A Pitx2-miRNA pathway modulates cell proliferation in myoblasts and skeletal-muscle satellite cells and promotes their commitment to a myogenic cell fate.

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Running Title: Pitx2-miRNA and satellite cells.
28 ABSTRACT

29 The acquisition of a proliferating cell status from a quiescent state as well as the shift between
30 proliferation and differentiation are key developmental steps in skeletal-muscle stem cells
31 (satellite cells) to provide proper muscle regeneration. However, how satellite-cell proliferation is
32 regulated, though, is not fully understood. Here, we report that the c-isoform of the transcription
33 factor Pitx2 increases cell proliferation in myoblasts by down-regulating the miRNAs mir-15b,
34 mir-23b, mir-106b, and mir-503. This Pitx2c-miRNA pathway also regulates cell proliferation in
35 early-activated satellite cells, enhancing the Myf5+ satellite cells and thereby promoting their
36 commitment to a myogenic cell fate. This study reveals unknown functions of several miRNAs in
37 myoblast and satellite-cell behaviour and thus may have future applications in regenerative
38 medicine.
The maintenance and repair of adult muscle tissue is 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 myofibres (1). Together with skeletal muscles, satellite cells originate from cells of the segmented paraxial mesoderm known as somites. Somite formation starts around 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 dorso-lateral 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 foetal body muscles (3, 4). Pax genes directly control the activation of the myogenic programme in the limb by binding and activating the myogenic regulatory factors Myf5 and Mrf4, followed by MyoD (5, 6, 7, 8). Pax7 is maintained in foetal myogenic precursors and satellite cells in adults, whereas Pax3 is downregulated during the foetal period (9), although the Pax3 locus remains active in a subset of satellite cells of particular muscles in the adult (10, 11). In adults, satellite cells can be recruited to supply myoblasts for routine muscle-fibre homeostasis, or for the more sporadic demands of myofibre 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, co-labelling 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 the redox regulation during foetal myogenesis have also been 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 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 in our lab have revealed that these roles of Pitx2c balancing proliferation vs. differentiation as well as signalling 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 post-transcriptional 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 miR-206 and miR-486 induce myoblast differentiation by down-regulating Pax7 (24). More recently, Gagan et al., 2011 (25), have identified a feed-forward loop where MyoD indirectly down-regulates its inhibitor 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 repressing 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 pre-differentiated state. Furthermore, miRNAs modulate stem-cell fate decisions and some miRNAs involved in satellite-cell quiescence and activation are starting to be identified (27, 103 28, 29). In the present study, we have further elaborated on 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.

MATERIAL AND METHODS

Microarrays, Statistical and bioinformatics analysis
In the present study, mirVana microarrays (Ambion) were used to profile the microRNA signature at different Pitx2c overexpression conditions, namely two different doses (400 and 800 ng/ml of CMV-Pitx2c plasmid, respectively) after 24 h of transfection. The data discussed here have been deposited in NCBI’s Gene Expression Omnibus (30) and are accessible through GEO Series accession number GSE53943 (31). 30 µg of total RNA was used to hybridize the distinct microRNA microarrays on each condition analysed. microRNA -Cy5 labelling, microarray hybridization and washing steps were performed according to 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 same probe was considered as an independent sample for each condition and, then, normalized using the "justvsn" function implemented in the "vsn" Bioconductor library (bioconductor.org) run in R software (r-project.org). The normalized data was then uploaded and analyzed in the TM4 microarray software suite (tm4.org), where a One-way ANOVA test was carried out; those microRNAs showing a false significant proportion < 0.05 were selected as significant and their expression levels used to obtain the gene hierarchical clustering (using Pearson absolute correlation and complete linkage algorithms).

DNA plasmids/siRNA transfections experiments and luciferase assays

DNA plasmids and siRNA transfections were performed in Sol8 myoblasts as previously described (19). The DNA plasmids used were CMV-Pitx2c and CMV-EGFP. For RNA interference siRNA-Pitx2c from Sigma was used (19). For luciferase assays the cyclin D1, cyclin D2 and Myf5 3'-UTRs were amplified from mouse genomic DNA and cloned into pGLuc-Basic vector (New England Biolabs). Cyclin D1, cyclin D2 and Myf5 3’-UTRs were amplified from mouse genomic DNA with specific primers bearing HindIII/BamHI restriction sites and cloned into pGLuc-Basic vector (New England Biolabs). PCR-based site-directed mutagenesis was performed using Stratagene QuikChange Site-Directed Mutagenesis kit, but using the enzymes and buffers from the BioRAD iPROOF PCR kit. Primers used for site directed mutagenesis (Table 1) introduced mutations on miR15b, miR106b, miR23b and miR-503 seed sequences.
present in 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 20µL of culture media; luciferase activity was measured 24h after transfection using BioLux™ Gaussia Luciferase Assay Kit or BioLux™ Cypridina Luciferase Assay Kit (New England Biolabs), respectively. In all cases, transfections were carried out in triplicates.

**Generation of conditional tissue-specific null mutant mice**

Pax3-Cre transgenic mice, purchased by Jackson Laboratory, were crossed into homozygous Pitx2floxed mice and heterozygotes Pax3Cre+/Pitx2fl+/ were backcrossed. The littermates were PCR screened with Pitx2- and Cre-specific primers (32) Cre-positive heterozygote mice were selected as wild-type controls (Pax3Cre+/−). 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**

Satellite cells were isolated as described Qu-Petersen et al., 2002 (33). Two populations of early-preplate (EP) cells were used: the EP cells after 3 days of culturing, which has been previously described as phenotypically EPq, relatively quiescent-early activated and the EP cells after 5 days of culturing, which have been previously described as phenotypically EPa, activated (33). Primary muscle cultures were prepared from young (3–4 m) normal mice using a modified version of a previously described preplate technique (34, 35, 36, 37, 38). 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 centrifuged and
resuspended in proliferation medium (PM). PM consists of DME containing 10% horse serum,
10% FBS, 0.5% chick embryo extract, and 1% penicillin–streptomycin (all reagents purchased
from (GIBCO BRL). Different populations of muscle-derived cells were isolated based on their
adhesion characteristics. The muscle cells were plated on collagen-coated flasks (collagen type
1; 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 (34, 35, 36, 37). After 24 h, the floating cells in pp2
were collected, centrifuged, and plated on new flasks (pp3). These procedures were repeated at
24-h intervals until serial preplates (pp5) were obtained. All cell populations (pp3-5) were
maintained in PM with daily changes. Based on the previous report (33) 30–40% of the cells in
pp2 and pp3 are known to be nonmyogenic, whereas up to 95% desmin_ cells can be found in
pp4 and pp5 (37). To further purify the myogenic cell population pp2 and pp3 were discarded and
only pp4 and pp5 were combined and termed the early-preplate cells (EP) population as
previously described (38).

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 respectively express viral
proteins needed for producing viral particles in packing cells (Lenti-X 293T), by using Lenti-X HTX
Packaging Systems and following manufacturer’s procedure (Clontech). Lentivirus was titred by
transducing Lenti-X 293T cells and by using the Lenti-X qRT-PCR Titration Kit (Clontech).
Freshly isolated satellite cells (EPq and EPa) 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.

Flow cytometry and fluorescence microscopy

Cells were fixed with 4% paraformaldehyde. ZsGreen1+ cells were analyzed by flow cytometry
using LSR-Fortessa cytometer (Beckman Coulter, Brea, California). DNA was stained with 4',6-
diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Fluorescence microscope images were
acquired with a confocal microscope 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 growing conditions.
Corresponding pre-miRNAs (Ambion) were transfected as described elsewhere (19).

Quantitative Reverse Transcriptase-PCR Analyses (qRT-PCR)

RNA isolation and (RT)-PCR were performed as described elsewhere (19) using standard
procedures. Total RNA was extracted from mouse muscle tissue, Sol8 cells, EPq cells, Epq cells
and differentiating myoblast by using the TriPure Isolation Reagent (Roche) according to the
supplier’s guideline. To minimize genomic DNA contamination, total RNA was treated with 20 U
of RNase-free DNase (Roche) for 1 hr at 37°C and then purified using a standard phenol–
chloroform protocol. One microgram of total RNA was reverse transcribed using Superscript
RNase H' reverse transcriptase (Invitrogen) or 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
within in MxPro Mx3005p PCR thermal cycler (Stratagene, Spain) and SYBR Green detection
system (DyNamo™ HS SYBER® GREEN qPCR Kit - Finnzymes). PCR reactions were
performed in 0.2-ml optical tubes (Cultek) in a 20-µl total volume containing Sybr Green Mix
(Finnzymes) and 2-µl of the reverse transcribed RNA. b-actin and Gapdh were used in parallel for
each run as an internal control. Amplification conditions were 95°C for 5 min; 40 cycles of 95°C
for 30 sec, annealing temperature for 30 sec and 72°C for 30 sec. The final cycle was 72°C for 7 min. Specific primers for each gene analyzed, annealing temperatures, and amplicon sizes are shown in the Table 2. 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. PCR size products were verified by 2% agarose gel electrophoresis. For each pool of cDNA used, Pitx2c expression was confirmed. 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 always run in triplicates using 5S as normalizing control, as recommended by the manufacturer. SyBR Green was used as quantification system on a Stratagene Q-Max 2005P qRT-PCR thermocycler. Relative measurements were calculated as described by Livak & Schmittgen (39) and control measurements were normalized to represent 100% as previously described (40). Specific primers for each miRNA analysed are shown in the Table 2.

In situ hybridization

In situ hybridization was performed in serial cryosections of limb muscles obtained from C57BL/10 by using double DIG-labeled LNA oligos (Exiqon) or antisense RNA probes was carried out as previously described (41). Combined immunohistochemical detection was performed as previously described (42) by using anti-Pax7 antibody (Developmental Hybridoma Bank).

Immunocytochemistry

Immunocytochemistry experiments in 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), anti-PHH3 (Millipore). For immunofluorescence the following fluorochrome conjugated secondary antibodies were used for
visualization: anti-rabbit Alexa-546 (Invitrogen). Nuclear staining was performed using DRAQ-5™ (Red Fluorescent Cell-Permeable DNA probe, Biostatus Limited). Immunofluorescent detection was performed by confocal analyses using a Leica TCS SL confocal microscope (Leica LCS Version 2.0). Quantification was performed at 10x of magnification for PHH3 positive cells and at 20x for Ki67 and Myf5 positive cells; each experimental point is represented by the average of the analysis of five different images for each independent experiment and repeated at least in three different biological samples to have a representative average.

Chromatin immunoprecipitation ChIP assay

ChIP assays were performed as described elsewhere (43) with modifications. Sol-8 cells were transfected with 8-µg of pcDNA-V5-Pitx2c plasmid in 100 mm dishes. After 24h of Pitx2c transfection, the cells were cross-linked with 1% formaldehyde for 10 minutes at 37°C. For chromatin immunoprecipitation the antibodies used were anti-V5 (clone V5-10, Sigma) or Pol II (8WG16) (Santa Cruz), antibody against mouse dystrophin was used as mouse IgG control. 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 sequences for mir15b, miR-106b, miR-503 and miR23b (Table 2). 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 internal positive control of the ChIP assays, which was observed in all DNA regions analysed.

Statistics

Data are presented as the mean with standard error bars representing the standard deviation. Data were analysed by Student’s t-test for significance and considered significantly different if p < 0.01.

RESULTS
Pitx2-mediated microRNA expression in skeletal myoblasts

Pitx2 is expressed in developing myoblasts very early in development and it has recently been demonstrated to play a pivotal role in regulating key myogenic steps. We have previously documented that Pitx2c is key in modulating proliferation vs. differentiation and balancing different progenitor cell populations during myogenesis. Furthermore, we have shown that Pitx2c post-transcriptionally modulates key myogenic transcription factors such as Pax3 by repressing miR-27 expression (18, 19). To further analyse the role Pitx2c in the post-transcriptional control of myogenesis, we have performed microRNA microarray analyses using Pitx2c overexpressing Sol8 myoblasts. Because we have previously demonstrated that Pitx2c-mediated effects on myoblast is dose-dependent, we have used two different doses of CMV-Pitx2c plasmid (400 and 800 ng/ml of CMV-Pitx2c plasmid) which previously affected myoblast proliferation and differentiation (19), thereby providing insights into the dose dependency of Pitx2-regulated miRNAs (31). Figure 1A illustrates hierarchical clustering of differentially expressed and statistically significant miRNAs after 24 h of cell transfection. From 497 miRNAs, 60 (~10%) displayed statistically significant differences, thus suggesting that they are regulated by Pitx2. Detailed analyses demonstrate at least four distinct expression patterns. Small proportions of miRNAs display higher levels (7/60; ~11%) or lower levels at both doses of CMV-Pitx2c plasmid transfections (Figure 1A). Most miRNAs (40/60; ~67%) display significantly lower expression levels only at Pitx2c doses previously shown to have more profound effects on myoblast phenotype (800 ng/ml of CMV-Pitx2c plasmid transfections) (Figure 1A) while only a minority (1/60; ~1%) increased at that dosage. The remaining miRNAs displayed a transient increase (2/60; ~3%) or decrease (3/60; ~5%) at low doses (400 ng/ml of CMV-Pitx2c plasmid transfections). Overall, these data illustrate that Pitx2 regulates the expression of different subsets of miRNAs. Importantly, most miRNAs regulated by Pitx2 in skeletal-muscle myoblasts display lower levels (50/60; ~83%) and only a small proportion (10/60; ~17%) were higher. We have validated our microarray expression data by using qRT-PCR. As illustrated in Figure 1B and Figure 2A miR-1, miR-15a, miR-15b, miR21, miR-23a, miR-23b, miR-106b, miR-130, miR133 and miR-503 displayed significantly lower levels after Pitx2c overexpression whereas...
siRNA against Pitx2 in Sol8 myoblasts resulted in up-regulation of miR-1, miR-15a, miR-15b, miR21, miR-23a, miR-23b, miR-106b, miR-130, miR133, and miR-503 (Figure 1C Figure 2B), supporting our microarray observations. Individual miRNAs can target a wide 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 the skeletal-muscle myoblasts, we have undertaken a gene-ontology analyses using DAVID software (44). These gene-ontology analyses revealed that the Pitx2 up-regulated and Pitx2 down-regulated miRNAs might modulate highly similar pathways: transcription, regulation of transcription, cell morphogenesis, and cell proliferation (Supplementary Table S1). In addition, cell projection, cell organization, cell motility, cell proliferation, and cell maturation were also revealed for Pitx2 down-regulated miRNAs (Supplementary Table S1). Overall, these data support the notion that cell behaviour and proliferation pathways induced by Pitx2 could be driven by microRNA expression in myoblasts.

A role for Pitx2-mediated microRNAs regulating myoblast cell proliferation

Previously we have demonstrated that over-expression 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 over-expression raises levels of Pax3 expression, which thus in turn inhibits Myod and myogenin expression and terminal differentiation, a process mediated by Pitx2 regulation of miR-27 (19). However, cell proliferation in Pitx2c-overexpressing myoblast is independent of Pax3 and/or miR-27 regulation (19). Additionally, a role of Pitx2 controlling myocyte number on the chick myotome has been reported (45) suggesting that the effect of Pitx2 on cell proliferation in myogenic cells is conserved in different vertebrate species. Thus, we focus our attention on whether deregulation of other miRNAs, mediated by Pitx2, can affect cell-cycle regulation. We bioinformatically analysed the putative microRNA targeting key genes of cell-cycle regulation, such as cyclin D1 (ccnd1) and cyclin D2 (ccnd2) and we found that more of 65% of the predicted target sites for microRNA in ccnd1 (8/12) and ccnd2 (11/16) 3’UTRs correspond to
four down-regulated miRNAs in Sol8 after Pitx2c over-expression (Figure 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 on the regulation of cell proliferation on skeletal-muscle cells has been not previously reported. 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 miR-503, we performed transfection experiments with these miRNAs on Sol8 myoblast at low confluence (10^5/well) and assessed cell proliferation by phospho-histone 3 immunolabelling after 24 h of transfection. In addition, cyclin D1 and cyclin D2 expression levels were measured by qRT-PCR. As reflected in Figures 4A and 4B, transfection experiments with a cocktail of these miRNAs, resulted in 80% lower expression levels of cyclin D1 and cyclin D2. In line with these findings, cells displayed lower phospho-histone 3 immunolabelled index and thus were clearly proliferating at a slower pace, as illustrated in Figures 4C-G. To test whether the predicted miR-15b, miR-23b, miR-106b, and miR-503 elements in the 3'-UTR of cyclin D1 and cyclin D2 were functional, we ligated these sequences downstream of the luciferase gene in the pGLuc-Basic vector and co-transfected independently with pre-miR-15b, pre-miR-23b, pre-miR-106b, and pre-miR-503 into Sol8 cells (Figures 4H-I). Luciferase activity for cyclin D1 and cyclin D2 3'-UTRs was approximately halved with co-transfection of pre-miR-15b, pre-miR-23b, pre-miR-106b, and pre-miR-503 compared with 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 cyclin D1 and cyclin D2 3'-UTRs eliminated such repression (Figures 4H-I). Transfection experiments with pre-miR-15b, pre-miR-23b, pre-miR-106b, and pre-miR-503a separately decreased the expression levels of cyclin D1 and cyclin D2 only 50-60% (Figure 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 miR-15b, miR-23b, miR-106b, and miR-503 genetic loci. Five conserved Pitx2 binding sites were identified ~6 kb
354 upstream of miR-15b and miR-106b genetic loci, four sites ~6 kb upstream of miR-503 genetic
loci and one site ~6 kb upstream of miR-23b gene locus (Figure 6A). To test the interaction of
355 Pitx2 with those putative binding sites, we performed chromatin immunoprecipitation (ChIP)
356 assays in Sol8 cells. Exogenous Pitx2 bound to the all-putative binding sites upstream of miR-
357 15b, miR-23b, miR-106b, and miR-503 genetic loci, as illustrated in Figure 6A. RNA polymerase
359 II occupancy suggests that all DNA regions tested are transcriptionally active (Figure 6B). Taken
360 together, these data point out a Pitx2-miRNAs pathway controlling the expression of key
361 regulatory cell-cycle genes, which in turn modulate cell proliferation in myoblasts.
362
363 Pitx2-mediated miRNAs and regulatory cell-cycle gene expression are de-regulated in
364 conditional tissue-specific Pitx2 mutant mice
365 First, we checked the expression pattern for miRNAs regulated by Pitx2c in serial section
366 obtained from wild-type mouse limb muscles by LNA-in situ Hybridization. As illustrated in
367 Figures 7A, miR-15b, miR-23b, miR-106b and miR-503 display a tissue expression pattern
368 compatible with marked expression on satellite cells. Secondly, to determine whether Pitx2c-
369 mediated miRNA regulation on myogenic cells is maintained in vivo, we used qRT-PCR to
370 analyse the expression levels of miRNA regulated by Pitx2c in conditional tissue-specific Pitx2
371 mutant mice by intercrossing a Pitx2 floxed mouse line with a Cre deleter mouse line, which
372 rendered muscle-lineage-specific Pax3Cre recombination. This conditional mutant mouse line is
373 currently being analysed and characterized (Lozano–Velasco et al., in progress). Because
374 Pax3cre+/Pitx2+/- homozygote mutant neonates were born alive but died soon after birth, in the
375 present work we analysed adult heterozygote and neonatal mutants. Notably, limb muscles from
376 Pax3cre+/Pitx2+/- heterozygote mice, in which Pitx2 expression is reduced by more than 50%
377 (Figure 8A), display higher expression levels of miRNAs regulated by Pitx2c (miR-15b, miR-23b,
378 miR-106b and miR-503) than Pax3Cre+/Pitx2+/- control limb muscles (Figure 7B). Moreover,
379 given that even in the absence of overt damage myonuclear turnover in rodents is 1-2% per week
380 (46) we also analysed the expression levels for cyclin D1 and cyclin D2 genes and we found that
381 were clearly down regulated in Pax3Cre+/Pitx2+/- animals in comparison with control mice
These observations are consistent with our in vitro findings revealing the existence of a Pitx2-miRNAs pathway controlling the expression of key regulatory cell-cycle genes in myogenic cells. Similarly to adulthood, limb muscles from Pax3cre+/Pitx2cre+/Pitx2cre+/Pitx2+/Pitx2-/- neonatal heterozygote and homozygote mice, which exhibited lower Pitx2c expression levels (Figure 8B), showed increased levels of miRNAs regulated by Pitx2c. Curiously, no changes of cyclin D1 and cyclin D2 expression levels were detected in neonatal mutants pointing out a different status for this Pitx2-miRNA pathway in neonates (Figures 7D-E).

Pitx2-miRNAs pathway-regulating cell proliferation is conserved in early-activated satellite cells

Given that it has been 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 (47), we next investigated whether the Pitx2-miRNAs pathway is also present in freshly isolated adult satellite cells. Because the adult satellite cells isolated from freshly dissected mouse-muscle tissue rapidly initiate the process of activation, leading to myogenic differentiation (48), 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 behaviour, as previously described (33, 49) as well as EPa-derived differentiating myoblasts fusing to myotubes (Figure 9A). As illustrated in the Figure 9B, EPq can thus be rendered as Pax7 high/low-proliferating cells and EPa cells as Pax7 low-expressing/high-proliferative satellite cells (50). Notably, Pitx2c expression levels are higher in the early-activated EPq cells as compared with long-term-activated satellite cells (EPa) and display low expression levels in the differentiating myotubes derived from satellite cells (Figure 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 on EPq and EPa satellite cells showed that Pitx2c overexpression leads to a clear
cyclin D1 and D2 up-regulation in early-activated satellite cells (EPq) but not in long-term activated satellite cells (EPa) (Figure 10A-C). Moreover, the number of Ki67 positive cells was significantly higher in EPq cells overexpressing Pitx2c as compared to cells transfected with the empty lentiviral vector (Figure 10D-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 analysed miRNA expression profiles on EPq and EPa cells overexpressing Pitx2c and our analyses showed that all miRNAs were dramatically down-regulated after Pitx2c overexpression (Figure 11A). Moreover, the miRNAs miR-15b, miR-23b, miR-106b, and miR-503 display an expression profile complementary to Pitx2c during the process of in vitro differentiation (Figure 11B), thus reinforcing the notion that Pitx2c acts negatively to regulate those 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 D2 expression in EPq cells may indicate that Pitx2c-miRNA pathway participate in cell-proliferation expression at an early step of activation but that other regulatory molecules contribute to maintain cell proliferation after activation is triggered. In accordance with that idea, in neonatal Pax3cre+/−/Pitx2c−/− homozygote mice, altered expression of Pitx2c-regulated miRNAs did not lead to cyclin D1 and D2 dysregulation (Figure 7). Since it has been previously showed that at neonatal stages many satellite cells are in a permanent stage of activation to ensure muscle growth (11), those finding emphasize the role of Pitx2 on cell proliferation in the onset of satellite cell activation but not when the cell activation processes have already taken place. Finally, as observed in Sol8 myoblasts, transfection experiments with a cocktail of these miRNAs in EPq satellite cells resulted in a clear down-regulation of cyclin D1 and cyclin D2 (Figure 11C-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 up-regulation reinforcing the notion that those miRNAs mediate the Pitx2c effects on cyclin D1 and cyclin D2 gene expression.
(Figure 12). Therefore, all together, these findings indicate that Pitx2c-miRNA pathway modulating cell proliferation is also present in satellite cells.

430 Pitx2c enhances Myf5+ satellite-cell population by regulating miR106b

431 After activation, satellite stem cells expand and undergo symmetric and asymmetric divisions in vivo and in vitro (51,52). Symmetric divisions result in the symmetric expansion of Pax7+/Myf5-
432 satellite cells retaining its stem-cell identity. Through asymmetric divisions, one daughter cell
433 retains its stem cell identity (Pax7+/Myf5-); and the other daughter cell upregulates myogenic
434 factor 5 (Myf5) and becomes Pax7-Myf5+, representing more committed myogenic progenitors
435 that participate in skeletal-muscle growth and regeneration (48, 52). Myf5 is one of the most
436 relevant transcription factors that play a key role as the determinant of the acquisition of a
437 myogenic cell fate in satellite cells (53,54). Therefore, in order to test whether Pitx2c-mediated
438 effects on cell proliferation can modulate the rate of myogenic commitment of satellite cells, we
439 analyse Myf5 expression by qRT-PCR and immunohistochemistry. As illustrated in Figure 13A,
440 Myf5 mRNA expression levels were up-regulated in Pitx2c-overexpressing satellite cells with
441 respect to control. In addition, the percentage of Myf5+ cells was significantly higher after Pitx2c
442 overexpression in satellite-cell cultures (Figure 13B-C). These results indicate that Pitx2c
443 increase the number of the Myf5+ satellite cells.

444 Notably, the bioinformatic analyses by TargetScan showed that Myf5 is a predicted target for the
445 Pitx2c-regulated miRNA miR-106b (www.targetscan.org). To validate Myf5 as a target for miR-
446 106b, we performed pre-miR-106b transfection experiments in satellite cells and, as displayed in
447 Figure 13D, miR-106b overexpression leads to Myf5 down-regulation. Luciferase reporter assays
448 further validated Myf5 as a direct target for miR-106b (Figure 13E). Additionally, pre-miR-106b
449 transfection in Pitx2c-overexpressing cells rescued Myf5 up-regulation to basal levels (Figure 14
450 A-C) supporting the idea that miR-106b is key in mediating the Pitx2c effect on Myf5 gene
expression. Thus, since we have shown 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 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 (18, 19). 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 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 miRNAs expression and also indispensable to regulate 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 controlling microRNA expression in myoblasts. It has been recently reported that Pitx2 positively regulates miR-17-92 and miR-106b-25 in the heart (55); 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. Gene-ontology analyses have revealed that the miRNAs regulated both positively and negatively by Pitx2c might lead to modulation of signalling pathway that control focal adhesion, adherens junction and actin cytoskeleton expression, providing the bases of abnormal cell fusion (18,19), whereas the
miRNAs down-regulated by Pitx2c also might modulate the cell-cycle progression in accordance with the previously reported Pitx2 functions in cell proliferation (56, 18, 57, 58, 19, 47). Here 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. Furthermore, we show evidence that this Pitx2-miRNA pathway 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 (59, 60, 61, 62) 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. (2013) (21) could be explained by the moderate Pitx2c expression achieved in their in vitro gain-of-function experiments as argued by the authors (21). 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 (47). 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 Pitx2c expression was higher in early-activated satellite cells than in long-term activated, and our in vitro Pitx2c gain-of-function experiments reveal that Pitx2c stimulates cyclin D1 and D2 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-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 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 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 (63), 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 myofibres by fusion to repair the damaged muscle or they can renew the satellite-cell population and return to a quiescent state (38). Quiescent satellite cells expressing paired box 7 (Pax7) but low or undetectable levels of the myogenic regulatory factors Myf5 and MyoD, upon activation undergo symmetric and asymmetric divisions. While symmetric expansion of Pax7+/Myf5 satellite stem cells assure maintenance of the Pax7+/Myf5- undifferentiated population, asymmetric divisions generate Pax7+/Myf5+ and Pax7+/Myf5- daughter cells (38). 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 (64) 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 (64). 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 (53). 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.

In conclusion, in this manuscript we report 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.

**Author Contribution**

Estefanía Lozano-Velasco: Collection and/or assembly of data

Daniel Vallejo: Collection and/or assembly of data
Acknowledgments

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References


44. http://david.abcc.ncifcrf.gov/


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53.- Kassar-Druchossoy L, Gayraud-Morel B, Gomès D, Rocancourt D, Buckingham M,
Shinin V, Tajbakhsh S. 2004. Mrf4 determines skeletal muscle identity in Myf5: Myod double-
mutant mice. Nature.431(7007):466-71

54.- Gayraud-Morel B, Chrétien F, Jory, A, Sambasivan R, Negroni E, Flamant P,


56.- Kioussi C, Briata P, Baek SH, Rose DW, Hamblet NS, Herman T, Ohgi KA, Lin C,


64.- Crist CG, Montarras D, Bckingham M. 2012. Muscle satellite cells are primed for myogenesis but maintain quiescence with sequestration of Myf5 mRNA targeted by microRNA-31 in mRNP granules. Cell Stem Cell. 11(1):118-26
Figure 1: A: Hierarchical clustering of statistically significant microRNA microarray expression profiles on Sol8 cell line transfected with 400 ng/ml and 800 ng/ml of CMV-Pitx2c plasmid at 24 h after transfection. Colour 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 Pitx2c plasmid as compared to controls. C: Expression profiles of the statistically significant miRNAs by qRT-PCR in Pitx2c silenced Sol8 cells (siRNA Pitx2c) as compared to controls.

Figure 2: 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 3: 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 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) as compared to control cells. C and D: Representative images of Sol8 cells transfected with miRNAs cocktail and control cell cultures. E and F: Representative images of immunohistochemical PHH-3 staining in Sol8-cell line overexpressing miRNAs as compared to controls. The inset represents close-ups of the corresponding immunostained cells. G: Quantification PHH-3+/total cells in Sol8- transfected cells as compared to controls after 24h of miRs transfection. H and I: Normalized luciferase activity of the 3'-UTR cyclin D1 and cyclin D2 luciferase reporter (WT cyclin D1 and wt cyclin D2 3'-UTR) with empty plasmid or pre-miRNAs shows loss of luciferase activity with expression of miR-15b, miR-23b, miR-106b, and miR-503. There is no loss of luciferase activity when the miRNAs seed sequences were mutated.
Figure 5: 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 6: A: ChIP assays: Pitx2 binds DNA regions upstream of miR-15b, miR-23b, miR-106b, and miR-503 genetic loci. Observed enrichment in Pitx2 binding within all miRNAs analysed. ***p<0.001, **p<0.01, *p<0.05. These experiments were performed in Sol8 cells. B: 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 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 present a tissue distribution very similar to satellite cells as illustrate by Pax7 co-staining for miR-106b. LNA probe with a scrambled sequence has been used to test the specificity of the probes. B: Expression profile for miR-15b, miR-23b, miR-106b, and miR-503 in Pax3cre+/−/Pitx2+− heterozygote mice (n=9) as compared to Pax3cre−/+ control mice (n=8). C: Cyclin D1 and D2 expression profile on Pax3cre−/+ heterozygote mice. D: Expression profile for miR-15b, miR-23b, miR-106b, and miR-503 in Pax3cre−/+/Pitx2+− heterozygote (n=6) and Pax3cre−/+/Pitx2−/+ homozygote neonates (n=5) as compared to Pax3cre−/+ control neonatal mice (n=6). E: Cyclin D1 and D2 expression profile on Pax3cre−/+/Pitx2+− heterozygote and Pax3cre−/+/Pitx2−/+ homozygote neonates vs. Pax3cre−/+ control neonatal mice.

Figure 8: 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 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-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 10: A: Representative images of EPq transfected with Lentivirus-Pitx2c-ZsGreen vector (LVX-Pitx2c). B: qRT-PCR for Pitx2c expression in EPq and EPa cells transfected with LVX-Pitx2c with respect to cells transfected with the empty LVX-ZsGreen lentiviral vector (LVX). C: Cyclin D1 and D2 expression in EPq and EPa Pitx2c-overexpressing cells respect to control cells. D: Representative images of immunohistochemical analyses for Ki67-positive cells on EPq transfected with Lentivirus-Pitx2c-ZsGreen vector (LVX) as compared to cells transfected with the empty LVX-ZsGreen lentiviral vectorLVX-Pitx2c. E: Percentage of Ki67+ cells on EPq transfected with Lentivirus-Pitx2c-ZsGreen vector respect to cells transfected with the empty LVX-ZsGreen lentiviral vector. EPq: early-activated satellite cells; EPa: long-term activated satellite cells.

Figure 11: A: Expression profile for miR-15b, miR-23b, miR-106b, and miR-503 in EPq and EPa Pitx2c-overexpressing cells with respect to control cells. B: Relative expression 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 D2 down-regulation.

Figure 12: 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).

Figure 13: A: Myf5 expression profile on EPq Pitx2c-overexpressing cells. B: Representative images of immunohistochemical analyses for Myf5-positive cells in EPq transfected with
Lentivirus-Pitx2c-ZsGreen vector (LVX) as compared to cells transfected with the empty LVX-ZsGreen lentiviral vector (LVX-control). C: Percentage of Myf5+ cells on EPq transfected with Lentivirus-Pitx2c-ZsGreen vector (LVX-Pitx2c) respect to cells transfected with the empty LVX-ZsGreen lentiviral vector (LVX-Pitx2). D: miR-106b overexpression leads to Myf5 up-regulation in EPq cells in E. F: Normalized luciferase activity of the 3'-UTR Myf5 luciferase reporter (WT Myf5 3'-UTR) with empty plasmid (Vector) or pre-miR-106b shows the loss of luciferase activity with expression of miR-106b. There is no loss of luciferase activity when the miR-106b seed sequence was mutated.

Figure 14: A: Pitx2c overexpression is maintained after Pre-miR-106 transfection in EPq cells. Pre-miR-106b transfection in EPq cells overexpressing Pitx2c (B) rescue Myf5 expression at the basal levels (control cells)(C).
Figure 1

A

B

C

miRs relative expression

miR-1 miR-15a miR-15b miR-21 miR-23a miR-23b miR-106b miR-130 miR-133 miR-503

0

5

10

15

50

100

150

Pitx2c-overexpression

Control

siRNA-Pitx2c

miR-1 miR-15a miR-15b miR-21 miR-23a miR-23b miR-106b miR-130 miR-133 miR-503

0

200

400

600

800

1000

miRs relative expression

siRNA-Pitx2c

Control

Pitx2 (800 ng/ml)

Pitx2 (400 ng/ml)

siRNA-Pitx2c
Figure 2

A  

Pitx2c

Control 400 ng/ul 800 ng/ul

mRNA expression levels

****

B  

siPitx2c

Control Pitx2c Pitx3

mRNA expression levels

***
Figure 4

A) mRNA expression levels of Cyclin D1

B) mRNA expression levels of Cyclin D2

C) Control

D) miRNAs

E) PHH3+ cells

G) % PHH3+ cells
Figure 5

A

- **miR15b**
  - Control
  - miR15b

- **miR106b**
  - Control
  - miR106b

- **miR23b**
  - Control
  - miR23b

- **miR503**
  - Control
  - miR503

B

- **Cyclin D1**
  - Control
  - miR-15b
  - miR-23b
  - miR-106b
  - miR-503

- **Cyclin D2**
  - Control
  - miR-15b
  - miR-23b
  - miR-106b
  - miR-503
Figure 6

A

miR15b ChiP

miR23b ChiP

miR106b ChiP

miR503 ChiP

B

RNApol2 ChiP

Gapdh Promoter

miR15b.1 miR15b.2 miR15b.3 miR15b.4 miR15b.5

miR23b

miR106b

miR503

miR15b'

miR23b'

miR106b'

miR503'

on July 7, 2017 by guest http://mcb.asm.org/ Downloaded from
Figure 7

A

Scramble
miR-23b
miR-15b

miR-503
miR-106b
miR-106b Pax7

B

miRNA-23b

miRNA-15b

miRNA-503

miRNA-106b

C

Cyclin D1

Cyclin D2

D

miRNA-503

miRNA-106b

E

Cyclin D1

Cyclin D2
Figure 8

A

B

mRNA expression levels

$\text{Pitx2c}$

(Adults)

mRNA expression levels

$\text{Pitx2c}$

(Neonates)
Figure 9

A  

B  

Cyclin D2  

Myf5  
Pax7  

C  

mRNA expression levels

EPq  

EPA  

Myotubes  

Figure 9
**Figure 10**

A: Image showing aggregates of cells with green fluorescence.

B: Graph showing mRNA expression levels of Pitx2c with error bars.

C: Graph showing mRNA expression levels of Cyclin D2 with error bars.

D: Images of cell nuclei stained with LVX and LVX-Pitx2c, with Ki67+ cells indicated.

E: Graph comparing Ki67+ cell counts between LVX and LVX-Pitx2c with error bars.
Figure 11

A. miR relative expression

miR-15b

miR-106b

miR-503

miR-23b

B. mRNA expression levels

C. miR relative expression

D. mRNA expression levels

Cyclin D1

Cyclin D2

Control

miRNAs Transfected

Transfected

Control
Figure 12

A. Pitx2c mRNA expression levels

B. miRNAs relative expression

C. Cyclin D1 mRNA expression levels

Cyclin D2 mRNA expression levels
Figure 14

A: mRNA expression levels of Pitx2c

B: miR-106b relative expression

C: mRNA expression levels of Myf5

** and *** indicate statistical significance.
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Table 2: Specific primers for each gene/miRNA analyzed, annealing temperatures, and amplicon sizes

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