Pitx2c Modulates Cardiac-Specific Transcription Factors Networks in Differentiating Cardiomyocytes from Murine Embryonic Stem Cells

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Key Words
Embryonic stem cells · Cardiomyocytes · Differentiation · Cell culture · Pitx2c

Abstract
Aim: The knowledge of the molecular signals that control cell differentiation into cardiomyocytes is critical to apply cell-based therapies and repair an injured heart. The transcription factor Pitx2 has essential roles in the development of different organs including the heart. Although a direct role of Pitx2 in the developing myocardium has recently been reported, the molecular pathways driven by Pitx2 as well as its cardiac target genes remain largely unexplored. The aim of this study was to unravel the molecular mechanisms driven by Pitx2 during the process of cardiomyocyte differentiation in vitro in mouse embryonic stem cell-derived cardiomyocytes.
Methods and Results: Pitx2c was overexpressed in the R1-embryonic stem cell line. mRNA levels and protein distribution of several specific cardiac genes were analyzed by real-time PCR and immunohistochemistry experiments in R1-embryonic stem cell-derived beating areas at different stages of in vitro differentiation. Our results show that overexpression of Pitx2c in embryonic stem cell-derived cardiomyocytes is able to dynamically upregulate several cardiac-enriched transcription factors such as Isl1, Mef2c and Gata4. Additionally, Pitx2c induces the expression of chamber-specific cardiac genes such as Tbx5, Nppa and Cx40. These data were validated in an in vivo model of Pitx2 loss of function.
Conclusion: Taken together, these results demonstrate that Pitx2 plays a major role reinforcing the transcriptional program of cardiac differentiation.

Abreviations

EBs	embryonic bodies
ESC	embryonic stem cell
FHF	first heart field PHH3 phospho-histone H3
RT	reverse transcriptase
SHF	second heart field

Introduction

Cardiogenesis is a complex process that requires the contribution of diverse cell types with specialized function. It has previously been well established that cardiomyocytes arise from two distinct cardiac progenitor populations during development, the first one contributing to the left ventricle (first heart field or FHF), and the second one to the right ventricle, outflow tract, and atria (second heart field or SHF) [Kelly and Buckingham, 2002]. The molecular mechanisms that govern cardiogenesis take place via the activation of multimeric transcription factor complexes. To date, those reported to be essential for normal cardiac development include members of the Nkx2, GATA, myocyte enhancer factor-2 (MEF-2) and T-box families [Brand, 2003; Dodou et al., 2004]. Recently, it has been demonstrated that all cardiomyocytes derive from Isl1+/Nkx2.5+ multipotent progenitor cells [Ma et al., 2008]. Nonetheless, it is clearly established that the transcription factor Isl1 is mainly expressed in and is required by the cells that are added to the developing heart tube from the second cardiogenic region known as the SHF [Galli et al., 2008].

Pitx2 is a homeobox transcription factor that plays an important role in cardiac morphogenesis [Gage et al., 1999]. Pitx2 is widely expressed in several embryonic tissues including cardiac and skeletal muscles [Gage et al., 1999]. The generation of Pitx2 null mice revealed early embryonic lethality with severe cardiac malformations [Gage et al., 1999], thus demonstrating the importance of Pitx2 during cardiogenesis. Expression analyses as well as epigenetic and genetic studies have demonstrated that Pitx2c is the major isoform involved in heart development [Schweickert et al., 2000; Liu et al., 2001]. Recent studies have revealed that Pitx2 could act by conferring left-right identity to SHF-derived myocardium [Ai et al., 2006; Galli et al., 2008]. Moreover, analyses of the Pitx2 conditional knockouts in the developing myocardium [Tessari et al., 2008] have provided evidence that Pitx2 acts first by driving left atrial morphological identity in the SHF and/or venous pole and later by regulating cardiomyocyte maturation. These results clearly indicate that Pitx2 plays an essential role in the process of myocardial differentiation. In the present work our main interest is to unravel the role of Pitx2c in the transcriptional regulation of differentiating and functionally active cardiomyocytes by using an in vitro embryonic stem cell (ESC) differentiation system. Our results place Pitx2-modulating cardiac molecular pathways and identify several putative Pitx2c cardiac target genes.

Materials and Methods

ESC Culture and Differentiation

R1-ESC line, derived from the 129 S1/SvImJ strain, were a kind gift from Dr. Andras Nagy (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ont., Canada). R1-ESCs were cultured and differentiated as described in detail in the online supplementary methods (www.karger.com/doi/10.1159/000323533). We designated day 0 as when embryonic bodies (EBs), obtained from hanging drops, were plated. In general, we identified contracting regions within the EBs between 3 and 4 days after plating (10–11 total days).

DNA Transfections

Two independent transfections were performed in R1-ESCs with different plasmids. To evaluate α-MHC promoter activity in this system, we stably transfected R1-ESCs with a pαMHC-EGFP plasmid containing a neomycin resistance gene driven by the SV40 promoter. To generate ESC-derived cardiomyocytes overexpressing Pitx2c, R1-ESCs were stably cotransfected with two plasmids: (a) PGK-Neo which contains a neomycin resistance gene driven by the PGK promoter and (b) a pαMHC-Pitx2c-IRES-puromycin. Transfections, construct preparation and subclone selection were done as described previously [Klug et al., 1996; Martinez-Fernandez et al., 2006] (see online supplementary methods).

Generation of Conditional Tissue-Specific Null Mutant Mice

Conditional tissue-specific Pitx2 mutants were generated by intercrossing Nppa-Cre transgenic mice [de Lange et al., 2003] and Pitx2 floxed mice [Gage et al., 1999]. Double heterozygous offspring were screened by PCR using Cre-specific and Pitx2-specific primers as described previously [Tessari et al., 2008] and in detail in online supplementary methods. The investigation performed conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the Bioethics Committee of the University of Jaén.

Immunohistology Staining and Chamber Diameter Measurements

EBs and Nppa-Cre+ Pitx2fl/fl or Nppa-Cre+ Pitx2–/– were fixed, embedded, sectioned and stained as described in the online supplementary methods.

RNA Isolation, Reverse Transcriptase Reaction and Quantitative Real-Time PCR

Because spontaneously contracting cardiomyocytes constitute only a small fraction of EBs, parts of EBs containing beating areas were dissected under a binocular microscope. Only dissected specimens were used to perform reverse transcriptase (RT) reactions. Beating areas were collected at three different times after contraction was initiated: 3 days (beginning of contraction), 7 days (4 days' beating) and 13 days after EB plating (10 days' beating). These times of culture have previously been considered as early, intermediate and late stages of ESC in vitro differentiation [Maltsev et al., 1994]. Total RNA isolation, RT reactions and quantitative real-time PCR were performed as described in detail in online supplementary methods.

Results

Cardiac Gene Expression Profiles in Beating Areas Obtained from R1-ESCs

The goal of this research is to identify the molecular mechanisms governed by Pitx2 in differentiating cardiomyocytes obtained from R1-ESCs. Cardiogenic foci in EBs derived from ESCs are characterized by spontaneous contractile activity [Klug et al., 1996]; thus, we have used for our studies isolated beating areas derived from R1-ESCs. Although characterization of gene expression signatures for cardiomyocytes derived from R1 or other ESC lines has been previously reported [Doss et al., 2007; Yamanaka et al., 2008], we performed a more extensive analysis of gene expression levels in isolated beating areas derived from the R1-ESC line, especially regarding the transcriptional

pathways that govern cardiac field differentiation. We first unraveled the basal cardiac transcriptional networks that regulate in vitro cardiac differentiation in R1-ESC-derived beating areas, especially with regard to the relationship of Isl1/Pitx2c with other transcription factors (fig. 1).

Nkx2.5 transcript levels were higher at early stages indicating that this cardiac transcription factor is induced early in this system of in vitro differentiation (fig. 1a). Isl1, Pitx2c and Gata4/6 mRNA expression patterns were similar, with their transcript levels maximal at intermediate stages. Other transcription factors considered as early markers of the SHF progenitors such as Foxh1 and Tbx1 were also assayed. Foxh1 expression was not detected in R1-ESC-derived beating areas (data not shown); however, Tbx1 expression was present at low levels without significant changes between the stages of in vitro differentiation analyzed (fig. 1a). mRNA levels for Mef2c were increased from early to late stages. Tbx5 and Tbx2, which are known to be involved during chamber-specific differentiation in vivo, displayed strong upregulation at early stages, decreasing to low levels at intermediate stages and rising again at later stages of in vitro differentiation. For structural cardiac genes, similar to what occurs in the mouse embryo [Lyons et al., 1990], we found that α-MHC, βMHC, Mlc2v and Mlc2a isoforms display a clear induction at early stages and maintain lower expression levels at later stages following a similar pattern to Nkx2.5 (fig. 1b).

As cardiomyocytes acquire a more mature phenotype, they initiate the expression of gap-junction genes such as Cx40 and Cx43 which are required for intercellular coupling [Delorme et al., 1997] as well as other genes such as atrial natriuretic factor (Nppa) [Houweling et al., 2005]. These genes (Cx40 and Nppa) display similar expression levels to that in native tissue in beating areas isolated at early stages of in vitro differentiation (online suppl. fig. 1). Gene profiles for these tissue-specific cardiac genes in R1-ESC-derived cardiomyocytes (fig. 1b) showed that Cx40 transcripts were present at similar low levels at all stages of in vitro differentiation. Nppa, which has been shown to be a target of Pitx2 [Ganga et al., 2003], displayed a very similar expression pattern to Pitx2, suggesting that this genetic interaction seems to be maintained in this in vitro system of cardiac differentiation. These results indicate that beating areas derived from R1-ESCs activate the cardiac transcription program and that cardiac genes are regulated in a time-dependent fashion in ESC-derived beating areas.

Since some of the transcription factors analyzed, such as Isl1 or T-box transcription factors, are not specific for differentiating cardiomyocytes, we cannot exclude that contaminating noncardiomyocytes within isolated beating areas could interfere with our analysis. To evaluate this possibility, we made RT-PCR analyses for the Nkx2.5 gene together with the endodermal (E-cadherin), ectodermal (Nestin) and skeletal muscle (MyoD) markers. We found, however, that a residual contamination of Ecadherin, Nestin and MyoD expression was detected (!10%) (fig. 2). Therefore, we can expect that the changes in gene expression profiles reported herein are mainly reflecting the changes occurring in differentiating cardiomyocytes. Furthermore, residual contamination of endodermal, ectodermal and skeletal muscle markers did not display variability at the different stages of in vitro culture (R1 + 3, R1 + 7 and R1 + 13) providing support to stage-to-stage comparisons.

Pitx2c Isoform-Specific Overexpression in ESC-Derived Cardiomyocytes

It has been previously demonstrated that Pitx2c is expressed in cardiac precursor cells as well as in differentiated cardiomyocytes [Ai et al., 2006; Nowotschin et al., 2006]. In the present work, we wanted to investigate the role of Pitx2c in the cardiac transcriptional machinery that acts in differentiating cardiomyocytes. For this purpose, we have generated a myocardial-specific Pitx2c overexpression model in ESCs by using the α-MHC promoter, which has been previously used as a strategy to select ESC-derived cardiomyocytes [Klug et al., 1996]. We examined first the α-MHC promoter activity in beating areas derived from ESCs by transfection with an αMHC-EGFP construct. After transfection, GFP fluorescence was observed in all beating areas of EBs derived from selected clones and only contractile foci were GFP positive. As shown in figure 3a, the size of EGFP-positive areas within EBs increased with culture time. To evaluate the role of Pitx2c in cardiomyocyte differentiation, we overexpressed mouse Pitx2c under the control of the cardiac specific α-MHC promoter in R1-ESC-derived cardiomyocytes. As shown in figure 3b, Pitx2c mRNA expression was increased in transfected puromycin-selected and isolated beating areas at early (R1 + 3), intermediate (R1 + 7) and late stages of differentiation (R1 + 13).

Pitx2c Overexpression in Nkx2.5(+) Cells from Spontaneous Beating Areas

In our study, quantitative RT-PCR analysis for Nkx2.5 showed that overexpression of Pitx2c did not alter Nkx2.5 mRNA levels at any stage of in vitro differentiation analyzed as compared to controls (fig. 3c). Nkx2.5 is indispensable for cardiac development [Brand, 2003], and previous publications have shown that most of the cells isolated from Nkx2.5-GFP ESC-derived EBs are cardiomyocytes, comprising all different types of cardiac cells (sinoatrial node, atrial and ventricular cells) [Hidaka et al., 2003; Nakashima et al., 2009]. Therefore, in order to analyze changes in expression levels in a more defined population of cardiomyocytes, mRNA expression levels for each gene were normalized to Nkx2.5. When Pitx2c RNA levels were normalized to Nkx2.5, a significant Pitx2c overexpression was only achieved, at similar levels, in beating areas at intermediate and late stages of in vitro differentiation (fig. 3d). Thus, all of our subsequent gene expression profiling was normalized to Nkx2.5 and analyzed only at intermediate and late stages.

Gene Expression Profile of Cell Cycle Genes in Pitx2c Overexpressing ESC-Derived Cardiomyocytes

Previous data from our laboratory have shown that overexpression of Pitx2c in Sol8 skeletal myoblasts inhibited myocyte differentiation and led to a significant upregulation of cell cycle genes such as cyclin D1, cyclin D2, and c-myc, providing these cells with a high proliferative capacity [Martinez-Fernandez et al., 2006]. To determine whether Pitx2c also acts by modulating expression of cell cycle genes in differentiating cardiomyocytes from ESCs, we performed real-time RT-PCR analyses for cyclin D1, cyclin D2, and c-myc mRNAs in beating areas derived from ESCs overexpressing Pitx2c. No changes in mRNA expression levels for these cell cycle genes were observed in Pitx2c-overexpressing cardiomyocytes compared to control cells at intermediate stages (R1 + 7 cells) (fig. 4a). In contrast to observations obtained in skeletal muscle, cyclin D1, cyclin D2 and c-myc were downregulated in beating areas overexpressing Pitx2c at late stages (R1 + 13 cells) (fig. 3a). Additionally, immunohistochemistry experiments using the anti-

PHH3 (phospho-histone H3) antibody as specific marker of mitotically active cells showed a decrease in the percentage of PHH3-positive nuclei in cells overexpressing Pitx2c at the late stage (fig. 4a). These results indicate that Pitx2c has no effect on proliferation at intermediate stages of in vitro differentiation but could have a role arresting proliferation at late stages. This effect was Pitx2c specific because cell cycle gene levels did not change in cells transfected with the α-MHC-EGFP construct only (online suppl. fig. 2).

Pitx2c Modulates SHF-Specific Transcriptional Pathways during the Process of in vitro Differentiation of ESC-Derived Cardiomyocytes in a Manner That Is Stage-Dependent

To determine whether Pitx2c acts by modulating expression of genes that are sequentially activated during cardiomyocyte differentiation, we analyzed expression levels of the transcription factor genes Mef2c, Isl1, Gata4/6 and Tbx2/5 and chamber-specific cardiac genes (Nppa, Cx40) in beating areas overexpressing Pitx2c (fig. 4b, c). Each stage analyzed was normalized to Nkx2.5. Our data show that Isl1, a marker for the SHF, was increased in beating areas overexpressing Pitx2c at intermediate stages of in vitro differentiation compared with control cells, but no differences were found at later stages (fig. 4b). However, other marker genes for the SHF such as Tbx1 did not display changes in gene expression after Pitx2c overexpression (data not shown), indicating that Pitx2c specifically affected Isl1 expression. Similar to Isl1 Mef2c, a direct transcriptional target of Isl1 in the SHF was also upregulated in beating areas overexpressing Pitx2c at intermediate stages (fig. 4b). Curiously, Pitx2c overexpression induced increases in the expression of Gata4 and Gata6 transcription factors, but only at late stages of in vitro differentiation (fig. 4b). We found that Pitx2c overexpression in beating areas results in upregulation of Tbx2 only at the intermediate stage. However, Tbx5 displayed a strong upregulation in beating areas overexpressing Pitx2c at intermediate and late stages of in vitro differentiation (fig. 4b). In line with the data presented above, immunohistochemistry experiments for Isl1 and Tbx5 revealed that the percentage of positive nuclei were higher in beating areas overexpressing Pitx2c compared to control cells (fig. 5). Thus, these data show that Pitx2c has an important role in modulating cardiac transcriptional machinery in EB-derived cardiomyocytes.

Curiously, cardiac myosin light and heavy chain isoforms that display cell-specific and stage-specific expression in developing atrial and ventricular myocytes did not change significantly after Pitx2c overexpression (fig. 4c). Therefore, this transcription factor is not implicated in the regulation of these contractile protein genes. Interestingly, coinciding with the findings for Tbx5, the cardiac chamber-specific genes Nppa and Cx40 were also upregulated after Pitx2c overexpression (fig. 4c). For Nppa, the only cardiac-specific gene previously described as a Pitx2 target [Ganga et al., 2003], a strong induction was detected in beating areas overexpressing Pitx2c at late stages of in vitro differentiation. Taken together, these results suggest that Pitx2c acts first by reinforcing the Isl1-Mef2c expression at intermediate stages and later by driving cardiomyocyte maturation during the process of in vitro cardiogenesis.

Lack of Function of Pitx2 in the Atrial Myocardium Leads to Misregulation of Cardiac Genes That Play Important Roles in Cardiac Myogenesis

In this study, we used an in vitro approach to overexpress Pitx2c under the control of the promoter of α-MHC, a gene that, in mice, is expressed dynamically. α-MHC expression is detected in all cardiomyocytes at early stages of development, it becomes confined to atrial cardiomyocytes at fetal stages and it is reexpressed in ventricular cardiomyocytes postnatally. Previous studies have shown that Pitx2 expression within the atrial chambers at fetal stages is mainly confined to left atrium during cardiac development [Campione et al., 2001]. Recently, Tessari et al. [2008] demonstrated that Pitx2 mediates left atrial molecular identity. Therefore, to analyze in vitro versus in vivo Pitx2c-regulatory mechanisms during cardiac development, we generated a Pitx2 conditional mutant using a Cre-LoxP approach to delete the homeodomain-coding region (present in all Pitx2 isoforms) of the Pitx2 gene in the developing atrial chambers (mainly in the left atrium). Pitx2 inactivation in the atrial myocardium, generated using a well-characterized atrial Cre recombinase driver [de Lange et al., 2003], results in embryonic hearts with dilated atrial chambers as illustrated in figure 6a and b, but no other morphogenetic defects were observed [Chinchilla et al., submitted]. The relative size of atrial cavities in Nppa-Cre/Pitx2–/– embryos revealed that dilatation was significantly higher in the left than in the right atrial compartment (fig. 6c). The atrial chambers of conditional mutants show a reduction of wall thickness (fig. 4e, 6d), despite the fact that the total number of nuclei was significantly higher compared to wildtype embryos (online suppl. fig. 3).

To validate our in vitro data, we performed a quantitative RT-PCR analysis in the conditional Pitx2 mouse mutant model using only left atrial chamber myocardium. We first evaluated Pitx2c deletion in the atria of these mutant mice by using specific primers in Pitx2c-specific exon 4 and in exon 5 which encode the homeodomain (online suppl. fig. 4). Although Pitx2c deletion is not complete, its expression is clearly diminished in the NppaCre/Pitx2–/– mice compared to the wild-type littermate controls.

Interestingly, cell cycle genes such as c-myc and cyclin D1 were upregulated in Nppa-Cre Pitx2–/– mice (fig. 6f, g). Immunohistochemical analysis using PHH3 antibody confirmed that the number of dividing cells in Nppa-Cre Pitx2–/– mice was significantly higher than in wild-type animals (fig. 7). These results are consistent with our in vitro Pitx2 gain-of-function data. Since Isl1 is poorly represented at fetal stages, we were not able to find changes of Isl1 expression in Nppa-Cre/Pitx2–/– mice (data not shown). However, for other early transcriptional factors that are still present at E16.5, such as Mef2c, we observed a clear decrease in its transcript levels and in the number of Mef2c-positive nuclei in Nppa-Cre-Pitx2–/– mice compared to the wild type (fig. 6h, 7, respectively). In line with our in vitro data, we also detected downregulation of Nppa expression (fig. 6i). These observations are consistent with the hypothesis that Pitx2 plays an important role in cardiac myocyte maturation.

Discussion

The Cardiac Muscle Transcriptional Program Is Dynamically Activated in R1-ESC-Derived Cardiomyocytes

It is well established that the different time courses of myocyte differentiation and the distinct regional contributions to the embryonic heart support the hypothesis

that Nkx2.5 acts as a critical transcription factor in the FHF and Isl1 as a transcriptional regulator in the SHF [Brand, 2003]. Our results show for the first time that Nkx2.5 and Isl1 are temporally regulated during in vitro differentiation of R1-derived cardiomyocytes. Therefore, in our in vitro system, Nkx2.5 displays early induction whereas Isl1 is induced at later stages, indicating that the temporal expression pattern of the FHF marker Nkx2.5 and the SHF marker Isl1 is conserved in beating areas obtained from R1-ESC. This Nkx2.5 expression profile is consistent with in vivo cardiogenesis (E10.5 at E18.5) (data not shown), suggesting that high mRNA levels of Nkx2.5 could be required in early cardiomyocytes. However, as cardiac differentiation progresses Nkx2.5 expression decreases within cardiomyocyte cells until it reaches basal levels. Moreover, these data are in accordance with other in vivo evidence suggesting that Nkx2.5 is the earliest cardiac transcription factor expressed in the FHF in vertebrates [Black, 2007].

It is established that the Isl1-Gata-Mef2c pathway is a central component of the transcriptional network directing the SHF contribution to the developing heart [Dodou et al., 2004]. We detected an increase in Mef2c during later stages of in vitro differentiation, following the increase in Isl1 and Gata4–6 levels; this suggests that this transcriptional pathway is maintained during in vitro differentiation of R1-ESC-derived cardiomyocytes. Furthermore, we documented similar expression profiles for Isl1 and Pitx2c, in line with previous in vivo data showing Isl1 and Pitx2c coexpression in the left portion of myocardial progenitors of the posterior SHF [Galli et al., 2008] and supporting the hypothesis that these gene products interact during cardiogenesis.

During mouse embryonic development, myosin genes are expressed as soon as the cardiac tube forms, and each cardiac myosin gene expression pattern is progressively modulated in a chamber-specific manner [Lyons et al., 1990]. Therefore, in concordance with previous in vivo data, we found high expression levels for myosin genes at early stages of in vitro differentiation decreasing at later stages. In summary, our data demonstrate that R1-ESCderived cardiomyocytes display a transcriptional modulation similar to that of FHF and SHF contribution during in vivo cardiogenesis.

Role of Pitx2c Modulating Cell Cycle Genes during Cardiomyocyte Differentiation

We have previously documented that Pitx2c promotes proliferation in undifferentiated skeletal myoblasts by inducing the expression of cell cycle-regulatory genes such as c-myc, cyclin D1 and cyclin D2 while inhibiting differentiation [Martinez-Fernandez et al., 2006]. In this report, our in vitro and in vivo data show that Pitx2 does not affect cell proliferation at intermediate stages, but it could be promoting the entry in the cell cycle arrest that has previously been observed in ESC-derived cardiomyocytes at later stages of in vitro differentiation [Yamanaka et al., 2008]. Recently, Gherzi et al. [2010] showed that Pitx2 is required for Ccnd1 (Cyclin D1) mRNA stabilization in proliferating myoblasts but contributes to Ccnd1 destabilization when the differentiation program is initiated [Gherzi et al., 2010]. Therefore, the distinct Pitx2mediated role in the proliferation rate between skeletal myoblasts and differentiating cardiomyocytes might be attributed to their distinct differentiation status. Moreover, our data are in line with those reported by Galli et al. [2008], supporting a role of Pitx2c-promoting cardiomyocyte cell cycle arrest.

Transcriptional Control of Cardiomyocyte Differentiation Mediated by Pitx2c

Several loss-of-function studies have provided evidence that Pitx2 plays a crucial role during cardiac development [Gage et al., 1999; Liu et al., 2001]. More recently, it has been demonstrated that Pitx2c play a crucial role in patterning cardiac progenitor cells that migrate into the developing heart as a part of the SHF [Ai et al., 2006; Galli et al., 2008]. However, the molecular mechanisms by which Pitx2c acts during cardiomyocyte differentiation remain largely unknown. Our analysis of Pitx2c gain of function in differentiating cardiomyocytes from ESCs revealed that this transcription factor modulates cardiac transcriptional pathways in a stage-dependent manner. We found that Pitx2c overexpression at intermediate stages of in vitro differentiation drives upregulation of Isl1 as well as its target gene Mef2c. Pitx2c-Isl1 interaction is supported by the previously reported coexpression of Pitx2c and Isl1 in the SHF [Galli et al., 2008]. Curiously, Isl1 and Mef2c expression did not display significant changes in ESC-derived cardiomyocytes at later stages of in vitro differentiation, suggesting that Pitx2c-mediated modulation of the Isl1-Mef2c pathway is stage dependent. In line with these in vitro data, Pitx2-dependent regulation of Mef2c was also observed in our in vivo model of Pitx2 loss of function (Nppa-Cre Pitx2–/– mice) where Mef2c was downregulated.

In silico analysis of the putative upstream transcriptional start site regulatory sequences allowed us to identify four highly conserved Pitx2-binding sites close to binding sites for other cardiac transcription factors in the Isl1 promoter, but only two Pitx2-binding sites on the Mef2c enhancer sequence (online suppl. fig. 5). These data support the hypothesis that Mef2c upregulation is driven as a consequence of the previous upregulation of Isl1, although a direct role of Pitx2 in modulating Mef2c transcriptional expression cannot be ruled out. The fact that Nkx2.5 expression is not modified after Pitx2c overexpression seems to indicate that Pitx2 is not crucial for FHF differentiation. However, Pitx2c-mediated modulation of the Isl1-Mef2c pathway at early stages suggests a role of Pitx2 in SHF, reinforcing several previously described observations [Ai et al., 2006; Galli et al., 2008].

During heart development, dynamic expression of the myosin isoforms α-MHC, β-MHC, Mlc2v and Mlc2a progressively define specific atrial or ventricular myocytes as well as their contractile patterns [Lyons et al., 1990]. We found that Pitx2c overexpression does not lead to changes in the expression levels of contractile protein genes suggesting that Pitx2 is not involved in atrial/ventricular cell specification during the process of in vitro cardiac differentiation.

The members of T-box transcription factor family Tbx2 and Tbx5 have been implicated in the transcriptional mechanism that governs in vivo cardiac-specific chamber expression controlling Nppa and Cx40 expression [Habets et al., 2002]. Interestingly, we found that Pitx2c overexpression increased the expression levels of Tbx2 and Tbx5 in beating areas at intermediate stages. Tbx5 induction was higher and maintained at late stages. This higher Tbx5 upregulation coincided with increases in Nppa and Cx40 expression in beating areas overexpressing Pitx2c at both stages of in vitro differentiation. Our data are consistent with the observation that Nppa expression was downregulated in Nppa-Cre+/Pitx2–/– mice. They are in concordance with previous data that demonstrate that Tbx5 acts as an activator of the cardiacspecific chamber genes, Nppa and Cx40 [Habets et al., 2002]. Additionally, these data are in line with previous work describing Nppa as a cardiac-specific Pitx2 target gene [Ganga et al.,

2003], validating therefore our Pitx2 gain-of-function ESC differentiation system. The in silico analysis showed no putative Pitx2 binding sites in the proximal (10 kb) Tbx2 transcriptional start site upstream sequence. However, since regulatory elements of Tbx2 might be located further upstream, we cannot rule out that Pitx2 might directly control Tbx2 expression. Experiments are underway to unravel a putative genetic Pitx2-Tbx2 interaction. Curiously, we found six highly conserved Pitx2-putative binding sites upstream and downstream of Tbx5 coding sequence and only one Pitx2-putative binding site upstream of the Nppa promoter sequence (online suppl. fig. 4). These findings indicate that Tbx5 might be a direct target of Pitx2c, and that increases in Nppa expression after Pitx2c overexpression could be due to Tbx5 upregulation as well as to a direct effect of Pitx2 on the Nppa gene as previously demonstrated [Ganga et al., 2003]. Taken together, these results suggest that Pitx2c plays an important role during the process of cardiomyocyte maturation, balancing Tbx2 and Tbx5 expression and secondarily modulating expression of cardiac chamber genes such as Nppa and Cx40.

In summary, the findings presented herein indicate that Pitx2 is a key regulator of transcription factor networks modulating cardiac chamber differentiation. When Pitx2c was overexpressed during ESC-derived cardiomyocyte differentiation, a set of cardiac marker genes were sequentially upregulated such as the Isl1-Mef2c transcriptional network. Similarly, Pitx2c overexpression leads to modulation of Tbx2/Tbx5 expression and subsequently of Nppa and Cx40. Pitx2 gene inactivation in the atrial myocardium further supports its role modulating these pathways in vivo. Thus our findings demonstrate a role for Pitx2 reinforcing specific transcription programs during the process of cardiac differentiation.

Acknowledgements

We thank Dr. Robert Kelly for critical discussion and comments on the manuscript.

This work was partially supported by grants LSHMCT-2005-018630 (VI FP Integrated Project Heart Failure and Cardiac Repair), BFU2008-01217 (Ministerio de Ciencia e Innovación, Gobierno de España), CTS-1614 and P08-CTS-3878 (Junta de Andalucia). G.E. Lyons is an Established Investigator of the American Heart Association.

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Figure legends

Fig. 1. Expression levels of different cardiac genes in beating areas derived from R1-ESCs. Quantitative RT-PCR analysis showing mRNA expression levels for the transcription factors Isl1, Tbx1, Pitx2c, Nkx2.5, Mef2c, Gata 4/6, and Tbx2/5 (a) and for tissuespecific cardiac genes α-MHC/β-MHC, Mlc2a/Mlc2v, Cx40 and Nppa (b) in beating areas at early (R1 + 3), intermediate (R1 + 7) and late (R1 + 13) stages of in vitro differentiation. For Gata4/6, Tbx2/5 each gene was analyzed independently and no comparative analysis was done between them, as the efficiency of the PCR reactions is different for each primer set. Fold changes are relative to the lower average expression of each gene, which was given the arbitrary value of 1. Error bars represent standard deviation.
* $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$.

Fig. 2. Quantitative RT-PCR analysis of gene expression profiles for Nkx2.5, E-cadherin, Nestin and MyoD genes as cardiac, endoderm, ectoderm and skeletal muscle markers in R1-ESC-derived isolated beating areas at R1 + 3, R1 + 7 and R1 + 13 stages.

Fig. 3. α-MHC promoter activity and Pitx2c overexpression using the α-MHC-Pitx2c-IRES-puromycin construct. GFP fluorescence was observed in beating areas (dotted areas) 3 days (R1 + 3), 7 days (R1 + 7) and 13 days (R1 + 13) after embryoid bodies were plated (a). Quantitative RT-PCR analyses of Pitx2c overexpression normalized to β-actin mRNA (b) and normalized to Nkx2.5 mRNA (d). c Nkx2.5 mRNA expression levels from beating areas derived from transfected R1-ESCs. BAs-Pitx2c = Transfected, puromycin-selected and isolated beating areas; CBAs = control beating areas. Error bars represent standard deviation. * $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$.

Fig. 4. Gene expression profiles in R1-ESC-derived cardiomyocytes overexpressing Pitx2c at (R1 + 7) and (R1 + 13) stages. Quantitative RT-PCR analysis of gene expression profiles for cell cycle genes cyclin D1, cyclin D2 and c-myc (a), Isl1, Mef2c, Gata4/6, and Tbx2/5 transcription factors (b) and the structural cardiac genes α-MHC/β-MHC, Mlc2a/Mlc2v, Connexin40 (Cx40) and Nppa (c) in R1-ESC-derived cardiomyocytes overexpressing Pitx2c (BAs-Pitx2c) compared with control beating areas (CBAs) at intermediate (R1 + 7) and late (R1 + 13) stages of in vitro differentiation. The data were normalized to Nkx2.5 gene expression. Error bars represent standard deviation.
* $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$.

Fig. 5. Representative images of immunohistochemical detection for PHH3, Isl1 and Tbx5, respectively, within MF20-positive areas (green) from control EBs and EB-overexpressing Pitx2c. Confocal microscope images were obtained from at least 4 distinct EBs in 3 independent experiments and PHH3-, Isl1or Tbx5-positive nuclei were scored within MF20-positive areas using the ImageJ software (http://rsb.info.hih.gov/ij/docs). Graphics show the percentage of PHH3-, Isl1and Tbx5-positive nuclei, respectively, in relation to the total nuclei (DRAQ5). CBAs = Control beating areas.

Fig. 6. a, b Paraffin-embedded sections of Nppa-Cre Pitx2–/– and wild-type embryos at stage E16.5 were stained with H&E for morphological analysis. c Estimated size of left

and right atrial compartments by measurements of longitudinal axis (arrows in a and b). d, e Desmin-stained transverse sections of E16.5 wild-type and Nppa-Cre Pitx2–/– hearts, respectively. Note that Nppa-Cre Pitx2–/– mice have dilated atria. f, g Gene expression profiles for cell cycle genes cyclin D1 (f) and c-myc (g) in the left atria of Nppa-Cre Pitx2–/– (null) and wild-type control mice at E16.5 were analyzed by quantitative RT-PCR. To avoid the contribution of nonmyocardial cells, cyclin D1 and c-myc mRNA levels were normalized to Nk2.5 mRNA. h, i Gene expression profiles for tissuespecific cardiac genes Nppa (i) and Mef2c (h) in Nppa-Cre Pitx2–/– and wild-type control mice at E16.5 analyzed by quantitative RTPCR. LA = Left atrium; RA = right atrium; wt = wild type. Error bars represent standard deviation. * $p < 0.01$; *** $p < 0.0001$.

Fig. 7. Representative images of immunohistochemical detection for PHH3 and Mef2c (respectively) in wild-type (wt) control mice at E16.5. Graphics show percentage of PHH3- and Mef2c-positive nuclei compared to total nuclei. Distinct regions of the developing atrial appendages (LA) were processed to count the total number of PHH3, and Mef2c stained nuclei with respect to total nuclei (hematoxylin staining) within a standardized area using the ImageJ software (http://rsb.info.hih.gov/ij/docs). ** $p < 0.001$; *** $p < 0.0001$.

Figure 1

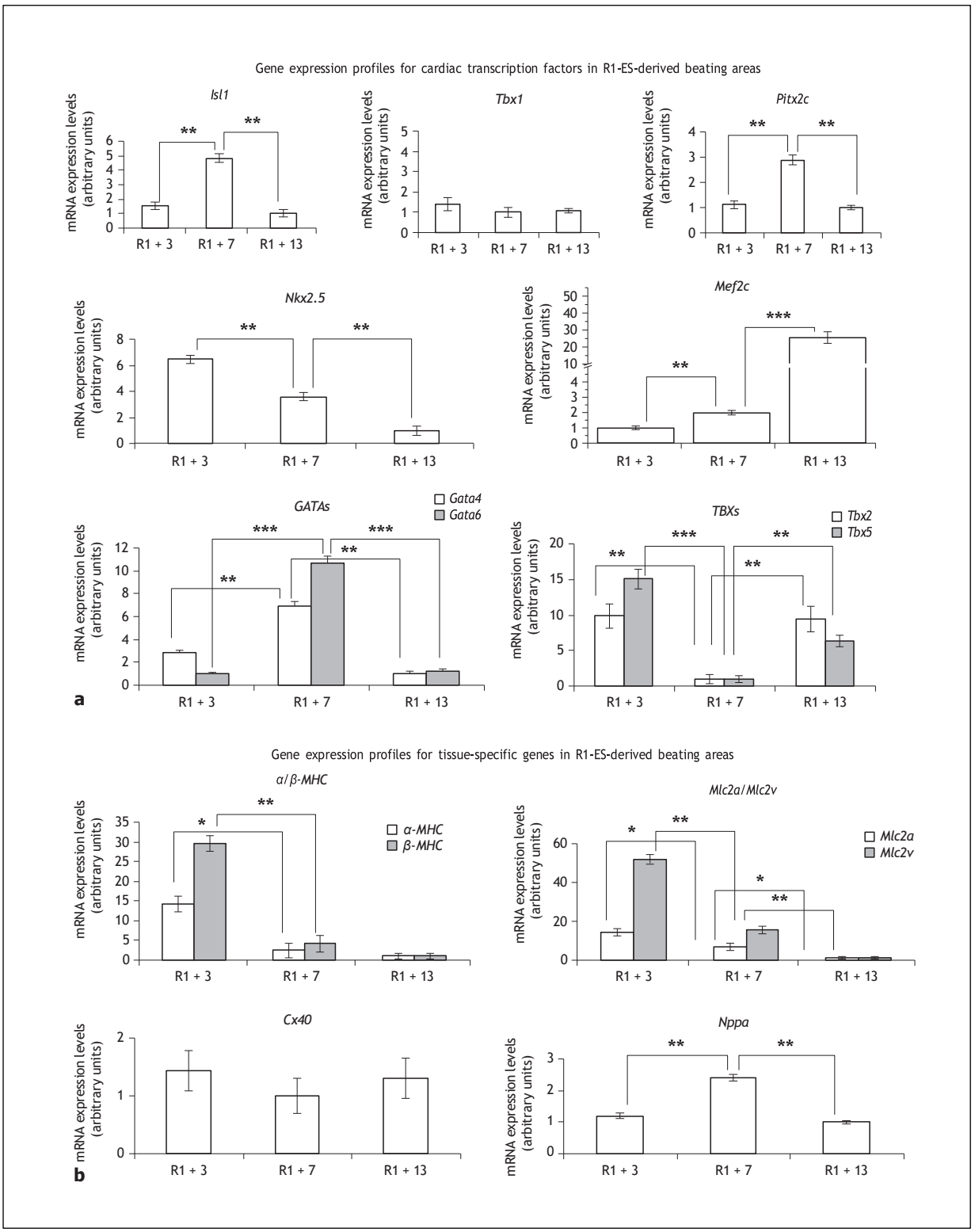

Gene expression profiles for cardiac transcription factors in R1-ES-derived beating areas
Isl1
Tbx1
Pitx2c
Nkx2.5
Mef2c
GATAs
Gata4
Gata6
TBXs
Tbx2
Tbx5
mRNA expression levels (arbitrary units)
R1 + 3
R1 + 7
R1 + 13
a
Gene expression profiles for tissue-specific genes in R1-ES-derived beating areas
α/β-MHC
α-MHC
β-MHC
Mlc2a/Mlc2v
Mlc2a
Mlc2v
Cx40
Nppa
b

Figure 2

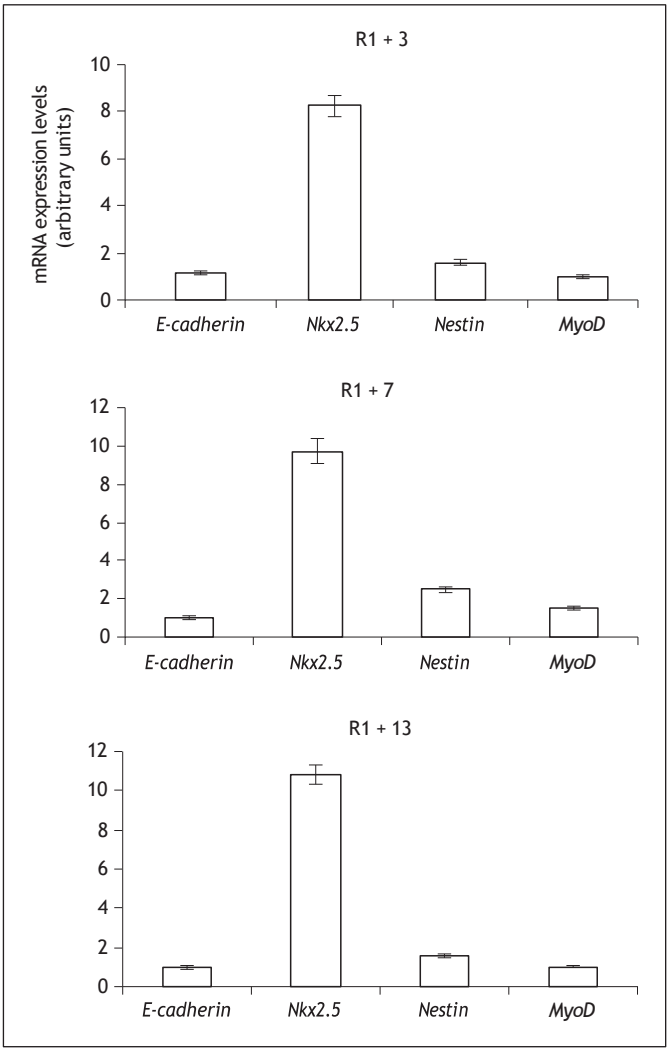

R1 + 3
mRNA expression levels (arbitrary units)
10
8
6
4
2
0
E-cadherin
Nkx2.5
Nestin
MyoD
R1 + 7
12
10
8
6
4
2
0
E-cadherin
Nkx2.5
Nestin
MyoD
R1 + 13
12
10
8
6
4
2
0
E-cadherin
Nkx2.5
Nestin
MyoD

Figure 3

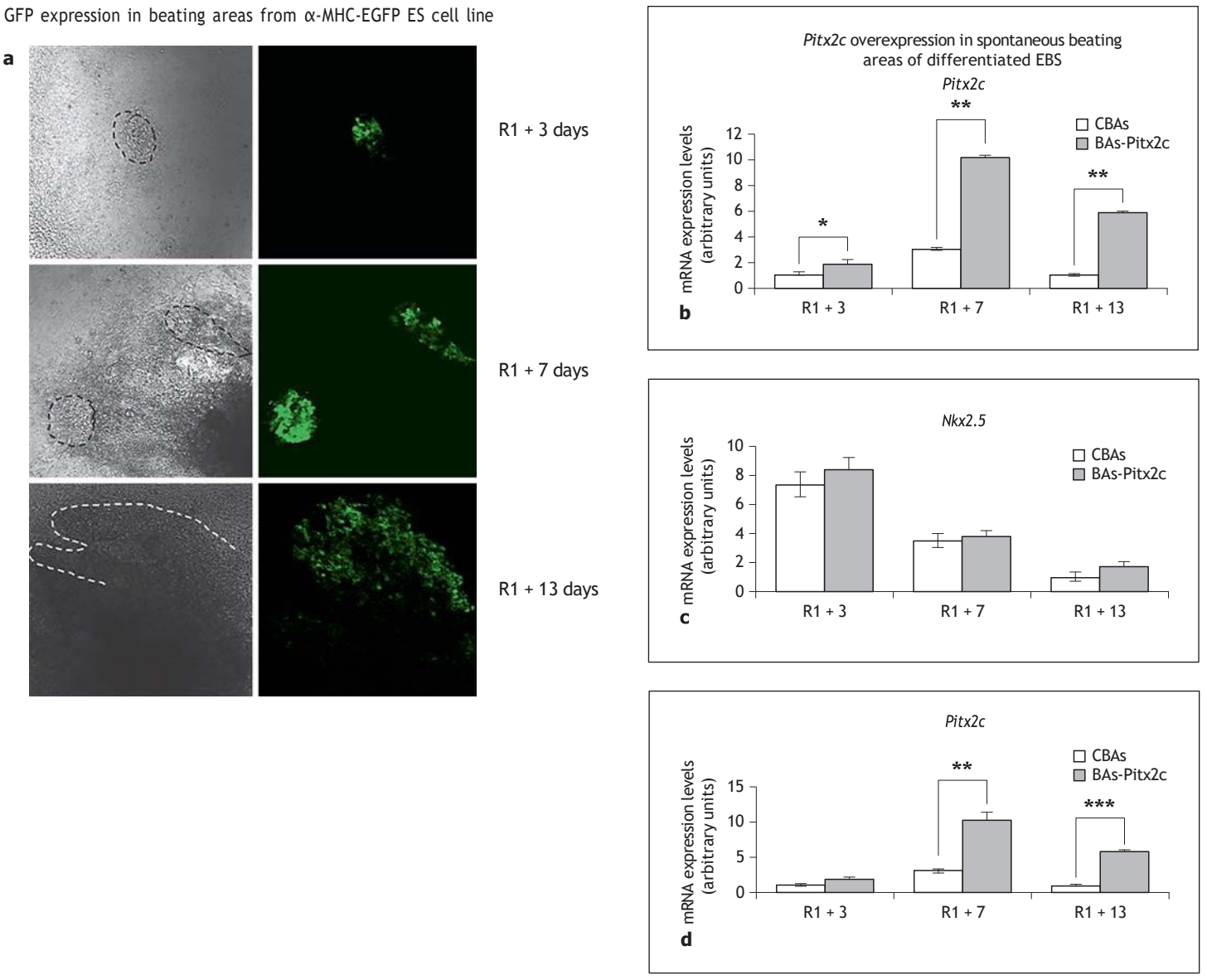

GFP expression in beating areas from α-MHC-EGFP ES cell line
a
R1 + 3 days
R1 + 7 days
R1 + 13 days
Pitx2c overexpression in spontaneous beating areas of differentiated EBS
Pitx2c
mRNA expression levels (arbitrary units)
CBAs
BAs-Pitx2c
R1 + 3
R1 + 7
R1 + 13
b
Nkx2.5
c
Pitx2c
d

Figure 4

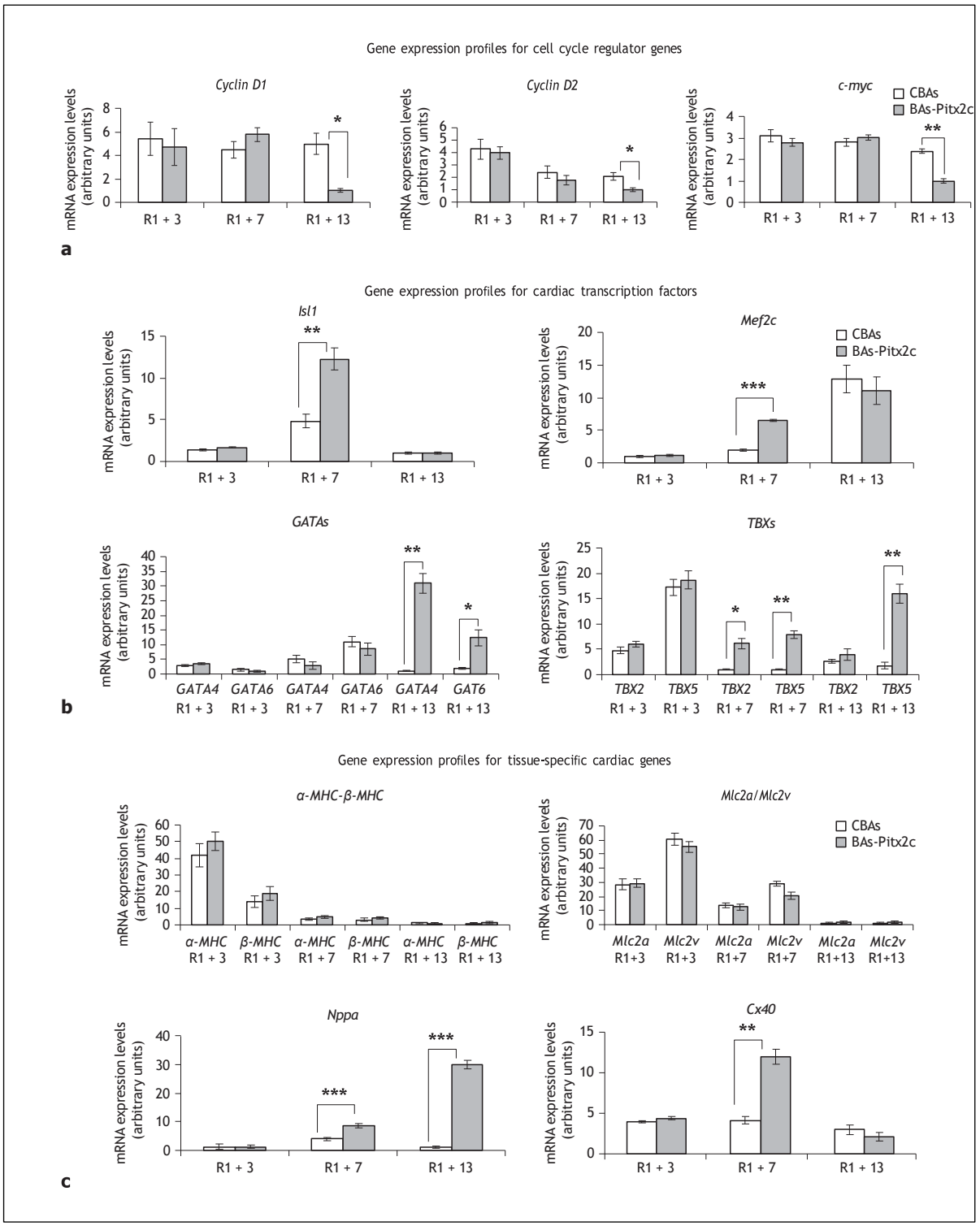
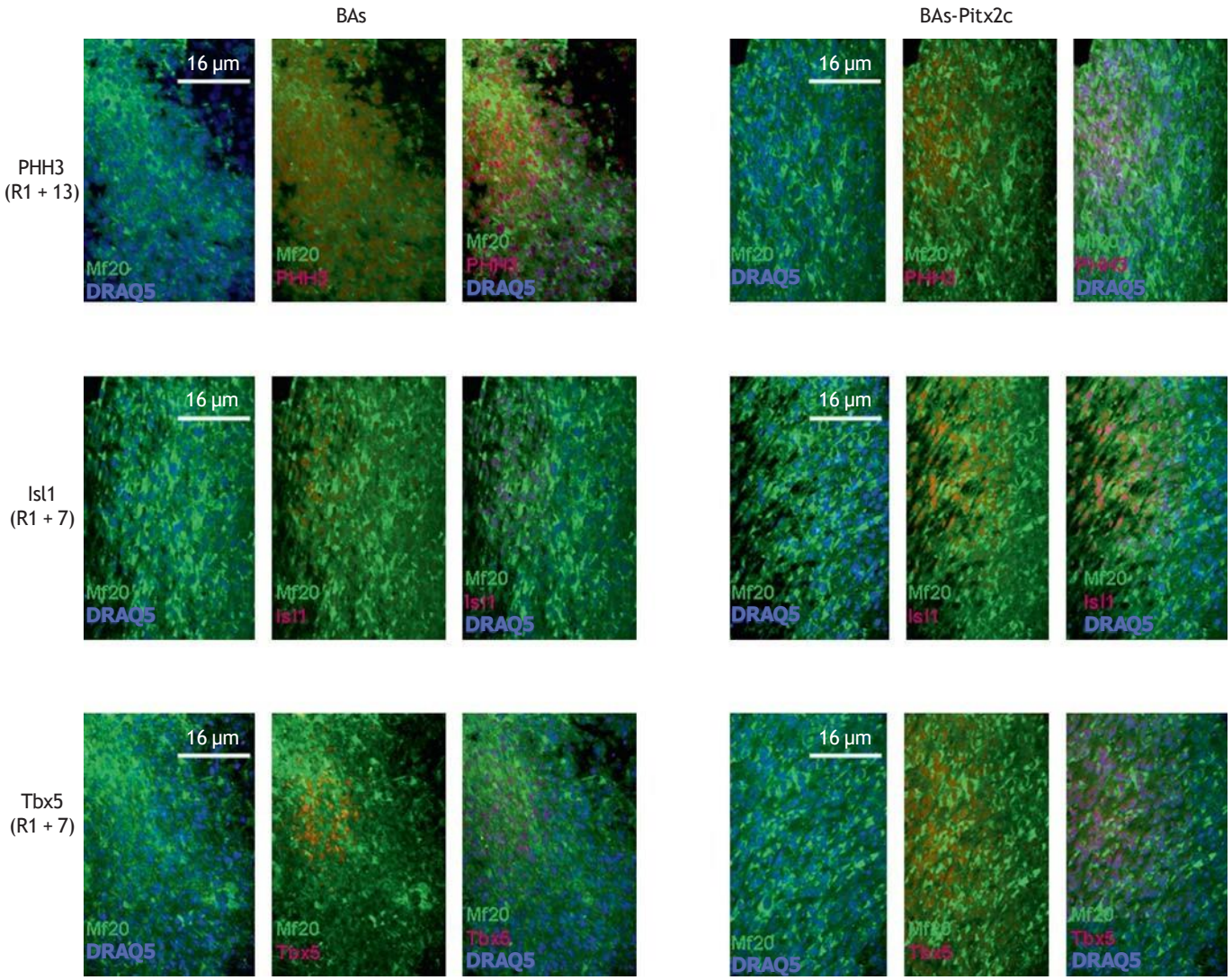

Gene expression profiles for cell cycle regulator genes
Cyclin D1
Cyclin D2
c-myc
CBAs
BAs-Pitx2c
mRNA expression levels (arbitrary units)
R1 + 3
R1 + 7
R1 + 13
a
Gene expression profiles for cardiac transcription factors
Isl1
Mef2c
GATAs
TBXs
GATA4
GATA6
GAT6
TBX2
TBX5
b
Gene expression profiles for tissue-specific cardiac genes
α-MHC-β-MHC
Mlc2a/Mlc2v
α-MHC
β-MHC
Mlc2a
Mlc2v
Nppa
Cx40
c

Figure 5


BAs
BAs-Pitx2c
PHH3
(R1 + 13)
Isl1
(R1 + 7)
Tbx5
(R1 + 7)
16 µm
Mf20
DRAQ5
PHH3
Isl1
Tbx5

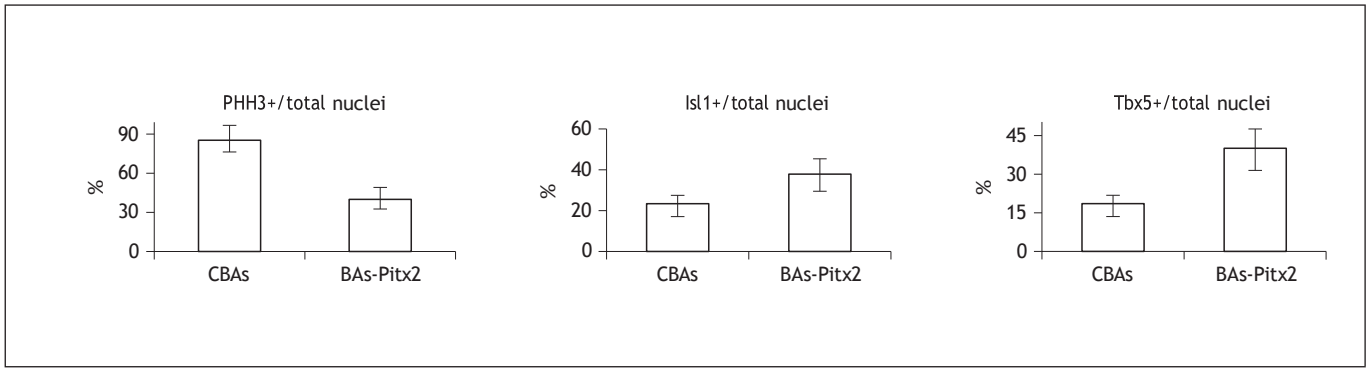
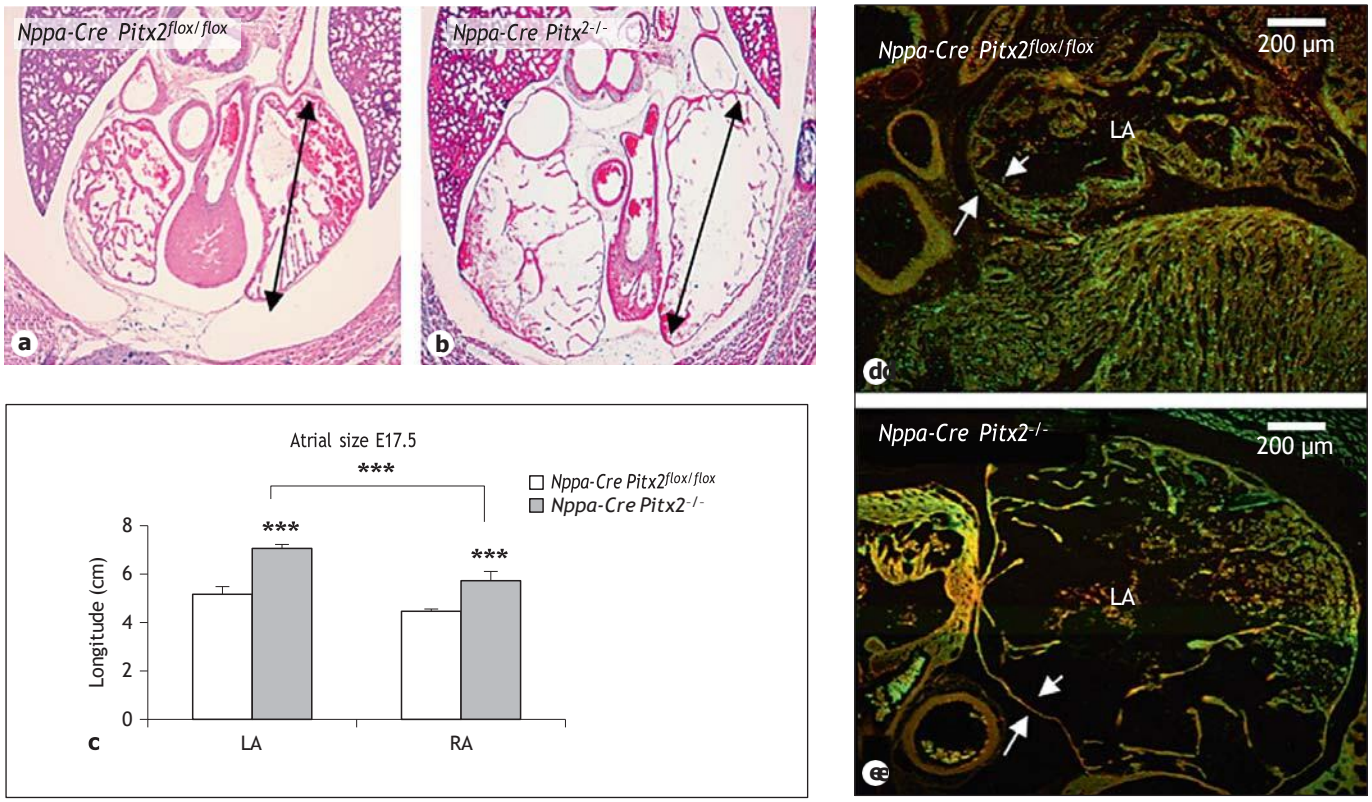
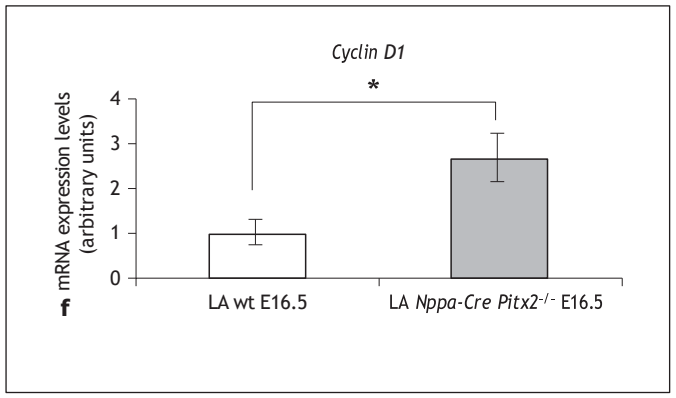
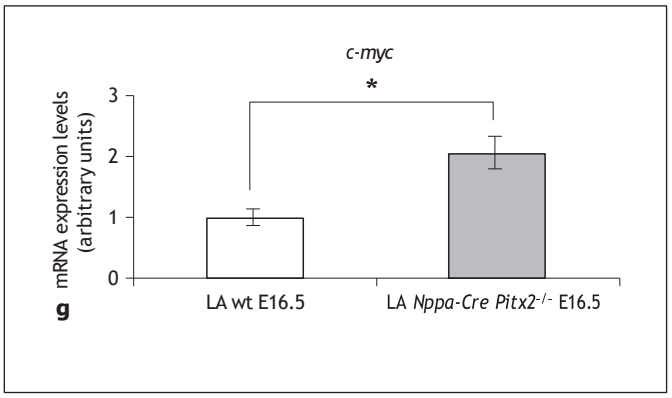
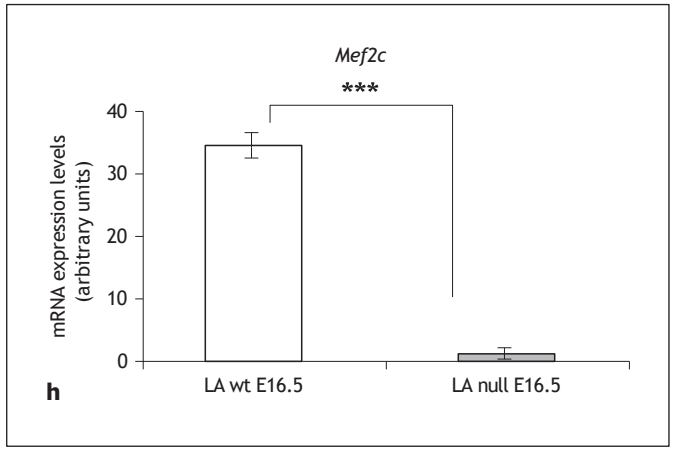

PHH3+/total nuclei
%
90
60
30
0
CBAs
BAs-Pitx2
Isl1+/total nuclei
%
60
40
20
0
CBAs
BAs-Pitx2
Tbx5+/total nuclei
%
45
30
15
0
CBAs
BAs-Pitx2

Figure 6


Nppa-Cre Pitx2flox/flox
Nppa-Cre Pitx2-/-
a
b
Nppa-Cre Pitx2flox/flox
200 µm
LA
d
Nppa-Cre Pitx2-/-
200 µm
LA
e
Atrial size E17.5
***
Nppa-Cre Pitx2flox/flox
Nppa-Cre Pitx2-/-
Longitude (cm)
8
6
4
2
0
***
***
LA
RA
c


Cyclin D1
*
mRNA expression levels (arbitrary units)
4
3
2
1
0
LA wt E16.5
LA Nppa-Cre Pitx2-/- E16.5
f


c-myc
*
mRNA expression levels (arbitrary units)
3
2
1
0
LA wt E16.5
LA Nppa-Cre Pitx2-/- E16.5
g


Mef2c
***
mRNA expression levels (arbitrary units)
40
30
20
10
0
LA wt E16.5
LA null E16.5
h

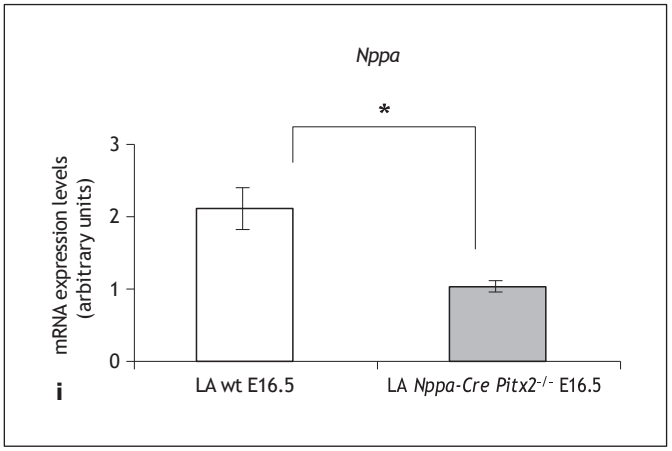
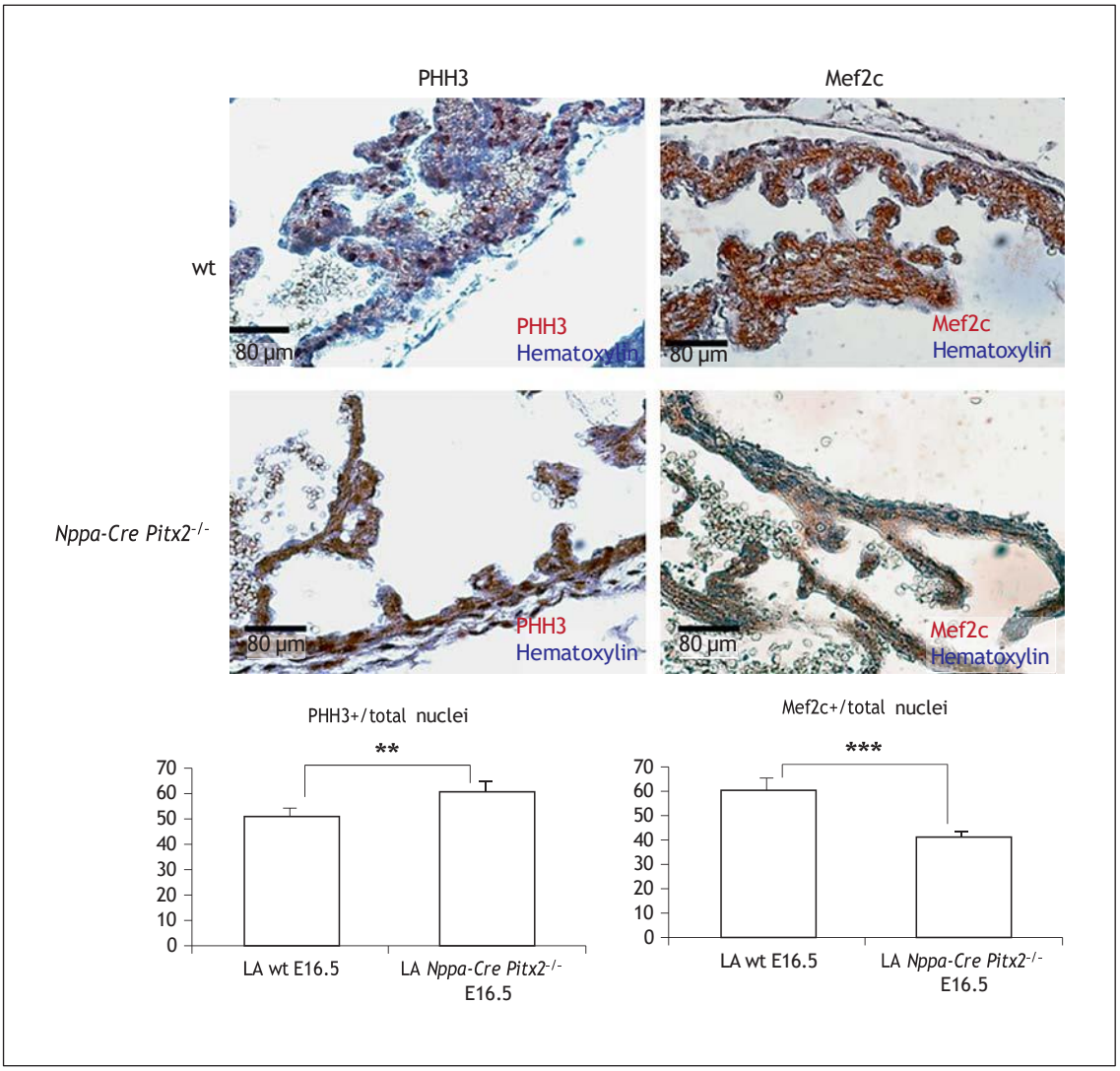

Nppa
*
mRNA expression levels (arbitrary units)
3
2
1
0
LA wt E16.5
LA Nppa-Cre Pitx2-/- E16.5
i

Figure 7


PHH3
Mef2c
wt
80 µm
PHH3
Hematoxylin
Mef2c
Hematoxylin
Nppa-Cre Pitx2-/-
PHH3+/total nuclei
**
70
60
50
40
30
20
10
0
LA wt E16.5
LA Nppa-Cre Pitx2-/-
E16.5
Mef2c+/total nuclei
***
LA wt E16.5
LA Nppa-Cre Pitx2-/-
E16.5