Developmental Biology 357 (2011) 165–178

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Pitx2c modulates Pax3+/Pax7+ cell populations and regulates Pax3 expression by repressing miR27 expression during myogenesis

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ARTICLE INFO

Article history:
Received for publication 7 January 2011
Revised 13 June 2011
Accepted 14 June 2011
Available online 1 July 2011

Keywords:
Pitx2
Myogenesis
Pax3
Pax7

ABSTRACT

Pitx2 is a paired-related homeobox gene that is expressed in muscle progenitors during myogenesis. We have previously demonstrated that overexpression of Pitx2c isoform in myoblasts maintained these cells with a high proliferative capacity and completely blocked terminal differentiation by inducing high Pax3 expression levels (Martinez et al., 2006). We now report that Pitx2c-mediated proliferation vs. differentiation effect is maintained during in vivo myogenesis. In vivo Pitx2c loss of function leads to a decrease in Pax3+/Pax7− cell population in the embryo accompanied by an increase of Pax3+/Pax7+ cells. Pitx2c transient-transfection experiments further supported the notion that Pitx2c can modulate Pax3/Pax7 expression. Pitx2c but not Pitx3 controls Pax3/Pax7 expression, although redundant roles are elicited at the terminal myoblast differentiation. Contrary to Pitx2c, Pitx3 does not regulate cell proliferation or Pax3 expression, demonstrating the specificity of Pitx2c mediating these actions in myoblasts. Furthermore we demonstrated that Pitx2c modulates Pax3 by repressing miR27 expression and that Pax3-miR-27 modulation mediated by Pitx2c is independent of Pitx2c effects on cell proliferation. Therefore, this study sheds light on previously unknown function of Pitx2c balancing the different myogenic progenitor populations during myogenesis.

Introduction

The skeletal muscles of the body (trunk and limbs) derive from transitory mesodermal structures called somites that are generated by a segmentation process of the paraxial mesoderm, known as somitogenesis, which involves complex cellular and molecular events. Somitogenesis starts at about E7.75 in the mouse embryo and continues until the species-specific number of somites is reached (Tam, 1981). As the somite matures, myogenic progenitor cells become confined to the dorso-lateral part of the somite: the dermomyotome. Skeletal myogenesis is then initiated in myogenic cells originating from the dermomyotome lips that differentiate to form primary muscle fibers. Subsequently, a progenitor population expressing Pax3 and Pax7 arises from the central portion of the dermomyotome and is maintained throughout embryogenesis within the developing skeletal muscles (Buckingham et al., 2003). These cells can either activate the myogenic program through the action of Myf5 and MyoD or remain as a proliferating progenitor population that resides within the muscle mass (Gros et al., 2005; Relaix et al., 2005). In late-stage fetal muscle, these cells adopt a satellite cell position, suggesting that they can become the pool of adult satellite cells responsible for postnatal skeletal muscle growth and integrity (Relaix et al., 2005). Although the precise influences of Pax7 and Pax3 on myogenic progression remain a subject of debate, it has been shown that Pax3 and Pax7 downregulation is required to properly complete myogenic differentiation (Buckingham and Vincent, 2009; Crist et al., 2009).

It has been previously reported that members of the Pitx gene family of the bicoid class of homeodomain proteins Pitx2 and Pitx3 are expressed in myotomes and migrating myoblasts (Logan et al., 1998; Zhao et al., 2004). A more detailed analysis on the expression of Pitx2 and Pitx3 during myogenesis revealed that Pitx2 is first expressed in muscle progenitor cells; this expression is followed and partly overlapping with the appearance of Pitx3 in differentiated muscle cells. *Pitx3* then is maintained in all skeletal muscles of the body and limbs while Pitx2 expression decreases thereafter (L'Honoré et al., 2007). Importantly, these authors showed that Pitx3 deficiency (Pitx3−/− mutant mice) is compensated by the maintenance of Pitx2 expression in all skeletal muscle, suggesting that Pitx2 and Pitx3 have redundant roles (L'Honoré et al., 2007). Additionally, recent reports have proposed that Pitx2 can act as an upstream activator of myogenesis in the extraocular muscles and to cooperate with the Myf5/Myf4 pathway to control somite-derived myogenesis (L'Honoré et al., 2010; Zacharias et al., 2010).

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doi:10.1016/j.ydbio.2011.06.039

We have previously demonstrated that overexpression of *Pitx2c* in myoblasts, the main *Pitx2* isoform expressed in this cell type, maintains these cells with a high proliferative capacity while cell fusion into myotubes and terminal differentiation were blocked. Pitx2c overexpression led to increased cell proliferation by modulating *c-myc*, *cyclin D1* and *cyclin D2* expression, without changes in *Pitx2a* and *Pitx2b* expression levels, indicating thus a c-isoform-specific effect on myocyte proliferation (Martínez-Fernández et al., 2006). Furthermore, Pitx2c overexpression increased *Pax3* expression, providing thus a means to inhibit terminal differentiation, observed by decreased expression levels of muscle-determining transcription factors (*MyoD* and *Myogenin)* and terminal differentiation markers (myosin isoforms). Thus, these data indicate that Pitx2c is implicated in the regulation of Pax3 expression in the myoblast although it remains to be explored in detail the genetic interactions between these two transcription factors.

Several recent reports have identified post-transcriptional control mediated by miRNAs as a crucial level of regulation of myogenesis. In this context, miRNAs have been shown to play crucial roles in muscle development and in regulation of muscle cell proliferation and differentiation (Chen et al., 2006; Kim et al., 2006). Regarding the skeletal muscle progenitor cells, Crist et al. (2009) recently demonstrated that miR-27b directly targets the 3-UTR of *Pax3* mRNA and affects *Pax*3 expression both in vitro and in vivo. These authors showed that this miRNA is expressed in the differentiating muscle masses (myotome) of the embryo as well as in differentiating satellite cells. They also demonstrated that miR27-mediated Pax3 down-regulation has consequences for the onset of skeletal muscle differentiation leading to robust entry into the myogenic differentiation program. More recently, Chen et al. (2010) have shown that miR-1 and miR-206 facilitate satellite cell differentiation by restricting their proliferative potential and regulating Pax7 expression. Therefore, together these reports indicate that miRNAs have a relevant role regulating the myogenic gene program transitions from proliferation to differentiation.

In this study, we further explore the role of Pitx2c in the activation of myogenic differentiation program as well as its putative interaction with *Pax3*. We find that in vivo Pitx2c loss of function leads to a clear decrease in Pax3+/Pax7− cell population accompanied by an increase of Pax3+/Pax7+ cells. *Pitx2c* transient-transfection experiments further support the notion that Pitx2c could modulate *Pax3*/*Pax7* expression in the myogenic precursor cells. Moreover we provide evidence that Pitx3 did not play a key role regulating cell proliferation or *Pax3* expression, demonstrating the specificity of Pitx2c mediating these actions in myoblasts. Furthermore, we show that Pitx2c modulates miR-27 expression thereby providing a mean for the control of Pax3 expression by Pitx2 during myogenesis.

Material and methods

Pitx2c mutants' embryos

Pitx2c−/− mutants were previously developed as an in vivo model of Pitx2c loss of function. Pitx2c−/− mutant embryos (Liu et al., 2002) were kindly provided from Nigel Brown laboratory (St George Hospital, London). For morphometric analyses, embryos at E10.5 and E12.5 stages were fixed in paraformaldehyde and stored in 70% ethanol. These embryos were examined and photographed using a light microscope (Olympus BX50 microscope, Japan) and camera (Olympus, Japan), limb length, limb area and body area were evaluated by using the image J software (NIH Image). The results were presented as mean ± standard error of the difference between populations and statistical significance was established by Student *t* test. For immunohistochemical analysis Pitx2c−/−; Pitx2c+/− and wild type (wt) littermate embryos at E10.5 and E12.5 stages were processed as previously described (Domínguez et al., 2005).

Cell culture

The Sol8 myoblast cell line when cultured in the presence of 10% FBS proliferates as mononucleated cells but terminally differentiate into skeletal myocytes and fuse to form multinucleated myotubes when grown to confluence and deprived of growth factors (Daubas et al., 1988; Yaffe and Saxel, 1977). Mitotic Sol8 mouse myoblasts (Daubas et al., 1988) were cultured in growth medium (GM), consisting of DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 50 U/ml penicillin–streptomycin. When the cells reached confluence, GM was switched to differentiation medium (DM), consisting of DMEM supplemented with 2% horse serum. Contractile myotubes were observed after 4 days. Growth and differentiation media were replaced by fresh medium every 48 h.

DNA plasmid and cell transfection assays

Plasmid constructs

p_CMV-Pitx2c. Pitx2c (NM_001042502.1) open reading frame (ORF) was amplified from Sol8 myoblasts cDNA by PCR with specific primers containing *HindIII* and *XbaI* restriction sequences. Subsequently, PCR product was inserted into the pcDNA3.1(+) plasmid (Invitrogen) by using *HindIII*/*XbaI*, to generate p_CMV-Pitx2c.

p_CMV-Pitx3. To generate the pCMV-Pitx3 plasmid, Pitx3 (NM_008852.4) ORF was removed from commercial pBluescript-Pitx3 vector (EUROFINS) by *HindIII* digestion and cloned into pEGFP-N1 (Clontech), in which the EGFP ORF was previously removed by *HindIII*/*XbaI* digestion.

Pitx2c and Pitx3 transfection assays

Three independent transfections were performed in Sol8 myoblasts with three different plasmids: CMV-Pitx2c, CMV-Pitx3 and CMV-EGFP. The cells were seeded at 10^5 cells/35 mm dish and transfected in serum free conditions for 5 h with 2, 4 and 8 μg of CMV-Pitx2c, CMV-Pitx3 or CMV-EGFP plasmids by using the Lipofectamine Reagent (Invitrogen) according to the supplier's guidelines. Fresh growth medium was added the morning after transfection, and cells were allowed to grow until they reached confluence. Then the medium was switched to a differentiation medium, and cultured for 4 days. The Sol8 myotubes generated from Sol8 myoblasts transiently transfected with CMV-EGFP were checked for EGFP expression.

siRNA

Sol8 cells and Sol8–Pitx2c-transfected cells (maintaining their proliferative capability after 4 days cultured under differentiation conditions) were transfected in serum free conditions for 5 h with siRNA-Pitx2c (Sigma), at different concentrations (25 nM and 50 nM) by using the Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) following the supplier's guideline.

miR-27 transfection assays

Sol8 cells were seeded at 10^5 cells/35 mm dish and grown for 24 h. Pre-miR-27b and antimiR-27b molecules (Ambion) were transfected by lipofectamine Reagent (Invitrogen) into Sol8 cells at 50 nM according to manufacturer's guidelines, respectively. Negative controls included non transfected cells as well as FAM-labeled pre-miR negative control transfected cells, which also allowed transfection efficiency evaluation. In all cases, transfection efficiencies were greater than 50%, as revealed by observation of FAM-labeled pre-miR. After 4 h of transfection, Sol8 cells were cultured in growth medium and collected at 24 h.

Total RNA extraction and reverse transcription

Total RNA was extracted from control and transfected Sol8 cells respectively, 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 h 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) and a 15-mer oligo-dT primer (Promega) for 1 h at 37 °C according to the manufacturer's protocol. As a reverse transcription negative control, each sample was subjected to the same process without reverse transcriptase.

Quantitative real-time PCR

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. β-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 s, annealing temperature for 30 s and 72 °C for 30 s. 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 1 (Supplementary material). Each PCR reaction was carried out in triplicate and repeated at least in three distinct biological samples to obtain 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. PCR size products were verified by 2% agarose gel electrophoresis. For each pool of cDNA used, Pitx2c overexpression was confirmed. For microRNAs, qRT-PCR was performed using Exiqon LNA microRNA qRT-PCR primers and detection kit according to manufacturer's guidelines. The LNA microRNA primers used are listed on Table 1. All reactions were always run in triplicates using 5S as normalizing control, as recommended by the manufacturers. SyBR Green was used as quantification system on a Stratagene Q-Max 2005P qRT-PCR thermocycler. Each PCR reaction was performed in triplicate and repeated at least in three distinct biological samples to obtain a representative average. For statistical analyses of data sets the unpaired Student's t-tests were used. Significance levels or p values are stated in each corresponding figure legend. Relative measurements were calculated and the controls were normalized to 100% as previously described (Domínguez et al., 2005). As PCR is an exponential system, a twofold increase or reduction in expression is the result of only 1 cycle. Based on these assumptions and to minimize experimental errors, we only consider for statistical analyses the results corresponding at least to twofold differences in gene expression.

Immunohistochemistry

The immunohistochemical reactions carried out in null Pitx2c−/−, Pitx2c+/− and control wildtype embryos were performed by using a sequential double stain kit which allows us to co-localize two different mouse primary antibodies (EnVison G/2 Doublestain kit, DAKO, Denmark) according to manufacturer's guide. Briefly, tissue sections were deparaffinized and incubated in blocking reagents. After incubation with the first primary antibody, immunohistochemical detection was performed by HRP-DAB system and, after treatment with Doublestain Block reagent and incubation with the second primary antibody, the AP-Permanent Red system leads us to visualize the second antigen. Immunohistochemical markers studied were: anti-Pax7 and anti-Pax3 (Hybridoma Bank, Iowa) diluted 1:10, as well as anti-PHH3 (Millipore) diluted 1:100.

For immuohistochemical analyses in culture cells, Pitx2c and Pitx3 transfected cells were fixed for 20 min at room temperature with 4% paraformaldehyde, and immunostained by using anti-myogenin antibody (sc-760, Santa Cruz Biothecnology) as previously described (Lozano-Velasco et al., 2011).

In situ hybridization

Embryos were harvested and fixed for 24 h in 4% paraformaldehyde, washed 3 times in PBS, and stored in methanol for a minimum of 24 h. Endogenous miRNA-27b was detected by using a locked nucleic acid (LNA) probe against miRNA-27b (Exiqon). Prehybridization and hybridization were performed with 60% formamide, at 57 °C. After the washes, embryos were incubated with anti-digoxigenin (DIG) antibody (Roche) overnight at 4 °C and washed extensively for 5 days before staining with BM Purple AP substrate (Roche) as previously reported (Crist et al., 2009).

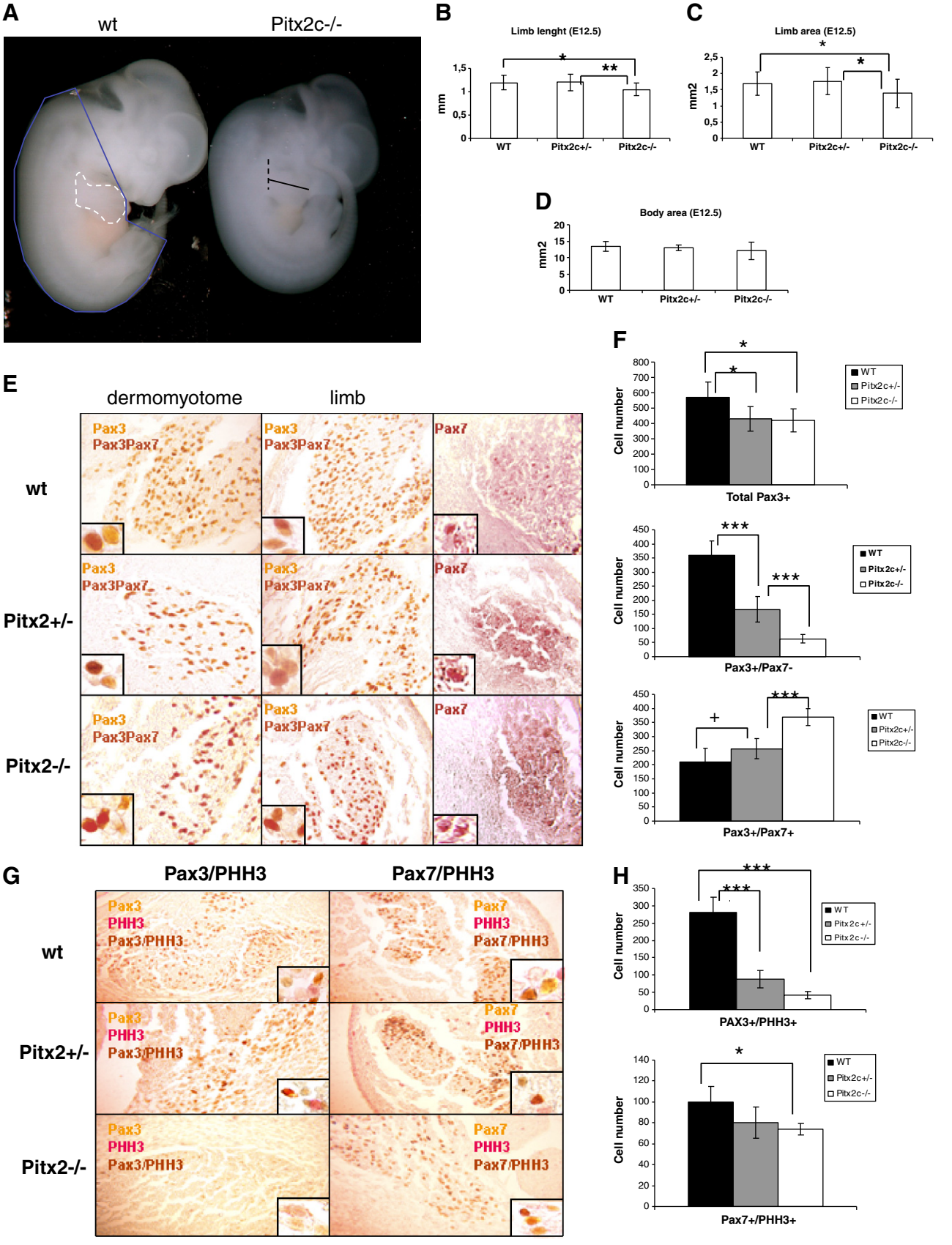
Results

Pitx2c−/− mutant mice exhibit a drastic decrease in Pax3+ precursors

We have previously reported in vitro data demonstrating that Pitx2c overexpression in Sol8 myoblasts leads to Pax3 up-regulation, thus maintaining these cells in a proliferative and undifferentiated state (Martínez-Fernández et al., 2006). During embryogenesis, Pax3 is expressed in all muscle progenitor cells of the early myotome and Pax7 expression is initiated in Pax3+ cells of the central part of the dermomyotome as well as in the muscle progenitors once they have migrated to the limb (Buckingham and Vincent, 2009). Therefore, in order to explore the role of Pitx2c during in vivo myogenesis, we have analyzed the limb phenotype as well as muscle progenitor cell distribution in Pitx2c−/− mutant embryos. Because skeletal muscle progenitor cells originate from demomyotome between E8.5 and E13.5 in the mouse embryo (Tajbakhsh, 2009), analyses of Pitx2c−/− mouse embryos were carried out at E10.5 and E12.5 developmental stages. At the E10.5 stage, the phenotype observed in Pitx2c−/− embryos was similar to that observed for Pitx2c+/− and wild type embryos (data not shown). However, at E12.5 limb length as well as limb area was reduced in Pitx2c−/− mutant (Fig. 1A–B–C) without changes in body area (Fig. 1D) indicating the presence of apparent limb morphological defects in these mutants.

To examine the different types of skeletal muscle progenitors in Pitx2c−/− mutant mice, sequential transverse sections from E12.5 embryos were taken at the level of forelimb (FL) and hindlimb (HL) buds and analyzed by immunohistochemical co-staining of Pax3 and Pax7 and the number of the total Pax3+ cells (Pax7− and Pax7+), Pax3+/Pax7− cells and co-stained Pax3+/Pax7+ cells was analyzed (Fig. 1E, F and Fig. 1A Supplementary data). These analyses showed that the total number of precursor cells expressing Pax3+ (Pax7− and Pax7+) was decreased in the Pitx2c−/− and Pitx2c+/− mice as compared to wild type and the analysis of Pax3+/Pax7− and Pax3+/Pax7+ cell populations revealed that this decrease is mainly due to a dramatic decrease of progenitor cells expressing only Pax3 (Pax3+/Pax7−) while the number of cells co-expressing Pax3 and Pax7 undergoes a mild increase in heterozygote mice but a robust increase in homozygote mutant mice as compared to wild type littermates (Fig. 1F and Fig. 1A Suppplementary data) suggesting a role of Pitx2c balancing the different skeletal muscle progenitor populations during myogenesis. To evaluate the proliferative capacity of Pax3+ or Pax7+ cells in Pitx2c−/− mutants, we performed co-staining with phosphohistone H3 (PHH3) and the number of Pax3+/PHH3+ and Pax7+/

PHH3+ cells was evaluated (Fig. 1G,H). Whereas the number of Pax7+/PHH3+ was moderately reduced in the mutant mice the number of Pax3+/PHH3+ was drastically reduced in Pitx2c−/− and Pitx2c+/− embryos as compared to wild type (Fig. 1H) indicating that Pitx2c acts controlling cell proliferation mainly in the Pax3+ progenitor cells.



Pitx2c modulates proliferation and Pax3 expression in a dose-dependent manner

Analyses of Pitx2c−/− embryos as well as our in vitro results indicate that Pitx2c regulates different genes involved in proliferation and differentiation of myogenic cells. To further investigate how Pitx2c perform those effects we now checked if Pitx2c-modulation on these genes is dose-dependent. For this purpose Sol8 myoblasts were transiently transfected with different doses of CMV-Pitx2c plasmid. As expected, the mRNA expression levels of Pitx2c increased in accordance with higher doses of the plasmid (Fig. 2B). We observed that a high percentage of Sol8 cells transfected with 2 μg of Pitx2c express the myogenic differentiation marker myogenin and fuse to form myotubes as cells transfected with control plasmid when they are cultured in differentiation medium (Fig. 2A, C). However Sol8 myoblasts transfected with 4 and 8 μg of Pitx2c plasmid did not fuse and maintained an undifferentiated phenotype as myogenin negative cells (Fig. 2A, C). To verify the specificity of Pitx2c blocking differentiation in myoblasts, Pitx2c-transfected cells were transfected with siRNA-Pitx2c which leads to severe down-regulation of Pitx2c (Fig. 1D) and, as expected, siRNA reversed the phenotype of Pitx2c-tranfected cells leading them to express myogenin and form myotubes reaching differentiated phenotype (Fig. 2E).

To characterize the proliferative capacity of Sol8 cells over-expressing *Pitx2c*, we counted the number of proliferating cells (PHH3+ cells) at different doses of Pitx2c-transfection. We found that the number of proliferating cells was increased when Pitx2c doses were higher (4 and 8 μg, Fig. 2F). Consistent with this result, expression levels of cell cycle regulators genes in Sol8 cells over-expressing *Pitx2c* increase according to *Pitx2c* dose transfection (Fig. 3A). Since overexpression can generate non-physiological levels of protein, we also test the expression levels of *cyclin D1*, *cyclin D2*, and *c-myc* after Pitx2c-RNA silencing (siRNA) in Pitx2c-transfected cells (Fig. 4A) and we found that Pitx2c-RNA silencing leads to a clear down-regulation of these cell cycle regulator genes. These results are in line with the reduction of the number of proliferative myogenic progenitor cells found in Pitx2c−/− mutant mice and reinforce the notion that Pitx2c modulates the expression of these cell cycle regulator genes in myoblasts.

Next we explored the effects of different doses of Pitx2c transfection on the expression levels of specific skeletal muscle transcription factors such as Pax3, Pax7, MyoD, and myogenin. qRT-PCR analysis revealed that *Pax3* expression levels increased with Pitx2c doses (Fig. 3B). However Pax7 gene was similarly down-regulated at all doses of Pitx2c transfection assayed (Fig. 3B). Thus, these data suggest a role of Pitx2c balancing Pax3–Pax7 expression in myoblasts. However, we found that Pitx2c-RNA silencing clearly decreased the *Pax3* expression levels in Pitx2c-transfected cells, but no changes were detected for *Pax7* expression levels after Pitx2c-RNA silencing (Fig. 4B) indicating a clear Pitx2c-dependence for *Pax3* expression modulation but not for *Pax7*. Similarly to *Pax7, MyoD* and *Myogenin* genes were down-regulated at low doses (2 μg) of *Pitx2c* transfection (Fig. 3B), when arrest of differentiation and increases in cell proliferation were not yet observed (as illustrated before in Fig. 2A, F). Moreover, we found that the expression levels of *MyoD* and *Myogenin* genes were up-regulated after Pitx2c-RNA silencing (Fig. 4B) in agreement with the reversion to a differentiated phenotype.

Differential role of Pitx2 and Pitx3 regulating proliferation vs differentiation in myoblasts

The earliest *Pitx* gene expressed during muscle cell differentiation is *Pitx2* which is expressed in proliferating muscle progenitors (Kioussi et al., 2002; L'Honoré et al., 2007; Logan et al., 1998). However *Pitx2* expression is progressively replaced by *Pitx3* in differentiated and postmitotic muscle cells, persisting in adult skeletal muscles (L'Honoré et al., 2007). These data suggest that a feedback mechanism exists to the maintain levels of *Pitx* transcription factors in muscle cells and that this mechanism can feedback onto the *Pitx2* gene, in the absence of *Pitx3,* resulting in complete compensation (L'Honoré et al., 2007).

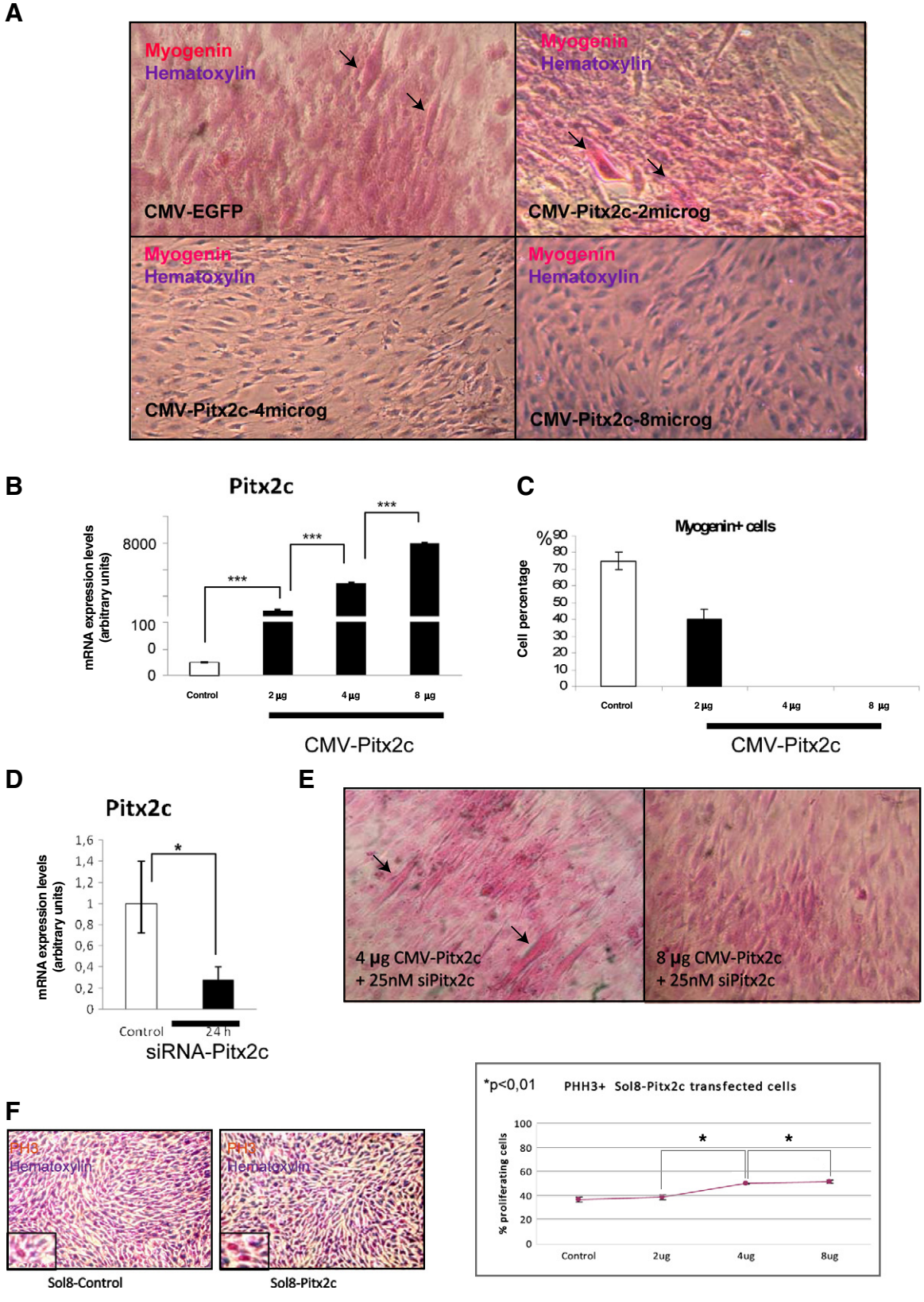
To unravel if Pitx2 and Pitx3 display redundant roles during myoblast and myofiber formation, we have performed transient transfection in the Sol8 cell line with different doses of CMV-Pitx3 plasmid. As expected mRNA expression levels of *Pitx3* were increasing in accordance with higher doses of the plasmid (Fig. 5B). Interestingly we observed that Pitx3 tranfection also blocked myogenin expression and myoblast differentiation but only at high doses of Pitx3 plasmid transfection (8 μg) (Fig. 5A, C). To characterize the proliferative capacity of Sol8 cells overexpressing Pitx3, we counted the number of proliferating cells (PHH3+ cells) in transiently transfected and control Sol8 cells. We found no differences in the proliferation rates between Sol8 cells overexpressing Pitx3 and control cells (Fig. 5D). In addition, the expression levels of cell cycle regulator genes *cyclin D1, cyclin D2*, and *c-myc* were examined in Sol8–*Pitx3* transient transfected cells as well as in control Sol8 cells by qRT-PCR and we found that mRNA levels of *cyclin D1, cyclin D2*, and *c-myc* were similar between *Pitx3* transfected cells and cells transfected with the control plasmid (Fig. 6A).

Real time RT-PCR analysis for the specific skeletal transcription factors Pax3, Pax7, MyoD and *Myogenin* revealed that mRNA expression levels of *Pax3* and *Pax7* display no statistically significant differences in Pitx3-tranfected cells as compared to the control cells (Fig. 6B) demonstrating that Pitx3 does not play a key role controlling Pax3–Pax7 expression in myoblasts. However, in accordance with Pitx3 mediated block of myoblast differentiation, the expression levels of differentiation genes, such as *MyoD* and *Myogenin* were clearly down-regulated in *Pitx3* overexpressing cells in comparison with control cells (Fig. 6B). These in vitro analyses reveal partial Pitx2c/Pitx3 redundancy controlling myoblast differentiation and demonstrate that only Pitx2c, but not Pitx3, modulates cell proliferation and Pax gene expression during myogenesis.

To further investigate the previously suggested feedback between Pitx2 and Pitx3 (L'Honoré et al., 2007) we analyzed by real time RT-PCR the expression levels of *Pitx2c* and *Pitx3* in the in vitro and in vivo models of gain and loss of function used in this study. For this analyses we first evaluated the basal expression levels of Pitx2 and Pitx3 in undifferentiated Sol8 cells and, as expected, the expression levels of Pitx2c were clearly higher (5 fold) than Pitx3 expression levels in Sol8 cells (Fig. 7A). Interestingly we found that although Pitx2c

Fig. 1. Analysis of Pitx2c−/− embryos: A: schematic representation of morphometric analyses in Pitx2c−/− mutant embryos (E12.5) carried out by evaluating limb length (black line; broken black line: proximal end of the limb) as well as limb and body areas (delineated by white broken line and blue line respectively) by using the image J software (NIH Image) in a total of 150 embryos (n = 40 for Pitx2c−/−, n = 70 for Pitx2c+/− and n = 40 for Pitx2+/+). B, C and D shows the results of the quantification of limb length, limb area and body area respectively. E: Representative image of immunohistochemical Pax3 and Pax7 co-staining showing close up views of stained cells in Pitx2c−/−, Pitx2c+/− and wild type littermate embryos. Pax7 staining alone is also shown. F: Quantification of the number of total Pax3+ cells (Pax7+ and Pax7-), Pax3+/Pax7− cells and cells co-expressing Pax3 and Pax7 (Pax3+/Pax7+) was carried out in the entire dermomyotome and entire limb of Pitx2c−/− (n = 8) Pitx2c+/− (n = 9) and wild type littermate (wt) (n = 11). $^{+}$ = P = 0.03; * = P < 0.01; *** = P < 0.0001. G: Representative image of immunohistochemical co-staining for Pax3 and PHH3 as well as Pax7 and PHH3 co-staining showing close up views of stained cells in Pitx2c−/−, Pitx2c+/− and wild type littermates embryos. Although the analysis was performed in limbs and dermomyotome, only dermomyotomes are shown. H: Quantification of the number of Pax3+/PHH3+ and Pax7+/PHH3+ cells was carried out in entire dermomyotomes and limbs of Pitx2c−/− (n = 6) Pitx2c+/− (n = 8) and wild type littermates (wt) (n = 10). * = P < 0.01; ** = P < 0.001.

overexpression in Sol8 cells did not lead to changes in the Pitx3 transcript levels (Fig. 7B), after Pitx2c-RNA silencing in Pitx2c-transfected cells as well as in Pitx2c−/− mutant mice the expression levels of Pitx3 were up-regulated (Fig. 7C, D). However, Pitx2c expression was strongly down-regulated in Pitx3-overexpressing cells (Fig. 7E). Therefore, these results clearly show that Pitx3 expression remains unchanged in the presence of high expression levels of Pitx2c (basal conditions and Pitx2c-overexpression)



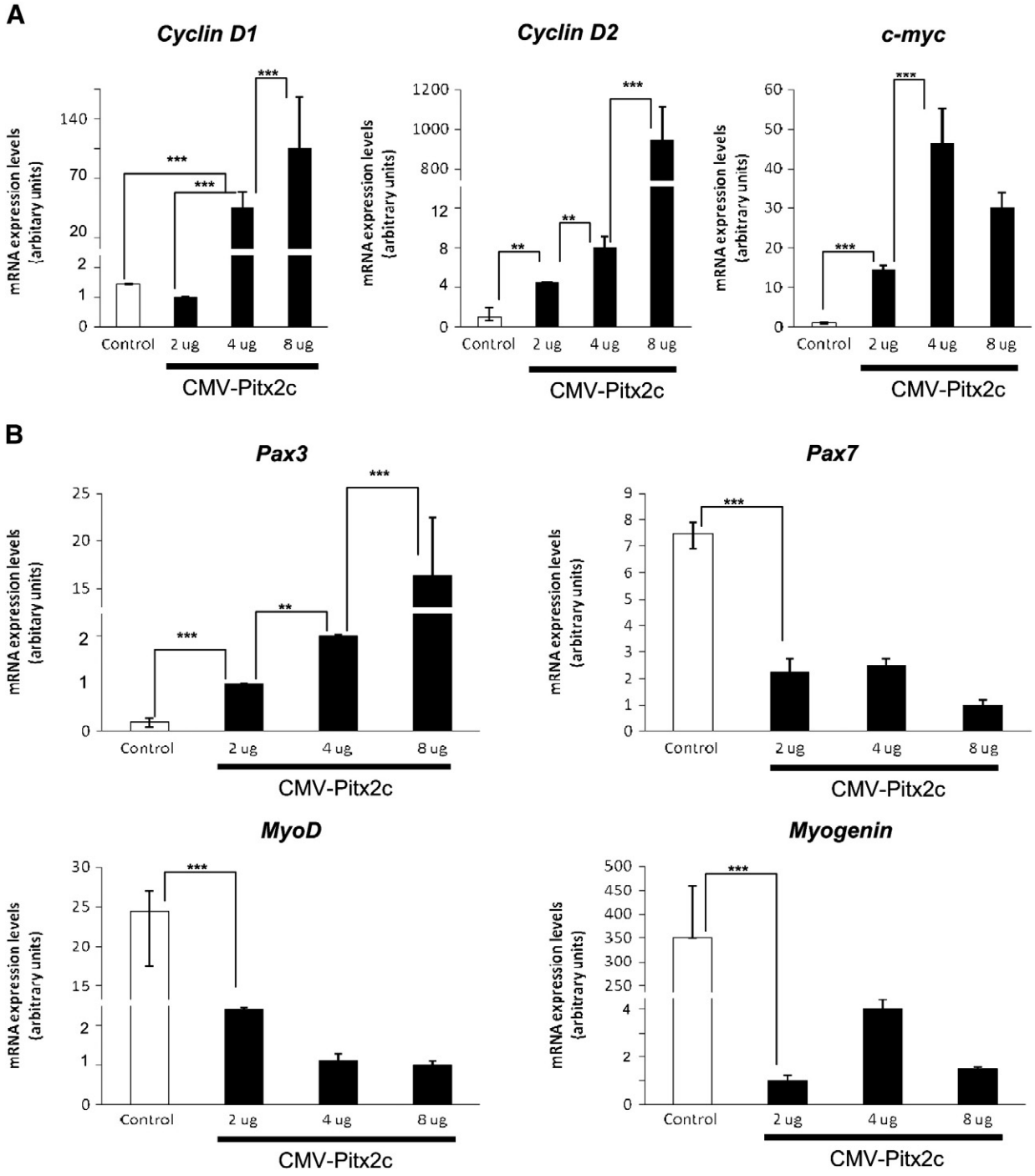


Fig. 3. Expression levels of proliferation and differentiation control genes in Sol8 cells overexpressing *Pitx2c*. A: Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis of mRNA expression levels for *Cyclin D1*, *Cyclin D2* and *c-myc* in Sol8 cells overexpressing *Pitx2c* (CMV-Pitx2c) at different doses and cultured under differentiation condition. B: qRT-PCR analysis of mRNA expression levels for *Pax3*, *Pax7*, *MyoD* and *Myogenin* in Sol8 cells overexpressing *Pitx2c* (CMV-Pitx2c) at different doses and cultured under differentiation condition. Error bars represent standard deviation, *P<0.01, **P<0.001; ***P<0.0001.

Fig. 2. Pitx2c transient transfection in Sol8 cells. A: Pitx2c overexpression in Sol8 cells cultured under differentiation conditions: Myogenin expression is detected and myotubes are formed (black arrows) in control myoblasts (CMV-GFP transfected cells) after 4 days in differentiation medium (DMEM + 2% horse serum [HS]). When Sol8 cells were transfected at low doses (2 μg CMV-Pitx2c) myogenin expression and myotubes were observed (black arrows) after 4 days in differentiation medium as control situation. In Sol8 Pitx2c-transfected cells at medium and high doses (4 μg and 8 μg CMV-Pitx2c) myogenin expression was almost undetected and thus no myotubes were observed after 4 days in differentiation medium. B: Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis of mRNA expression levels for Pitx2c expression levels in Sol8-Pitx2c transfected cells: the Pitx2c expression levels increased in a dose-dependent manner. C: Quantification of myogenin positive cells in Sol8 cells transfected at different doses of *Pitx2c*. D: qRT-PCR analysis of *Pitx2c* mRNA expression levels after 24 h of siRNA tranfection in Pitx2c-overexpressing Sol8 cells E: siRNA in Sol8 *Pitx2c*-transfected cells at medium and high doses: myogenin positive myotubes were observed (black arrows) after 2–3 days of siRNA transfection D: Proliferative capacity of Sol8 cells overexpressing Pitx2c: after 4 days in differentiation medium the number of proliferative cells (PHH3+) was higher in Sol8 cells transfected at medium doses (4 μg) as compared to low doses (2 μg) and at high doses (8 μg) as compared to the medium doses (4 μg). Error bars represent standard deviation, *P<0.01.

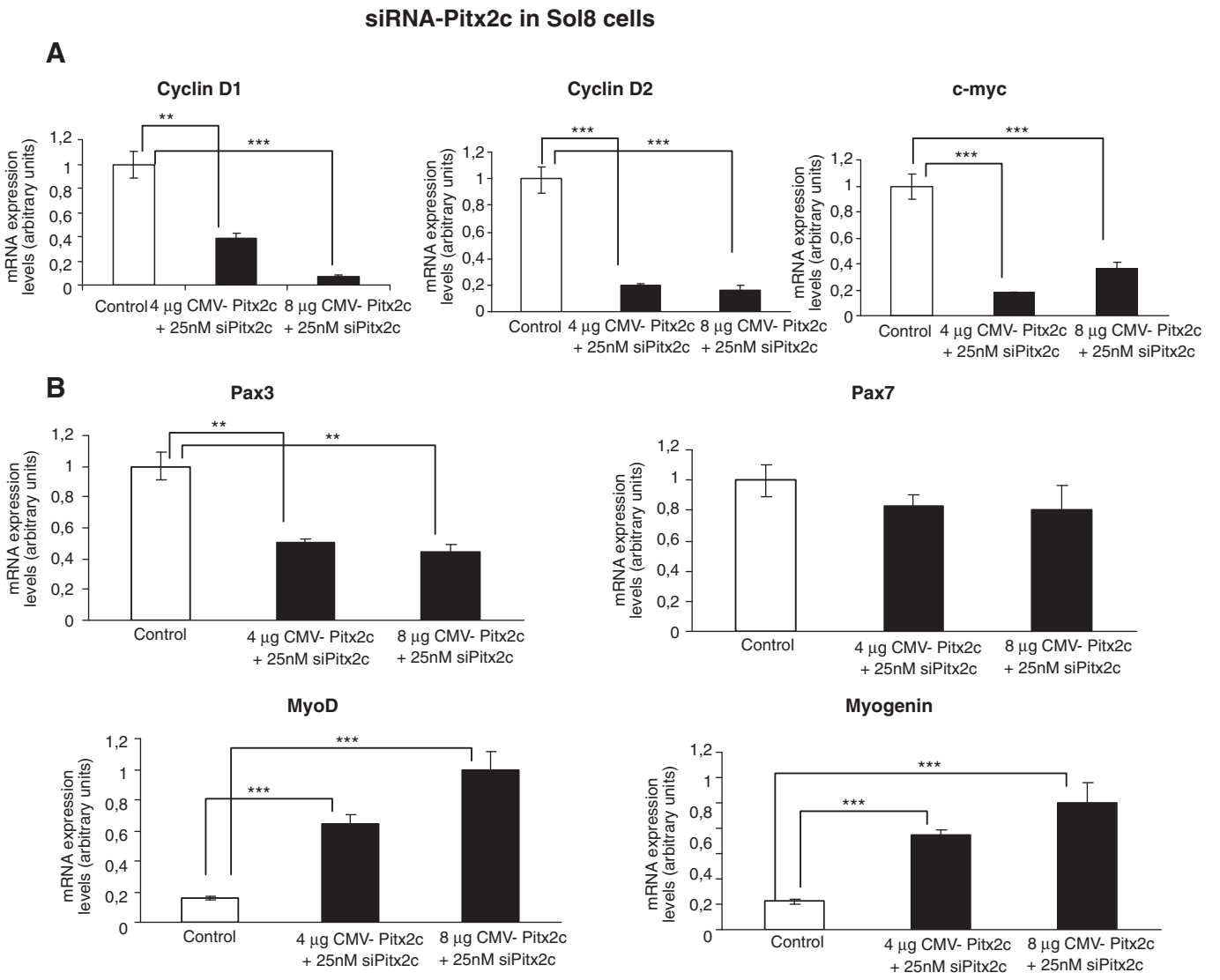


Fig. 4. Expression levels of proliferation and differentiation control genes in Sol8 cells overexpressing Pitx2c after siRNA for Pitx2c. A: Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis of mRNA expression levels for *Cyclin D1*, *Cyclin D2* and *c-myc* after siRNA in Sol8 *Pitx2c*-transfected cells at medium and high doses. B: qRT-PCR analysis of mRNA expression levels for *Pax3*, *Pax7*, *MyoD* and *Myogenin* after siRNA in Sol8 Pitx2c-transfected cells at medium and high doses. Error bars represent standard deviation, *P<0.01, **P<0.001; ***P<0.0001.

but Pitx3 increases when the Pitx2c expression levels decrease. Importantly, high expression levels of Pitx3 lead to a decrease in Pitx2c mRNA expression levels.

Pitx2 regulates Pax3 by modulating miR-27

microRNAs (miRNAs) are a class of gene regulators that are endogenously produced as small non-protein coding RNAs. These highly conserved ~22 nucleotide RNAs negatively regulate gene expression at the post transcriptional level by homologous interactions mainly with the 3′-untranslated region (UTR).

It has been recently reported that miR-27b is expressed in the differentiating skeletal muscle of the embryonic myotome and in activated satellite cells of adult muscle (Crist et al., 2009). In adult muscle satellite cells, miR-27b is expressed when Pax3 transcripts are still present, just before differentiation. In these myogenic progenitor cells, the inhibition of miR-27 delays the onset of skeletal muscle differentiation, in keeping with previous results that indicate an inhibitory effect of Pax3 on this process (Boutet et al., 2007; Crist et al., 2009). To further analyze the Pitx2c–Pax3 interaction, we have performed microRNA-microrarrays in Sol8–Pitx2c transfected cells at 4 and 8 µg vs. control cells. This analysis revealed a set of miRNAs up-regulated and a set of miRNA down-regulated at both doses of Pitx2c transfection. Among the miRNAs that were down-regulated, we found miR-27 (Lozano-Velasco et al., in preparation). Two different pre-miR-27 have been described, miR-27a and miR-27b, having just one single mismatch on their mature miRNA sequence yet equal seed sequence and thus the same predicted miRNA gene targets. We have analyzed the expression levels of miR-27a and miR-27b in Sol8 Pitx2c overexpressing cells and we observed that both miRNAs were down-regulated in a dose-dependent manner in these cells (Fig. 8A). However the expression levels of miR-27a and miR-27b in Sol8-Pitx3 transient transfected cells did not display significant differences in comparison with the control (Fig. 8B) demonstrating the specificity of Pitx2c modulating miR-27 expression in myoblasts. Moreover, in line with previous results demonstrating a role of miR-27 controlling Pax3 expression (Crist et al., 2009), Sol8 cells transfected with pre-miR-27 showed a clear *Pax3* down-regulation while *Pax3* up-regulation was observed when Sol8 cells were transfected with anti-miR-27b (Fig. 1B-C Supplementary data). Interestingly, anti-miR-27 restored the expression of *Pax3* in Sol8 myoblats transfected with siRNA-Pitx2c (Fig. 8C) supporting the notion that miR-27 plays a central role mediating the Pitx2c effect on Pax3 gene expression.

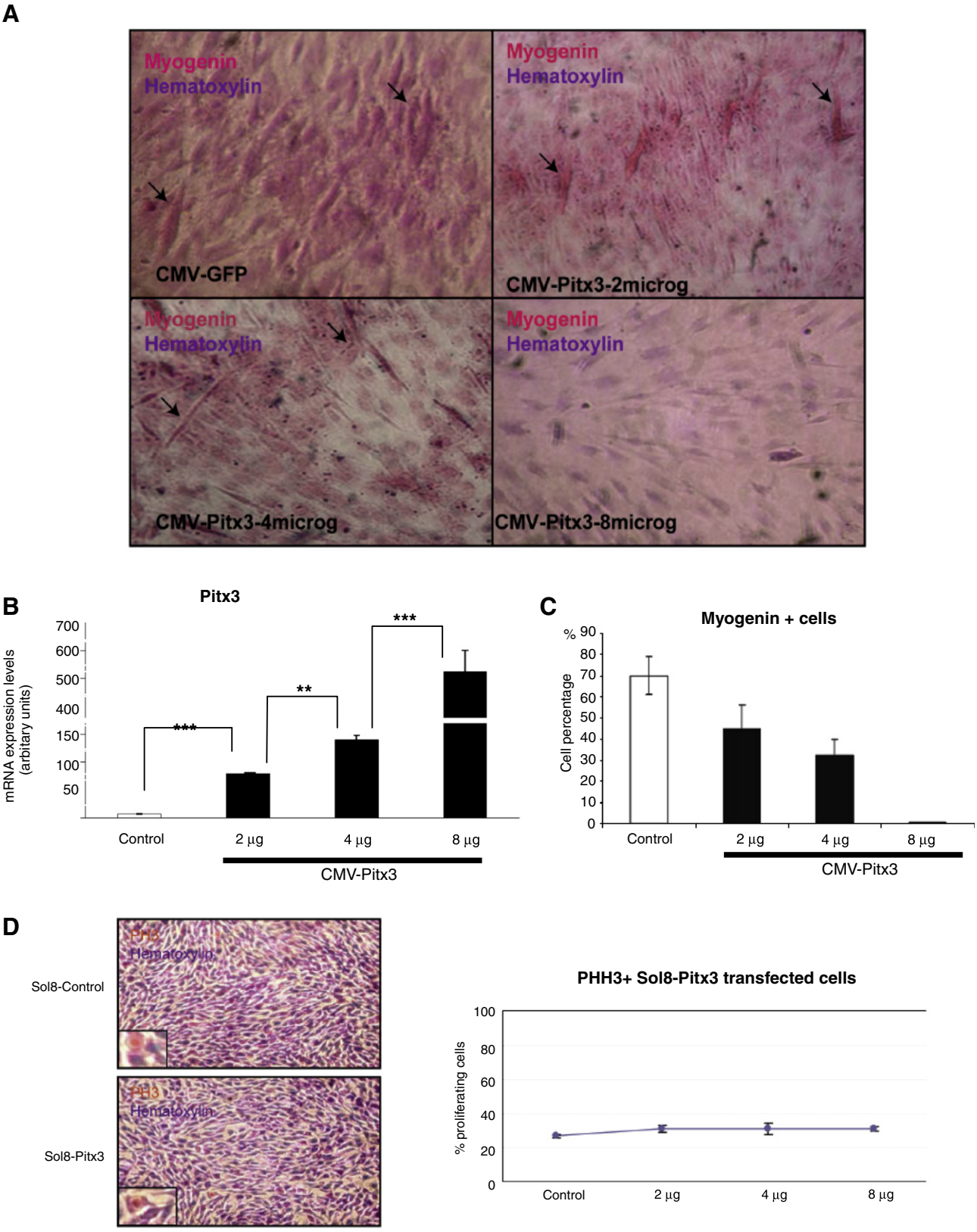


Fig. 5. Pitx3 transient transfections in Sol8 cell line. A: Pitx3 overexpression in Sol8 cells cultured under differentiation conditions: Myotubes expressing myogenin were formed (black arrows) in control myoblasts (CMV-GFP transfected cells) after 4 days in differentiation medium. In Sol8 Pitx3-transfected cells at low and medium doses (CMV-Pitx3—2 µg and CMV-Pitx3—4 µg respectively) myotubes expressing myogenin were also observed (black arrows) after 4 days in differentiation medium. However, Sol8 Pitx3-transfected cells at high doses (CMV-Pitx3—8 µg) did not fuse, myogenin expression was almost absent, and thus no myotubes were observed after 4 days in differentiation medium. B: qRT-PCR analysis of mRNA expression levels for *Pitx3* in Sol8-*Pitx2c* transfected cells after 4 days in differentiation. C: Quantification of myogenin positive cells in Sol8 cells transfected at different doses of Pitx3. D: Proliferative capacity of Sol8 cells overexpressing Pitx3: After 4 days in differentiation medium the number of proliferative cells (PHH3+) remains unchanged in the cells transfected with different doses of Pitx3 as compared to control cells. Error bars represent standard deviation, *P<0.01, **P<0.001; ***P<0.0001.

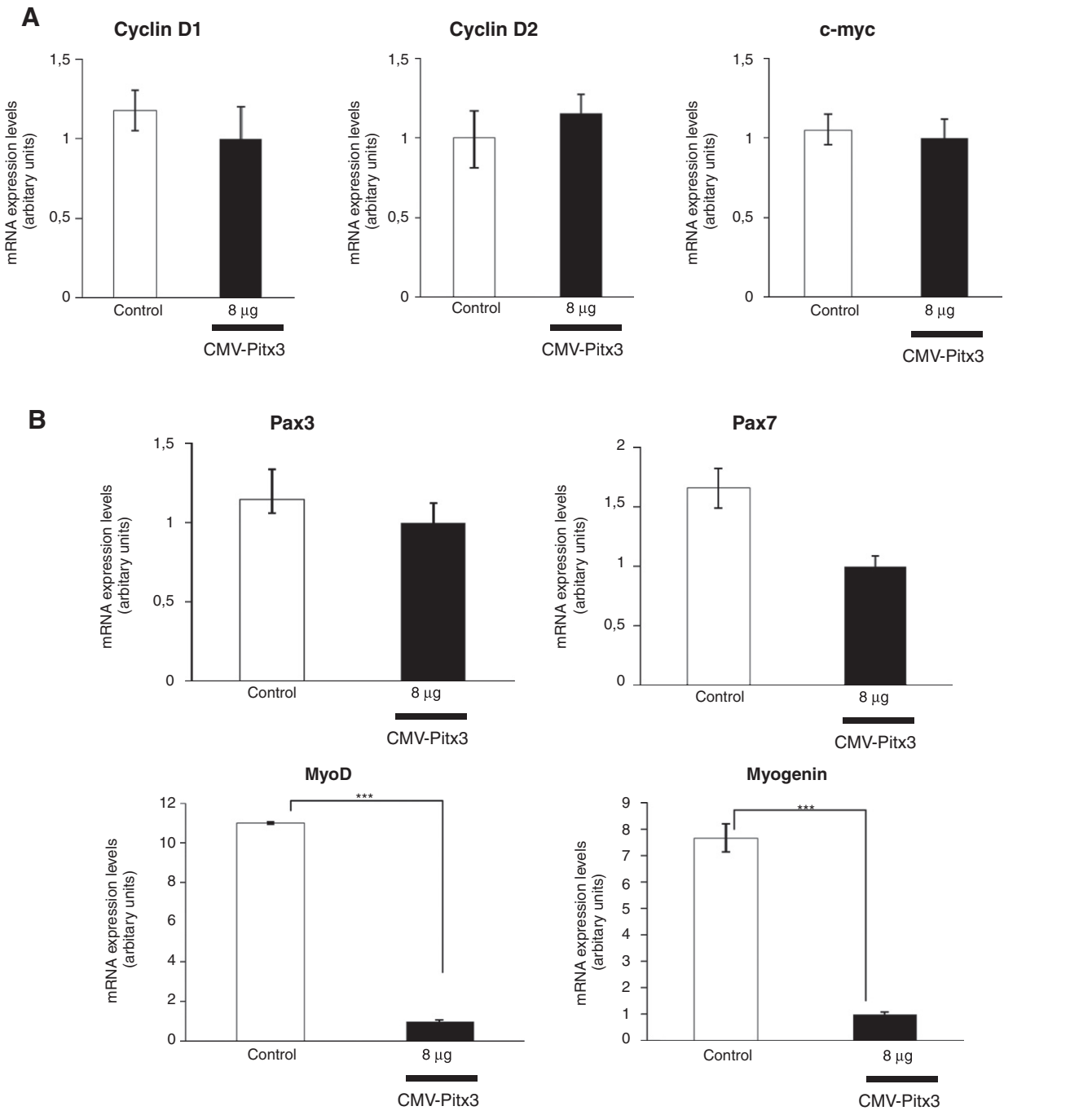


Fig. 6. Expression levels of proliferation and differentiation control genes in Sol8 cells overexpressing Pitx3. A: Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis of mRNA expression levels for *Cyclin D1, Cyclin D2* and *c-myc* in Sol8 cells overexpressing Pitx3 (CMV-Pitx3) at different doses and cultured under differentiation condition. B: qRT-PCR analysis of mRNA expression levels for *Pax3, Pax7, MyoD* and *Myogenin* in Sol8 cells overexpressing Pitx3 (CMV-Pitx3) at different doses and cultured under differentiation condition. Error bars represent standard deviation, ***P<0.0001.

To determine if Pitx2c-mediated miR-27 regulation is maintained in vivo we have analyzed the expression of miR-27 in Pitx2c mutant embryos at embryonic day 10.5 (E10.5) by in situ hybridization as well as by real time RT-PCR. We found that, in accordance with our in vitro results, miR-27 expression is up-regulated in the somites and limb buds of Pitx2c−/− mice (Fig. 8D). Taken together these results showed that Pitx2c up-regulates Pax3 expression by repressing miR-27 expression.

Pax3 is required for the survival and migration of cells from the hypaxial dermomyotome to the limb buds and it has been previously suggested that it could be implicated in proliferation of these muscle progenitor cells (Bajard et al., 2006; Buckingham and Relaix, 2007; Zhou et al., 2008). Therefore, to evaluate if Pitx2c-mediated effects on cell proliferation are independent or not of miR-27-Pax3 modulation we analyze the number of proliferating cells (PHH3+) in Sol8 cells transfected with miR-27 and anti-miR-27. No changes were observed between miR-27-transfected cells and control situation (Fig. 1D, Supplementary data). Moreover, the expression levels *of cyclin D1, cyclin D2*, and *c-myc* were similar between miR-27-transfected and control cells with (Fig. 2, Supplementary data). Therefore, we can conclude that Pitx2c-mediated changes on cell proliferation are independent of miR-27.

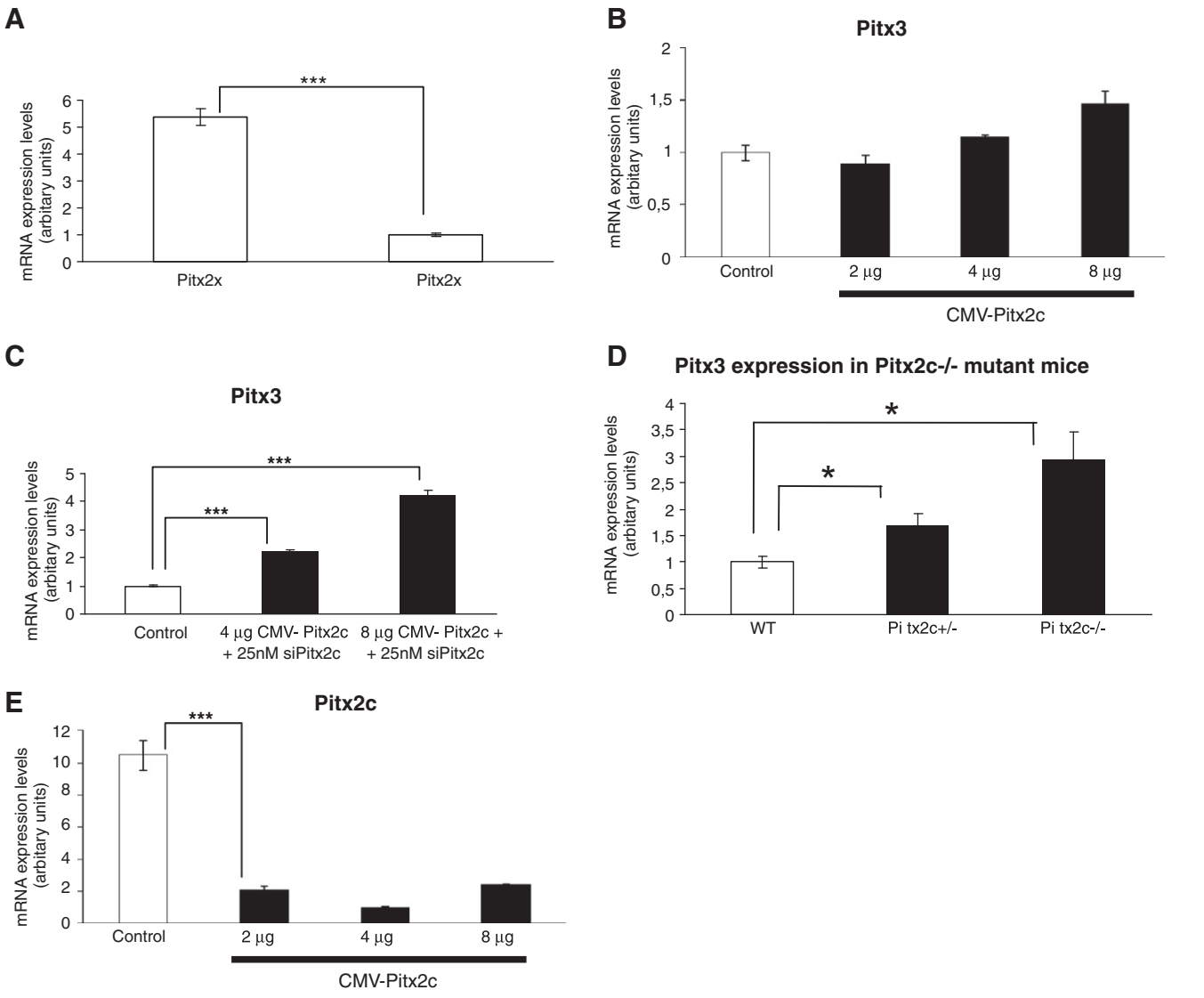


Fig. 7. A: Pitx2c vs Pitx3 expression in Sol8 myoblasts. B: qRT-PCR analysis of mRNA expression levels for Pitx3 in Sol8-Pitx2c transfected cells (CMV-Pitx2c) at different doses. C: qRT-PCR analysis of Pitx3 mRNA expression levels after siRNA tranfection in Pitx2c-overexpressing Sol8 cells. D: qRT-PCR analysis of Pitx3 mRNA expression levels in Pitx2c−/− mutant mice. E: qRT-PCR analysis for Pitx2c, in Sol8-Pitx3 transfected cells (CMV-Pitx3) at different doses. Error bars represent standard deviation, *P<0.01, **P<0.001; ***P<0.0001.

Discussion

Pitx2 expression is observed in muscle progenitor cells during musculature development (Dong et al., 2006; Shih et al., 2007) co-labeling with Pax3+ and Pax7+ myotome cells (L'Honoré et al., 2007). We have previously 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 (Martínez-Fernández et al., 2006). Now, we demonstrate that these roles of Pitx2c balancing proliferation vs. differentiation as well as signaling through Pax3 are also observed during in vivo myogenesis.

The dermomyotome contains multipotent progenitor cells of different cell types; the early myotome and embryonic myofibers originate from Pax3+ cells. In the central portion of the dermomyotome and in the muscle progenitors that have migrated to the limb, Pax7 expression is initiated in Pax3+ cells (Buckingham and Vincent, 2009). Here, we demonstrate that the total number of progenitor cells expressing Pax3+ is similarly reduced in Pitx2c heterozygous and null mutant mice and we found that this reduction is mainly due to a dramatic decrease of the cells that express only Pax3 (Pax3+/Pax7− cells) whereas the numbers of Pax3+/Pax7+ cells were significantly enhanced. Interestingly heterozygous Pitx2 mutant displays intermediate Pax3+/Pax7− and Pax3+/Pax7+ cell populations as compared to wild type and Pitx2c null mutant, suggesting that the presence of one or two alleles could determine the balance between Pax3+ and Pax7+ cell populations. In addition, analysis of Pitx2c−/− mice revealed that cell proliferation was dramatically diminished in Pax3+ cell populations demonstrating that Pitx2c controls proliferation mainly in a subset population of myogenic progenitor cells. Together, these results indicate that Pitx2c can play a role balancing the different muscle progenitor populations during in vivo myogenesis and are in line with our previous in vitro results as well as several works which have provided evidence that Pitx2 increases cell proliferation of myogenic progenitor cells during in vivo myogenesis (Abu-Elmagd et al., 2010; L'Honoré et al., 2007; Martínez-Fernández et al., 2006). In this context it is interesting to highlight that, in many muscles, quiescent satellite cells co-express Pax7 and Pax3 and their expression is maintained in proliferating cells derived from activated satellite cells before the activation of myogenic program (Buckingham and Relaix, 2007). Moreover, previous works have demonstrated that

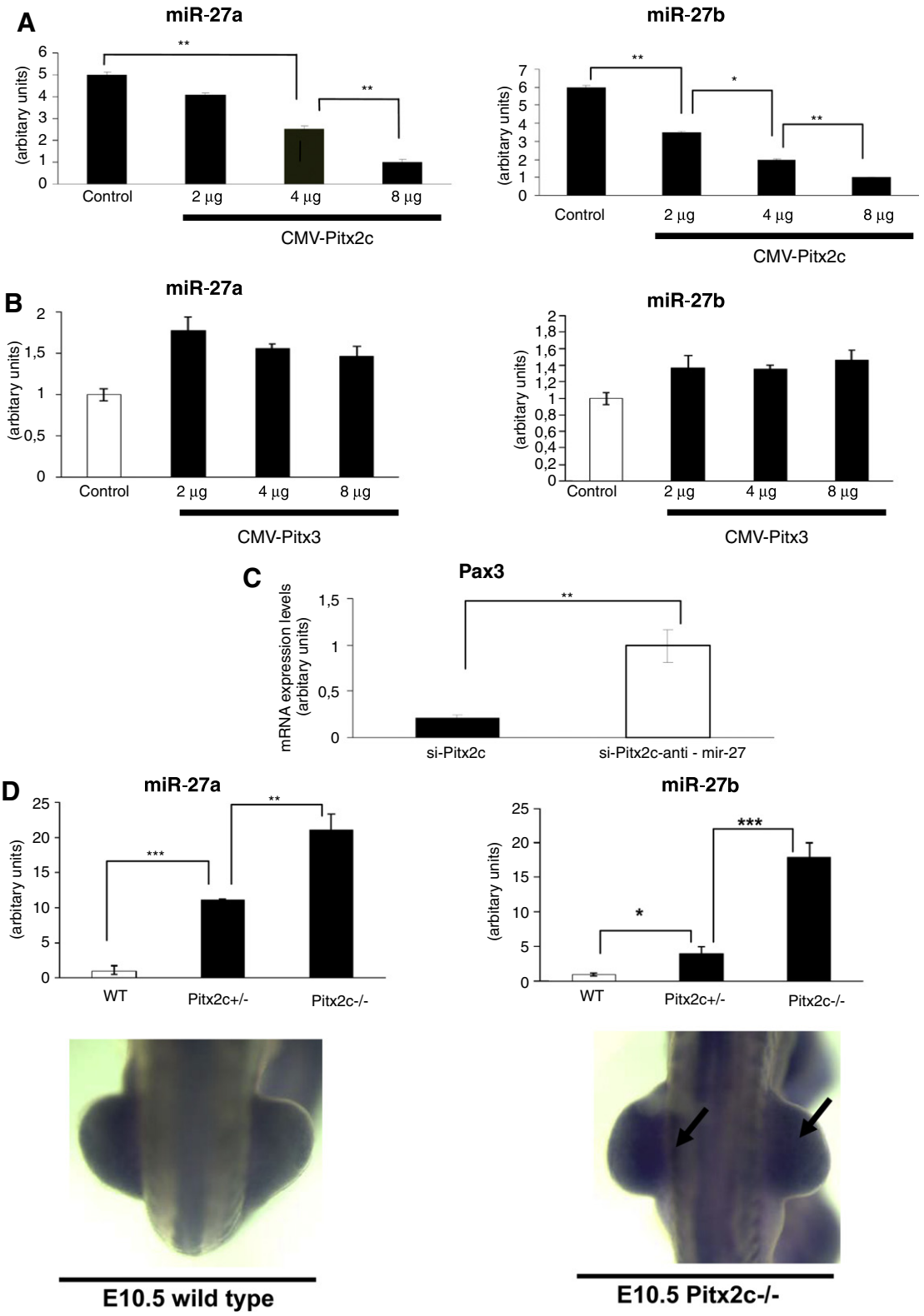


Fig. 8. microRNAs expression in Sol8 transfected cells. A: qRT-PCR analysis for miR-27a and miR-27b in Sol8-Pitx2c transfected cells (CMV-Pitx2c) at different doses. B: qRT-PCR analysis for miR-27a and miR-27b in Sol8-Pitx3 transfected cells (CMV-Pitx3) at different doses. C: qRT-PCR analysis for *Pax3* in Sol8 cells transfected with siRNA-*Pitx2c* (si-Pitx2c) and in Sol8 cells transfected with anti-miR-27 after 24 h of si-Pitx2c (siPitx2c-anti-miR-27). D: qRT-PCR analysis for miR-27a and miR-27b in the limb buds of Pitx2c−/− embryos and LNA-in situ hybridization against miR-27 in E10.5 embryo Pitx2−/− mice compared with control mice. Error bars represent standard deviation, *P<0.01, **P<0.001; ***P<0.0001.

Pitx2 continues to be expressed in satellite cells of adult extraocular muscles (Zhou et al., 2008) and we have found that Pitx2c is present in some satellite cells of adult limb muscles (data not shown). Therefore the data presented herein indicate a role of Pitx2c in balancing Pax3+–Pax7+ cells which can have a great impact in regenerative myogenesis.

In addition, and in line with the increase of the number of cells co-expressing Pax3+ and Pax7+ in the in vivo model of Pitx2c loss of function, our in vitro analysis revealed that overexpression of Pitx2c leads to *Pax7* down-regulation together to *Pax3* up-regulation providing additional evidence of a role of Pitx2c regulating Pax3–Pax7 expression in myogenic cells. However, transient transfection of Sol8 myoblasts at different doses of Pitx2c demonstrated that Pitx2c-mediated effects on cell proliferation and *Pax3* regulation was dose-dependent while decreases in the expression levels of *Pax7* did not show a clear Pitx2c dose-dependency. Furthermore, in vitro Pitx2c-loss of function (siRNA) leads to *Pax3* down-regulation but no changes in the *Pax7* expression levels were observed. Together, these findings lead us to think that the effects of Pitx2c over Pax7 expression could be indirect and mainly due to the effect of Pitx2c maintaining the progenitors cells in a more undifferentiated stage. It nonetheless remains to be explored in detail how Pitx2c balances Pax3 vs Pax7 expression in myogenic cells. Based on the Pitx2c effect blocking myoblast differentiation, we can speculate that *MyoD* and *Myogenin* down-regulations are a consequence of the maintenance of an undifferentiated stage. Recently, L'Honoré et al. (2010) have pointed out a crucial role of Pitx2 in the onset of *MyoD* gene expression in limb muscle progenitors and they propose that Pitx2 acts downstream of Pax3 during skeletal myogenesis. Here we provide evidence that Pitx2c may be balancing Pax3 vs Pax7 expression in the myogenic presursors. Together these observations suggest that Pitx2 may play a dual role in myogenic progenitor cell fate maintenance and myogenic progenitor cell differentiation.

Together with Pitx2 the related Pitx3 gene is also expressed in somites (Zhao et al., 2004) and a muscle-specific promoter responsible for its expression in myotome and muscle has been identified (Coulon et al., 2007). Pitx2 is first expressed in muscle progenitor cells of the dermomyotome and myotome. The onset of myoblast differentiation is concomitant with the expression of Pitx3 and its expression is maintained in all skeletal muscles while Pitx2 expression decreases thereafter (L'Honoré et al., 2007). Pitx3 on its own is not required for myogenesis, since the absence of Pitx3 does not significantly perturb muscle development and its expression is completely compensated by the maintenance of Pitx2 expression in all skeletal muscles. Thus a partially redundant role of Pitx2 and Pitx3 has been previously proposed (L'Honoré et al., 2007). In this study we show that high doses of Pitx3 are capable of blocking differentiation in Sol8 myoblasts, revealing a partial redundancy of Pitx2c and Pitx3 in regulating terminal differentiation in myoblasts. Conversely to Pitx2c, proliferative capability, cell cycle gene expression as well as *Pax3* and *Pax7* expressions were not altered in Sol8–Pitx3 overexpressing cells; however we found that *Pitx2c* was clearly down-regulated in Pitx3-overexpressing cells. Based on our in vitro results after Pitx2c-siRNA we can expect that, secondarily to Pitx2c down-regulation, the proliferative capability, cell cycle genes and Pax3 expression can be also altered in these cells. Therefore, we cannot exclude the possibility that Pitx3 can be compensating, at least in part, the effects of Pitx2c on proliferative capability and *Pax3* expression in Pitx3-overexpressing cells. Furthermore our results show that, as described during muscle development (L'Honoré et al., 2007), the Pitx3 expression levels increase when Pitx2c expression levels decrease in line with the hypothesis of L'Honoré et al. (2007) who proposed the existence of a feedback mechanism between these two genes. However, our results demonstrate that Pitx2–Pitx3 feedback is unidirectional and only Pitx3 can modulate Pitx2 expression.

MicroRNAs (miRNAs) are a class of regulatory RNAs of ~22 nucleotides that post-transcriptionally regulate gene expression. Increasing evidence points to the potential role of miRNAs in various biological processes. Some of these miRNAs are expressed in a lineage restricted manner; this is the case for the muscle specific "myomiRs" which include miRNA-1 and miRNA-133, miRNA-1(miR-1) and miRNA-133 (miR-133) are clustered on the same chromosomal loci and are transcribed together in a tissue-specific manner during development having distinct roles in modulating skeletal muscle proliferation and differentiation in cultured myoblasts in vitro and in *Xenopus laevis* embryos in vivo (Chen et al., 2006). More recently Crist et al. (2009) nicely demonstrated that the 3′-UTR of Pax3 mRNA is a target of miR-27. These authors have shown that miR-27b is expressed in both myotome and activated satellite cells of adult muscle. Furthermore, overexpression of a miR-27b transgene in Pax3-positive cells in the embryo leads to down-regulation of Pax3, resulting in interference with progenitor cell migration and in premature differentiation. Thus, these authors conclude that miR-27b regulates Pax3 protein levels and this downregulation ensures rapid and robust entry into the myogenic differentiation program (Crist et al., 2009). Therefore, to further investigate how Pitx2c can modulate proliferation vs differentiation in myogenic cells we performed microRNA-microarray analyses in Sol8 cells overexpressing *Pitx2c*. Interestingly, miR-27 was present among the battery of microRNA genes that were down-regulated in Pitx2c-overexpressing cells with respect to the control. Here we show that, as it occurs for Pax3 expression, miR27 expression is down-regulated in Pitx2c-overexpressing Sol8 myoblasts in a dose-dependent manner. Interestingly, analyses of miR-27 expression in Pitx2c mutant mice provide evidence that loss-of function of Pitx2c leads to miR-27 up-regulation. Furthermore, anti-miR-27 was able to rescue the decrease in *Pax3* expression due to Pitx2c interference in myoblasts. miR-27a and miR-27b are located into two different miRNAs clusters within the mouse genome and we have identified several putative Pitx2-binding sites 20 kb upstream of both miR-23b-27b-24-1 and miR23a-27a-24-2 clusters suggesting that Pitx2 could directly regulate the transcription of these miRNAs clusters. Transcriptional regulation of mRNAs is an emerging field and our understanding of the regulation and transcription of mRNAs genes is still limited. Therefore, based on our in vitro and in vivo data we conclude that Pitx2c indirectly controls Pax3 expression by repression of miR-27 expression. It nonetheless remains to be explored in detail the genetic interactions between Pitx2 and miR-23b-27b-24-1 and miR23a-27a-24-2 clusters. Recent findings have demonstrated that Pitx2 can act in a subset of myogenic cells as transcriptional activators collaborating with several muscle regulatory factors (MRFs) to induce myoblast differentiation (L'Honoré et al., 2010; Zacharias et al., 2010). However we show here that Pitx2c functions as a repressor of miR-27 in myogenic precursors cells. Taken together we hypothesize that Pitx2 could be acting first as a transcriptional repressor of miRNAs to maintain cell fate in myogenic progenitor cells and subsequently collaborate to activate the myogenic differentiation program in myoblasts.

Additionally Crist et al. (2009) showed that interference with miR-27b function results in the maintenance of Pax3 expression, leading to more proliferation and a delay in the onset of differentiation. Thus, we explore the possibility that effects on cell proliferation mediated by Pitx2c could also be due to miR-27b repression and we found that miR-27-overexpression in Sol8 myoblasts did not lead to any change in cell proliferation demonstrating that Pitx2c modulates cell proliferation independently to miR-27 modulation.

In summary, in this study we provide evidence for the first time that Pitx2c can play a role in balancing the different myogenic progenitor cell populations during in vivo myogenesis. Therefore, Pitx2c loss of function leads to a decrease in Pax3+ cell population in the embryo accompanied by an increase of Pax3/Pax7+ cells. Our in vitro results using different doses of Pitx2c overexpression further support the notion that Pitx2c could modulate specific cell populations by regulating Pax3–Pax7 expression. Additionally, we demonstrated that Pitx2c modulates Pax3 by repressing miR-27 expression and that Pax3-miR-27 modulation mediated by Pitx2c is independent of Pitx2c effects on cell proliferation. Based on the key role of Pax3/

Pax7 positive cells on the maintenance of satellite cells' self-renewal in the adult skeletal muscle we can conclude that these findings may have future applications in regenerative medicine.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.06.039.

Funding

This work was partially supported by grants BFU2008-01217, BFU2009/11566 (Ministerio de Ciencia e Innovación, Gobierno de España), CTS-1614, P08-CTS-3878 (Junta de Andalucia).

Conflict of interest

None declared.

Acknowledgments

We specially thank Dr. Nigel Brown (Division of Basic Medical Sciences, St. George Hospital, University of London, UK) for supplying the Pitx2c null embryos.

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