Pax3-cre*$^{+/-}$/*Pitx2*$^{-/-}$ homozygote (n = 5) neonates compared to *Pax3-cre*$^{+/-}$ control neonatal mice (n = 6). (E) Cyclin D1 and cyclin D2 gene expression profiles in *Pax3-cre*$^{+/-}$/*Pitx2*$^{+/-}$ heterozygote and *Pax3-cre*$^{+/-}$/*Pitx2*$^{-/-}$ homozygote neonates versus *Pax3-cre*$^{+/-}$ control neonatal mice.

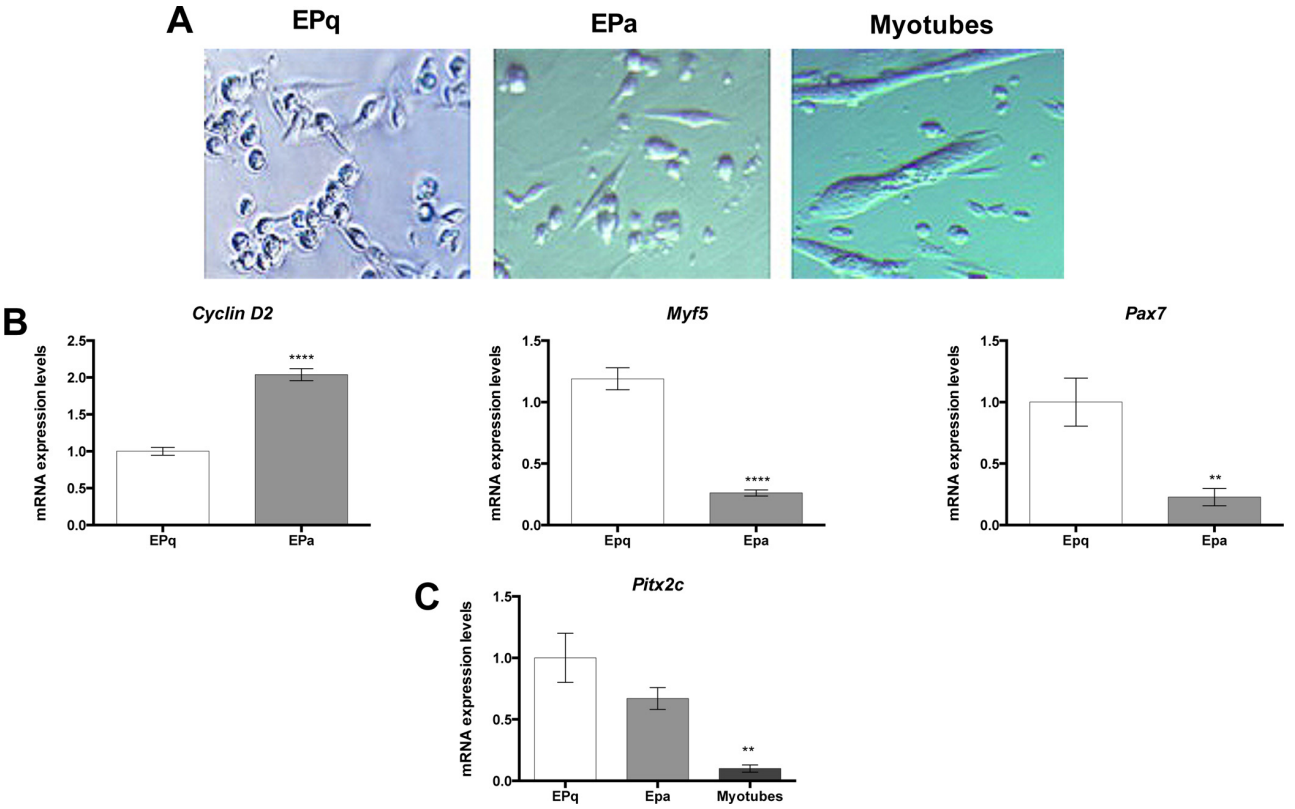


FIG 9 (A) Representative images of early-plated relatively quiescent/early-activated satellite cells (EPq), early-plated long-term-activated satellite cells (EPa), and EPa-derived differentiating fusing-myoblast cultures. (B) mRNA expression levels of the cyclin D2, *Myf5*, and *Pax7* genes in EPq and EPa cells. (C) mRNA expression levels of *Pitx2c* in EPq cells and EPa cells and myoblasts.

Pax3-cre$^{+/-}$*/Pitx2*$^{+/-}$ animals in comparison with control mice (Fig. 7C). These observations are consistent with our *in vitro* findings revealing the existence of a *Pitx2*-miRNA pathway controlling the expression of key regulatory cell cycle genes in myogenic cells. Similar to those from adult mice, limb muscles from *Pax3-cre*$^{+/-}$*/Pitx2*$^{-/-}$ neonatal heterozygote and homozygote mice, which exhibited lower Pitx2c expression levels (Fig. 8B), showed increased levels of miRNAs regulated by *Pitx2c*. Curiously, no changes in cyclin D1 and cyclin D2 gene expression levels were detected in neonatal mutants, pointing out a different status for this *Pitx2*-miRNA pathway in neonates (Fig. 7D and E).

The *Pitx2*-miRNA pathway regulating cell proliferation is conserved in early-activated satellite cells. Given that it was previously shown that Pitx2 can be detected in proliferating myoblasts during adult myogenesis (20), and it appears to help maintain a proliferating pool of myogenic precursor cells in extraocular muscles (45), we next investigated whether the *Pitx2*-miRNA pathway is also present in freshly isolated adult satellite cells. Because adult satellite cells isolated from freshly dissected mouse muscle tissue rapidly initiate the process of activation, leading to myogenic differentiation (36), we first evaluated the expression profile for *Pitx2c* and miRNAs during the *in vitro* process of satellite cell activation and differentiation. Therefore, in the present study, we used early-plated early-activated satellite cells (EPq) and early-plated long-term-activated satellite cells (EPa) isolated from mouse hind limbs according to their adhesion characteristics and proliferation behavior, as previously described (31, 46), as well as EPa-derived differentiating myoblasts fusing to myotubes (Fig. 9A). As illustrated in Fig. 9B, EPq cells can thus be rendered *Pax7* high/low-proliferating cells, and EPa cells can be rendered *Pax7* low-expressing/high-proliferating satellite cells (47). Notably, *Pitx2c* expression levels are higher in early-activated EPq cells than in long-term-activated satellite cells (EPa) and display low expression levels in differentiating myotubes derived from satellite cells (Fig. 9C). This *Pitx2c* expression profile might indicate a Pitx2c requirement just before reaching high levels of cell proliferation during the process of satellite cell activation. Indeed, gain-of-function experiments with EPq and EPa satellite cells showed that *Pitx2c* overexpression leads to a clear upregulation of the cyclin D1 and D2 genes in early-activated satellite cells (EPq) but not in long-term-activated satellite cells (EPa) (Fig. 10A to C). Moreover, the number of Ki67-positive cells was significantly higher in EPq cells overexpressing *Pitx2c* than in cells transfected with the empty lentiviral vector (Fig. 10D and E). These results indicate that *Pitx2c* could regulate proliferation during satellite cell activation.

Next, to test whether *Pitx2c* also acts to control miR-15b, miR-23b, miR-106b, and miR-503 expression in satellite cells, we analyzed miRNA expression profiles on EPq and EPa cells overexpressing *Pitx2c*, and our analyses showed that all miRNAs were dramatically downregulated after *Pitx2c* overexpression (Fig. 11A). Moreover, the miRNAs miR-15b, miR-23b, miR-106b, and

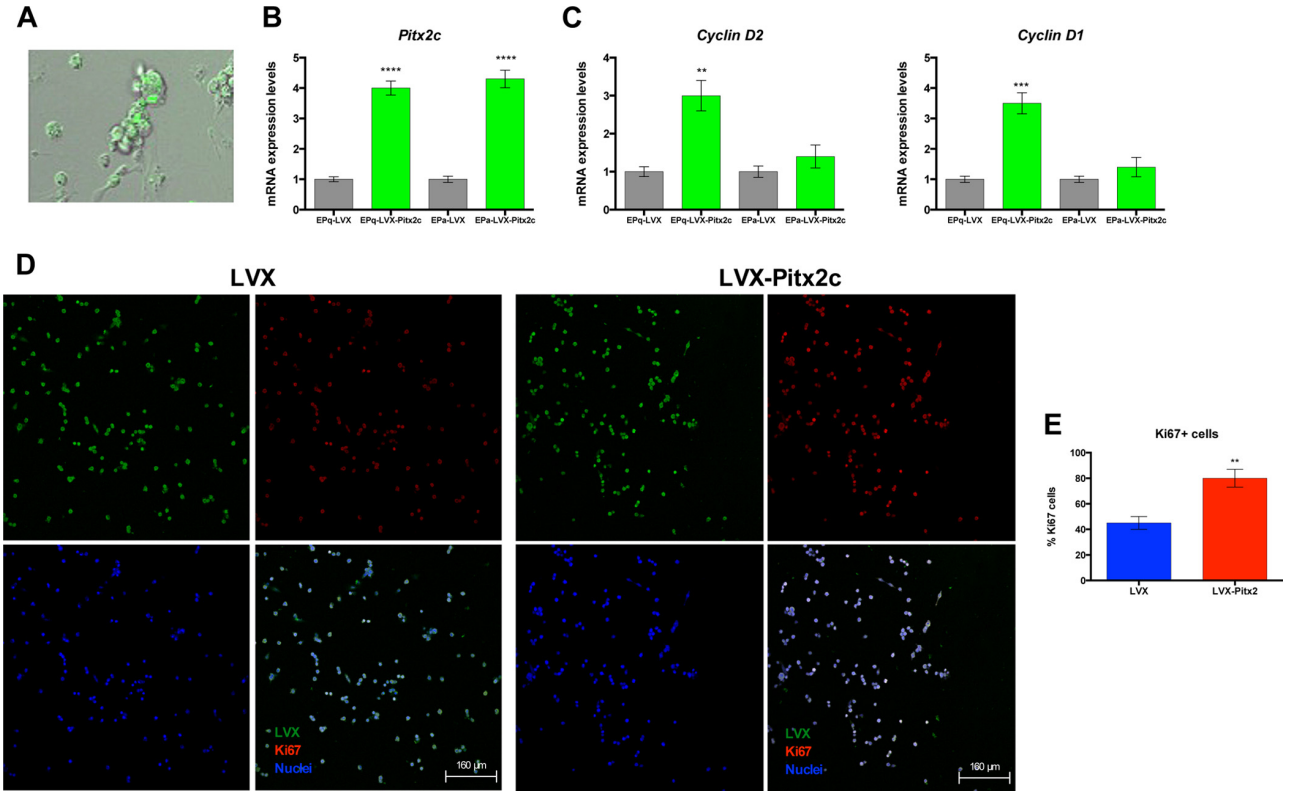


FIG 10 (A) Representative images of EPq cells transfected with a lentivirus-Pitx2c-ZsGreen vector (LVX-Pitx2c). (B) qRT-PCR for *Pitx2c* expression in EPq and EPa cells transfected with the LVX-Pitx2c vector with respect to cells transfected with the empty LVX-ZsGreen lentiviral vector (LVX). (C) Cyclin D1 and cyclin D2 gene expression in EPq and EPa Pitx2c-overexpressing cells with respect to control cells. (D) Representative images of immunohistochemical analyses for Ki67-positve cells in EPq cells transfected with the lentivirus-Pitx2c-ZsGreen vector (LVX) compared to cells transfected with the empty LVX-ZsGreen lentiviral vector (LVX-Pitx2c). (E) Percentage of Ki67$^+$ cells in EPq cells transfected with the lentivirus-Pitx2c-ZsGreen vector with respect to cells transfected with the empty LVX-ZsGreen lentiviral vector.

miR-503 displayed an expression profile complementary to *Pitx2c* during the process of *in vitro* differentiation (Fig. 11B), thus reinforcing the notion that *Pitx2c* acts negatively to regulate these miRNAs in activated satellite cells. The fact that this *Pitx2c*-miRNA pathway is present in EPq and EPa cells but has effects only on cyclin D1 and cyclin D2 gene expression in EPq cells may indicate that the *Pitx2c*-miRNA pathway participates in cell proliferation at an early step of activation but that other regulatory molecules contribute to maintaining cell proliferation after activation is triggered. In accordance with this idea, in neonatal *Pax3-cre*$^{+/-}$/*Pitx2*$^{-/-}$ homozygote mice, altered expression of *Pitx2c*-regulated miRNAs did not lead to cyclin D1 and cyclin D2 gene dysregulation (Fig. 7). Since it was previously shown that many satellite cells at neonatal stages are in a permanent stage of activation to ensure muscle growth (11), these finding emphasize the role of *Pitx2* in cell proliferation at the onset of satellite cell activation but not when the cell activation processes have already taken place. Finally, as observed for Sol8 myoblasts, transfection experiments with a cocktail of these miRNAs in EPq satellite cells resulted in a clear downregulation of the cyclin D1 and cyclin D2 genes (Fig. 11C and D). Moreover, transfection experiments with pre-miR-15b, pre-miR-23b, pre-miR-106b, and pre-miR-503 in *Pitx2c*-overexpressing cells rescued cyclin D1 and cyclin D2 gene upregulation, reinforcing the notion that these miRNAs mediate the *Pitx2c* effects on cyclin D1 and cyclin D2 gene expression (Fig. 12). Therefore, altogether, these findings indicate that the *Pitx2c*-miRNA pathway that modulates cell proliferation is also present in satellite cells.

***Pitx2c* enhances the Myf5$^+$ satellite cell population by regulating miR-106b.** After activation, satellite stem cells expand and undergo symmetric and asymmetric divisions *in vivo* and *in vitro* (48, 49). Symmetric divisions result in the symmetric expansion of Pax7$^+$/Myf5$^-$ satellite cells, retaining their stem cell identity. Through asymmetric divisions, one daughter cell retains its stem cell identity (Pax7$^+$/Myf5$^-$), and the other daughter cell upregulates myogenic factor 5 (Myf5) and becomes Pax7$^+$ Myf5$^+$, representing more committed myogenic progenitors that participate in skeletal-muscle growth and regeneration (36, 49). Myf5 is one of the most relevant transcription factors that plays a key role as a determinant of the acquisition of myogenic cell fate in satellite cells (50, 51). Therefore, in order to test whether *Pitx2c*-mediated effects on cell proliferation can modulate the rate of myogenic commitment of satellite cells, we analyzed *Myf5* expression by qRT-PCR and immunohistochemistry. As illustrated in Fig. 13A, *Myf5* mRNA expression levels were upregulated in *Pitx2c*-overexpressing satellite cells with respect to the control. In addition, the percentage of Myf5$^+$ cells was significantly higher after *Pitx2c* overexpression in satellite cell cultures (Fig. 13B and C). These

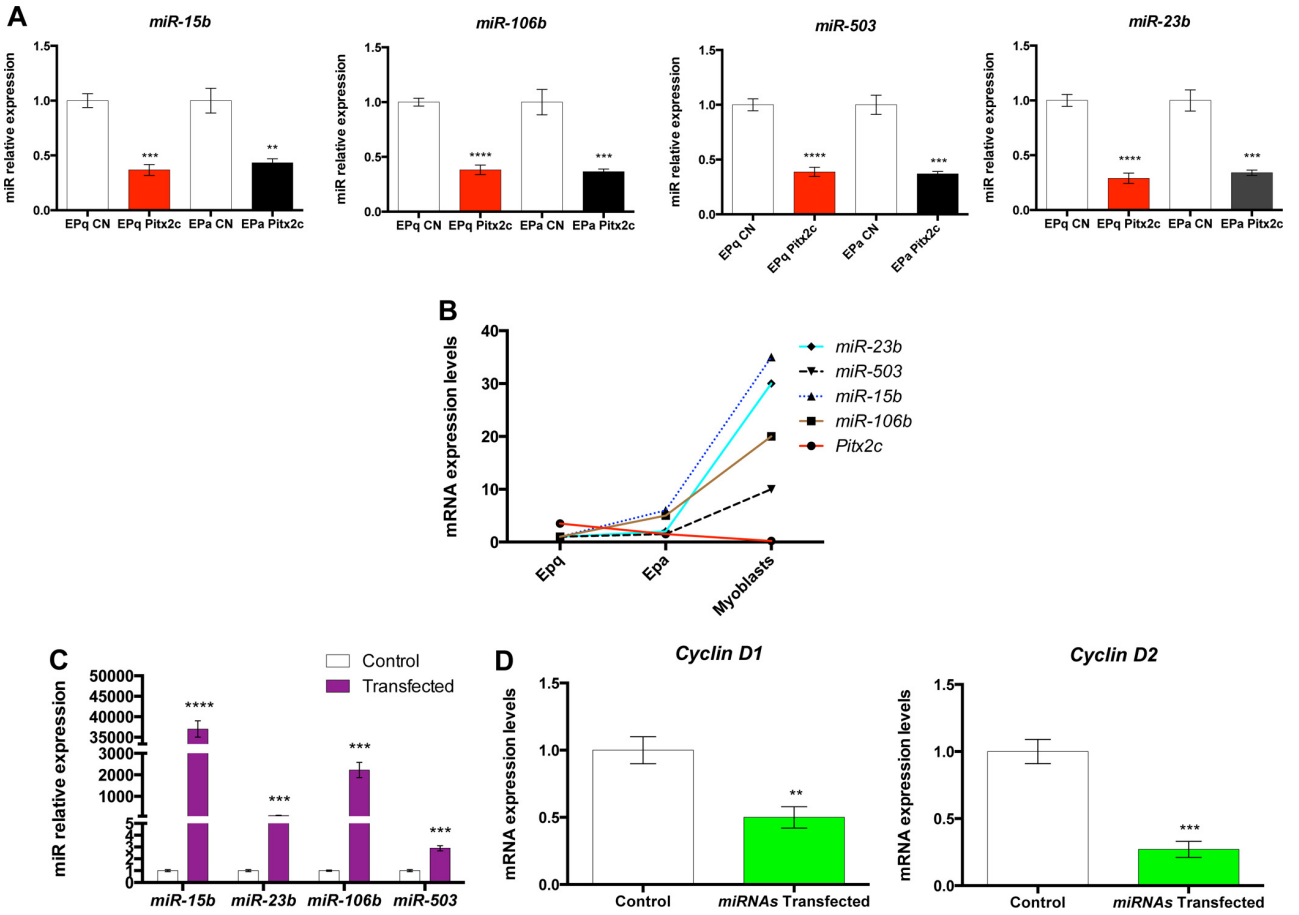


FIG 11 (A) Expression profiles for miR-15b, miR-23b, miR-106b, and miR-503 in EPq and EPa *Pitx2c*-overexpressing cells with respect to control (CN) cells. (B) Relative expression levels of of *Pitx2c* as well as of miR-15b, miR-23b, miR-106b, and miR-503 during myogenic progression. (C and D) miR-15b, miR-23b, miR-106b, and miR-503 overexpression in EPa cells leads to cyclin D1 and cyclin D2 gene downregulation.

results indicate that *Pitx2c* increases the number of Myf5$^+$ satellite cells.

Notably, bioinformatic analyses by TargetScan showed that *Myf5* is a predicted target for the *Pitx2c*-regulated miRNA miR-106b (http://www.targetscan.org/). To validate *Myf5* as a target for miR-106b, we performed pre-miR-106b transfection experiments in satellite cells, and as displayed in Fig. 13D, miR-106b overexpression leads to *Myf5* downregulation. Luciferase reporter assays further validated *Myf5* as a direct target for miR-106b (Fig. 13E). Additionally, pre-miR-106b transfection in *Pitx2c*-overexpressing cells rescued *Myf5* upregulation to basal levels (Fig. 14A to C), supporting the idea that miR-106b is key in mediating the *Pitx2c* effect on *Myf5* gene expression. Thus, since we describe above that miR-106b is a *Pitx2c*-regulated miRNA, we propose that *Pitx2c* enhances *Myf5* expression in satellite cells by regulating miR-106b.

DISCUSSION

Pitx2 is a homeobox transcription factor that has been shown to regulate skeletal-muscle development (15, 16). We previously documented that the c-isoform of *Pitx2* plays a pivotal role in modulating proliferation versus differentiation during myogenesis, balancing the Pax3$^+$/Pax7$^+$ myogenic population *in vivo*, and regulating key myogenic transcription factors such as Pax3 by repressing miR-27b (18, 19). In the present study, we investigated the role of *Pitx2* in controlling microRNA expression in myogenic cells by identifying a subset of *Pitx2c*-regulated miRNAs controlling cell proliferation in myoblasts and demonstrating that this *Pitx2c*-miRNA pathway controls cell proliferation as well as myogenic commitment of satellite cells.

Our analyses revealed that most miRNAs display lower levels after *Pitx2c* overexpression, as revealed by microarray analyses and further validated by qRT-PCR. Importantly, *Pitx2* is sufficient to induce impaired miRNA expression and is also indispensable for regulation of the expression levels of these microRNAs, as revealed by *Pitx2*-silencing experiments. Thus, these data demonstrate the pivotal role of the homeobox transcription factor Pitx2 in controlling microRNA expression in myoblasts. It was recently reported that Pitx2 positively regulates miR-17-92 and miR-106b-25 in the heart (52); however, we found *Pitx2*-mediated negative regulation of miRNA expression in myoblasts. These different functional requirements for Pitx2 underline the differences between cardiac and skeletal myogenesis. Therefore, this study represents the first available description of *Pitx2*-regulated

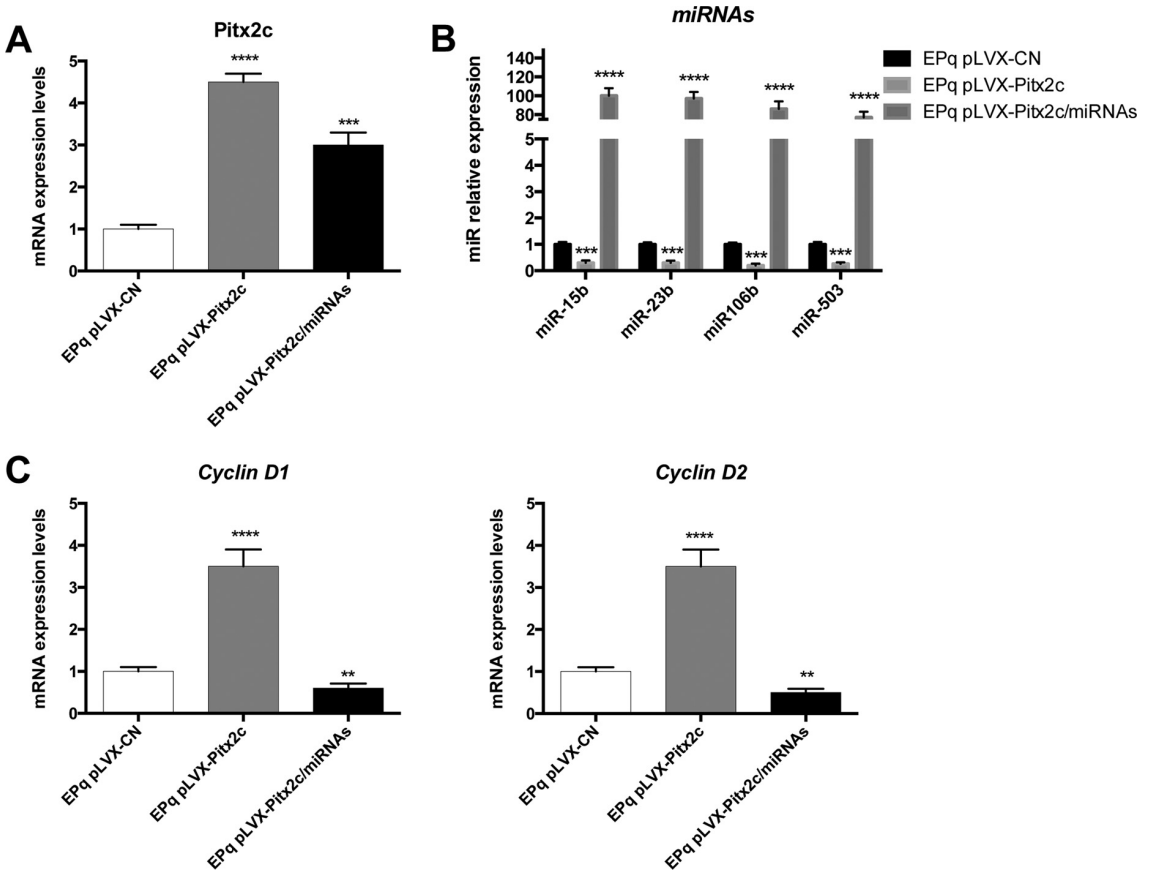


FIG 12 (A) *Pitx2c* overexpression is maintained after pre-miR-106 transfection in EPq cells. (B and C) Pre-miR-106b transfection in EPq cells overexpressing *Pitx2c* (B) rescues cyclin D1 and cyclin D2 gene expression at the basal levels (control cells) (C).

microRNA expression in myogenic cells, providing new insights into the microRNA-mediated mechanisms during myogenesis. Gene ontology analyses have revealed that miRNAs regulated both positively and negatively by *Pitx2c* might lead to modulation of the signaling pathways that control focal adhesion and adherens junction and actin cytoskeleton expression, providing the bases for abnormal cell fusion (18, 19), whereas the miRNAs downregulated by *Pitx2c* also might modulate cell cycle progression in accordance with the previously reported *Pitx2* functions in cell proliferation (18, 19, 45, 53–55). Here, we show that a subset of these *Pitx2c*-downregulated miRNAs, such as miR-15b, miR-106b, miR-23b, and miR-503, targeting cyclins together have dramatic effects on myoblast proliferation *in vitro*, providing a means for the previously reported *Pitx2c* functions in cell proliferation. Furthermore, we show evidence that this *Pitx2*-miRNA pathway that controls cell cycle genes in myogenic cells is also present *in vivo*. Although it has been demonstrated that miR-15b, miR-23b, miR-106b, and miR-503 can regulate the cell cycle (56–59) in different cell types, their functions regarding the regulation of myoblast cell proliferation have not been previously reported.

The role of Pitx2 in satellite cell proliferation and/or differentiation is recently emerging and is controversial. *Pitx2* expression is detected in proliferating satellite cells (20), but the constitutive expression of any *Pitx2* isoform suppresses satellite cell proliferation, with the cells undergoing greater myogenic differentiation (21). Nevertheless, the divergence of the *Pitx2c* effects on satellite cell proliferation found by Knopp et al. (21) could be explained by the moderate *Pitx2c* expression achieved in their *in vitro* gain-of-function experiments, as argued by those authors (21). Even more recently, it was reported that the knockdown of *Pitx2* in satellite cells isolated from extraocular muscles decreased their proliferation rate, and a similar trend was seen for satellite cells isolated from tibialis anterioris muscle (45). Here, we demonstrate that enhanced *Pitx2c* expression boosted cell proliferation in freshly isolated satellite cells, reinforcing the contention that Pitx2 positively regulates cell proliferation in satellite cells.

Notably, we found that the *Pitx2c* expression level was higher in early-activated satellite cells than in long-term-activated satellite cells, and our *in vitro Pitx2c* gain-of-function experiments revealed that *Pitx2c* stimulates cyclin D1 and cyclin D2 gene expression, accelerating cell proliferation during early satellite cell activation. Moreover, we have demonstrated that such *Pitx2c* effects on satellite cell proliferation are mediated by the *Pitx2c*-downregulated miRNAs miR-15b, miR-106b, miR-23b, and miR-503. Recent evidence suggests a role of miRNAs in the regulation of satellite cell fate and self-renewal (27, 29), and it has been reported that miR-106b inhibition augments the number of $Pax7^+$ cells (27). Our findings point out previously unknown functions of miR-15b, miR-106b, miR-23b, and miR-503 in satellite cell proliferation. Since one of the key prerequisites for the triggering

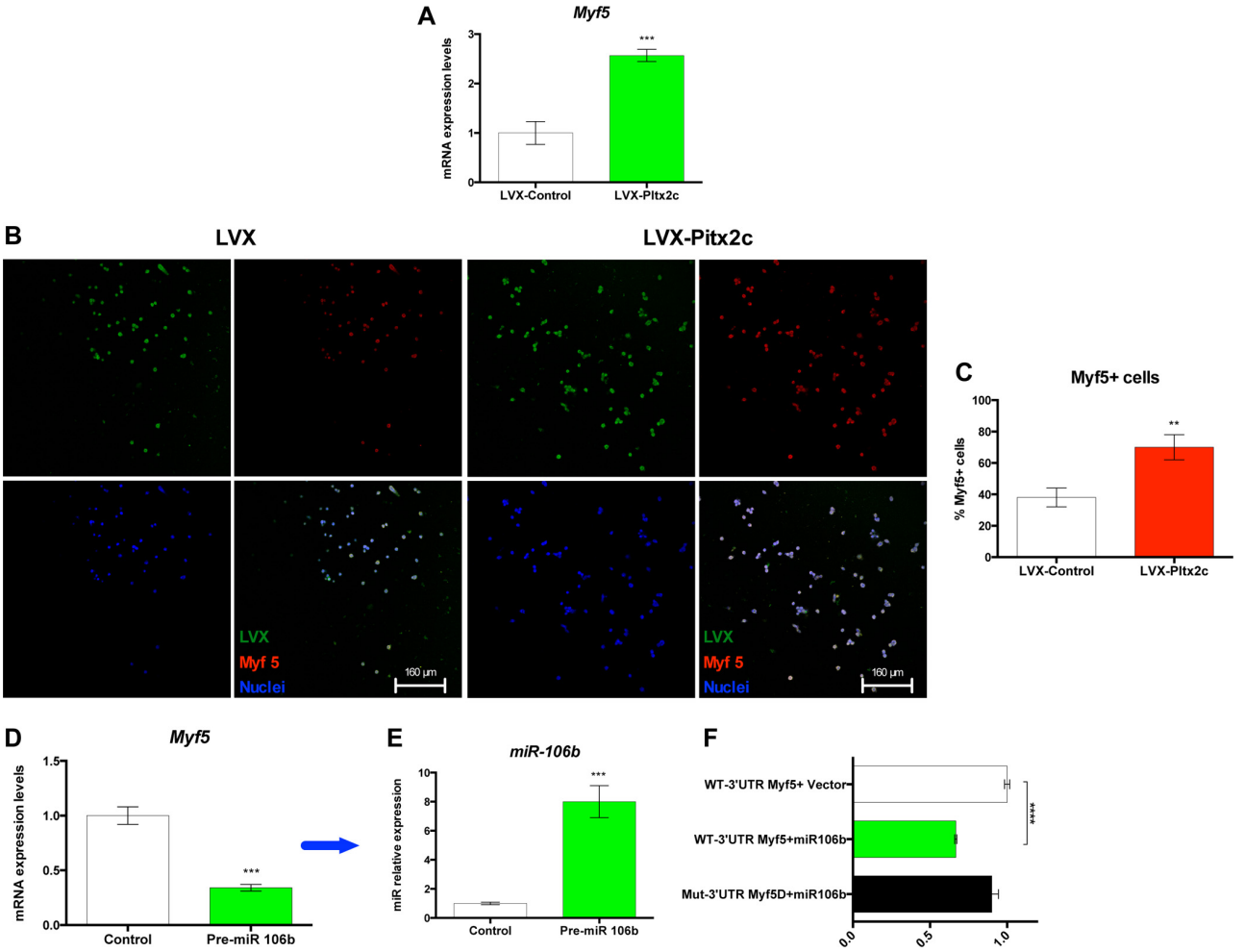


FIG 13 (A) *Myf5* expression profile in EPq *Pitx2c*-overexpressing cells. (B) Representative images of immunohistochemical analyses for Myf5-positive cells in EPq cells transfected with the lentivirus-*Pitx2c*-ZsGreen vector (LVX) compared to cells transfected with the empty LVX-ZsGreen lentiviral vector (LVX-Pitx2c). (C) Percentage of Myf5$^+$ cells in EPq cells transfected with the lentivirus-Pitx2c-ZsGreen vector (LVX-control) with respect to cells transfected with the empty LVX-ZsGreen lentiviral vector (LVX-Pitx2). (D and E) miR-106b overexpression leads to *Myf5* upregulation in EPq cells. (F) Normalized luciferase activity of the 3′-UTR *Myf5* luciferase reporter (wild-type *Myf5* 3′ UTR) with an empty plasmid (vector) or pre-miR-106b shows the loss of luciferase activity with expression of miR-106b. There was no loss of luciferase activity when the miR-106b seed sequence was mutated.

of cell proliferation at the onset of satellite cell activation is proper cell cycle progression (60), the existence of the *Pitx2*-miRNA pathway controlling the expression of key regulatory cell cycle genes in early-activated satellite cells reveals a role of *Pitx2* in satellite cell activation. Although muscle satellite cells are promising targets for cell therapies, the paucity of satellite cells that can be isolated or expanded from adult muscle tissue is limiting; thus, our findings provide new molecular tools to overcome such a bottleneck.

Proliferating satellite cells have a binary fate decision to make: they can differentiate into myoblasts and intercalate into myofibers by fusion to repair the damaged muscle, or they can renew the satellite cell population and return to a quiescent state (36). Quiescent satellite cells expressing paired box 7 (*Pax7*) but with low or undetectable levels of the myogenic regulatory factors Myf5 and MyoD undergo symmetric and asymmetric divisions upon activation. While symmetric expansion of Pax7$^+$/Myf5 satellite stem cells ensures the maintenance of the Pax7$^+$/Myf5$^-$ undifferentiated population, asymmetric divisions generate Pax7$^+$/Myf5$^+$ and Pax7$^+$/Myf5$^-$ daughter cells (36). *Myf5* induction demarcates the entry of satellite stem cells into the myogenic program. Our analyses showed that *Pitx2c* can increase *Myf5* expression by regulating miR-106b, thus enhancing the Myf5$^+$ satellite cell population and revealing a role for *Pitx2c* in promoting satellite cell populations that are more primed for myogenic commitment. The importance of miRNAs in the posttranscriptional regulation of *Myf5* in satellite cells is beginning to emerge. Recently, Crist et al. (61) reported that although many quiescent satellite cells transcribe *Myf5*, they do not enter myogenesis because of miR-31. Thus, miR-31 interacts with the 3′ UTR of *Myf5* mRNA and therefore can prevent its translation into a quiescent cell, but it is rapidly downregulated early during activation, leading to a rapid accumulation of Myf5 protein (61). Here, we demonstrate that downregulation of miR-106b leads to increased *Myf5* expression

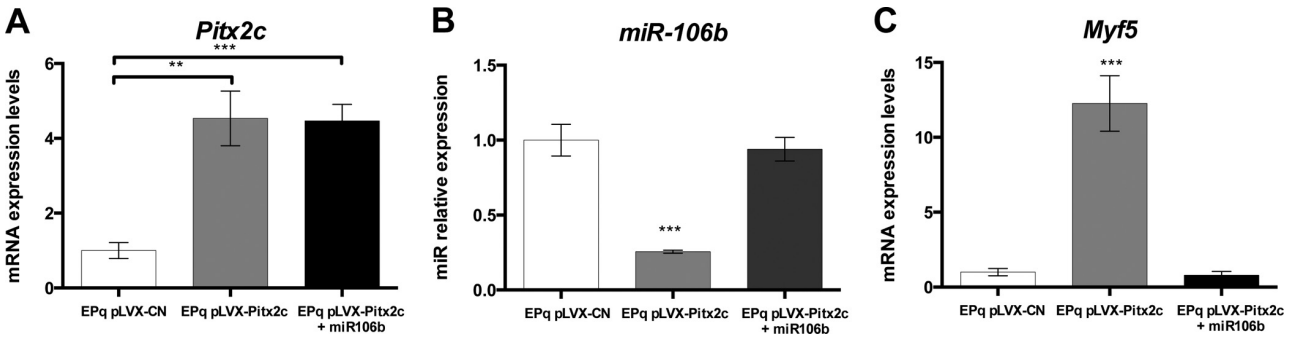


FIG 14 (A) *Pitx2c* overexpression is maintained after pre-miR-106 transfection in EPq cells. (B and C) Pre-miR-106b transfection in EPq cells overexpressing *Pitx2c* (B) rescues *Myf5* expression at basal levels (control cells) (C).

in early-activated satellite cells, thus providing additional information concerning the role of miRNAs in the posttranscriptional control of myogenic progression in adult myogenesis.

On other hand, a mechanism linking *Myf5* levels to muscle stem cell heterogeneity was also recently proposed. A detailed analysis of satellite cell behavior in *Myf5* haploinsufficient mice revealed the duality in the functional role of *Myf5*, as a promoter of muscle fate and also as being incompatible with terminal differentiation, raising questions about the precise role of this transcription factor during different cell states in myogenic lineage progression (50). In the present study, we describe a *Pitx2c*–miR-106b pathway controlling *Myf5* expression, providing new insights into the molecular mechanisms that control satellite cell behavior. These findings might thus have future applications in modulating satellite cell fate during muscle regeneration.

In conclusion, in this paper, we report a subset of microRNAs regulated by *Pitx2*, with previously unknown functions in myogenic cells, which have profound effects on myoblast proliferation. Notably, we found that this *Pitx2*-miRNA pathway regulating cell proliferation is conserved in freshly isolated satellite cells, providing developmental cues that enhance the commitment of satellite cells to myogenic lineage differentiation by downregulating miR-106b expression. Overall, the present study describes a previously unknown *Pitx2*-miRNA pathway controlling cell proliferation in myogenic cells, providing new targets to enhance the regenerative capacity of limb skeletal-muscle myogenic precursor cells for the treatment of skeletal-muscle diseases.

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Estefanía Lozano-Velasco collected and/or assembled data; Daniel Vallejo collected and/or assembled data; Francisco J. Esteban performed data analysis and interpretation; Chris Doherty collected and/or assembled data; Francisco Hernández-Torres collected and/or assembled data; Diego Franco performed data analysis and interpretation and manuscript writing; Amelia Eva Aránega conceived of and designed the study, performed data analysis and interpretation, provided financial support, and performed manuscript writing.

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Figure S1, Related to Figure 3: RNA polymerase II occupancy in tested DNA regions upstream of *miR-15b, miR-23b*, *miR-106b* and *miR-503*. Notably, all of these DNA regions have similar RNA polymerase II occupancy levels than Gapdh promoter used as control

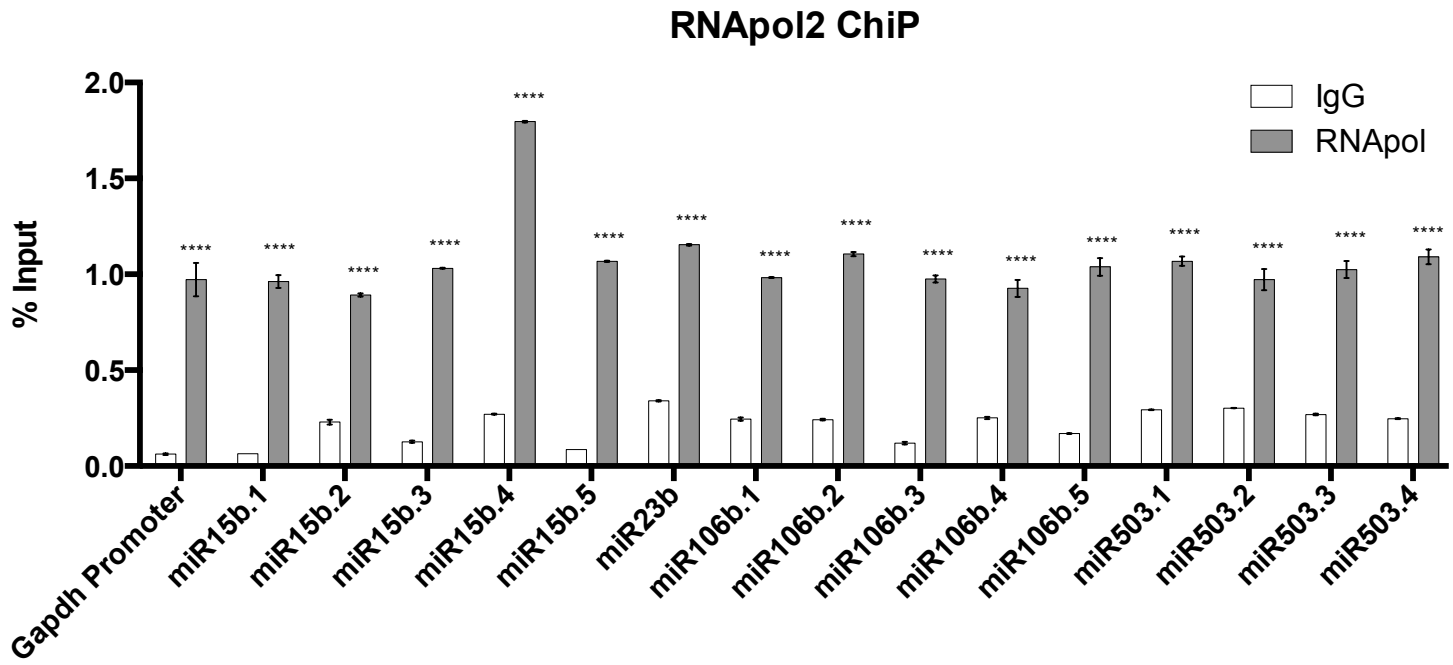


Figure S2, Related to Figure 1: A: qRT-PCR analyses showing Pitx2c overexpression after transfection with two different doses of *CMV-Pitx2c* plasmid (400 and 800 ng/ml). **B:** mRNA expression levels for Pitx2c and Pitx3 after siRNA against *Pitx2* in Sol8 myoblasts.

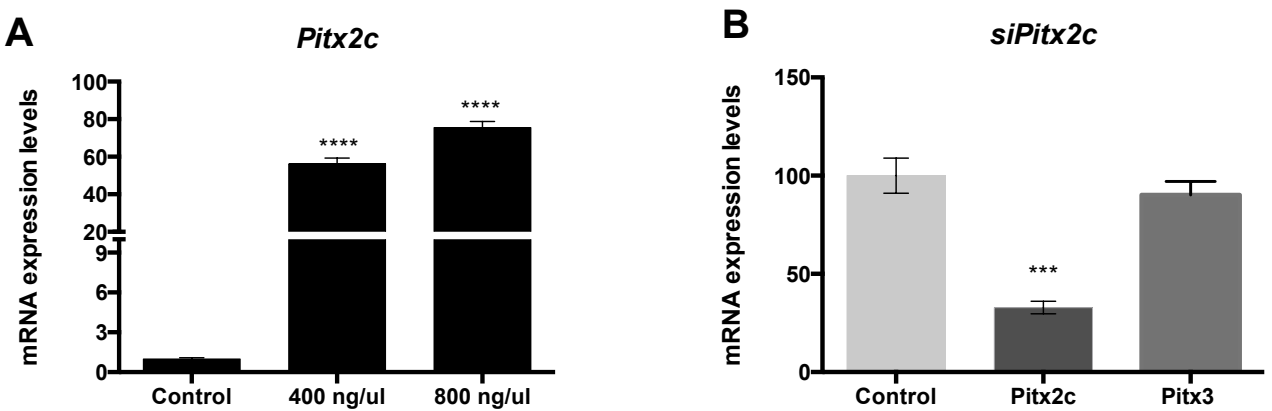


Figure S3-Related to Figure 2 : Schematic representation of the putative *microRNA* binding sites in *cyclin D1* (*ccnd1*) and *cyclin D2* (*ccnd2*) as revealed by TargetScan algorithm (www.targetscan.org)

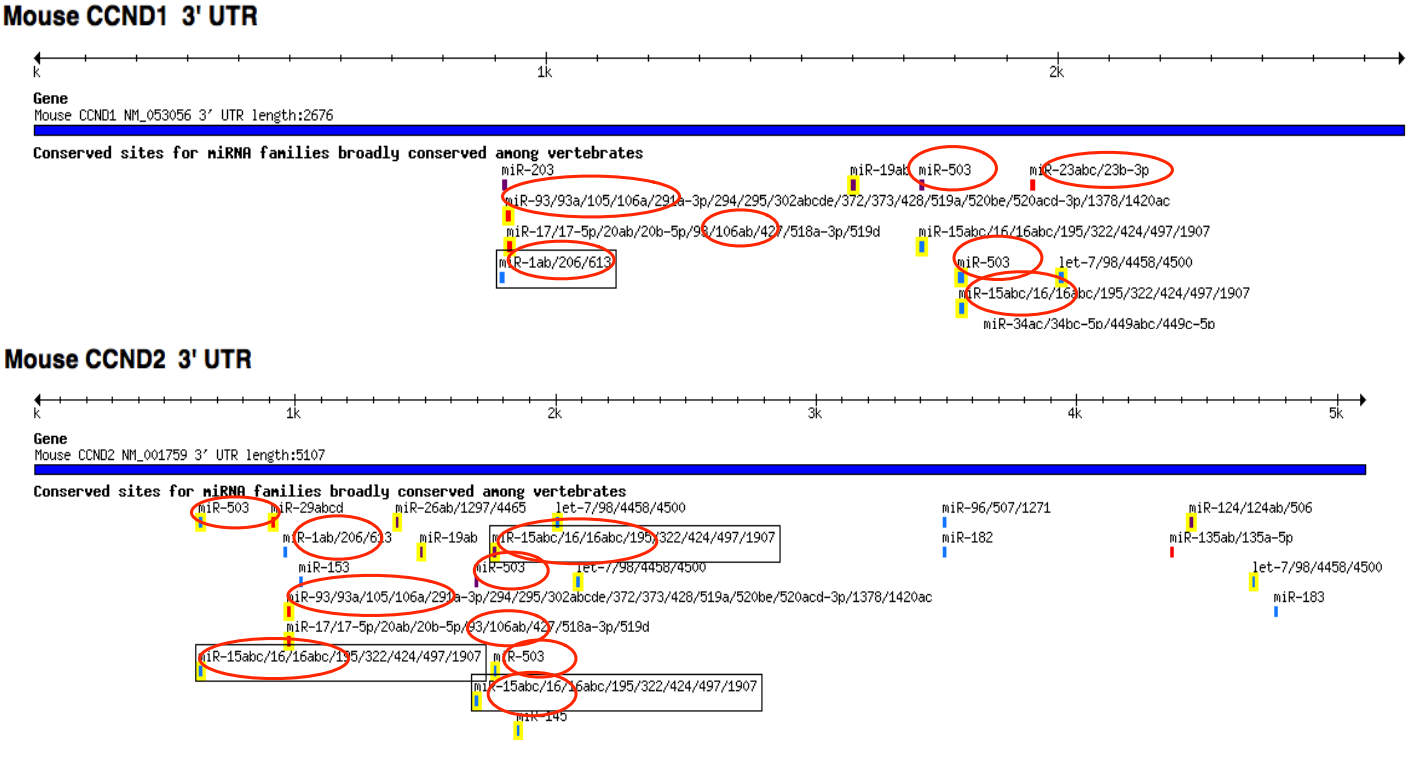


Figure S4, Related to Figure 2: A: qRT-PCR analyses showing *miRNA* overexpression after pre-miRNAs independent transfections. **B:** mRNA expression levels for *cyclin D1 and cyclin D2* were 50-60% lower when *miRNAs* were transfected separately in Sol8 cells.

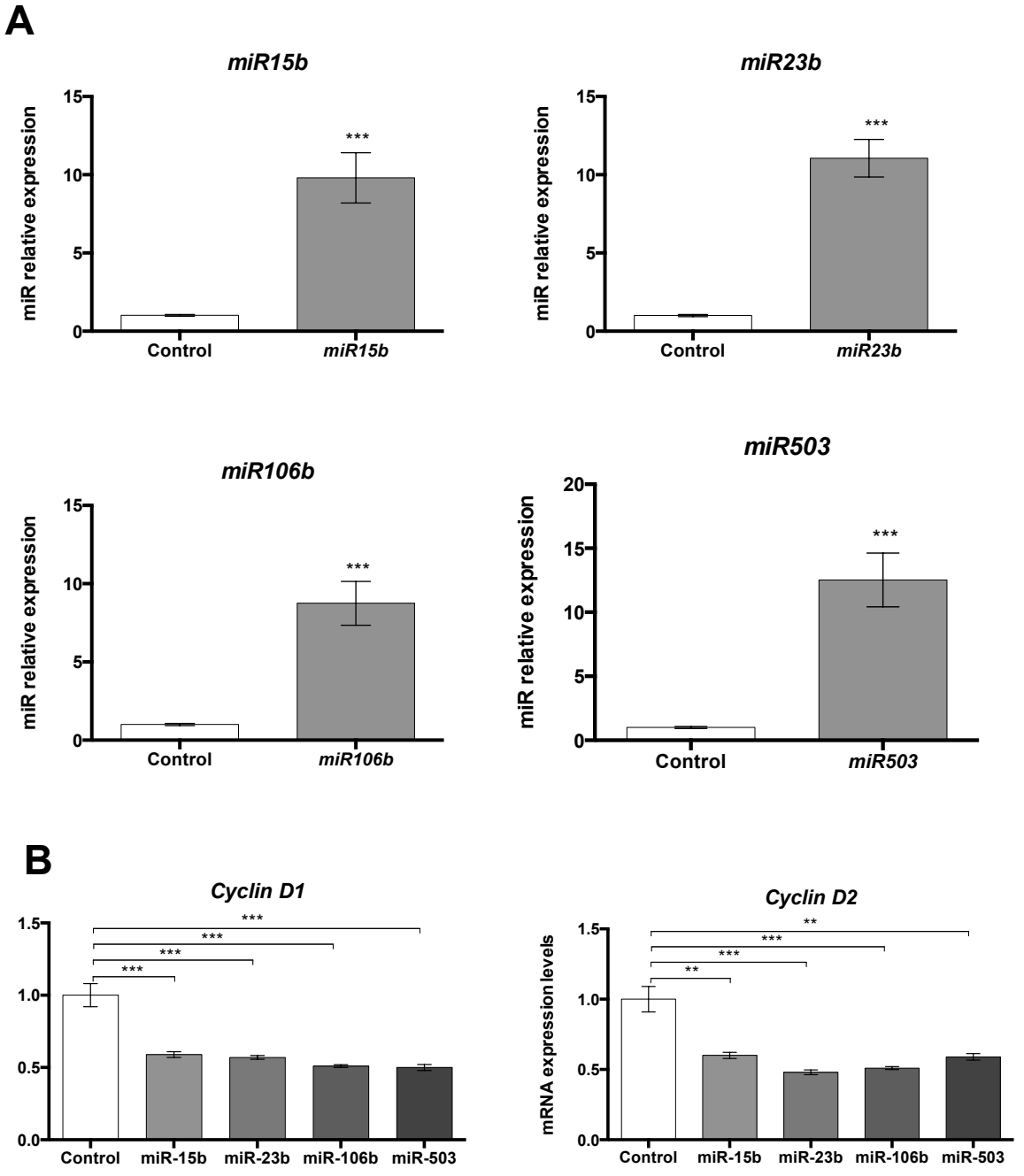


Figure S5, Related to Figure 4: **A**: qRT-PCR for *Pitx2c* expression in *Pax3cre*$^{+/-}$/*Pitx2*$^{+/-}$ heterozygote adults mice. **B**: qRT-PCR for *Pitx2c* expression in *Pax3cre*$^{+/-}$/*Pitx2*$^{+/-}$ heterozygote and *Pax3cre*$^{+/-}$/*Pitx2*$^{-/-}$ homozygote neonates.

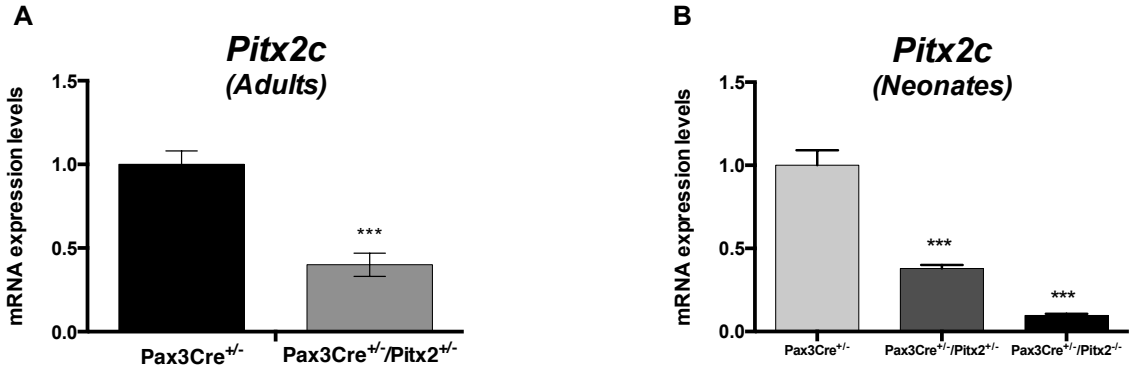


Figure S6, Related to Figure 5: **A:** Representative images of early-plated relatively quiescent/early-activated satellite cells (EPq), early-plated long-term-activated satellite cells (EPa) and EPa-derived differentiating fusing-myoblasts cultures respectively**. B:** mRNA expression levels for *Cyclin D2*, *Myf5* and *Pax7* in EPq and EPa cells. **C:** mRNA expression levels for *Pitx2c* in EPq, EPa cells and myoblasts.

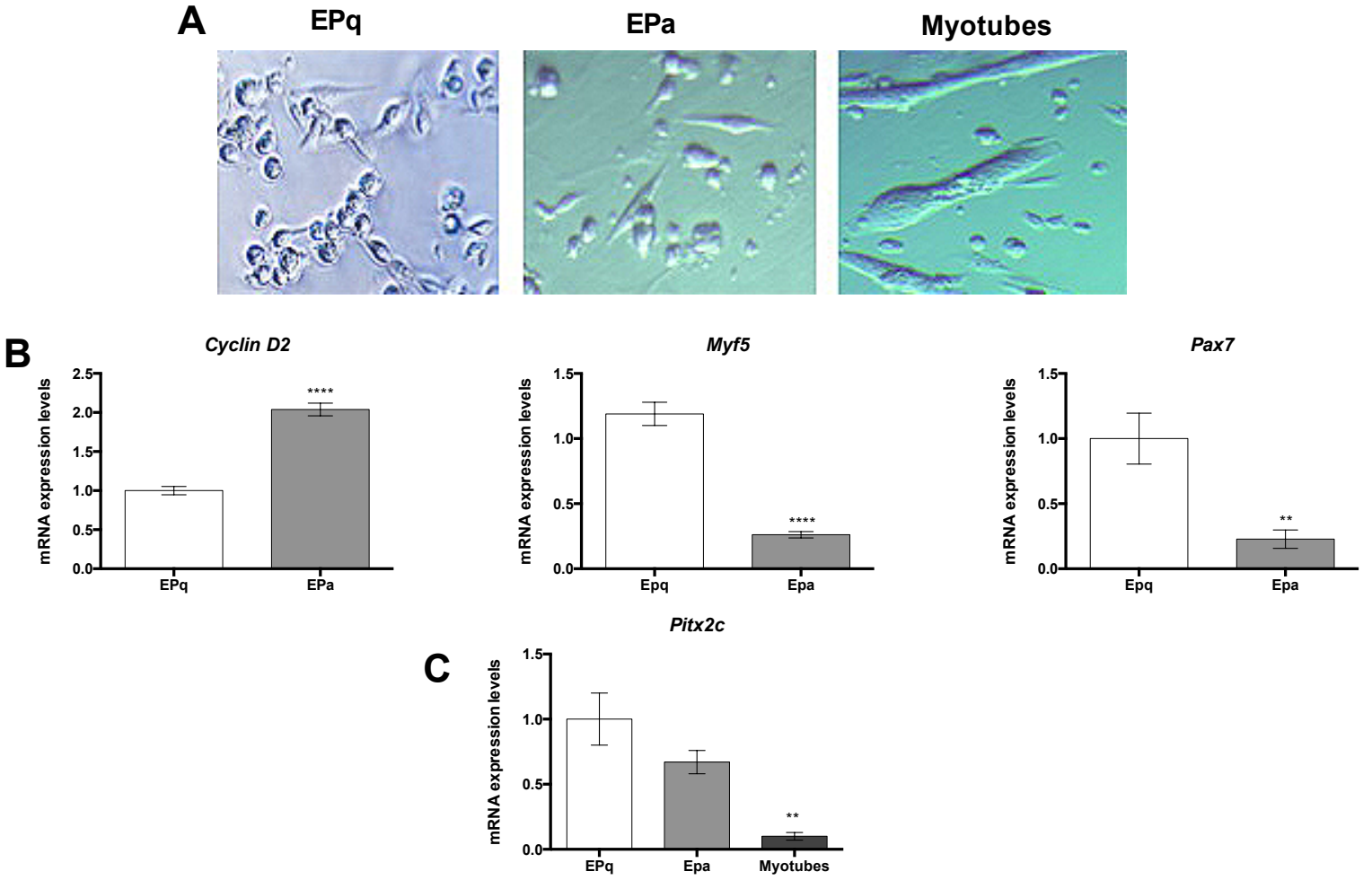
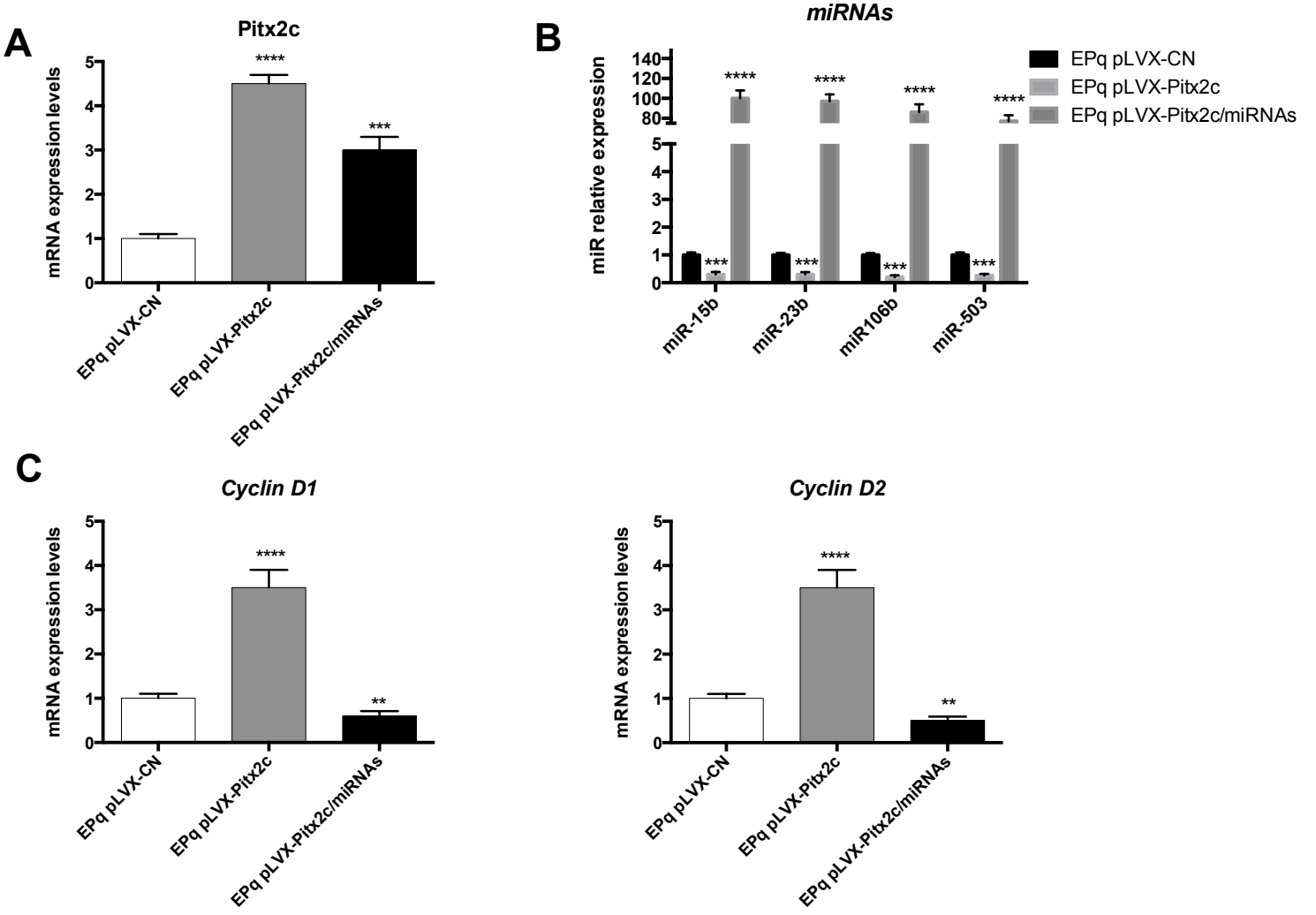


Figure S7-Related to Figure 6: A: Pitx2c overexpression is maintained after Pre-miR-106 transfection in EPq cells. *Pre-miR-106b* transfection in EPq cells overexpressing *Pitx2c* **(B)** rescue *CyclinD1* and *CyclinD2* expression at the basal levels (control cells) **(C)**.



CHAPTER III: RESULTS (II)

Stem Cell Reports

Pitx2 enhances the regenerative potential of dystrophic skeletal muscle stem cells

--Manuscript Draft--

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Abstract:	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 found 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 restoring dystrophin expression by regulating miR-31. These Pitx2-mediated effects finally lead to improve 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.

Professor Christine Mummery
Editor-in-Chief, Stem Cell Reports

January 9th, 2018

Dear Dr. Mummery

Dear Christine Mummery

Please find enclosed the revised version of our manuscript STEM-CELL-REPORTS-D-17-00256 (Pitx2 enhances the regenerative potential of dystrophic skeletal muscle stem cell). We greatly appreciate the opportunity to submit this new version of the manuscript. Based on the reviewers' comments, we have now completely revised the manuscript including new data that address the reviewers' concerns. We are really grateful for the expert comments and excellent advice we have received.

No part of this paper has been published previously either in whole or in part, or is under consideration for publication elsewhere, and all authors have read and approved this version of the manuscript and are prepared to take public responsibility for its contents.

With our appreciation in advance, we look forward to your decision on this paper in due time. Please do not hesitate to contact me meanwhile if there are any questions regarding our manuscript.

Sincerely yours,
Amelia E. Aránega Jiménez

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Pitx2 enhances the regenerative potential of dystrophic skeletal muscle stem cells

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Running Title: *Pitx2* and dystrophic skeletal muscle stem cells

Key words: Pitx2, muscular dystrophy, *miR-31*

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 found 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 restoring dystrophin expression by regulating *miR-31*. These Pitx2-mediated effects finally lead to improve 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.

Highlights

-Impaired myogenic potential of dystrophic satellite cells is recued by *Pitx2c*

- *Pitx2c* promotes muscle regeneration in dystrophic DMD/mdx mice.

-*Pitx2c* restores dystrophin expression in DMD/mdx mice by down-regulating *miR-31*

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 up-regulation 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 fibres derived from ES 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 myofibre 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 foetal myogenesis (L'Honoré et al., 2007; Zacharias et al., 2010; L´Honoré et al., 2010; L'Honoré, 2014). Previous works from our laboratory showed that Pitx2c is the main Pitx2-isoform expressed in myoblasts playing a pivotal role modulating proliferation *vs* 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-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 behaviour 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 myofibres, represses *miR-31* reaching dystrophin restoration, 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, Knoop 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 analysing MyoD and Ki67 expression in isolated and culture satellite cells before they reach confluence. We observed that the number of Ki67+ and MyoD+ positive nuclei was higher in satellite cells overexpressing *Pitx2c* as compared to 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 increase 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 **(Figure S1F-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-F)**. 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 analysed the expression profile of *Pitx2c* expression after induction of skeletal muscle damage by cardiotoxin (CTX) injection in mice. As illustrated in **Figure 1A**, we found that *Pitx2c* expression increased 5 fold at day 1 after muscle damage induction**.** However, qRT-PCR analyses revealed that *Pitx2c* expression 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 TA muscles; Pitx2c staining was also detectable in some myonuclei as previously reported (Herbet et al, 2103) **(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 **(Figure 1E-G).**

In addition to *Pitx2c*-decreased expression **(Figure 2A)**, and in agreement with previous recent reports (Chat et al., 2015; Dumont et al., 2015), we also observed that dystrophic satellite cells exhibited a clear defect to form myotubes **(Figures 2B and C)** together with a lower *in vitro* differentiation potential as observed by a decreased proportion of MHC-positive cells and fusion index **(Figures 2D and E)**. Interestingly, *in vitro* experiments of Pitx2c gain of function on dystrophic deficient satellite-cells **(Figure 3A)** lead 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-positive cells and fusion index **(Figures 3B-E).** 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.

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 tibialis anterior muscle of DMD/mdx mice were transfected with the bicistronic lentiviral construction pLVX-*Pitx2c*-IRES-ZsGreen**.** Transfected satellite cells were then 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 ZS Green to localize *Pitx2c*-overexpressing cells after transplantation. After 15 days of cell transplantation, quantification of ZsGreen+ myofibres indicate that the number of ZS-Green+ cells forming myofibres 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-D)**. Moreover, *Pitx2c* overexpression induced a shift in the distribution of the regenerating fibre 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 down-regulating *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 down regulated 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 up-regulated 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 analysed 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 Caccharelli et al. (2011) have reported the identification of a microRNA—*miR-31*—that represses dystrophin expression by targeting its 3' untranslated region (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 down-regulated by *Pitx2c* in a previously reported microarray analyses in Sol8 myoblasts (Lozano-Velasco et al., 2015). We have further validated *Pitx2c*-mediated *miR-31* down-regulation 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* down-regulation **(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 **Figure 5B-C**.

According to those results, we found that *miR-31* expression significantly declined in muscles injected with *Pitx2*c-overexpressing cells compared with controls **(Figure 6A)**. As expected, a sharp rise in dystrophin-mRNA expression levels was also detected **(Figures 6B**). To analyse further how *miR-31* regulates dystrophin expression in our system, we first performed *in vitro* experiments blocking transcription by using alpha-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 fibres that express dystrophin [*31,32*]. The revertant fibres express dystrophins that arise for alternatively splicing transcripts lacking the mutant exon by exon skipping [*33*]. Thus, we tested by qRT-PCR the relative levels of dystrophin *mRNA* lacking the mutant exon by analysing 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 *Pitx2*c-overexpressing cells (**Figures 6D-E**). These findings indicate that, in our system *miR-31* downregulation mediated by Pitx2c lead 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 fibres (Thanh et al., 1995). As showed in **Figures 6F and G**, 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 fibres was significantly higher in the muscles injected with Pitx2c-overexpressing cells **(Figures 6H and I)**. Moreover, *Pitx2c*-overexpression in differentiated myotubes derived from DMD/mdx-satellite cells leads to increase dystrophin mRNA expression **(Figure 6J)** indicating that dystrophin restoration in the recipient muscle is a result of increase in expression of the gene 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% to 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 down-regulated 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 *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 but blocks terminal differentiation. 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, Knoop et al. (2013) showed that *Pitx2c* enhances *in vitro* myogenic differentiation but reduces proliferation in satellite-cell-derived myoblasts In this study, we 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 behaviour may be altered, depending of the method used for cell isolation (Montarras et al., 2005; Qu-Petersen et al., 2002). Therefore, taking all these results into consideration, we propose that Pitx2c effects on cell proliferation has different impact 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* up-regulation during satellite-cell activation *in vitro* (Lozano-Velasco et al., 2015 . This *Pitx2c* up-regulation was accompanied with 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*. 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-functions regulating the redox state during fetal myogenesis has been previously described (L'Honoré et al., 2014), *Pitx2c* overexpression on dystrophic satellite cells has not 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 *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 fibres. These revertant fibres 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* down-regulation when dystrophic satellite cells overexpressing *Pitx2c* were transplanted into the muscles of DMD/mdx mice and, consequently, the levels of dystrophin were significantly restored. These findings indicate that *Pitx2*-mediated *miR-31* down-regulation leads to dystrophin restoration in cell-transplanted muscle. Our analyses indicate that *miR-31* acts by promoting dystrophin *mRNA* degradation, and that *miR-31* down-regulation mediated by Pitx2 augments the total amount of dystrophin *mRNA*, overall the revertant dystrophin *mRNA* and thus revertant dystrophin protein. Dummont et al. (2015) have previously reported that dystrophin-deficient satellite cells exhibit a clear defect in asymmetric cell division (Dumont et al., 2015), we therefore can not rule out that increased cell division observed in transplanted dystrophin-deficient *Pitx2c*-overexpressing cells can be linked to dystrophin restoration. In addition, since it have 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* down-regulation. 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* repress *miR-31*, leading to dystrophin restoration 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 months old mice (C57BL/10ScSn and C57/Bl10ScSn-Dmdmdx/J(DMD/mdx) as well as from human Becker's muscle dystrophy patient (male, 35 year old) were performed by magnetic separations according to the manufacturer's protocol. Further details are provided in Supplemental Material. After separation, freshly isolated satellite cells were cultured as described (Garriga et al., 2000).

Pitx2c overexpression and siRNA-Pitx2c

Pitx2c overexpression and *siRNA-Pitx2c* in satellite cells was performed as described [18]. Briefly, 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 manufacturer's procedure (Clontech). Detailed information is provided in the Supplementary Material and Methods.

Quantitative Reverse Transcriptase-PCR Analyses (qRT-PCR)

RNA isolation and (RT)-PCR were performed as described elsewhere (Lozano-Velasco et al.,2011), using standard procedures. Detailed information is provided in the Supplementary Material and Methods.

Cardiotoxin (CTX) and muscle injury

Cardiotoxin was prepared by dissolving a freshly opened tube in PBS at 10 μ M as described (Lepper et al.,2009). The tibialis anterioris muscles (TA) of 4 months old C57BL/10ScSn (n=4) mice were injected with 50ul 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 -80ºC.

Cell transplantation experiments

4 months old DMD/mdx mice (n=12) pretreated with the immunosuppressant FK506 (Kinoshita et al., 1994) were intramuscularly injected into the TA muscle with cardiotoxin (Lepper et al., 2009) into the TA muscle to increase damage. Freshly isolated satellite cells were isolated from 4 months old donors DMD/mdx and infected with lentiviral vectors as previously described (Benchaoir et al, Cell Stem Cell, 2007). For the histological, immunohistochemistry and mRNA analysis, donors DMD/mdx cells infected with pLVX empty lentival vector were injected (5 x 10^4 in 50 ul F12 medium) into the right TA muscle and used as controls (n=6), whereas donors DMD/mdx infected with pLVX-*Pitx2* lentivral vector where injected (5 x 10^4 in 50 ul 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 -80ºC.

For the Treadmill test, both TA muscles of 4 months old recipients DMD/mdx control mice (n=8) were injected intramuscularly with 5 × 10^4 cells infected with pLVX empty lentivector 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 exercise-tolerance test as described below.

microRNA and anti-microRNA transfection assays

Satellite cells were cultured under growing conditions. Corresponding pre-miRNAS (Ambion) were transfected as described elsewhere (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 Supplementary Material and Methods.

Cross-section area

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

Western blot

Western-blot analyses were performed as previously described (Camici et al, 2007). Detailed information is provided in the Supplementary Material and Methods.

Chromatin immunoprecipitation ChIP assay

ChIP assays were performed as described 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 *mir31*. As controls, normal rabbit IgG replaced the anti-V5 antibody to reveal nonspecific immunoprecipitation of chromatin.

Alpha amanitin

RNA pol-II satellite cells were inhibited by adding α-amanitin (#A2263, Sigma-Aldrich) to the cell-culture medium to a final concentration of 4 μg/ml, as described elsewhere (Daimi et al., 2015).

Exercise-tolerance test

An exhaustion treadmill was performed to evaluate the endurance of the mice by using the motorized treadmill LE8708 single mouse, Treadmill, PanLab, Harvard Apparatus supplied with shocker plate as described elsewhere (Benchaouir et al., 2007).

Conflict of interest: none declared

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Author contribution statement

DV: conceived and carried out experiments; FHT and ELV: contributed to cell transplantation and qRT-PCR experiments.; AC and CC: performed collection of biopsies from dystrophic patients; DF: contributed to the project design and critical reading of the manuscript; AEA: carried out experiments, provided over all project supervision, and produced the manuscript and figures.

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FIGURE LEGENDS

Figure 1: Pitx2c during muscle regeneration and DMD: A) Pitx2c expression peak at day 1 after cardiotoxin injection in C57/BL3 mice. B) Pitx2c 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 anterioris muscles isolated from 4 moths old C57/BL3 mice. Yellow arrows:

Pax7+/Pitx2c+ cells; green arrows: Pax7+ cells; red arrows: Pitx2c+ myonuclei D) Representative images of immunohistochemistry for Pitx2c and CD34 in uninjured tibialis anterioris muscles isolated from 4 moths old C57/BL3 mice. Yellow arrows: CD34+/Pitx2c+ cells; red arrows: CD34+ cells; green arrow: Pitx2c+ myonuclei E) Representative images of immunohistochemistry for Pitx2c and Pax7 in injured tibialis anterioris muscles isolated from 4 moths old C57/BL3 mice (3 days after injury). Yellow arrows: Pax7+/Pitx2c+ cells. F) Representative images of immunohistochemistry for Pitx2c and Pax7 in uninjured tibialis anterioris muscles isolated from 4 moths old DMD/mdx mice. Yellow arrows: Pax7+/Pitx2c+ cells; green arrows: Pax7+ cells; red arrows: Pitx2c+ myonuclei G) Percentage of $Pitx2c^{+}$ cells 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. All the experiments were repeated at least in three different biological samples to have a representative average. **p<0.001, ***p<0.0001, ****p<0.00001.

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 vs. 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 vs. 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 vs. 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 *vs.* satellite cells isolated from Dmd/mdx mice (day 14 of culture). E) MHC-positive cells and fusion index: Five random fields of view from each group were scored for the presence of MHC-positive cells. Values obtained in the control group (LVX) were set at 100%. The fusion index was calculated as follows: (MF20-stained myocytes containing ≥2 nuclei/total number of nuclei) × 100. Values and error bars are means of standard deviation of at least three independent experiments. ***p<0.0001; ****p<0.00001.

Figure 3: Pitx2c overexpression in dystrophic deficient satellite cells rescues their myogenic differentiation potential: A) Pitx2c expression satellite cells isolated from 4-month-old DMD/mdx mice transfected with LVX-Pitx2c vs. LVX particles. B) Ratio of number of myotubes through in vitro differentiation in Pitx2c-overexpressing cells vs. control. C) Representative images of formed myotubes of satellite cells isolated from C57/BL6 mice and dystrophic satellite cells overexpressing Pitx2c (LVX-Pitx2c) vs. 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) vs. control cells (LVX) (day 14 of culture). All the experiments were repeated at least in three different biological samples to have a representative average. *p<0.01, **p<0.001, ***p<0.0001, ****p<0.00001.

Figure 4: Cell transplantation of dystrophic satellite cells overexpressing Pitx2c into DMD/mdx recipient enhances the number and size of the newly

formed myofibres: 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) vs. 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) vs. control Lvx-ZsGreen muscles (n=6). D) Representative images for ZsGreen+ myofibers in Lvx-Pitx2c-ZsGreen muscles vs. Lvx-ZsGreen muscles an negative control (uninjected muscle). E) Cross-sectional area in tibialis anterioris (TA) muscles of Lvx-Pitx2c-ZsGreen muscles (n=6) vs. control Lvx-ZsGreen muscles (n=6). *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) vs. control cells (Lvx-ZsGreen). Data are presented as means +/- s.d. for at least three independent experiments **B)** Pitx2 binds DNA regions upstream of *miR-31* genetic locus. **B**) RNA polymerase II occupancy in tested DNA regions upstream of *miR-31*. **C)** For ChiP analysys experiments were repeated at least two times with similar results. A representative example of a ChIP analysis is shown. Error bars display the SD of the mean.**p<0.001,***p<0.0001 ,****p<0.00001.

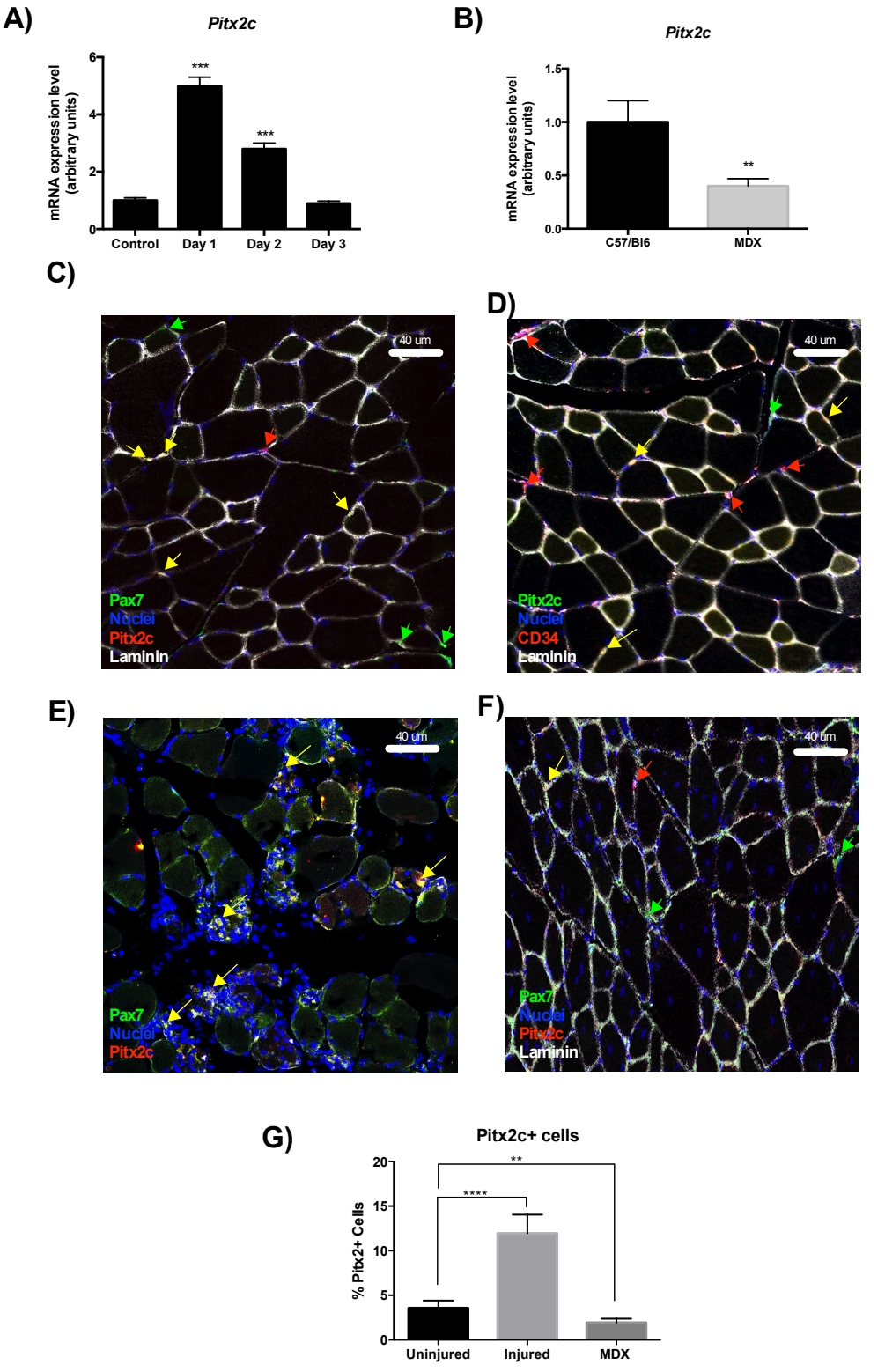
Figure 6: Pitx2c-overexpression down-regulates miR-31, leading to dystrophin restoration in transplanted muscles: A) miR-31 expression in muscle transplanted with Pitx2c-overexpressing cells (Lvx-Pitx2c-ZsGreen) vs. control muscles (Lvx-ZsGreen). B) mRNA levels for dystrophin in muscles

transplanted with Pitx2c-overexpressing cells (Lvx-Pitx2c-ZsGreen) vs. control muscles (Lvx-ZsGreen). C) pre-miR31 transfection clearly decreases *mRNA* levels for dystrophin after alpha-amanitin treatment and, blocking endogenous miR-31 by a*nti-miR-31* transfection, mRNA levels for dystrophin are increased. D) RT-PCR analysis for exon 18–35 spliced dystrophin mRNAs. E) RT-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) vs. control muscles (Lvx-ZsGreen). G) Quantification of western-blot analysis. H) Representative images for Dystrophin+ myofibres in muscles transplanted with Pitx2c-overexpressing cells (Lvx-Pitx2c-ZsGreen) vs. control muscles (Lvx-ZsGreen). I) Quantification of Dystrophin+ myofibres in muscles transplanted with Pitx2c-overexpressing cells (Lvx-Pitx2c-ZsGreen) vs. control muscles (Lvx-ZsGreen). J) qRT-PCR analyses for dystrophin expression in differentiating satellite cells overexpressing *Pitx2c* (LVX-*Pitx2c*) *vs.* 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 and DMD/mdx mice transplanted with control dystrophic satellite cells (n=8). Values and error bars are means of standard deviation of at least three independent experiments. *p<0.01,**p<0.001, ,***p<0.0001 ,****p<0.00001.

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 increase the amount of dystrophin mRNA trancripts lacking the mutant exon and finally to dystrophin restoration. Together, Pitx2c effects lead to improve muscle regeneration.

Figure 1


A)
Pitx2c
mRNA expression level (arbitrary units)
Control
Day 1
Day 2
Day 3
B)
Pitx2c
mRNA expression level (arbitrary units)
C57/Bl6
MDX
C)
40 um
Pax7
Nuclei
Pitx2c
Laminin
D)
40 um
Pitx2c
Nuclei
CD34
Laminin
E)
40 um
Pax7
Nuclei
Pitx2c
F)
40 um
Pax7
Nuclei
Pitx2c
Laminin
G)
Pitx2c+ cells
% Pitx2+ Cells
Uninjured
Injured
MDX

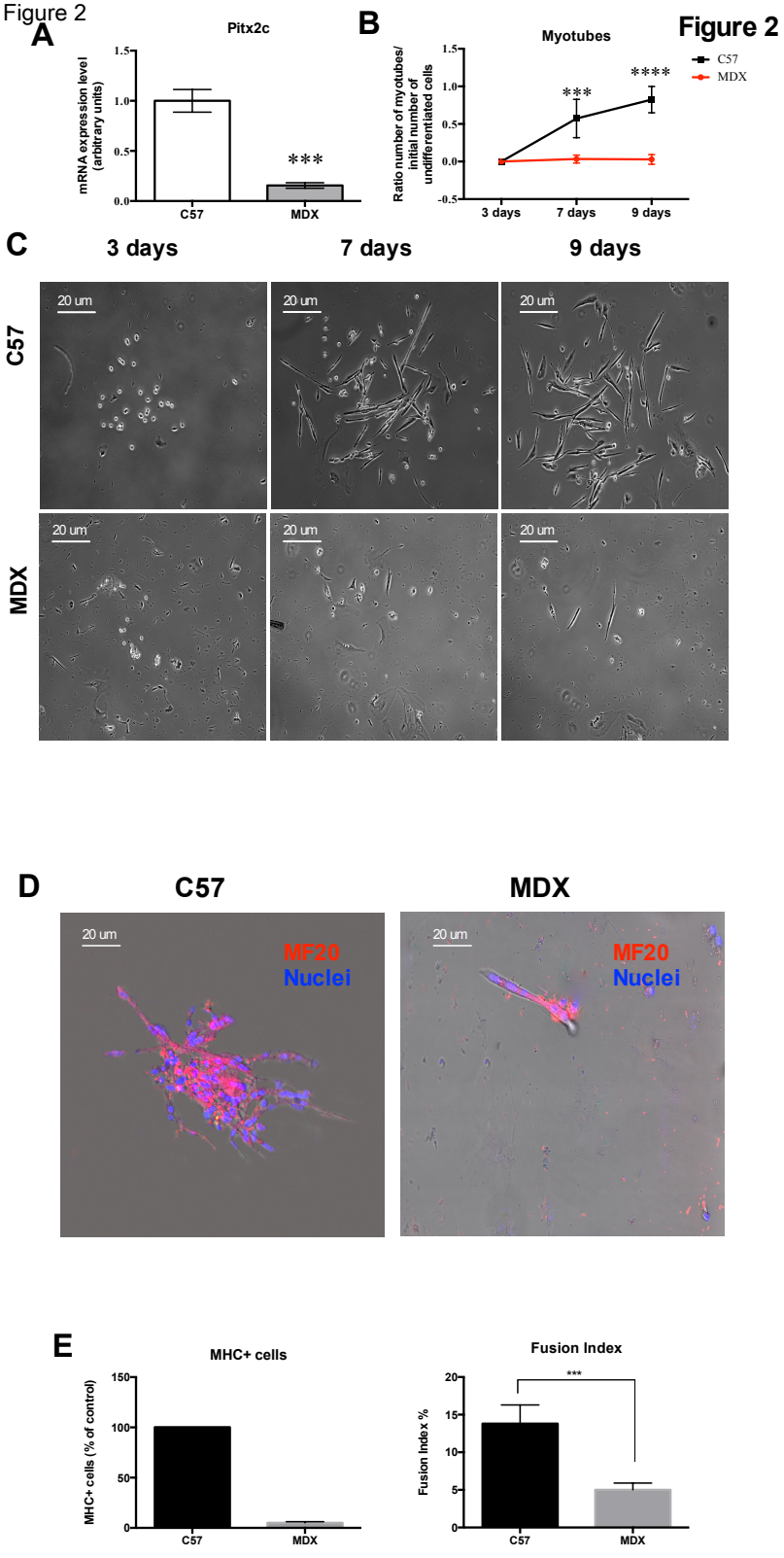

A
Pitx2c
mRNA expression level (arbitrary units)
1.5
1.0
0.5
0.0
***
C57
MDX
B
Myotubes
Ratio number of myotubes/ initial number of undifferentiated cells
1.5
1.0
0.5
0.0
-0.5
***
****
3 days
7 days
9 days
C57
MDX
Figure 2
C
3 days
7 days
9 days
C57
MDX
20 um
D
C57
MDX
20 um
MF20
Nuclei
E
MHC+ cells
MHC+ cells (% of control)
150
100
50
0
C57
MDX
Fusion Index
Fusion Index %
20
15
10
5
0
***
C57
MDX

Figure 3

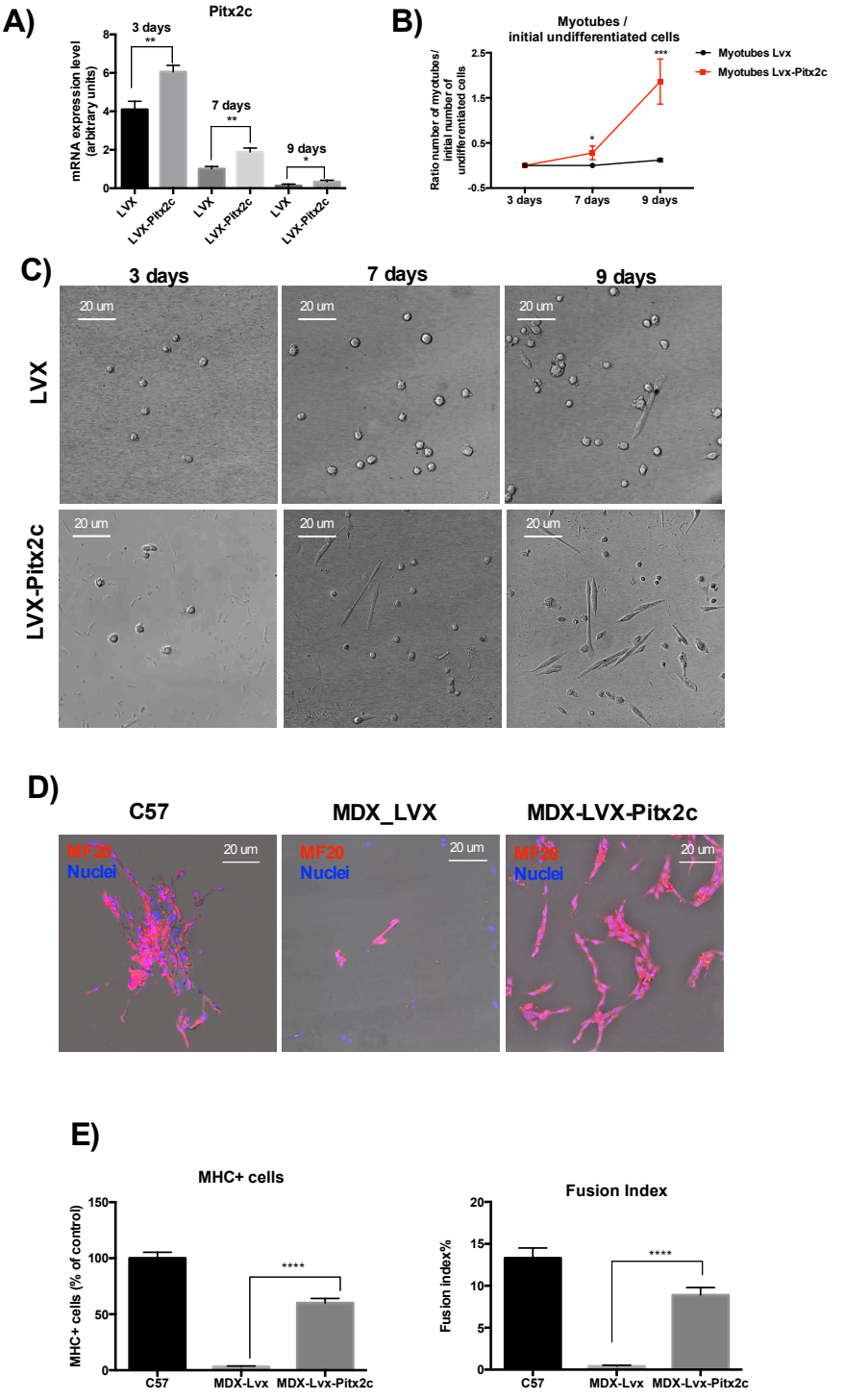

A)
Pitx2c
mRNA expression level (arbitrary units)
3 days
7 days
9 days
LVX
LVX-Pitx2c
B)
Myotubes / initial undifferentiated cells
Ratio number of myotubes/ initial number of undifferentiated cells
Myotubes Lvx
Myotubes Lvx-Pitx2c
3 days
7 days
9 days
C)
3 days
7 days
9 days
LVX
LVX-Pitx2c
20 um
D)
C57
MDX_LVX
MDX-LVX-Pitx2c
MF20
Nuclei
20 um
E)
MHC+ cells
MHC+ cells (% of control)
Fusion Index
Fusion index%
C57
MDX-Lvx
MDX-Lvx-Pitx2c

Figure 4

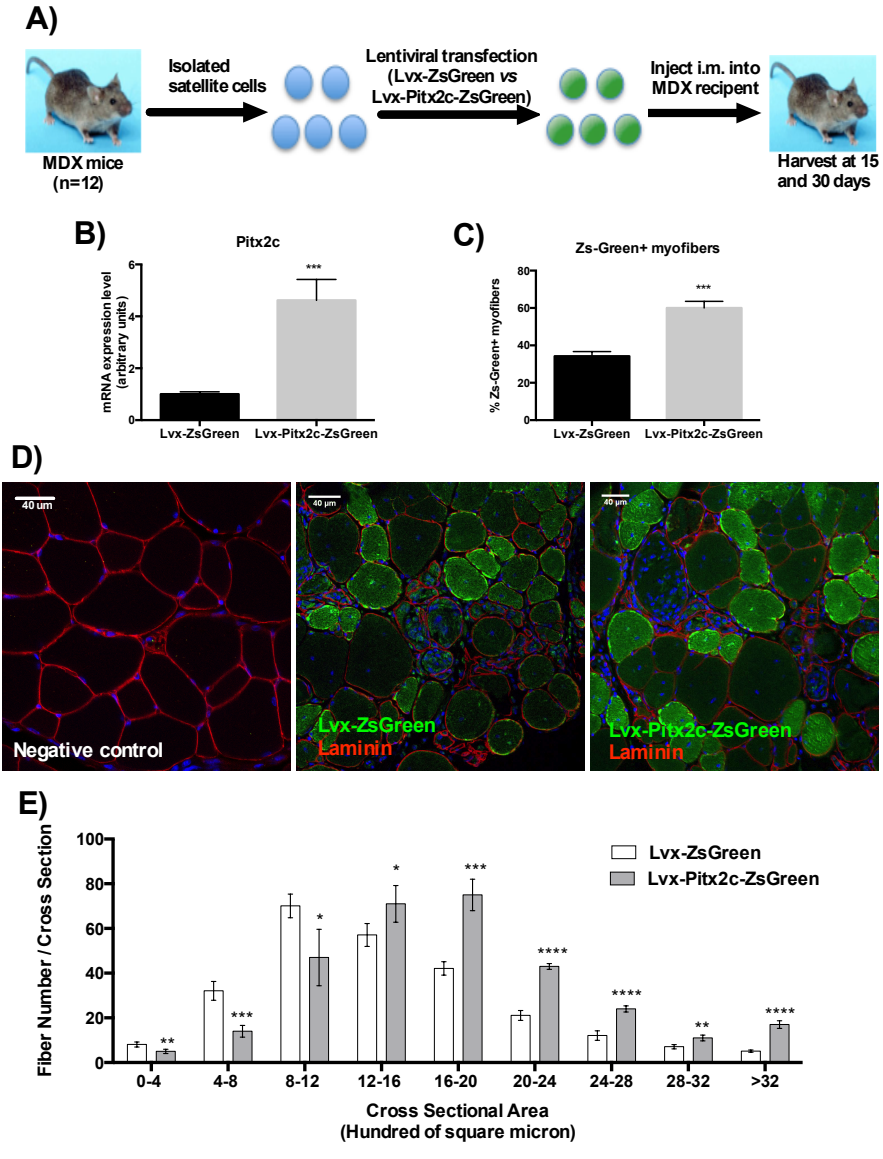
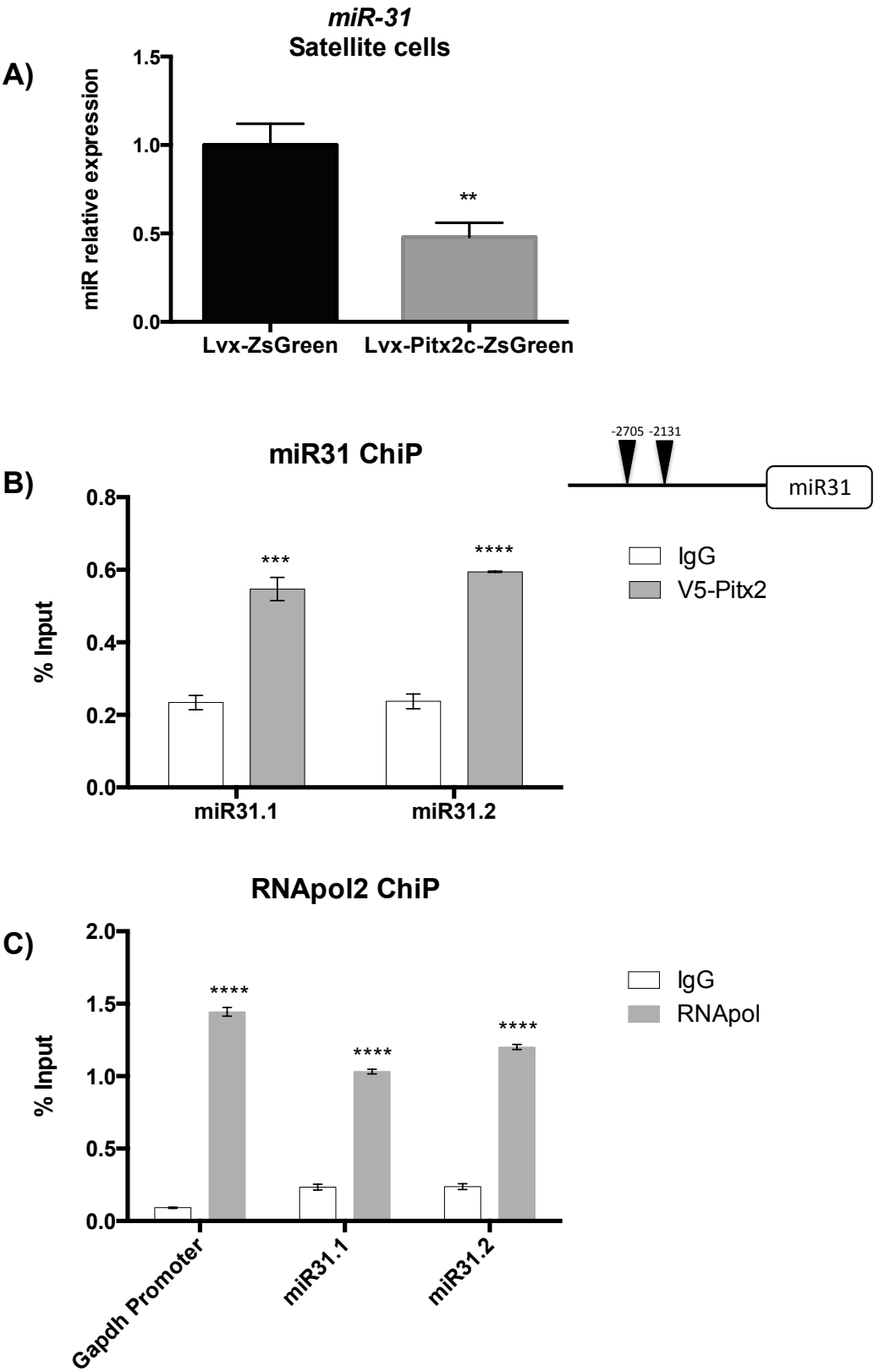

A)
MDX mice
(n=12)
Isolated
satellite cells
Lentiviral transfection
(Lvx-ZsGreen vs
Lvx-Pitx2c-ZsGreen)
Inject i.m. into
MDX recipent
Harvest at 15
and 30 days
B)
Pitx2c
mRNA expression level
(arbitrary units)
***
Lvx-ZsGreen
Lvx-Pitx2c-ZsGreen
C)
Zs-Green+ myofibers
% Zs-Green+ myofibers
***
Lvx-ZsGreen
Lvx-Pitx2c-ZsGreen
D)
40 um
Negative control
Lvx-ZsGreen
Laminin
Lvx-Pitx2c-ZsGreen
Laminin
E)
Fiber Number / Cross Section
Lvx-ZsGreen
Lvx-Pitx2c-ZsGreen
0-4
4-8
8-12
12-16
16-20
20-24
24-28
28-32
>32
Cross Sectional Area
(Hundred of square micron)

Figure 5


A)
miR-31
Satellite cells
miR relative expression
1.5
1.0
0.5
0.0
**
Lvx-ZsGreen
Lvx-Pitx2c-ZsGreen
B)
miR31 ChiP
-2705 -2131
miR31
IgG
V5-Pitx2
% Input
0.8
0.6
0.4
0.2
0.0
***
****
miR31.1
miR31.2
C)
RNApol2 ChiP
IgG
RNApol
% Input
2.0
1.5
1.0
0.5
0.0
****
****
****
Gapdh Promoter
miR31.1
miR31.2

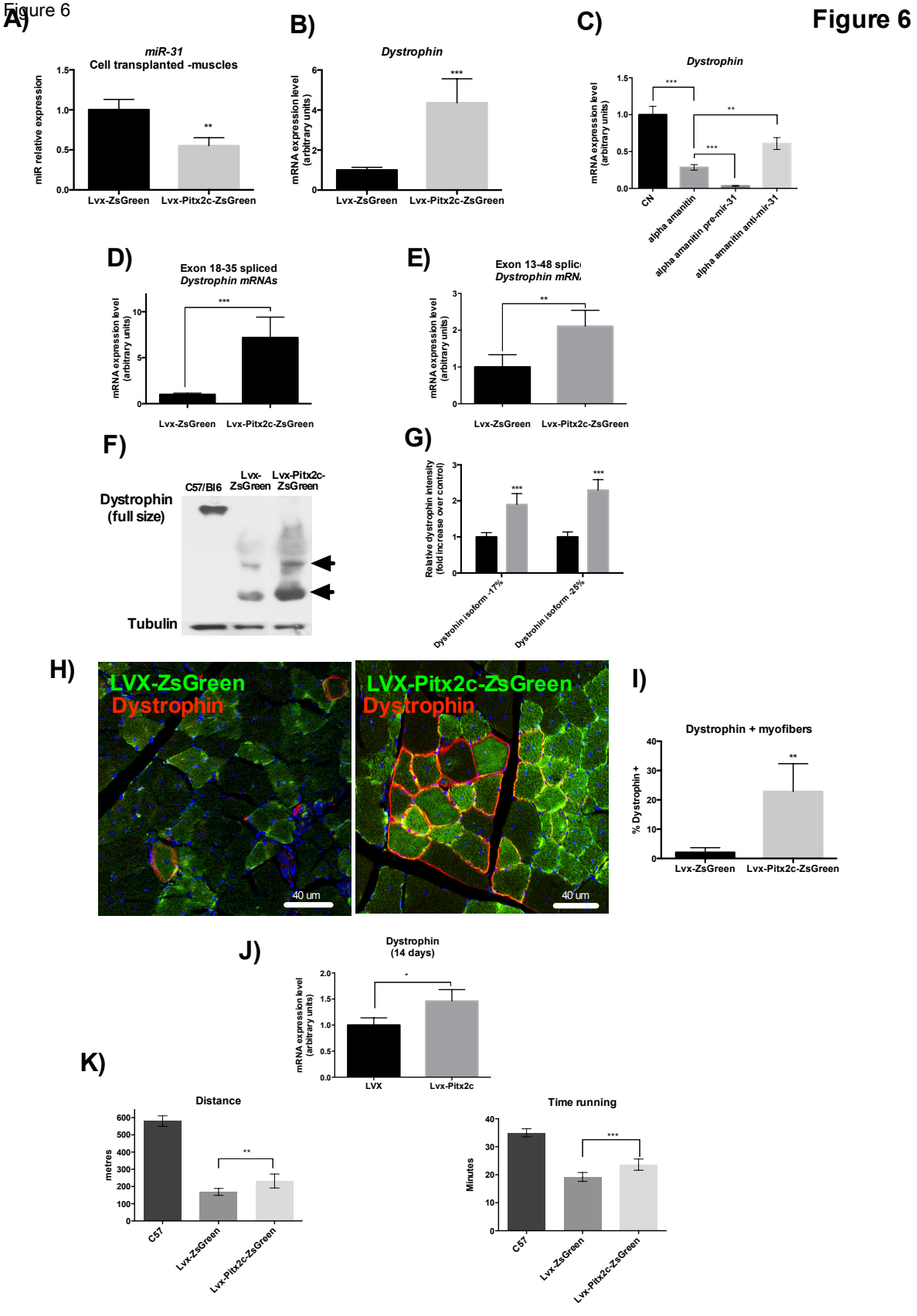

A)
miR-31
Cell transplanted -muscles
miR relative expression
Lvx-ZsGreen
Lvx-Pitx2c-ZsGreen
B)
Dystrophin
mRNA expression level (arbitrary units)
Lvx-ZsGreen
Lvx-Pitx2c-ZsGreen
C)
Dystrophin
mRNA expression level (arbitrary units)
CN
alpha amanitin
alpha amanitin pre-mir-31
alpha amanitin anti-mir-31
D)
Exon 18-35 spliced
Dystrophin mRNAs
mRNA expression level (arbitrary units)
Lvx-ZsGreen
Lvx-Pitx2c-ZsGreen
E)
Exon 13-48 splic
Dystrophin mRN
mRNA expression level (arbitrary units)
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)
Dystrophin isoform ~17%
Dystrophin isoform ~25%
H)
LVX-ZsGreen
Dystrophin
40 um
LVX-Pitx2c-ZsGreen
Dystrophin
40 um
I)
Dystrophin + myofibers
% Dystrophin +
Lvx-ZsGreen
Lvx-Pitx2c-ZsGreen
J)
Dystrophin
(14 days)
mRNA expression level (arbitrary units)
LVX
Lvx-Pitx2c
K)
Distance
metres
C57
Lvx-ZsGreen
Lvx-Pitx2c-ZsGreen
Time running
Minutes
C57
Lvx-ZsGreen
Lvx-Pitx2c-ZsGreen

Figure 7

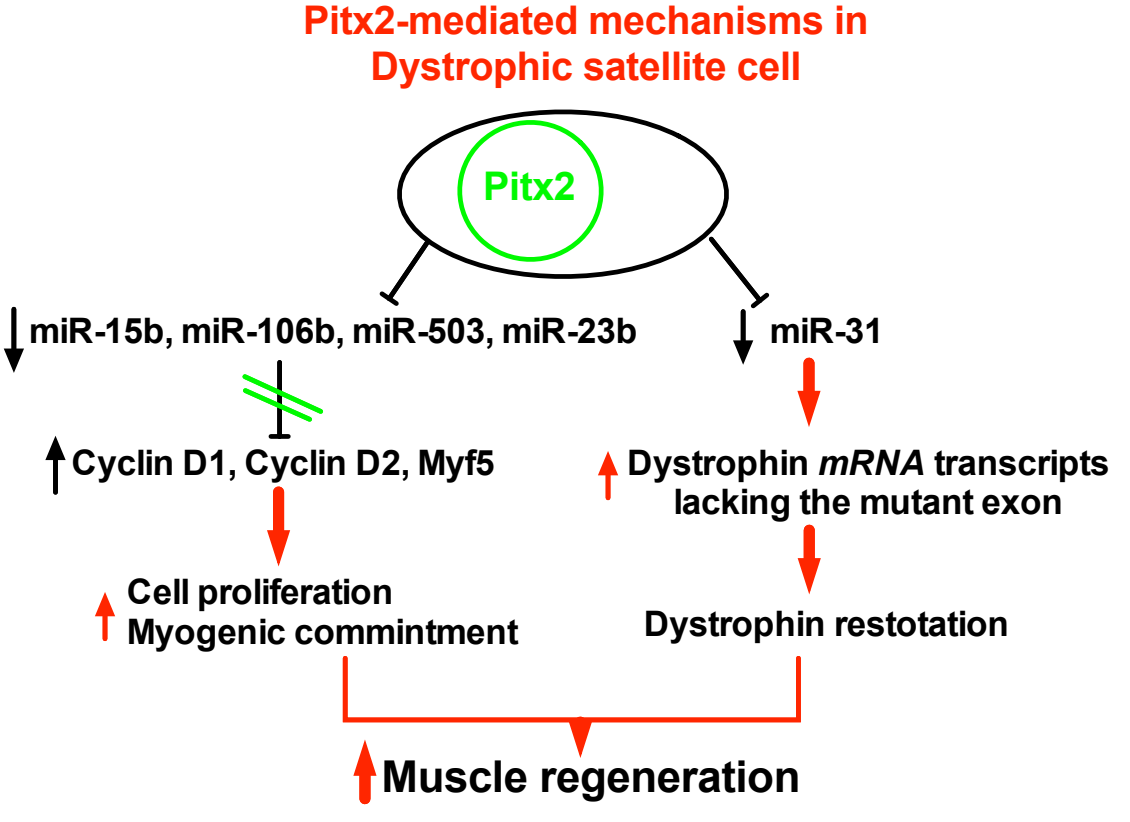

Pitx2-mediated mechanisms in
Dystrophic satellite cell
Pitx2
miR-15b, miR-106b, miR-503, miR-23b
miR-31
Cyclin D1, Cyclin D2, Myf5
Dystrophin *mRNA* transcripts
lacking the mutant exon
Cell proliferation
Myogenic commintment
Dystrophin restotation
Muscle regeneration

Table S1: Specific primers for each gene/miRNA analyzed, annealing temperatures, and amplicon sizes

microRNA	primer
hsa-miR-31	AGGCAAGAUGCUGGCAUAGCU
hsa-miR-15b	UAGCAGCACAUCAUGGUUUACA
hsa-miR-23b	AUCACAUUGCCAGGGAUUACC
hsa-miR-106b	UAAAGUGCUGACAGUGCAGAU
hsa-miR-503	UAGCAGCGGGAACAGUUCUGCAG

Gene	Primer	Annealing Tª	Amplicon Size
Gusb	Sense 5'- ACGCATCAGAAGCCGATTAT-3' Antisense 5'-ACTCTCAGCGGTGACTGGTT -3'	60º C	212 pb
Gapdh	Sense 5'-TCT TGC TCA GTG TCC TTG CTG G-3' Antisense 5'- TCC TGG TAT GAC AAT GAA TAC GC- 3'	60º C	180 Pb
Pitx2c	Sense 5´-CCT CAC CCT TCT GTC ACC AT-3´ Antisense 5´-GCC CAC ATC CTC ATT CTT TC-3	60º C	175 pb
Myf5	Sense 5'-TGA GGG AAC AGG TGG AGA AC-3' Antisense 5'-AGC TGG ACA CGG AGC TTT TA-3'	60º C	187 pb
Dmd (Dystrophin)	Sense 5´-TGG TTG GCA GTC AAA CTT CA-3' Antisense 5 -TCA TCT GCC ATG TGG AAA G -3'	60º C	192 pb
Dmd Ex13-48 (Dystrophin)	Sense 5´- CAG CTG CTT TGG AAG AAC AA -3' Antisense 5 - AGG AGA GAG CCA CAG AAG CAG -3'	60º C	133 pb
Dmd Ex18-35 (Dystrophin)	Sense 5´- CGA AAA GAA GGC AAC ATC TCA -3' Antisense 5 - CTT TCT TGC CCA ACA CCA TT -3'	60º C	135 pb
Ccnd1 (Cyclin D1)	Sense 5´-TTG ACT GCC GAG AAG TTG TG-3´ Antisense 5´-CTG GCA TTT TGG AGA GGA AG-3´	60º C	154 pb
Ccnd2 (Cyclin D2)	Sense 5´-ATG CTG CTC TTG ACG GAA CT-3´ Antisense 5´-ATG CTG CTC TTG ACG GAA CT-3´	60º C	201 pb
Sod1 *(superoxide dismutase 1)*	Sense 5´- GAG ACC TGG GCA ATG TGA CT -3´ Antisense 5´- TAA CCC TAA CGC GTC ATT TG -3´	60º C	220 pb
Sod2 *(superoxide dismutase 2)*	Sense 5´- GCA AGG AAC AAC AGG CCT TA -3´ Antisense 5´- TCG TGA TAC TGA CCC CAC GA -3´	60º C	245 pb

Supplemental Experimental Procedures

Pitx2c overexpression and siRNA-Pitx2c

Lentivirus was titred by transducing Lenti-X 293T cells and by using the Lenti-X qRT-PCR Titration Kit (Clontech). Freshly isolated satellite cells were transduced with lentiviral vectors coding for *Pitx2c* (LVX-Pitx2c-ZsGreen) or the empty lentiviral vector (pLVX-IRES-ZsGreen1) cDNA, at a multiplicity of 50 to 100 genome units, determined by qPCR, adsorption of the lentiviral vectors was for 8-24 h on culture after viral particles were added. Transduction was monitored in all experiments by flow cytometry and by fluorescence microscopy. For the siRNA-Pitx2 transfection assays, satellite cells were transfected in serum-free conditions for 16 h with siRNA-Pitx2c (Sigma), at concentrations of 85 nM by using the Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) following the supplier's guideline.

Quantitative Reverse Transcriptase-PCR Analyses (qRT-PCR)

Total RNA was extracted from mouse muscle tissue, satellite cells, by using the TriPure Isolation Reagent (Roche) according to the supplier’s guideline. Each PCR reaction was carried out at least 5 times to ensure a representative average. The relative level of expression of each gene was calculated as the ratio of the extrapolated levels of expression of each gene and Gapdh level. Each PCR reaction was performed in triplicate and repeated at least in three different biological samples to have a representative average.

For *microRNAs* qRT-PCR were performed by using Exiqon LNA microRNA primers and detection kit according to manufacturer’s guidelines. All reactions

were consistently run in triplicates using 5S as normalizing control, according to manufacturer recommendations. SyBR Green was used as the quantification system on a Stratagene Q-Max 2005P qRT-PCR thermocycler. Relative measurements were calculated as described Livak et al. (2001) and control measurements were normalized to represent 100%, as previously reported Dominguez et al. (2005). Specific primers for each gene analyzed, annealing temperatures, and amplicon sizes are shown in the **Table 1.**

Immunocytochemistry and Immunohistochemistry

Tissue specimens were sliced using a cryostatic microtome to 10 µm thick and mounted onto silane–treated glass slides. For IHC, the tissue sections were incubated in blocking buffer with either 2% bovine serum diluted in PBS at room temperature for 30 min. The blocking buffer was removed and primary antiserum was applied. Primary antibodies used were against Pax7 (DSHB), Myod (clone 5.8A, DAKO), Ki67(Abcam), Myf5 (Santa Cruz biotechnology), MF20 (DSHB) Dystrophin (Abcam) and Laminin (Sigma). The antibody against Pitx2c was used as described (Hernández-Torres et al, 2008). The antibodies were diluted into fresh 2% bovine serum in PBS and applied to the section and incubated overnight at 4°C in a humidified chamber. The antibody solutions were removed and the samples were washed with PBS (3x) at room temperature. Fluorescently labelled secondary antibodies diluted in bovine serum-PBS were applied to the samples and incubated at room temperature for 2 h in a dark plastic chamber to exclude light. The antibody solutions were then removed and washed with PBS.

Finally, the sections were incubated with DAPI at room temperature, washed three times in PBS. Images were captured using the Leica TCS SP2 confocal system.

Satellite cells isolation

The hindlimb muscles of young mice were removed, and the bones were dissected. The muscle was then minced into a coarse slurry using scalpels. The muscle tissue was enzymatically dissociated at 37 C in 0.2% collagenase-type XI (Sigma-Aldrich) for 1 h, and then centrifuged at 3,500 rpm for 5 min. The cells were collected, incubated in dispase (GIBCO BRL), prepared as 2.4 units/1 ml HBSS (GIBCO BRL), for 45 min, and then incubated for 30 min in 0.1% trypsin-EDTA (GIBCO BRL) diluted in HBSS. After the enzymatic dissociation, the muscle cells were magnetically labelled as follows. In general, magnetic labelling was performed for 15 min at 4°C in 0.1 ml of a 1 : 5 dilution of the according MicroBeads Satellite Cell Isolation Kit (Miltenyi Biotec 130-090-485). Previously, the lysis of red blood cells was made (Miltenyi Biotec 130-094-183). Columns were pre-equilibrated and placed in appropriate magnets and cell suspension was applied onto the column. The flow-through containing unlabeled cells was collected, representing the enriched satellite cells. Anti-Integrin α-7 MicroBeads was used to further increase the purity of isolated satellite cells (Miltenyi Biotec 130-104-261).

Western blot

For western blot analysis, equal amounts of denatured total protein extracts (60 µg) were loaded and separated in 4–12% SDS-

polyacrylamide gel. Proteins in the gel were transferred to a PVDF membrane (Amersham Pharmacia Biotech., NJ, USA) and then blocked. Polyclonal antibodies to Dystrophin (1/300, Abcam), and a monoclonal antibody to α-tubulin (Sigma, St Louis, MO, USA), as the internal control, were used for the detection of the respective proteins. Antibody reaction was revealed by chemiluminescence detection procedures according to the manufacturer's recommendations (ECL kit, Amersham Corp., Buckinghamshire, UK)

Supplemental References

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Method**25**, 402-408.

Dominguez, J.N., Navarro, F., Franco, D., Thompson, R.P., and Aránega A. E. (2005).Temporal and spatial expression pattern of beta1 sodium channel subu nit during heart development. Cardiovasc Res. 65, 842-850.

Hernández-Torres, F., Pedrajas, J. R., Aránega, A. E., and Navarro, F. (2008). Expression in bacteria of small and specific protein domains of two transcription factor isoforms, purification and monospecific polyclonal antibodies generation, by a two-step affinity chromatography procedure. Protein Expr Purif. 60(2), 151-156.

Figure S1

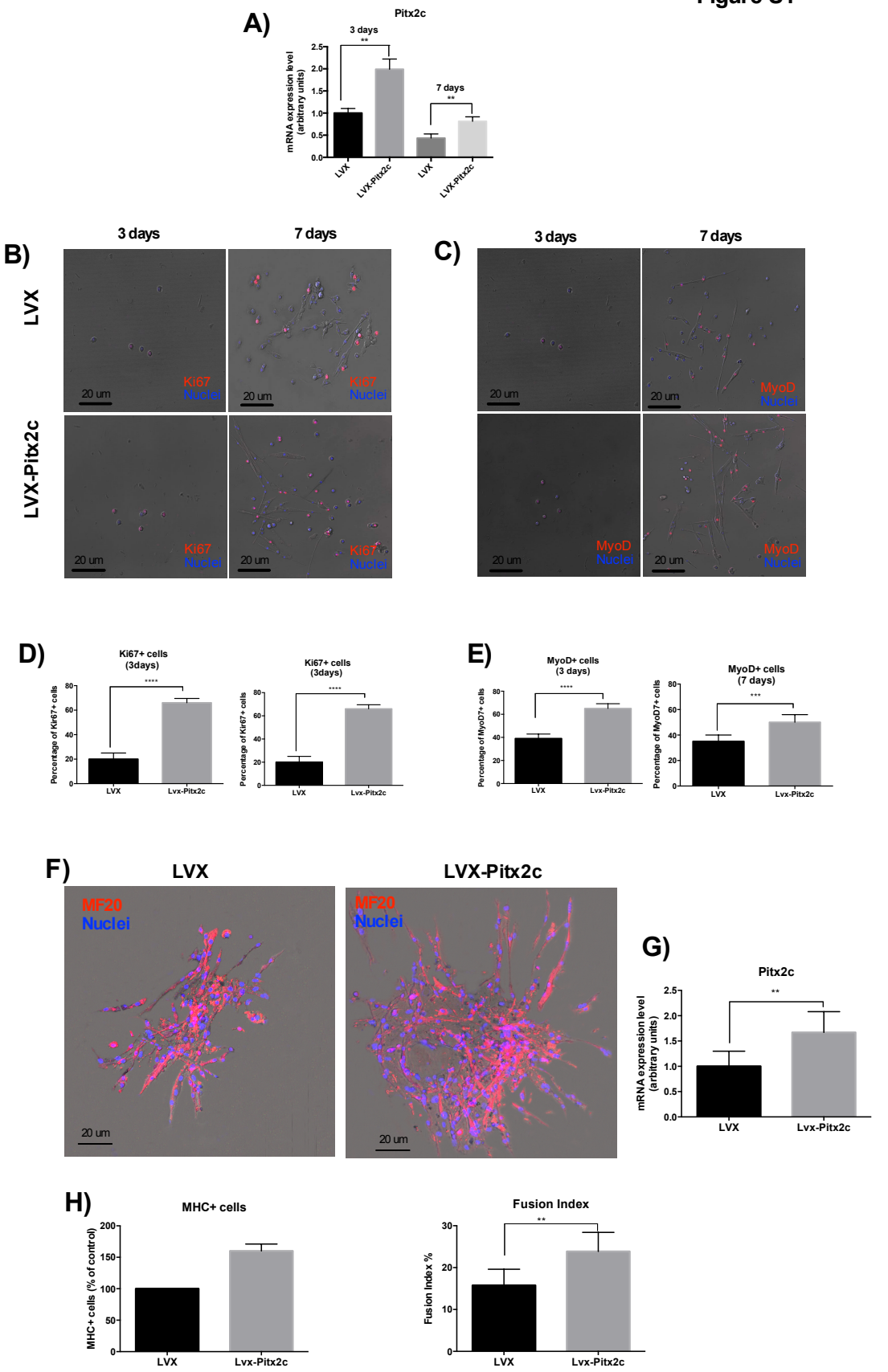

A)
Pitx2c
3 days
7 days
mRNA expression level (arbitrary units)
LVX
LVX-Pitx2c
B)
3 days
7 days
LVX
LVX-Pitx2c
Ki67
Nuclei
20 um
C)
MyoD
D)
Ki67+ cells (3days)
Percentage of Ki67+ cells
E)
MyoD+ cells (3 days)
MyoD+ cells (7 days)
Percentage of MyoD7+ cells
F)
MF20
G)
H)
MHC+ cells
MHC+ cells (% of control)
Fusion Index
Fusion Index %

Figure S2

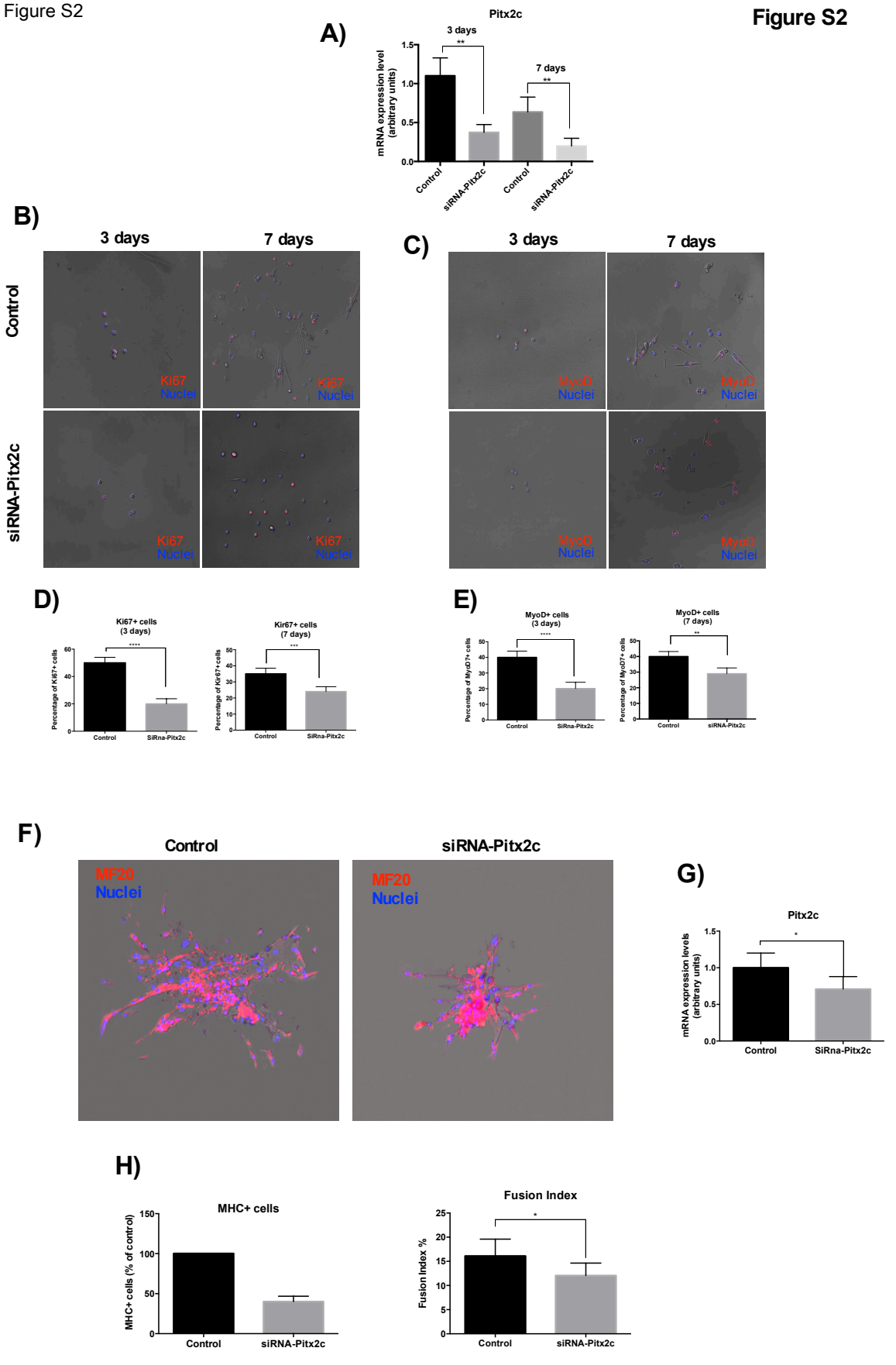
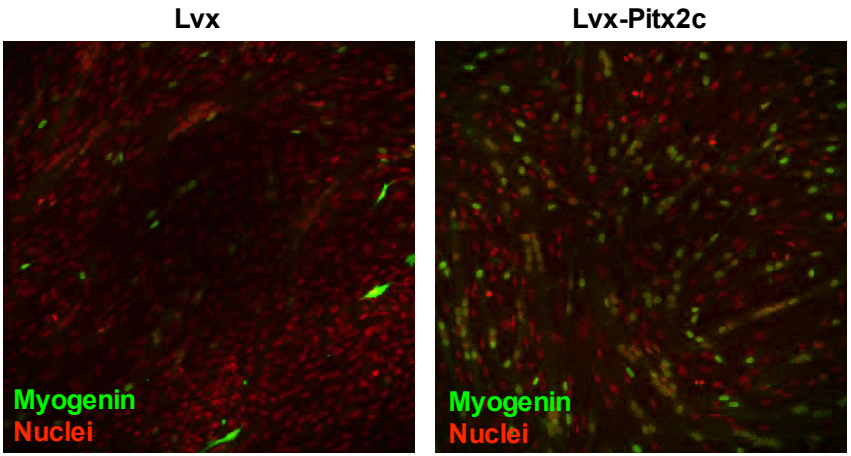
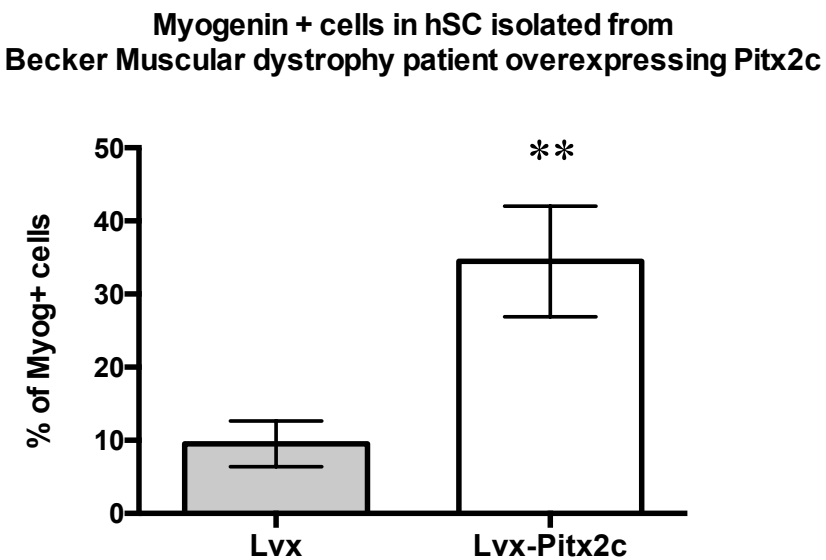

A)
Pitx2c
3 days
7 days
mRNA expression level (arbitrary units)
Control
siRNA-Pitx2c
B)
3 days
7 days
Control
siRNA-Pitx2c
Ki67
Nuclei
C)
MyoD
D)
Ki67+ cells (3 days)
Kir67+ cells (7 days)
Percentage of Ki67+ cells
E)
MyoD+ cells (3 days)
MyoD+ cells (7 days)
Percentage of MyoD7+ cells
F)
Control
siRNA-Pitx2c
MF20
Nuclei
G)
Pitx2c
mRNA expression levels (arbitrary units)
H)
MHC+ cells
MHC+ cells (% of control)
Fusion Index
Fusion Index %

Figure S3


Lvx
Lvx-Pitx2c
Myogenin
Nuclei
Myogenin
Nuclei


Myogenin + cells in hSC isolated from
Becker Muscular dystrophy patient overexpressing Pitx2c
% of Myog+ cells
50
40
30
20
10
0
**
Lvx
Lvx-Pitx2c

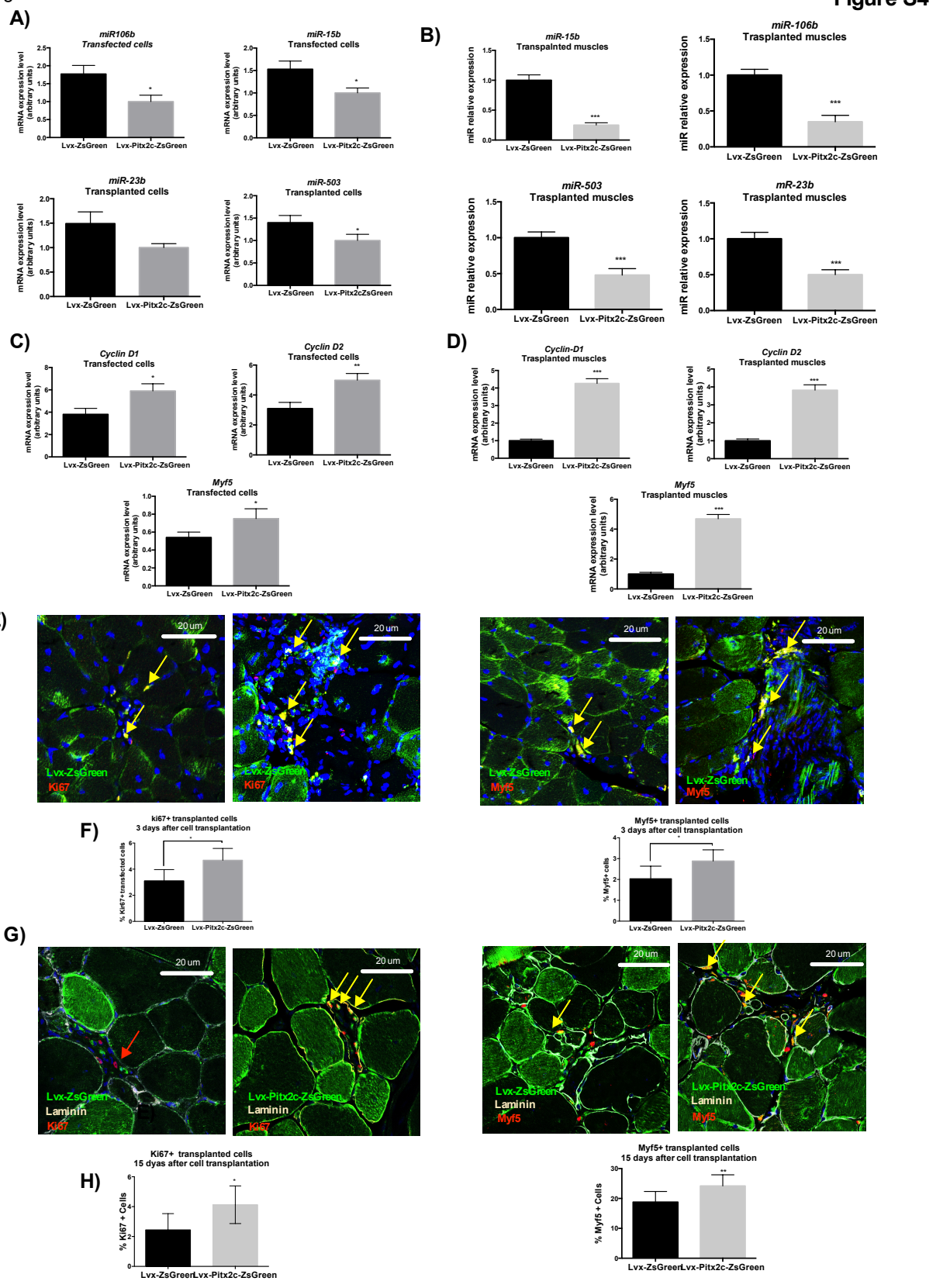


Figure S4
A)
miR106b
Transfected cells
miR-15b
Transfected cells
miR-23b
Transplanted cells
miR-503
Transplanted cells
mRNA expression level (arbitrary units)
Lvx-ZsGreen
Lvx-Pitx2c-ZsGreen
B)
miR-15b
Transpalnted muscles
miR-106b
Trasplanted muscles
miR-503
Trasplanted muscles
mR-23b
Trasplanted muscles
miR relative expression
C)
Cyclin D1
Transfected cells
Cyclin D2
Transfected cells
Myf5
Transfected cells
D)
Cyclin-D1
Trasplanted muscles
Cyclin D2
Trasplanted muscles
Myf5
Trasplanted muscles
E)
20 um
Lvx-ZsGreen
Ki67
Myf5
F)
ki67+ transplanted cells
3 days after cell transplantation
% Ki67+transfected cells
Myf5+ transplanted cells
3 days after cell transplantation
% Myf5+ cells
G)
Laminin
H)
Ki67+ transplanted cells
15 dyas after cell transplantation
% Ki67 + Cells
Myf5+ transplanted cells
15 days after cell transplantation
% Myf5 + Cells

Figure S5

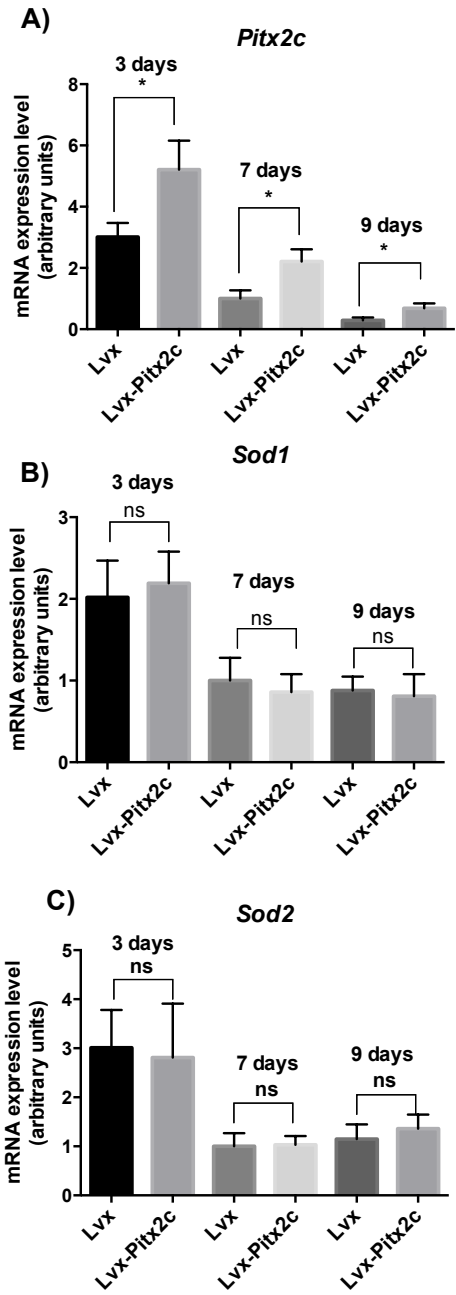


Figure S5
A)
Pitx2c
3 days
*
7 days
*
9 days
*
mRNA expression level (arbitrary units)
8
6
4
2
0
Lvx
Lvx-Pitx2c
Lvx
Lvx-Pitx2c
Lvx
Lvx-Pitx2c
B)
Sod1
3 days
ns
7 days
ns
9 days
ns
mRNA expression level (arbitrary units)
3
2
1
0
Lvx
Lvx-Pitx2c
Lvx
Lvx-Pitx2c
Lvx
Lvx-Pitx2c
C)
Sod2
3 days
ns
7 days
ns
9 days
ns
mRNA expression level (arbitrary units)
5
4
3
2
1
0
Lvx
Lvx-Pitx2c
Lvx
Lvx-Pitx2c
Lvx
Lvx-Pitx2c

Supplemental figure legends

Figure S1: *Pitx2c* overexpression in satellite cells enhances the number of myogenic commitment cells leading to increase differentiation. A) *Pitx2c* expression in isolated satellite cells transfected with LVX-*Pitx2c vs.* LVX particles at 3 and 7 days of culture. B) Representative images of Ki67+ cells (days 3 and 7 of culture) in *Pitx2c*-overexpressing cells *vs.* control. **C)** Representative images of MyoD+ cells (days 3 and 7 of culture) in *Pitx2c*-overexpressing cells *vs.* control. D and E) Percentages of Ki67+ and MyoD+ cells respectively (days 3 and 7 of culture). F) Representative images of immunohistochemistry for MF20 in differentiating satellite cells overexpressing *Pitx2c* (LVX-*Pitx2c*) *vs.* control cells (LVX) (day 14 of culture). G) *Pitx2c* expression in differentiating satellite cells transfected with LVX-*Pitx2c vs.* LVX particles at 14 days of culture. H) MHC-positive cells and fusion index. All the experiments were repeated at least in three different biological samples to have a representative average. ,**p<0.001, ,***p<0.0001 ,****p<0.00001.

Figure S2: *siRNA-Pitx2c* in satellite cells diminishes the number of nuclei during myogenic differentiation leading to decreased myogenesis. A) Pitx2c expression in isolated satellite cells transfected with *siRNA-Pitx2c vs.* control cells at 3 and 7 days of culture. B) Representative images of Ki67+ cells. (days 3 and 7 of culture) in transfected cells with *siRNA-Pitx2c vs.* control cells. C) Representative images of MyoD+ cells (days 3 and 7 of culture) in transfected cells with *siRNA-Pitx2c vs.* control cells. D and E) Percentages of Ki67+ and MyoD+ cells respectively (days 3 and 7 of culture). F) Representative images of immunohistochemistry for MF20 in differentiating satellite cells transfected with *siRNA-Pitx2c vs.* control cells (day 14 of culture). G) *Pitx2c* expression in

differentiating satellite cells transfected with *siRNA-Pitx2c vs.* control cells at 14 days of culture. H) MHC-positive cells and fusion index. All the experiments were repeated at least in three different biological samples to have a representative average. *p<0.01,**p<0.001, ,***p<0.0001 ,****p<0.00001.

Figure S3: Pitx2c overexpression significantly in human satellite cells isolated from a Becker muscular dystrophy patient.

Figure S4: Pitx2c-overexpression in transplanted dystrophic satellite cells leads to increased cell proliferation and a higher number of myogenic committed cells due to the activation of the Pitx2-miR15b-miR106b-miR503-miR23b pathway. A) miR15b-miR106b-miR503-miR23b expression in Pitx2c-overexpressing cells (Lvx-Pitx2c-ZsGreen) vs. control cells (Lvx-ZsGreen) before transplantation B) miR15b-miR106b-miR503-miR23b expression in muscles transplanted with Pitx2c-overexpressing cells (Lvx-Pitx2c-ZsGreen) vs. control muscles (Lvx-ZsGreen). C) Cyclyn D1, Cyclin D2 and Myf5 expression in Pitx2c-overexpressing cells (Lvx-Pitx2c-ZsGreen) vs. control cells (Lvx-ZsGreen) before transplantation. D) Cyclyn D1, Cyclin D2 and Myf5 expression in muscles transplanted with Pitx2c-overexpressing cells (Lvx-Pitx2c-ZsGreen) vs. control muscles (Lvx-ZsGreen). E) Representative images for Ki67+ and Myf5+ cells in muscles transplanted with Pitx2c-overexpressing cells (Lvx-Pitx2c-ZsGreen) vs. control muscles (Lvx-ZsGreen) 3 days after cell tranplantation. Arrows shown donor Ki67+ and donor Myf5+ cells in yellow F) Quantification of Ki67+ and Myf5+ cells in muscle transplanted with Pitx2c-overexpressing cells (Lvx-Pitx2c-ZsGreen) vs. control muscles (Lvx-ZsGreen) 3 days after cell transplantation. G) Representative images for Ki67+and Myf5+ cells in muscles transplanted with Pitx2c-overexpressing cells (Lvx-Pitx2c-ZsGreen) vs. control muscles (Lvx-

ZsGreen) 15 days after cell tranplantation. Arrows shown donor Ki67+ and donor Myf5+ cells in yellow. H) Quantification of Ki67+ and Myf5+ cells in muscle transplanted with Pitx2c-overexpressing cells (Lvx-Pitx2c-ZsGreen) vs. control muscles (Lvx-ZsGreen. All the experiments were repeated at least in three different biological samples to have a representative average *p<0.01,**p<0.001, ***p<0.0001.

Figure S5: RT-PCR analysis for genes encoding antioxidant enzymes in *Pitx2c* overexpressing dystrophic satellite cells.

CHAPTER IV: FINAL DISCUSSION

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SUMMARY

Pitx2 in the transcriptional regulation of miRNAs in myoblasts and satellite cells.

Pitx2 is a homeobox transcription factor that has been shown to regulate skeletal-muscle development [1,2]. We have previously documented that c-isoform of *Pitx2* plays a pivotal role in modulating proliferation *vs.* differentiation during myogenesis, balancing Pax3+/Pax7+ myogenic population *in vivo*, and regulating key myogenic transcription factors such as *Pax3* by repressing *miR-27b* [3,4]. Within the present study, we investigated the role of *Pitx2* controlling *microRNA* expression in myogenic cells identifying a subset of *Pitx2c*-regulated *miRNAs* controlling cell proliferation in myoblasts and demonstrating that this *Pitx2c-miRNA* pathway controls cell proliferation as well as myogenic commitment of satellite cells. Our data demonstrate the pivotal role of the homeobox transcription factor *Pitx2* controlling *microRNA* expression in myoblasts. It has been recently reported that *Pitx2* positively regulates *miR-17-92* and *miR-106b-25* in the heart [5]; however, we found a *Pitx2*-mediated negative regulation of *miRNA* expression in myoblasts. These different functional requirements for *Pitx2* underline the differences between cardiac and skeletal myogenesis. Therefore, this study represents the first available description of *Pitx2*-regulated *microRNA* expression in myogenic cells, providing new insights into the *microRNA* mediated mechanisms during myogenesis.

In particular we show that a subset of these *Pitx2c*-down-regulated *miRNAs* such as *miR-15b*, *miR-106b*, *miR-23b*, and *miR-503* targeting cyclins together have dramatic effects on myoblast proliferation *in vitro*, providing a means for the previously reported *Pitx2c* functions in cell proliferation. The role of *Pitx2* in satellite-cell proliferation and/or differentiation is recently emerging and is controversial. *Pitx2* expression is detected in proliferating satellite cells [6] but the constitutive expression of any *Pitx2* isoform suppresses satellite-cell proliferation, with the cells undergoing greater myogenic differentiation [7]. Nevertheless, the divergence of the *Pitx2c* effects on satellite-cell proliferation

found by Knopp et al. (2013) [7] could be explained by the moderate *Pitx2c* expression achieved in their *in vitro* gain-of-function experiments as argued by the authors [7]. Even more recently it has been reported that the knockdown of *Pitx2* in satellite cells isolated from extraocular muscles slowed their proliferation rate and a similar trend was seen for satellite cells isolated from tibialis anterioris muscle [8]. Here we demonstrate that enhanced *Pitx2c* expression boosted cell proliferation in freshly isolated satellite cells, reinforcing the contention that *Pitx2* positively regulates cell proliferation in satellite cells.

In addition, we also have demonstrated a *Pitx2c* effects on satellite-cell proliferation mediated by the *Pitx2c*-down-regulated *miRNAs miR-15b*, *miR-106b*, *miR-23b*, and *miR-503*. Recent evidence suggests a role of *miRNAs* in the regulation of satellite-cell fate and self-renewal [9,10] and it has been reported that *miR-106b* inhibition augments the number of Pax7+ cells [9]. Our findings point out previously unknown functions of *miR-15b*, *miR-106b*, *miR-23b*, and *miR-503* on satellite-cell proliferation. Since one of the key prerequisites to trigger cell proliferation at the onset of satellite-cell activation is a proper cell-cycle progression [11], the existence of the *Pitx2-miRNA* pathway controlling the expression of key regulatory cell-cycle genes in early-activated satellite cells reveals a role of *Pitx2* in satellite-cell activation. Although muscle satellite cells are promising targets for cell therapies, the paucity of satellite cells that can be isolated or expanded from adult muscle tissue is limiting; thus our findings provide new molecular tools to overcome such a bottleneck.

Myf5 induction demarcates the entry of satellite stem cells into the myogenic programme. Our analyses showed that *Pitx2c* can increase *Myf5* expression by regulating miR-106b, thus enhancing the Myf5+ satellite-cell population and revealing a role for *Pitx2c* promoting satellite-cell populations more primed for myogenic commitment. The importance of *miRNAs* in the post-transcriptional regulation of *Myf5* in satellite cells is beginning to emerge. Recently Crist et al., 2012 [12] have reported that although many quiescent satellite cells transcribe *Myf5*, they do not enter myogenesis because of *miR-31*. Thus, *miR-31* interacts with the *3'-UTR* of *Myf5 mRNA* and therefore can prevent its translation into a

quiescent cell, but it is rapidly down-regulated early during activation, leading to rapid accumulation of *Myf5* protein [12]. Here we demonstrate that down-regulation of *miR-106b* leads to increased *Myf5* expression in early-activated satellite cells, thus providing additional information concerning the role of *miRNAs* in the post-transcriptional control of myogenic progression in adult myogenesis.

On other hand, a mechanism linking *Myf5* levels to muscle stem-cell heterogeneity has also been recently proposed. A detailed analysis of satellite-cell behaviour in *Myf5* haploinsufficient mice has revealed the duality in the functional role of *Myf5*, as a promoter of muscle fate, and also as incompatible with terminal differentiation, raising questions about the precise role of this transcription factor during different cell states in a myogenic lineage progression [13]. In the present study, we describe a *Pitx2c-miR-106b* pathway controlling *Myf5* expression providing new insights into the molecular mechanisms that control satellite-cell behaviour. These findings might thus have future applications that can modulate satellite-cell fate during muscle regeneration.

Pitx2 on the regenerative potential of satellite cells

In this work we have identified Pitx2c as an essential positive regulator of muscle regeneration in mice. Also, we found that *Pitx2c* is down-regulated 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 *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.

We have previously shown that overexpression of *Pitx2c* in Sol8 myoblasts maintain them with high proliferative capacity but blocks terminal differentiation [3]. More recently we have demonstrated that *Pitx2c* increases proliferation in

early-activated satellite cells and promotes their commitment to a myogenic cell fate. In this study, we observed that *Pitx2c* promotes both satellite-cell proliferation and their differentiation. Taking all these results into consideration, we propose that Pitx2c effects on cell proliferation has different impact at distinct stages of muscle stem cell lineage commitment, promoting myogenic lineage progression upon early activation but blocking terminal differentiation in committed myoblasts.

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 [14] 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 [15]. Here we show that *Pitx2c*-overexpression in dystrophic satellite cells increase their regenerative potential *in vivo*. Our analyses revealed that the *Pitx2-miRNA* pathway, previously described by us, 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-functions regulating the redox state during fetal myogenesis has been previously described [16], *Pitx2c* overexpression on dystrophic satellite cells has not impact on the expression of genes encoding antioxidant enzymes suggesting differences between adult and fetal myogenesis. Therefore, we postulate that, in our system, activation of *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.

The dystrophic muscles contain sporadic small clusters of dystrophin expressing revertant fibres. These revertant fibres 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 [17]. On other hand, recent studies have identified the post-transcriptional control of gene expression as a crucial level in the regulation of myogenesis [18–

[20]. 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 [21–23]. 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 [24]. 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* down-regulation when dystrophic satellite cells overexpressing *Pitx2c* were transplanted into the muscles of DMD/mdx mice and, consequently, the levels of dystrophin were significantly restored. These findings indicate that *Pitx2*-mediated *miR-31* down-regulation leads to dystrophin restoration in cell-transplanted muscle. Our analyses indicate that *miR-31* acts by promoting dystrophin *mRNA* degradation, and that *miR-31* down-regulation mediated by Pitx2 augments the total amount of dystrophin *mRNA*, overall the revertant dystrophin *mRNA* and thus revertant dystrophin protein. In addition, since it have been shown that manipulation of *miR-31* levels affects satellite cell differentiation by regulation of Myf5 protein [12], 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* down-regulation. 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.

CONCLUSIONS

1.-We have identified a subset of *microRNAs* regulated by *Pitx2*, with previous unknown functions on myogenic cells, which have profound effects on myoblast proliferation. Notably, we found that this *Pitx2-miRNA* pathway regulating cell proliferation is conserved in freshly isolated satellite cells, providing developmental cues that enhance the commitment of satellite cells to the myogenic lineage differentiation by down-regulating *miR-106b* expression. Overall, the present study describes a previous unknown *Pitx2-miRNA* pathway controlling cell proliferation in myogenic cells, providing new targets to enhance the regenerative capacity of limb skeletal-muscle myogenic precursor cells for the treatment of skeletal-muscle diseases.

2.- 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* repress *miR-31*, leading to dystrophin restoration and finally improving muscle regeneration. This study reveals the previously unknown function of *Pitx2* in skeletal-muscle repair and may help to develop therapeutic strategies for muscular disorders.

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