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Pax3 and Tbx5 specify whether PDGFRα+ cells assume skeletal or cardiac muscle fate in differentiating ES cells

Alessandro Magli[1], **Erin Schnettler**[1], **Scott A Swanson**[2], **Luciene Borges**[1], **Kirsta Hoffman**[1], **Ron Stewart**[2], **James A Thomson**[2], **Susan A. Keirstead**[3], and **Rita C. R. Perlingeiro**[1]

[1]Lillehei Heart Institute, Department of Medicine, University of Minnesota, Minneapolis, MN USA

[2]Morgridge Institute for Research, Madison, WI 53707, USA

[3]Stem Cell Institute, Department of Integrative Biology and Physiology, University of Minnesota, Minneapolis, MN USA

Abstract

Embryonic stem (ES) cells represent an ideal model to study how lineage decisions are established during embryonic development. Using a doxycycline-inducible mouse ES cell line, we have previously shown that expression of the transcriptional activator Pax3 in early mesodermal cells leads to the robust generation of paraxial mesoderm progenitors that ultimately differentiate into skeletal muscle precursors. Here we show that the ability of this transcription factor to induce the skeletal myogenic cell fate occurs at the expenses of the cardiac lineage. Our results show that the PDGFRα+FLK1− sub-fraction represents the main population affected by Pax3, through down-regulation of several transcripts encoding for proteins involved in cardiac development. We demonstrate that although Nkx2-5, Tbx5 and Gata4 negatively affect Pax3 skeletal myogenic activity, the cardiac potential of embryoid body (EB)-derived cultures is restored solely by forced expression of Tbx5. Taking advantage of this model, we employed an unbiased genome wide approach to identify genes whose expression is rescued by Tbx5, and which could represent important regulators of cardiac development. These findings elucidate mechanisms regulating the commitment of mesodermal cells in the early embryo and identify the Tbx5 cardiac transcriptome.

Keywords

Embryoid bodies; myogenesis; mesoderm; cardiac; stem cell plasticity

Corresponding Author: Rita C. R. Perlingeiro PhD, University of Minnesota, Nils Hasselmo Hall, 312 Church St. S.E., Minneapolis 55455, MN, USA, perli032@umn.edu, Phone: 612 625 4984; Fax: 612 624 8118.

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AUTHORSHIP CONTRIBUTIONS

A.M. designed and performed research, analyzed data, and wrote the manuscript; E.S. L.B. and K.H. performed research; S.S performed bioinformatic analyses; R.S. and J.A.T. supervised the bioinformatic analyses; S.A.K. performed the electrophysiology study and analyzed the data; R.C.R.P. contributed with interpretation of the data and wrote the manuscript.

INTRODUCTION

Embryonic development is a highly regulated process requiring rapid activation and repression of several transcriptional programs that ultimately result in the generation of specialized/differentiated cell types. Transcription factors represent one of the main players during this process and their presence is usually accompanied by a change in gene expression. Pax genes belong to one of several families of transcription factors expressed during embryonic development (reviewed in [1]). These transcription factors are characterized by the presence of the Paired Domain (PD), a DNA binding module highly conserved across different species [2, 3]. Nine members of this family have been described in mammals (named Pax1 to 9), and all these genes represent important regulators of embryonic development [1]. Among the Pax genes, Pax3 is expressed in the somites, neural tube, and neural crest cells [4]. In the somites, Pax3 regulates the migration, differentiation and survival of the skeletal myogenic precursors that will ultimately form the diaphragm and the muscles of the limb and tongue [1]. Pax3-target genes include the Hepatocyte Growth Factor Receptor c-Met, the myogenic determinant Myf5, as well as other important developmental regulators [5–8].

Recent studies involving the controlled *in vitro* differentiation of mouse pluripotent stem cells into muscle progenitors, by our group and others, have suggested that Pax3-mediated induction of the skeletal myogenic lineage is followed by repression of cardiac myogenesis [9, 10]. During mouse embryogenesis, heart development starts after gastrulation and depends on two waves of distinct progenitors arising from the lateral plate mesoderm, which will contribute to the first (FHF) and secondary heart field (SHF), respectively. In addition to these two waves of progenitors, cardiac neural crest cells (NCC) also play an important role in heart morphogenesis [11]. At E7.5, cells of the first heart field form a crescent-like structure that at E8.0 develops into the primordial heart tube. After this event, cells from the secondary heart field migrate into this primitive structure and contribute to the formation of the chambers (reviewed by [12]). These processes are regulated by several transcription factors which, if mutated or absent, lead to cardiac malformations in both mice and humans [12]. Due to the ability of mesodermal precursors to change fate based on the expression of lineage specific master regulators [13, 14], Pax3-induced repression of the cardiac program may be the result of the down-regulation of important key regulators of cardiac myogenesis. To gain better insight into the nature of this process, we analyzed how Pax3 represses the cardiac program during EB differentiation and characterized gene expression in different mesodermal sub-fractions. Furthermore, we tested whether critical transcription factors of the cardiac program were capable of reversing Pax3-induced repression during mesoderm patterning and, using a genome-wide approach, we identified a subset of candidate genes that might be involved in the regulation of cardiac development. Such studies are critical to further elucidate the regulation of lineage-specific transcriptional programs during embryonic development.

MATERIAL AND METHODS

Plasmids and cell line generation

The inducible lentiviral constructs encoding Nkx2-5, Tbx5 and Gata4 were obtained by subcloning the respective ORFs into the pSAM2-iresGFP or pSAM2-ires mRFP vectors [15]. Lentiviral constructs for Vsnl1 knockdown were obtained from the Biomedical Genomic Center at the University of Minnesota. These constructs are pLKO.1-puro vectors containing shRNA directed toward Vsnl1. The inducible cell lines encoding full length Pax3 (iPax3), Pax3 Paired-domain deletion mutant (iPax3 ΔPD), Pax3-iresGFP and iresGFP were described previously [9]. Lentiviruses were produced by co-transfection of the transfer vector and the packaging constructs (pVSV-G, pREV and pΔ8.74 [16]) in HEK 293T cells. Transfections were performed using Lipofectamine LTX with Plus Reagent (Invitrogen) following manufacturer instructions. Supernatants containing the lentiviral particles were collected 36h after transfection, passed through a 0.45μm filter and applied to the ES cell cultures. To prevent ES cell differentiation, 1,000 U/mL LIF (Millipore) was added to the media. Cells were spin-infected at 1100g for 1h and 30 minutes at 30°C and then incubated in the presence of the lentivirus for an additional 6h at 37°C. After, media was replaced with ESC culture media. In order to achieve good transduction efficiency (around 60%), iPax3 and control (A2lox-cre) cell lines were transduced twice with pSAM2 lentiviruses.

Embryo dissection

Time-pregnant wild-type (WT) CD1 mice were purchased from Charles River Laboratories. Embryos were removed from the decidua and the Reichert membrane and dissected in PBS supplemented with 10% FBS. At E7.5 dpc, embryos were staged according to morphologic criteria. E7.25–E7.75 embryos were used for experiments.

Cell cultures

HEK 293T cells, used for transfection, were maintained in DMEM (Gibco) supplemented with 10% Fetal Bovine Serum (FBS), penicillin/streptomycin (Gibco) and 2 mM Glutamax (Gibco). Mouse embryonic stem cells were cultured on irradiated mouse embryonic fibroblasts (MEFs) in Knockout DMEM (Gibco) supplemented with 15% ES qualified FBS (Gibco), penicillin/streptomycin, 2 mM Glutamax, 0.1 mM β-mercaptoethanol, and 1,000 U/mL LIF (Millipore), at 37°C in 5% CO2. For differentiation, ES cells were trypsinized, resuspended in EB differentiation media (EBM) and pre-plated for 30 min on gelatin-coated dishes to remove MEFs. After counting, cells were diluted in EBM at 10000 cells/ml, and plated as 10μl drops in non-adherent 15 cm Petri dishes to induce EB formation. EBs were harvested after 48h and plated on non-adherent 10 cm dishes on a swirling rotator in EBM at 37°C in 5% CO2. EBM consisted of Iscove-modified DMEM (Gibco) supplemented with 15% ES qualified FBS (Gibco), penicillin/streptomycin, 2 mM Glutamax, 200 μg/mL iron-saturated transferrin (Sigma), 4.5 mM monothioglycerol (Sigma) and 50 μg/mL ascorbic acid (Sigma). Doxycycline (Sigma – D9891) was dissolved in sterile PBS at a concentration of 1 mg/ml and stored at 4°C. Induction of the transgene was achieved by adding the doxycycline stock to the culture media at a final concentration of 0.8 μg/ml. Sorted or unsorted cells obtained from day 5 EBs were replated on gelatinized flasks and cultured at 37°C in 5% CO2 in EBM for 5 days.

FACS analysis

Day 5 EBs were harvested, washed twice with PBS and then trypsinized for 1.5 min in a 37°C water-bath with continuous shaking. Trypsin was inactivated by adding 4 volumes of PBS supplemented with 10% FBS (PBSF) and the cells were resuspended and filtered through a 70 μm strain to remove cell clumps. Cells were washed once with PBS and then incubated for 5 minutes in PBSF in the presence of Fc Block (1μl/4 million cells – E-bioscience). Staining was performed by adding 0.5 μl of each antibody per 1 million cells and incubating on ice for 25 min. We used PE-conjugated anti-mouse CD140a (PDGFRα) and APC-conjugated anti-mouse CD309 (FLK-1) (both from E-bioscience). Cells were washed twice with PBS and then resuspended in PBSF containing propidium iodide to exclude dead cells. Samples were analyzed and sorted using a FACSAria II (BD Biosciences).

RNA isolation, gene expression and RNAseq

Cells and total EBs were resuspended in Tryzol (Invitrogen) and processed following the manufacturer's instructions. RNAs from total EBs were retro-transcribed using Thermoscript (Invitrogen). RNAs from sorted cells were retro-transcribed using Superscript Vilo (Invitrogen). Gene expression analyses were performed using an amount of cDNA solution corresponding to 12.5 ng of starting RNA for each reaction. qRT-PCR analysis was performed using TaqMan Universal PCR Master Mix and TaqMan probes (Applied Biosystems). For RNAseq, 300ng of total RNA for each sample was treated with DNaseI amplification grade (Invitrogen) following the manufacturer's instructions. Sequencing libraries were generated from 100 ng of total RNA using the TruSeq RNA Sample Preparation kit (Illumina) using half the reaction volumes required by the Illumina protocol and quantitated using the Qubit fluorometer (Life Technologies) following the manufacturer's instructions. The libraries were then pooled using 50 ng/sample for a 51+7 cycle Single Read run on the HiSeq 2500 (Illumina) either by high output or rapid run sequencing.

The sequencer outputs were processed using Illumina's CASAVA-1.8.2 basecalling software. Demultiplexing assigned ~650 million reads across the 24 samples, ranging from 19 million to 35 million reads per sample. ~25 million reads were not successfully assigned. Of the assigned reads, about 1 million were discarded for low quality or the presence of sequencing adapters in the reads.

Each sample's reads were then processed using RSEM version 1.2.3 (with bowtie-0.12.9 for the alignment step) [17, 18]. Percentage of reads mapped to the transcriptome ranged from 72% to 85%.

The R package EBSeq [19] was used to identify genes expressed differentially between the 3 A2lox+Tbx5 samples, and all 21 other samples. Those genes reported by EBSeq to have a posterior probability of differential expression of .95 or greater were then filtered to select only those with a median-normalized TPM greater than 7 in at least one sample, and a fold change of at least 4 between the mean of the median-normalized Expected Counts of the 3 A2lox+Tbx5 samples, versus that of the other 21 samples. Using the R cor() function,

Spearman correlations (rho) were then calculated for each pair of samples over the set of 169 selected genes, ranked by their median-normalized Expected Counts (per EBSeq). The samples were then clustered using the R hclust() function, with the "single" algorithm applied to the distance metric (1- rho2). The R heatmap.2() function was used to display the resulting clusters, with each cell of the heatmap representing the calculated rho2.

Electrophysiology

Whole cell current clamp recordings were obtained from spontaneously contracting cells in a chamber that was continuously perfused with extracellular solution containing 146 mM NaCl, 3 mM KCl, 10 mM HEPES, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 1 mM Na pyruvate, and 10 mM D-glucose (pH 7.4,m, NaOH). Patch pipettes contained 140 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA, 5 mM HEPES, 1 mM glutathione, 3 mM ATP-2K, 2 mM glucose, 0.5 mM GTP-Na (pH 7.2, KOH). Recordings were made at room temperature using pipettes with resistances ranging from 2–5 Mohm, a Multiclamp 700A amplifier, Digidata 1322A, and pClamp 9.2 acquisition software and data was analyzed with Clampfit (Molecular Devices, Sunnyvale, CA). Junction potentials and electrode resistance were nulled, Data was acquired at 10 kHz, and filtered at 2 kHz for analysis. Carbachol and norepinephrine (Sigma-Aldrich, St. Louis, Missouri) were dissolved in distilled water at 1000 fold concentrations, and diluted in extracellular solution on the day of the experiment.

Western blot and immunofluorescence analysis

Proteins were extracted from Total EBs or single cells using RIPA buffer (150 mM NaCl, 50 mM Tris HCl pH 7.5, 1 mM EDTA, 1% Triton, 1% Sodium Deoxicholate, 0.1% SDS) supplemented with Protease inhibitors (Complete – Roche) and quantified with Bradford reagent (Sigma). Protein samples were prepared in Laemmli buffer and loaded on gels for SDS-PAGE. Proteins were transferred onto PVDF membranes (Millipore) for detection with the indicated antibodies. Immunofluorescence staining was performed by fixing cells with 4% Paraformaldehyde/PBS for 10 minutes at 4°C, permeabilization with 0.1% Triton/PBS and blocking with 5% BSA/PBS before incubating with the primary antibodies. Samples were rinsed with PBS, blocked with 5% BSA/PBS and then incubated with the secondary antibody. After washing, samples were mounted on the slides using Prolong Gold with DAPI (Molecular Probes). Pictures were acquired with Axioimager M1 fluorescence microscope (Zeiss).

Antibodies used in this study included anti-MYOD (BD Biosciences – 554130), anti-cTNI (Abcam – ab47003), anti-GAPDH (Abcam – ab8245), anti-MyHC (Clone MF20 – Developmental Studies Hybridoma Bank), HRP-conjugated anti-mouse and anti-rabbit IgG (Amersham), Alexa488-conjugated anti-rabbit IgG and Alexa555-conjugated anti-mouse IgG (Molecular Probes).

Statistical analysis

Student's one-tailed t-test was used to analyze differences between non-induced and dox-induced samples whereas differences between multiple samples were assessed by ANOVA.

RESULTS

Pax3 represses the cardiac lineage during ES cell differentiation

Recent findings from our group [9] and others [10] suggest that the Pax3-mediated induction of the skeletal myogenic lineage in differentiating mouse ES and iPS cells may occur at the expense of the cardiac differentiation program. Using a doxycycline-inducible Pax3 ES cell line, we have shown that induction of Pax3 during EB differentiation resulted in an increased frequency of the PDGFRα^{+}FLK1^{-} population in day 5 EBs (Figure 1A and [9, 20]). We show here that this is associated with the up-regulation of several paraxial mesoderm/myogenic genes, such as Paraxis (Tcf15), Myf5, M-cad and c-Met, and the concomitant down-regulation of several cardiac genes, including Nkx2-5, Gata4, Tbx5, Hand1, Mef2C, Pitx1 and Myl2 (Figure 1B). When cultures were maintained until day 10 as a monolayer, Pax3 promoted skeletal muscle differentiation while completely suppressing terminal cardiac differentiation, as shown by immunofluorescence and western blot analyses for MyoD and cardiac Troponin I (cTnI), respectively (Figure 1C–D). To exclude the possibility that repression of the cardiac program was caused by off-target effects of doxycycline, we used an inducible ES cell line expressing a deletion mutant of Pax3 that is unable to induce skeletal myogenesis [9]. As expected, the cardiac differentiation potential of EB-derived cells was unaffected by the expression of this mutant (Figure S1A). Similar results were obtained with a control cell line expressing only Ires-GFP (Figure S1B). This repression seems specific to the cardiac program since Pax3 did not exert repression on the expression levels of several hematopoietic genes (Figure S1C), a mesoderm lineage that is well recapitulated during EB development [21].

Pax3 affects the commitment of the PDGFRα^{+}FLK1^{-} fraction

We have previously documented that Pax3 has a differential ability to induce the myogenic program within the mesodermal subpopulation on day 5 EBs: PDGFRα+ cells are responsive but FLK1+ cells are not) [9, 20]. Since these two markers recapitulate well the subdivision of mesoderm into lateral (which give rise to hematopoietic, endothelial and cardiac lineages) and paraxial (comprising the dermal, axial skeletal and skeletal myogenic progenitors) [22], we determined whether Pax3 would affect cardiac gene expression within these mesodermal sub-fractions. As shown in Figure 2A, genes involved in the commitment of the cardiac lineage, such as Gata4, Nkx2-5, Tbx5, Pitx1, Hand1 and Baf60c (reviewed by [12, 23]), were mainly expressed in the PDGFRα^{+}FLK1^{-} (P+F−) and PDGFRα^{+}FLK1^{+} (P+F+) fractions of non-induced differentiating ES cells (white bars). In contrast, Runx1 (expressed in hematopoietic-endothelial progenitors) and Shh (a marker of the axial mesoderm) were mainly expressed in the PDGFRα^{-}FLK1^{+} (F+) and PDGFRα^{-}FLK1^{-} (NEG) fractions (Figure S1D), confirming our ability to distinguish between different mesodermal sub-fractions.

Interestingly, Pax3 repressed the cardiac program selectively in the PDGFRα^{+}FLK1^{-} fraction (P+F−), with only a minor effect in the PDGFRα^{+}FLK1^{+} (P+F+) fraction (Figure 2A, black bars). Because Pax3 activity was found predominantly within the PDGFRα^{+}FLK1^{-} fraction, we next investigated whether this cell population would become irreversibly and terminally committed to the skeletal myogenic lineage upon Pax3

expression. Taking advantage of our doxycycline inducible cell line, we analyzed the effect of a pulsed Pax3 induction on the cardiac potential of the PDGFRα$^+$FLK1$^-$ cell fraction (Figure 2B). As shown in Figure 2C and 2D (as well as in Figure 1C–D), in the absence of Pax3 expression (no dox), the PDGFRα$^+$FLK1$^-$ sub-fraction generated cardiac cTnI$^+$ cells whereas these were totally abolished by constant Pax3 induction (+dox), as evidenced by the expression of MyoD (Figure 2D, middle panel) or MyoG (Figure 2C) and absence of cTnI (Figure 2C and 2D, middle panel). However, when transient expression of Pax3 was applied to these cultures (pulse between days 3 and 5 of EB differentiation – Figure 2B), we observed the presence of cTnI$^+$ cells (Figure 2D, right panel), evidenced also by gene expression analysis (Figure 2C). This indicates that the PDGFRα$^+$FLK1$^-$ fraction retained the ability to differentiate into the cardiac lineage, although in a less efficient manner than the non-induced control (Figure 2C and 2D, left panel). The same trend was observed following staining with the pan-Myosin Heavy Chain (MF20) antibody, which recognizes both the skeletal and cardiac isoforms of MyHC (Figure S1E). Therefore, these findings indicate that Pax3-mediated repression of the cardiac program is still reversible in day 5 EB-derived PDGFRα$^+$FLK1$^-$ cells and this is in agreement with our previous observations showing the necessity of continuous Pax3 expression for the activation of the skeletal myogenic program.

Tbx5 rescues the cardiac lineage

The plasticity of lineage-committed mesodermal cells to transdifferentiate into other cell types has been reported by Takeuchi and Bruneau [24], which showed that over-expression of a combination of transcription factors and chromatin modifiers resulted in the cardiac commitment of non-cardiac mesoderm. Thus, it could be the case that Pax3 might specify the skeletal myogenic lineage at the expense of the cardiac fate. Considering the increasing interest in the mechanisms regulating cellular reprogramming, which is mainly affected by the expression of key transcription factors, we decided to use this model to investigate how lineage commitment is achieved from mesodermal precursors and to identify potential new regulators of cardiac commitment. We designed experiments to determine whether reintroduction of Gata4, Nkx2-5, or Tbx5 would be able to rescue the Pax3-mediated repression of the cardiac program. We began by up-regulating each single transcription factor using doxycycline-inducible lentiviruses in both iPax3 and control (A2lox-cre) ES cell lines. Since these lentiviral constructs contained a bicistronic cassette expressing the gene of interest as well as GFP, we were able to selectively analyze the cells expressing Pax3 and the cardiac gene transduced with each of these viruses. As shown in Figures 3 and S2, up-regulation of Gata4 and Nkx2-5 did not affect the ability of Pax3 to induce the PDGFRα$^+$FLK1$^-$ population. In contrast, the expression of Tbx5 was sufficient to repress the ability of Pax3 to increase the frequency of the PDGFRα$^+$FLK1$^-$ cell fraction (Figure 3A and B), resulting in levels comparable to the non-induced controls. Noticeably, overexpression of Tbx5 in the control cell line tended to further reduce the percentage of PDGFRα$^+$FLK1$^-$ cells (Figure 3B). Next, we isolated the GFP$^+$ cell fraction from these cultures to analyze the expression levels of genes involved in skeletal and cardiac differentiation. As expected, Pax3 induction in cells transduced with the control virus resulted in the up-regulation of Myf5 and c-Met (Figure 3C). This was not the case for the cell lines transduced with the inducible lentiviruses encoding Nkx2-5, Tbx5 and Gata4. Co-

expression of Pax3 with either one of the three cardiac transcription factors negatively affected the expression of Pax3 targets. On the other hand, Tbx5 but not Gata4 or Nkx2-5 was able to prevent the down-regulation of Hand1 and induce the expression of Mesp1, Bmp2 and Isl1 (Figure 3C). Hand1, Mesp1, Bmp2 and Isl1 represent important regulators of cardiac development [25–28].

Activation of the cardiac program in non-mesodermal cells, as shown by Srivastava and colleagues [29], requires combined expression of multiple transcription factors. Therefore, we reasoned that co-expression of Gata4, Tbx5 and Nkx2-5 might enhance the rescue of the cardiac program in iPax3 cells. As we will discuss later, we observed that Tbx5 is able to up-regulate Gatat4 levels (Figure 5A and Figure S2B). Taking advantage of this observation, we generated a lentiviral bicistronic construct encoding Nkx2-5 and the monomeric RFP (mRFP). This construct allowed the combined expression of Tbx5 (GFP^+) and Nkx2-5 ($mRFP^+$) or Gata4 (GFP^+) and Nkx2-5 ($mRFP^+$) in both Control and iPax3 ES cells. Analysis of untreated and dox-treated;GFP+;mRFP+ cells from day 5 EBs showed that co-expression of Nkx2-5 with either Tbx5 or Gata4 does not significantly affect the frequency of PDGFRa+FLK1− cells (Figure 3B and S2A) or the expression of Bmp2, Hand1, Isl1 and Mesp1 (Figure 3C). Since Nkx2-5, Tbx5 and Gata4 play an important role in cardiac development, we hypothesized that their over-expression would overcome the Pax3-mediated repression of the cardiac program. Nonetheless, when GFP^+ or GFP^+;$mRFP^+$ cells purified from day 5 EBs were cultured as a monolayer to evaluate their final commitment towards the skeletal myogenic (MyoD+) or the cardiac (cTnI+) lineage, we found that only Tbx5 was able to rescue the cardiac program, as shown by both immunostaining and qRT-PCR analyses (Figure 4A and B). More importantly, Tbx5 abolished the ability of Pax3 to induce the skeletal myogenic lineage at the expense of the cardiac program. In agreement with the gene expression data shown in Figure 3, co-expression of Nkx2-5 and Tbx5 did not improve the Tbx5-mediated rescue of the cardiac differentiation of iPax3 cells (Figure 4B and S2C). On the other hand, although both Nkx2-5 and Gata4 negatively affected the Pax3-induced differentiation towards the skeletal muscle lineage, single or combined expression of these two transcription factors did not rescue the Pax3-mediated repression of the cardiac program (Figure S2C).

To further characterize the ability of Tbx5 to rescue the cardiac program in the presence of Pax3, we analyzed the electrophysiological properties of the cardiomyocytes generated upon its over-expression. Whole-cell recordings demonstrated that spontaneously contracting cells in the control, Tbx5 only, and Pax3+Tbx5 groups exhibited spontaneous action potentials (Figure 4C). The frequency of action potentials was reduced by bath application of the cholinergic agonist carbachol (10 μM; Figure 4D) in the majority of cells of all three groups. On the other hand, treatment with the adrenergic agonist norepinephrine (100 μM) increased action potential frequency in most of examined cells (Table 1). These responses to parasympathetic and sympathetic agonists are expected in spontaneously active cardiomyocytes. The action potentials recorded in all three groups of cells displayed characteristics similar to those seen in developing cardiomyoyctes [30], including long action potential duration at 90% repolarization (APD90 - Table 1). While some cells had action potentials that appeared immature, with less negative maximum diastolic polarizations (MDP), lower peak amplitudes, and smaller maximum depolarizing slopes, and

shorter durations, others had action potentials that looked like more mature atrial-like or ventricular-like with more negative MDP, larger action potential amplitudes, faster depolarizing slopes, pronounced plateau phases, and very long APD90s (Figure 4E, Table 01). Together these data support the conclusion that Tbx5 rescued the cardiac phenotype in Pax3-transduced differentiating ES cells..

Identification of genes rescued by Tbx5

Based on these results, we hypothesized that in our experimental model, Tbx5, but not Nkx2-5 and Gata4, would be able to up-regulate a specific subset of genes necessary for the cardiac commitment that is down-regulated after induction of Pax3. To identify these genes, we performed a global gene expression analysis using RNAseq technology. In particular, we analyzed the RNAs from Ctrl, Pax3 only, Tbx5 only, Pax3+Tbx5, Gata4 only and Pax3+Gata4 total day 5 EBs as described in Table S1. Since Gata4 was not able to rescue the cardiac program, we hypothesized that important Tbx5-induced genes would not be up-regulated upon Gata4 expression. For the analysis, RNAs from 3 independent biological replicates for each group were sequenced using the Illumina HiSeq 2500 platform and the resulting reads were mapped to the mouse transcriptome for gene expression estimation. These data were then normalized to allow the comparison of gene expression levels between different samples. Next we performed a differential expression (DE) analysis to identify genes up- or down-regulated between the A2lox+Tbx5 samples and all other samples treated as a single experimental condition. After that we filtered the data to exclude low expressed genes using a cutoff of TPM>7 (where TPM stands for Transcript Per Million). Spearman correlations for each pair of samples across the resulting set of 169 genes were calculated, and the resulting ρ^2 values are shown in Figure S3, with the samples clustered using $(1 – \rho^2)$ as the distance metric. Samples cluster into their original types, indicating that the selected genes represent good markers to discriminate among the sample groups. Importantly, this analysis evidenced also the correlations between different groups and more specifically that samples formed three major clusters based on the expression of Tbx5, Gata4 or the empty vector (Figure S3). The effect of Pax3 expression was only evident within each cluster. Furthermore, clustering of the selected genes based on their pairwise Pearson correlations across the 24 samples highlighted the presence of multiple sub-clusters of gene within each group of sample replicates (Figure S4). Based on the ability of each group to differentiate toward the skeletal or the cardiac lineage, as showed in Figure 3 and 4, we hypothesized that when compared to the “Ctrl” group, genes important for cardiac development would be down-regulated in the “Pax3 only”, “Gata4 only” and “Pax3+Gata4” groups, up-regulated in the “Tbx5 only” group and expressed at comparable levels in the “Pax3+Tbx5” group. Notably, we could identify about 30 genes that followed this pattern of expression (Figure S4, yellow square and Table S2). This group includes the transcription factors Gata5 and Phox2a, the calcium binding protein Vsnl1 and Pvalb, the adenosine receptor Adora1, the Ephrin receptor B1 and Pnmt, one of the enzymes involved in cathecolamine synthesis. To confirm the reliability of these data, we validated the expression of some candidate genes by qRT-PCR. As shown in Figure 5A and S5A, all the genes tested (Adora1, Gata5, Phox2a, Pnmt and Vsnl1) were repressed by Pax3, up-regulated by Tbx5 and more importantly, these genes showed an expression level similar to the Ctrl or slightly up-regulated when both Pax3 and Tbx5 were induced. Gene expression analysis of these genes in “Gata4 only” and

"Pax3+Gata4" groups confirmed that this effect was specific for Tbx5 expression (Figure S5B). Interestingly, we also observed that Tbx5 over-expression positively affects the levels of Gata4 and to a lesser extent Gata6 (Figure 5A). A similar correlation has been shown in E8.0 embryos expressing decreased levels of Tbx5 [31, 32], suggesting that Tbx5 might regulate the expression/maintenance of the transcript encoding Gata4 in the cardiac crescent.

Although Tbx5 and Gata4 have been reported to cooperate in the regulation of cardiac differentiation, our data indicate that they activate a different set of genes in day 5 EBs. This is supported by the analysis of the RNAseq data of the groups expressing each of the single transcription factors (Figure S5C and Table S2 - Ctrl vs. Tbx5 only and Ctrl vs Gata4 only). Each transcription factor up-regulated about 100 genes, but only few of them were in common. This analysis also highlights the ability of Tbx5 to regulate the cardiac commitment by affecting the levels of a subset of transcription factors, including Tbx3 (Figure S5D), Tbx2, and Sox10 (Table S2).

Based on the above findings, Tbx5 appears to regulate the cardiac commitment of early mesodermal progenitors. To validate our observation *in vivo*, we analyzed the gene expression levels of the above mentioned genes in E6.5 and E7.5 total mouse embryos (Figure 5B). Noteworthy, we observed a marked up-regulation of Tbx5 and Gata5 between these two time points. An opposite trend was observed for the expression of Mesp1 and Adora1, which decreased between E6.5 and E7.5. In agreement with previous reports, Mesp1 is transiently expressed in mesodermal cardiac progenitors and it is quickly down-regulated after E7.0 [25]. Although to a lesser extent, Phox2a and Vsnl1 were also significantly more expressed in E7.5 embryos. A similar trend was observed for Actc1 and Nppa1, two known Tbx5-target genes [31, 32]. In contrast, we could not detect a difference in expression between these two time points for Gata4 and Gata6. To assess whether the genes identified with our approach play a role in the regulation of the cardiac program, we promoted their down-regulation using lentiviral-based shRNA constructs. Among the evaluated genes, we found two shRNA clones that efficiently down-regulated the calcium binding protein Vsnl1 (Figure S5E). Interestingly, knockdown of Vsnl1 negatively regulated cardiac differentiation, as shown by gene expression analysis of the cardiac marker Myl2 in transduced cultures (Figure S5E).

DISCUSSION

Transcription factors represent the major effectors of cell fate decisions during development, and for the homeostasis of adult tissues. Our data from differentiating ES cells suggest that Pax3-dependent activation of the skeletal myogenic program requires the repression of cardiac genes. Pax3 induction in this system results in an almost complete repression of the cardiac program, both at gene expression and protein levels (Figure 1), while leaving the hematopoietic lineage unaffected (Figure S1). In agreement with our findings, similar observations were reported in mouse iPS cells using either Pax3 or Pax7 [10]. Since differentiating ES cells contain precursors of different cell types, we extended our analysis to mesodermal sub-populations based on the expression of PDGFRα and FLK1, as described by Sakurai and colleagues [22]. Interestingly, we found that the PDGFRα^{+}FLK1^{-} sub-fraction was the main population affected by Pax3 (Figure 2A) and, following a pulsed

expression of Pax3, this sub-population was still able to generate cTnI+ cells (Figure 2D and E). Furthermore, while Nkx2-5 and Gata4 impaired only Pax3-dependent skeletal myogenesis, forced expression of Tbx5 in the inducible Pax3 ES cell line was able to rescue the cardiac differentiation (Figures 4A and B), as further confirmed by electrophysiological analysis of Tbx5-induced cardiomyocytes (Figure 4C–E and Table 01). Our data showing the ability of Tbx5 to promote cardiac differentiation from ES cells is corroborated by a previous report [33]. Tbx5, Gata4 and Nkx2-5 are well known to regulate several steps of cardiac development and accordingly, knock-out animals for each of these genes show severe cardiac defects [31, 34, 35]. Similarly, Tbx5 and Gata4, through interaction with the chromatin remodeler Baf60c, have been reported to induce the transdifferentiation of mouse non-cardiac mesoderm into heart tissue [24]. Although forced expression of these transcription factors has the potential to directly convert fibroblasts into cardiomyocytes [29], we did not observe enhanced rescued by co-expressing Tbx5 (or Gata4) and Nkx2-5 (Figure 4). Noteworthy, Tbx5 alone appear to be sufficient to repress the skeletal myogenic and promote the cardiac lineage, as evidenced by the up-regulation of Mesp1, Bmp2 and Isl1 in day 5 EBs, along with the rescue of Hand1 (Figure 3). Tbx5-dependent induction of Bmp2 may be associated with the repression of the skeletal myogenic program, as shown in chick embryos by Brand and colleagues [36]. Mesp1 has been recently showed to improve the efficiency of the Tbx5:Gata4:MEF2C-mediated direct reprogramming of human fibroblasts to cardiomyocytes [37], suggesting its requirement to activate the cardiac program in refractory cells. Since Mesp1 represents the earliest marker of cardiac mesoderm [25], these observations suggest that activation of the cardiac program in non-cardiac cells might require a transition through a cardiac mesodermal stage.

Using the ES/EB model to investigate how cardiac commitment is achieved in early mouse embryos, we identified a subset of genes up-regulated by Tbx5 (Figure 5) that could account for the rescue of the cardiac phenotype observed in Figure 4. As expected, this list contains genes that had already been reported to be expressed in the cardiac crescent in mouse (Vsnl1 and Gata5) or frog (Phox2a) developing embryos [38–40]. Gata5, in association with Gata4 and Gata6, is important for proper cardiac development, and its over-expression in ES cells differentiated, in serum-free conditions, efficiently promotes cardiac fate [14, 41]. Importantly, our screening identified a Tbx5-dependent up-regulation of Gata4 and Gata5 in cells from day 5 EBs, which suggests a positive correlation between Tbx5 and Gata4/5 expression. Differential Gata4 mRNA expression in the cardiac crescent has also been reported using an allelic series of mouse embryos expressing different levels of Tbx5. Notably, in the same article the authors identified Vsnl1 as an highly sensitive Tbx5 dose-dependent gene [32].

The list of candidates identified in our screening also contains genes reported to be involved in the regulation of cardiac function. Pnmt is the final enzyme in the pathway for catecholamine synthesis, playing a main role in the stimulation of heart rate and force of cardiac contraction. This enzyme, produced in the adult as well as in the developing myocardium, is required for proper cardiac development, and its expression can be already detected in E9.5 rat embryos [42–44]. Adora1, one of the adenosine receptors, has been shown to contribute to ischemic tolerance of the heart [45], and together with the other

adenosine receptor subtypes, plays a beneficial role in the modulation of cardiac functions [46].

Interestingly, a recent genome wide study using mouse ES cells, which were induced to differentiate towards the cardiac lineage, identified several groups of genes differentially expressed between four time points: ES cells (ESC), mesodermal precursors (MES), cardiac progenitors (CP) and fully differentiated cardiomyocytes (CM) [47]. Not surprisingly, when we compared our gene list with these published data (Figure S5F), we found that the majority belonged to the categories of genes specifically enriched in cardiac progenitors (47%) and cardiomyocytes (25%). The rest of genes were divided between the mesoderm-specific (13%), up-regulated in mesoderm and cardiac progenitors (9%), and up-regulated in cardiac progenitors and cardiomyocytes (3%). Therefore these data confirm the ability of our system to identify genes expressed in specific stages of cardiac development.

Lastly, in agreement with the *in vitro* data, our gene expression analysis using total E6.5 and E7.5 embryos confirmed the up-regulation of some of the candidate genes (Gata5, Vsnl1 and Phox2a) between these two time points (Figure 5). This coincides with the expression of Tbx5, and supports the hypothesis that Tbx5 might regulate their expression. This notion is corroborated by our preliminary finding showing that knockdown of Vsnl1 negatively affects Tbx5-mediated cardiac differentiation. This gene has been studied in the context of neuronal differentiation, where it is known to modulate cyclic nucleotide-dependent signaling and ultimately cellular differentiation [48]. Interestingly, Vsnl1 expression has been recently described during heart development [39], further supporting the likelihood of a function in the regulation of cardiac differentiation.

CONCLUSIONS

In this study, by combining the expression of different critical transcription factors, we were able to modulate the fate of differentiating ES cells, and identify potential new regulators of cardiac development. Ultimately, these data will be valuable in elucidating the mechanisms controlling cellular reprogramming.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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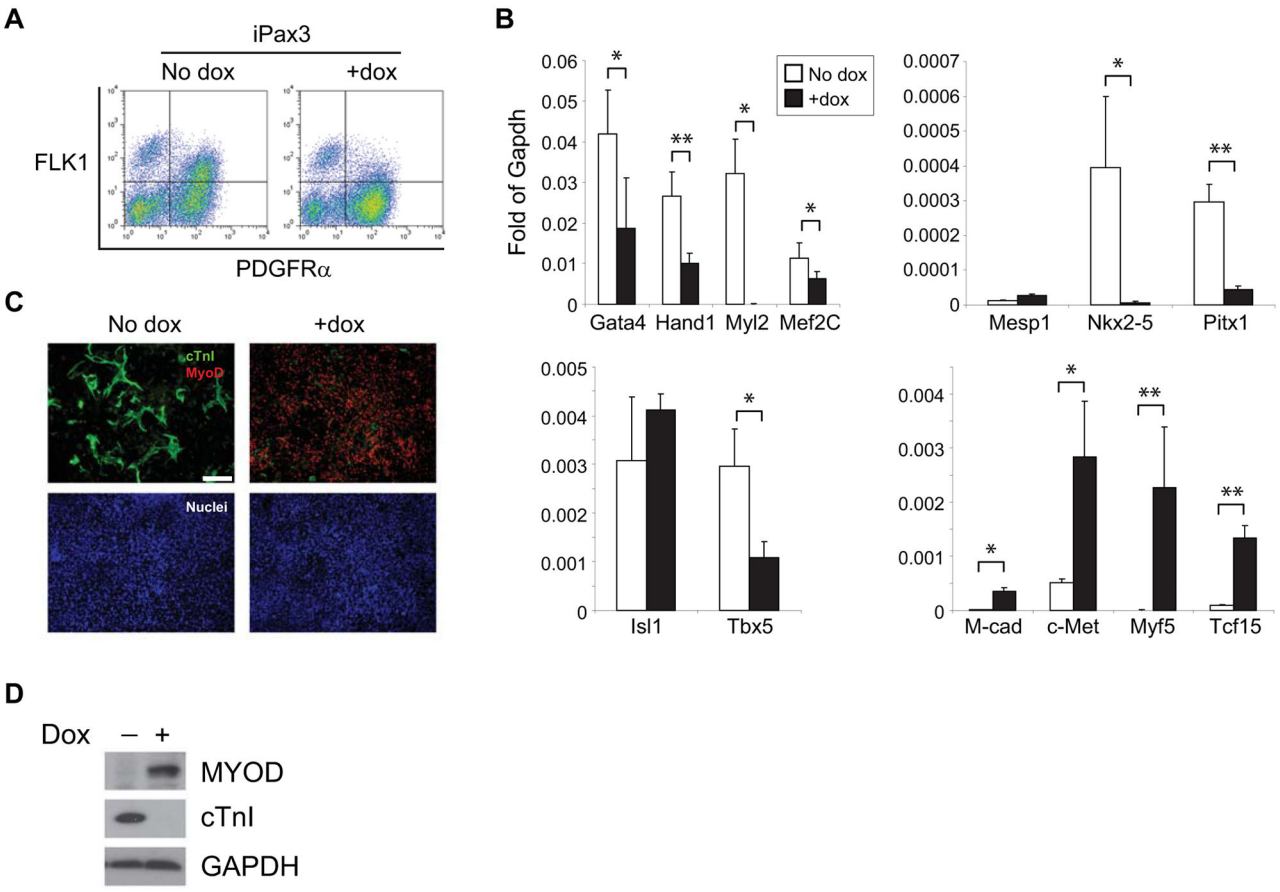

Figure 1.

Pax3 represses several cardiac transcription factors as well as terminal cardiac differentiation. A) Expression of Pax3 during EB differentiation increases the frequency of the PDGFRα^{+}FLK1^{-} population. B) Gene expression analysis of total day 5 EBs. RNAs were extracted, retrotranscribed and used for quantitative RT-PCR. Bars represent average ±SD for at least 3 independent experiments. *p<0.05, **p<0.01. C) Inducible Pax3 cells from day 5 EBs (untreated and Dox-treated) were cultured as a monolayer for 5 days to allow terminal differentiation. Cells were fixed and stained with anti-MYOD (in red), anti-cardiac TROPONIN I (in green), Dapi (in blue), and imaged on epifluorescence microscope. Bar: 100μm. D) Concomitantly, cells were lysed for protein extraction and western blot analysis using the indicated antibodies. GAPDH was used as loading control.

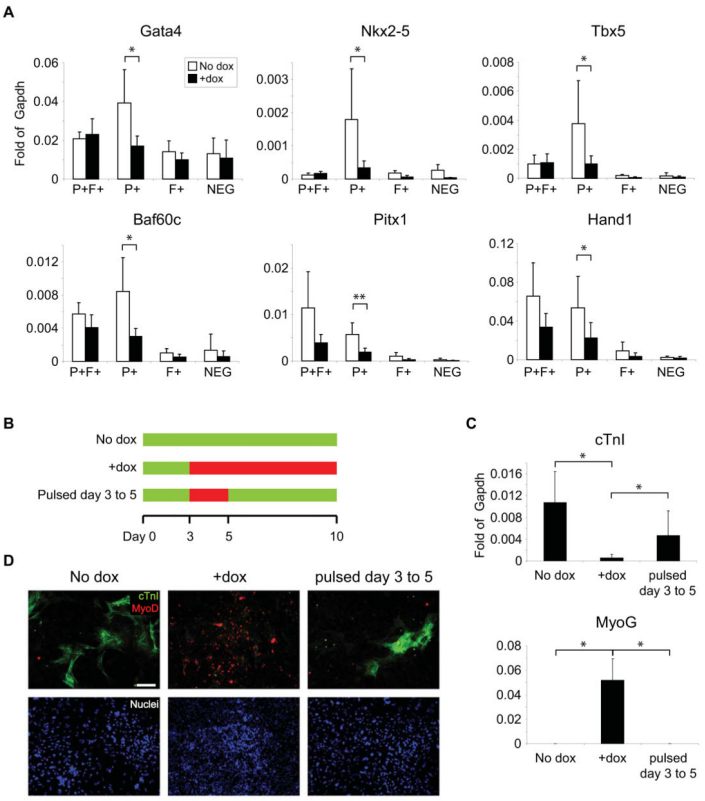

Figure 2.

Pax3 selectively downregulates the expression of cardiac developmental genes in the PDGFRα^{+} sub-fraction. A) Gene expression analysis in purified mesodermal sub-fractions. Cells from day 5 dox-induced or non-induced EBs were stained using antibodies against FLK1 and PDGFRα, and subsequently sorted as indicated in the graphs. RNAs were extracted, retrotranscribed and used for quantitative RT-PCR. Bars represent average ±SD for at least 3 independent experiments. *p<0.05, **p<0.01. Abbreviations: P+F+, PDGFRαFLK1 double positive cells; P+, PDGFRα single positive cells; F+, FLK1 single positive cells; NEG, double negative cells. B) Scheme representing the induction of Pax3 at different time points in day 5 to evaluate the ability of these cells to differentiate toward skeletal or cardiac muscle. Day 5 EB-derived PDGFRα single positive cells from induced (+dox), induced transiently for 2 days (pulsed from day 3 to 5), and non-induced (no dox) cultures were plated as monolayer for 5 days to allow terminal differentiation. C) Cells were resuspended in Trizol for RNA extraction followed by qRT-PCR to measure the expression of MyoG and Tnni3 (cTnI) or D) fixed and stained with anti-MYOD (in red), anti-cardiac TROPONIN I (in green), Dapi (in Blue), and imaged on epifluorescence microscope. Scale Bar: 100μm. Bars represent average ±SD of at least 3 independent experiments. *p<0.05

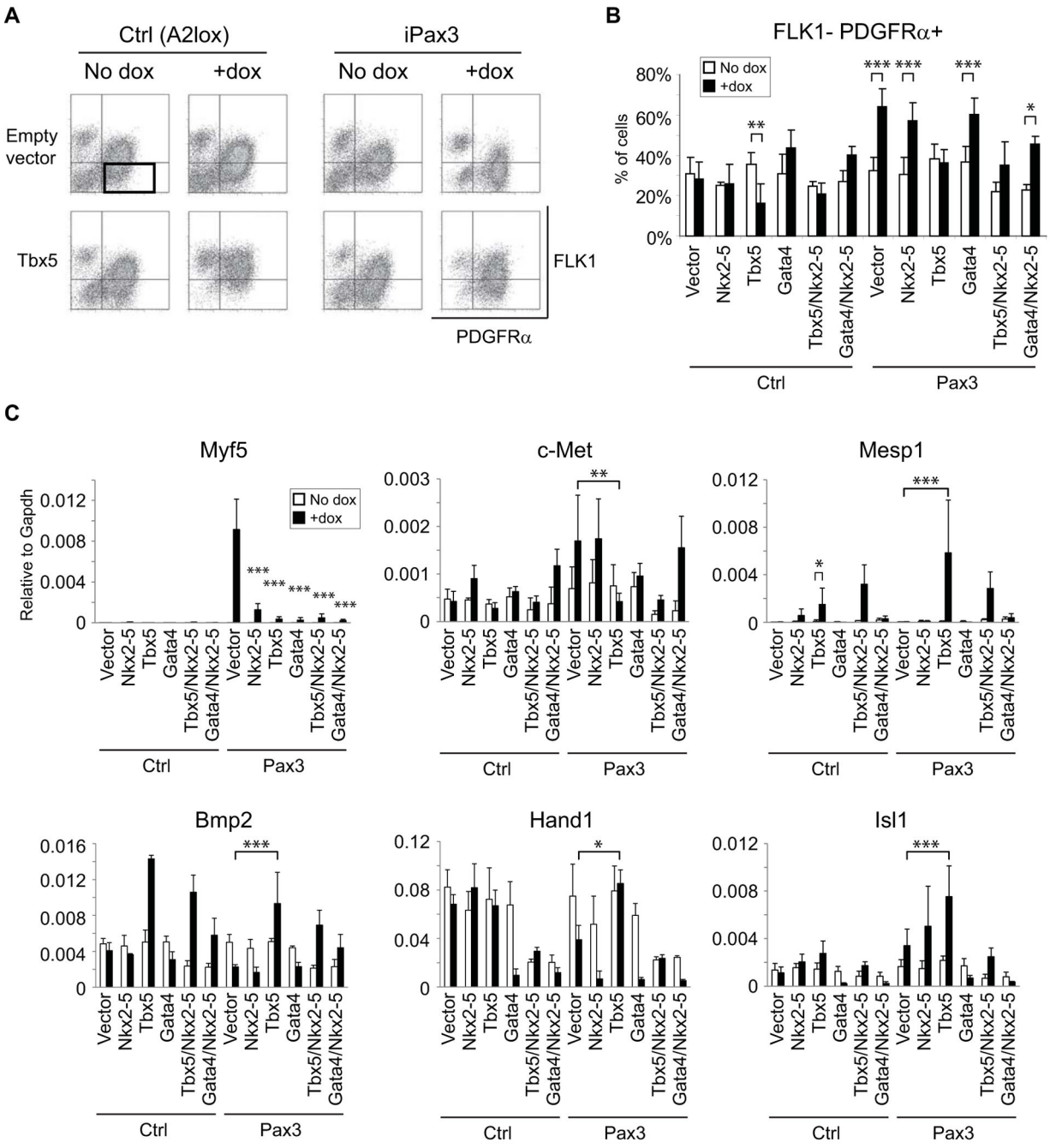

Figure 3.

Tbx5 induces the up-regulation of several cardiac markers in day 5 EBs. Inducible Pax3 and control (wild-type) ES cell lines were transduced with inducible lentiviral vectors encoding Gata4, Nkx2-5, Tbx5, or control vector. A) FACS plots for the expression of PDGFRα and FLK1 in day 5 EBs that had been treated or not with doxycycline for 2 days. B) Quantification of PDGFRα^{+}FLK1^{-}GFP^{-} (no dox, white bars) and PDGFRα^{+}FLK1^{-}GFP^{+} (+dox, black bars) sub-populations from the FACS plot shown in Panel A. Bars represent average ±SD of at least 3 independent experiments. C) Total EBs (no dox), sorted GFP

single positive or GFP:mRFP double positive cells (+dox) from day 5 cultures were analyzed by qRT-PCR for the indicated genes. Bars represent average ±SD of at least 3 independent experiments.

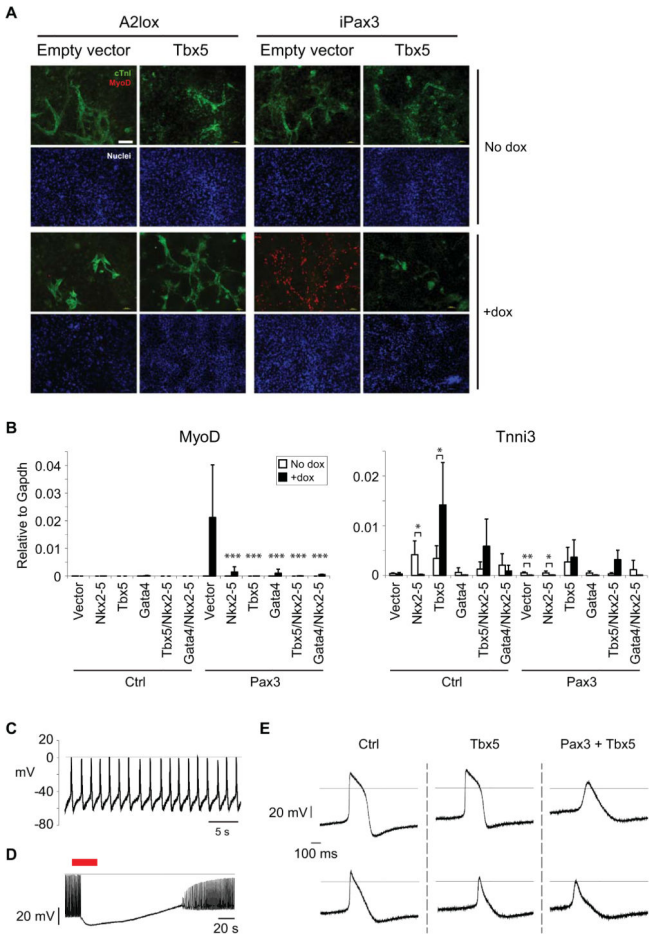

Figure 4.

Tbx5 rescues cardiac differentiation at the expense of the skeletal myogenic program. A) Inducible Pax3 ES cells and control ES cells transduced with inducible Tbx5-iresGFP or IresGFP (Control) lentiviruses were cultured as a monolayer for 5 days to allow terminal differentiation. In particular, total EBs from non-induced cultures were compared to GFP^+ cells from dox-induced cultures and plated as monolayer on day 5 of EB differentiation. Doxycycline was withdrawn after 2 days in monolayer culture. Cells were fixed and stained with anti-MYOD (in red), anti-cardiac TROPONIN I (in green), Dapi (in blue), and imaged on epifluorescence microscope. Bar: 100μm. B) Concomitantly, cells from all groups were resuspended in Trizol for gene expression analysis with the indicated Taqman probes. Bars represent average ±SD of at least 3 independent experiments. Tbx5 is able to induce the cardiac muscle program at the expense of the skeletal myogenic lineage. *p<0.05, **p<0.01, ***p<0.001. C) Spontaneous action potentials in a representative cardiomyocyte from Pax3+Tbx5 cultures. D) Membrane potential was reversibly hyperpolarized and spontaneous action potentials in a representative cardiomyocyte from Pax3+Tbx5 cultures were inhibited in response to bath application of 10 μM carbachol (represented on the graph by a red bar). E) Examples of action potentials recorded in Control, Tbx5, and Pax3+Tbx5 cultures. In C, D and E time scale is represented by a black bar on the graph and the horizontal line indicates 0 mV.

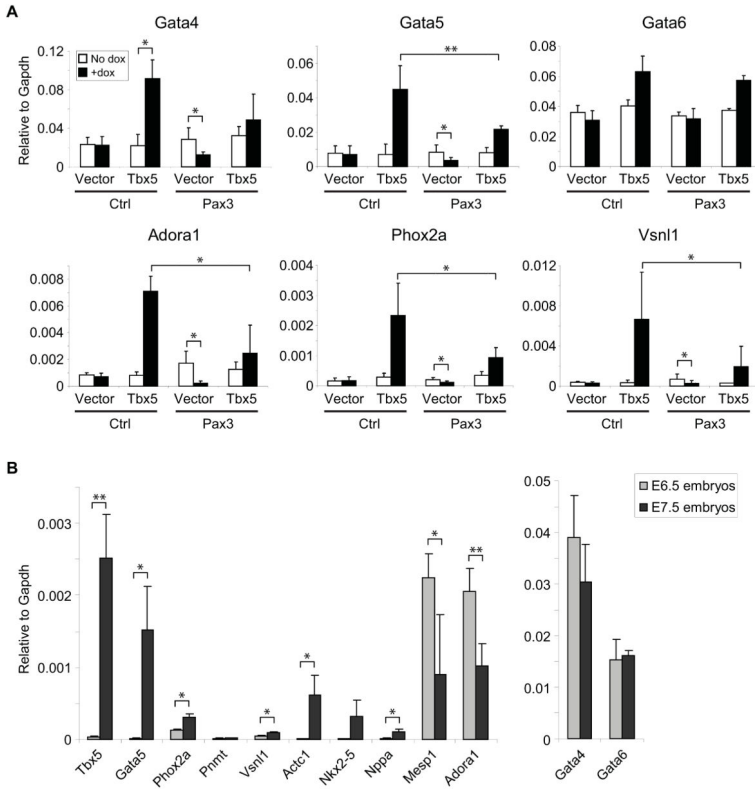

Figure 5.

Validation of candidate genes identified by RNA-seq. A) Total EBs (no dox) or sorted GFP positive cells (+dox) from day 5 cultures (from A2lox Ctrl and A2lox Pax3 cells transduced with a lentivirus encoding IresGFP only or Tbx5-IresGFP) were analyzed by qRT-PCR for the indicated genes. Bars represent average ±SD of at least 3 independent experiments. B) Total RNA from pulled E6.5 or E7.5 total embryos was retrotranscribed and used for measuring the levels of the indicated gens. Bars represent average ±SD of at least 3 independent experiments. *p<0.05, **p<0.01

Table 01

AP characteristics

Sample (n)		Max Rise Slope (V/sec)	Peak Amp (mV)	MDP (mV)	APA (mV)	APD90 (msec)	Response to Carbachol	Response to Norepinephrine
Control (5)	Mean	25.26	25.40	−71.81	97.21	275.82	3 / 4 cells	4 / 4 cells
	Range	4.85 59.20	8.47 38.46	−64.60 −82.99	73.08 110.24	217.19 419.74		
Tbx5 (7)	Mean	11.46	17.38	−46.09	82.65	213.79	7 / 7 cells	6 / 7 cells
	Range	1.33 48.11	5.67 42.86	−54.37 −81.33	64.95 107.45	159.53 289.44		
Pax3+Tbx5 (6)	Mean	2.44	5.39	−56.60	61.98	246.92	4 / 5 cells	4 / 4 cells
	Range	1.15 3.74	−2.45 16.33	−51.05 −64.02	54.51 68.70	197.49 286.58		

n = number of cells analyzed for each sample

Maximal Rise Slope

Peak Amplitude

MDP = maximal diastolic polarization

APA = action potential amplitude

APD90 = action potential duration at 90% repolarization