Expression patterns and immunohistochemical localization of Pittx2B transcription factor in the developing mouse heart

FRANCISCO HERNANDEZ-TORRES*, DIEGO FRANCO, AMELIA E. ARANEGA and FRANCISCO NAVARRO*
Department of Experimental Biology, Faculty of Experimental Sciences, University of Jaen, Jaen, Spain.

ABSTRACT The Pittx2 gene is involved in the establishment of vertebrate left-right axis with an important role in subsequent heart organogenesis. Mutations in the Pittx2 gene have been associated with Axenfeld-Rieger syndrome, which is characterized by ocular, craniofacial, and umbilical anomalies, as well as cardiac defects. In addition, recent data have unravelled a molecular link between Pittx2 loss of function and atrial fibrillation (AF), supporting an important role of Pittx2 not only in development but also in heart homeostasis. Three Pittx2 isoforms have been described in mice: Pittx2A, Pittx2B, and Pittx2C. During heart organogenesis, PITX2C seems to play a determinant role in left–right signalling from early somitogenesis onwards. However the participation of the Pittx2A and/or Pittx2B isoforms during cardiogenesis is controversial. Here we report for the first time that the Pittx2a and Pittx2b isoforms are jointly expressed with the Pittx2c isoform during heart development. Interestingly, in terms of relative quantification of mRNA, the Pittx2b and Pittx2c isoforms display similar expression profiles during cardiogenesis, decreasing with further development but maintaining their expression until adult stages. Moreover, a detailed analysis of PITX2B protein during cardiac development shows that PITX2B is dynamically expressed in the developing ventricular septum and asymmetrically expressed in the tricuspid valve primordia, suggesting a putative role of the PITX2B isoform during ventricular septation as well as in the maturation of the right portion of the atrioventricular canal.

KEY WORDS: Pittx2 isoforms, heart development, mouse and ventricular septation

The Pittx2 gene is a member of the Bicoid-like homeobox family (Semia et al., 1996). During the last fifteen years several studies reported the implication of Pittx2 in the correct establishment of vertebrate left-right axis. Left-right asymmetry is established through a molecular cascade that gives rise to Pittx2 asymmetric expression in the Lateral Plate Mesoderm (LPM) at early stages of development (Logan et al., 1998). This expression determines left/right polarity of mesoderm-derived organs such as heart, gut, and stomach (Campione et al., 1999; Logan et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998), supporting that Pittx2 could be the molecular transducer of embryonic left–right signalling at the organ level during early stages of development. Mutations in the Pittx2 gene have been associated with Axenfeld-Rieger syndrome, which is characterized by ocular, craniofacial, and umbilical anomalies (Semia et al., 1996) as well as cardiac defects, such as atrial septal defects, atrio-ventricular valve defects, and conduction abnormalities (Cunningham et al., 1998; Mammi et al., 1998; Tsai, Grajewski, 1994).

The Pittx2 gene is transcribed into three distinct isoforms in mice: Pittx2a, Pittx2b, and Pittx2c. Pittx2a and Pittx2b share the same promoter while Pittx2c uses an alternative promoter upstream of exon 4 (Gage et al., 1999; Schweickert et al., 2000). The analysis of expression patterns for the Pittx2 isoforms during heart development has been characterized in zebrafish, chicken and mouse (Campione et al., 1999; Campione et al., 2001; Franco et al., 2003). These studies have pointed out that Pittx2c plays a determinant role in left–right signalling during cardiogenesis (Campione et al., 1999; Schweickert et al., 2000; Liu et al., 2001). In addition, a molecular

Abbreviations used in this paper: Pittx2, pituitary homeobox 2 gene; AF, atrial fibrillation; LPM, lateral plate mesoderm; OFT, outflow tract; AS, alternative splicing.

*Address correspondence to: Francisco Navarro-Gómez. Department of Experimental Biology, Faculty of Experimental Sciences, University of Jaen, Paraje de Las Lagunillas, s/n, 23071, Jaen, Spain. Tel: +34-953-212771. Fax: +34-953-211875. E-mail: fnogomez@ujaen.es or Francisco Hernández-Torres. Department of Experimental Biology, Faculty of Experimental Sciences, University of Jaen, Paraje de Las Lagunillas, s/n, 23071, Jaen, Spain. Tel: +34-953-213056. Fax: +34-953-211875. E-mail: fraheto@ujaen.es

Supplementary Material (one figure) for this paper is available at: http://dx.doi.org/10.1387/ijdb.140224fh

Accepted: 5 February 2015.
link between Pitx2c and AF has been recently reported (Chinchilla et al., 2011; Kirchhof et al., 2011; Wang et al., 2010). Taken together, all these data suggest an important role of Pitx2c in heart development and homeostasis. However, Pitx2a and/or Pitx2b contribution during cardiogenesis cannot be completely discarded. Actually, a role for PITX2A during outflow tract (OFT) development has been suggested (Kioussi et al., 2002). In addition, Hjalt et al., (Hjalt et al., 2000), by using an antibody which could detect all PITX2 protein isoforms, detected the presence of PITX2 protein in the right atrial and ventricular wall at stage E12.5 in mouse, contradicting the Pitx2c mRNA expression pattern previously described (Camphione et al., 1999; Logan et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998). Moreover, analysis of the fate of Pitx2 daughter cells in mutant embryos that only express Pitx2a and Pitx2b isoforms (Pitx2 δabccrene -/- and Rosa26 reporter trans-heterozygotes) (Liu et al., 2002) revealed that fewer lacZ-positive cells were found in the right ventricular and inner curvature myocardium of Pitx2 mutant embryos at both 14.5 dpc and 18.5 dpc. Finally, comparative anatomical analysis of hearts from Pitx2 specific null mutant mice (Pitx2abc -/- (Gage et al., 1999), Pitx2ab -/- (Liu et al., 2001) and Pitx2c -/- (Liu et al., 2002)) leads to speculate that Pitx2a and/or Pitx2b could play a role in the development of the OFT (Franco et al., 2003). In spite of all these evidences, the implication of Pitx2a and or Pitx2b isoforms during cardiogenesis still remains elusive.

Here we show, for the first time, experimental data demonstrating that Pitx2a and Pitx2b isoforms are also expressed during heart development. In addition, we report a detailed analysis of PITX2B protein during cardiac development showing that PITX2B is dynamically expressed in the developing ventricular septum and asymmetrically expressed in the tricuspid valve primordia.

### Results

Pitx2a, Pitx2b and Pitx2c are dynamically expressed during mouse heart development

In order to analyse the mRNA expression pattern of each Pitx2 isoform during cardiogenesis, we performed qRT-PCR experiments in embryonic, neonatal and adult mouse whole hearts. As shown in Fig. 1 the three murine Pitx2 isoforms are expressed during cardiogenesis. Pitx2c expression levels are clearly higher than Pitx2a and Pitx2b isoforms, displaying different dynamic expression profiles. Thus, Pitx2c presents a maximal expression level at stage E11.5, decreasing at E13.5 and E15.5, increasing again at E17.5 and, subsequently decreasing at neonatal and adult stages where Pitx2c displayed its lowest expression levels. On the other hand, Pitx2b presents a maximal expression level at stage E9.5, decreasing strongly at E11.5 and maintaining similar expression levels until adulthood. Although Pitx2c is always expressed at higher levels than Pitx2b, the difference in terms of fold-times varies through heart development. Thus this difference is 7.3x, 65.4x, 66.6x, 187.5x, 150x, 73.3x, 4x and 4.8x for the stages of development E9.5, E11.5, E13.5, E15.5, E17.5, N1, N15 and adult, respectively. Finally, Pitx2a, the lowest expressed isoform, also presents a maximal expression level at E9.5 stage, followed by a strong decrease at stage E11.5 but, contrary to Pitx2c and Pitx2b, Pitx2a expression was not detected from E13.5 stage onwards.

Taken together, our results demonstrate for the first time that, in spite of Pitx2c presenting the highest levels of mRNA expression, Pitx2a and Pitx2b are coexpressed simultaneously during cardiogenesis. Moreover we also show that, although Pitx2a, Pitx2b and Pitx2c are coexpressed during early cardiogenesis, only Pitx2b and Pitx2c maintain their expression during embryonic, foetal, neonatal and adult stages.

### PITX2B protein expression profile during cardiogenesis

The presence of Pitx2b mRNA during heart development and its maintenance until adult stage suggests an

---

**Fig. 1. Pitx2 mRNA expression levels in cardiogenesis.** (A) Schematic representation of the strategy followed in order to measure specifically each Pitx2 isoform mRNA. Arrows indicate the primers annealing place used to amplify each isoform. (B) Pitx2a, Pitx2b and Pitx2c mRNA expression levels during mouse heart development as measured by quantitative RT-PCR. Data are expressed as ratio of each Pitx2 isoform mRNA to β-actin mRNA. Embryonic day (E) 8.5 to 175; N1, 1 day neonate mouse heart; N15, 15 day neonate heart; AD, adult mouse heart.
important role of this isoform in cardiogenesis. Thus, in order to analyse the distribution of murine PITX2B protein during cardiac development, we performed immunohistochemistry assays by using specific antibodies against PITX2B protein (Hernández-Torres et al., 2008). In order to demonstrate the specificity of antiPITX2B antibodies, we used immortalized cardiomyocytes isolated from mice at stage E9.5 (p38α+/+ and p38α−/−) (Adams et al., 2000) and demonstrated that lack of p38α Map Kinase avoid Pitx2a, Pitx2b and Pitx2c mRNA expression (Supplementary Fig. 1). In addition the results in Supplementary Fig. 1 B-F, demonstrate the specificity of antiPITX2B antibodies. Consequently, the use of these antiPITX2B antibodies allowed us to quantify the proportion of PITX2B positive nuclei in the heart at different developmental stages. As illustrated in Fig. 2 the PITX2B positive cardiac cell profile is in agreement with the mRNA expression profile described above. Thus, the number of PITX2B positive cells is higher at early stages of development, showing a maximal number of positive cells at stage E9.5. Subsequently, the proportion of PITX2B positive nuclei decreases until foetal and neonatal stages, where the proportion of PITX2B positive nuclei is around 6–7%.

Changes in PITX2B protein distribution within different cardiac compartments during development

Previous experiments have clearly determined that Pitx2c mRNA is asymmetrically distributed in heart, and is restricted to specific heart compartments as development progresses (Campione et al., 1999; Campione et al., 2001; Franco et al., 2003). Thus, in order to determine the distribution of PITX2B positive cells among different cardiac regions during heart development, we performed quantitative analyses of PITX2B positive cells within the different cardiac compartments from E9.5 to neonatal stages (Fig. 4 A-I). In agreement with the data presented above, the number of Pitx2b positive cells in the different heart regions is higher during earlier stages and subsequently decreases with further development. However, this pattern differs depending on the specific heart areas. Thus, while left and right atria show a constant and homogeneous decrease, the ventricular chambers display some regional variations. At stage E13.5 the proportion of Pitx2b positive cells within ventricular chambers is around 14–16%, but around 28–31% in the atrial chambers and interventricular septum. Notably these results indicate that the decrease in Pitx2b positive cells at E13.5...
is larger in the left and right ventricles as compared with atrial and interventricular septum compartments. However at E15.5 we observe a drastic decrease in the number of Pitx2b positive cells in the interventricular septum (only 4%) compared with a less extensive decrease in atrial (~10%) and ventricular chambers (~11–12%). Contrary to this, at E17.5 and neonatal stages, comparable proportions of Pitx2b positive nuclei (~5–6%) are shown in all cardiac structures (Fig. 4I). Overall, these findings indicate that during the developmental window comprising interventricular septum formation (E11.5-E15.5) (Miquerol et al., 2012) PITX2B positive cells displayed a dynamic distribution within this ventricular compartment. At E13.5, we found that a major proportion of PITX2B ventricular positive nuclei are located in the interventricular septum and these cells shift to minimal PITX2B expression at E15.5 when ventricular septation is fully completed (Miquerol et al., 2012).

In addition, and based on recent reports that pointed out a possible participation of PITX2 in the development of atrioventricular (AV) canal (Tessari et al., 2008), we investigated whether PITX2B could be involved in AV canal formation by analysing the number of PITX2B positive cells in those structures. At stages E9.5 and E11.5 the proportion of PITX2B positive mesenchymal cells in AV cushions is clearly low, around 4% (Fig. 5 A,B,E-H). Nevertheless, at stage E13.5, when the mural and septal portions of valve primordia can be observed (Lincoln et al., 2004), differences in the expression profile suggests a role for PITX2B in the establishment of different components of the tricuspid valve during development, and this is the only sign of asymmetry for PITX2B protein distribution that we have detected during heart development.

Discussion

It is well established that Pitx2 plays a crucial role in the establishment of vertebrate left-right axis determination, acting as a molecular transducer of embryonic left–right signalling at the organ level during early stages of development, including the heart (Franco et al., 2003). Previous studies have shown that, although Pitx2a and Pitx2b isoforms are symmetrically co-expressed with Pitx2c in certain regions of the embryo, only Pitx2c is asymmetrically expressed in the lateral plate mesoderm (LPM), heart and gut (Kitamura et al., 1999; Liu et al., 2001; Schweickert et al., 2000).

The importance of Pitx2 in heart development gathered strength when, concurrently, Pitx2 specific null mice mutants (Pitx2abc -/- (Gage et al., 1999), Pitx2ab -/- (Liu et al., 2001) and Pitx2c -/- (Liu et al., 2002)) were developed. Pitx2 mutant mice for all three isoforms (Δabcnull) display several cardiac malformations such as right sino-atrial isomerism and abnormalities of atrioventricular and ventriculoarterial connections. Since similar cardiac malformations have been described in Pitx2c mutant mice while no cardiac anomalies have been detected in Pitx2a/b mutant mice, the relevance of Pitx2c isoform in the heart has been extensively accepted (Liu et al., 2001; Liu et al., 2002). However, several sets of controversial data support a putative role for Pitx2a/b in this process. First, Kioussi et al., have suggested a role for PITX2A during outflow
In agreement with previous works, our qRT-PCR analysis showed that Pitx2c mRNA expression patterns during heart development are largely more sensitive than any ISH previously used to analyse Pitx2 isoform expression (Campione et al., 1999; Schweickert et al., 2000). Therefore, it is not surprising that Pitx2a and Pitx2b expression have not been detected before in the developing heart.

Although Pitx2, especially Pitx2c, mRNA expression patterns during cardiogenesis are apparently well known (Franco et al., 2003), PITX2 protein distribution in heart development is still poorly understood and seems to be contradictory (Hjalt et al., 1998). Third, analysis of the fate of Pitx2 daughter cells in mutant embryos that only express Pitx2a and Pitx2b isoforms (Pitx2 δabccrene -/- and Rosa26 reporter trans-heterozygotes) (Liu et al., 2002) revealed that fewer lacZ-positive cells were found in the right ventricular and inner curvature myocardium of Pitx2 mutant embryos at both 14.5 dpc and 18.5 dpc. And fourth, comparative anatomical analysis of hearts from embryos at both 14.5 dpc and 18.5 dpc. Therefore, we report for the first time that Pitx2a and Pitx2b isoforms are co-expressed with Pitx2c isoform during heart development. Interestingly, in terms of relative quantification of mRNA, while Pitx2a expression is only detected at early stages, Pitx2b displays a similar expression profile to that for Pitx2c, suggesting relevance for Pitx2 during cardiogenesis. Since Pitx2a and Pitx2b share the same promoter and the mature Pitx2a and Pitx2bmRNAs are originated from a common RNA molecule that undergoes a process of alternative splicing (AS), we could speculate that posttranscriptional regulation mechanisms could balance the amount of Pitx2a and Pitx2b isoforms. In fact, recent findings indicate that AS is controlled by the presence of post-transcriptional RNA operons or regulons (Keene, 2007). Pitx2a and Pitx2b expression is significantly lower than Pitx2c expression and current qRT-PCR techniques are largely more sensitive than any ISH previously used to analyse Pitx2c isoform expression (Campione et al., 1999; Schweickert et al., 2000). Therefore, it is not surprising that Pitx2a and Pitx2b expression have not been detected before in the developing heart.

Here, we report for the first time that Pitx2a and Pitx2b isoforms are co-expressed with Pitx2c isoform during heart development. In agreement with previous works, our qRT-PCR analysis showed that Pitx2c is largely the most abundant isoform during heart development, maintaining low expression levels in the adult heart.
2000). Here, we report for the first time the expression pattern of a specific PITX2 protein isoform during heart development. Our results show how PITX2B protein is differentially expressed within cardiac compartments during heart development. Thus, in contrast to Pitx2c (Campione et al., 1999; Schweickert et al., 2000), PITX2B distribution is ubiquitous in the atrial chambers, suggesting that PITX2B is not mainly involved in left-right atrial identity. However, within ventricular compartments, PITX2B protein progressively accumulates in the interventricular septum between E11.5 and E13.5 coinciding with the developmental window in which ventricular septation takes place (Miquerol et al., 2012). Liu et al., previously showed the presence of Pitx2-daughters cells in the interventricular septum and atrioventricular cushions (Liu et al., 2002). As suggested by the authors, one interpretation of these data is that Pitx2c daughter cells can expand to populate other hearts regions. Nevertheless, the contribution of cell populations expressing Pitx2a or Pitx2b isoforms in those cardiac tissues cannot be ruled out. In addition, it has been previously shown that Pitx2abc null mice exhibit ventricular septation defects (Gage et al., 1999) whereas Pitx2c mutants did not (Liu et al., 2002). These data suggest that the isoforms PITX2A and PITX2B could have a redundant function with PITX2C during heart development (Liu et al., 2001). In addition, we cannot rule out the possibility that different PITX2 isoforms could be coexpressed in the same cardiac cells, according to the fact that different isoforms of PITX2 can act together forming homo and heterodimers leading to transcriptional synergism (Cox et al., 2002; Saadi et al., 2003).

Current studies suggest that Pitx2-mediated signalling during cardiogenesis is conducted within three different cell types: the myocardium, the cardiac neural crest (CNC) cells, and the pharyngeal arch mesenchyme (Campione et al., 1999; Chinchilla et al., 2011; Franco et al., 2003; KIoussi et al., 2002; Liu et al., 2002; Tessari et al., 2008), minimizing the possible role that Pitx2 a, b or c, may have during development of epicardial and endocardial derivatives. In addition, it was previously reported only that Pitx2c expression is never detected in endocardium (Liu et al., 2002). Notably, we reported here the presence of PITX2B in epicardium and endocardium during heart development, opening new putative roles for this gene during heart organogenesis. Concerning the possible role of Pitx2 in myocardium, Tessari et al., (Tessari et al., 2008) reported how myocardial Pitx2 expression delineates the remodelling of the left atrioventricular canal, contributing to atrio-ventricular septation. Interestingly our results show that in the atrioventricular canal compartment PITX2B protein is mainly present in the mural primordium of the mitral valve during heart development, suggesting a role of PITX2B in the maturation of the right portion of the atrioventricular canal. Although Liu et al., (Liu et al., 2002) speculated that Pitx2 daughter cells from myocardium invade the AV cushions and valves, in mice it has been also proposed that the leaflets and tendinous cords of the tricuspid valve are all generated from mesenchyme, derived from the endocardium with no substantial contribution from cells of the myocardial lineage (de Lange et al., 2004). In whatever manner this cells arrives to the AV cushions, we cannot determine the source of cells that participate in this process since we have detected the presence of PITX2B in endocardium, myocardium and epicardium. Finally, we must stress that this PITX2B expression pattern in the atrioventricular canal constitute the only sign of asymmetry for PITX2B distribution.

Fig. 5. Quantitative analysis of PITX2B positive cells in the AV canal structures. Quantification of PITX2B positive cells in the AV canal structures at stage 9.5 (A), stage 11.5 (B) and stage 13.5; (C) Tricuspid valve primordium; (D) Mural valve primordium. AVCC Atrio-ventricular canal cushions. (E-K) PITX2B protein distribution in the AV canal structures from E9.5 to E13.5. Nucleus and PITX2B stain are showed in red and green respectively. (A) 10X transverse section of mouse embryos heart at stage E9.5. (F) White square on image E amplified 40X. (G) 10X transverse section of mouse embryos heart at stage E11.5. (H) White square on image G amplified 40X. (I) 10X transverse section of mouse embryos heart at stage E13.5. (J) PITX2B protein distribution in Tricuspid Valve primordia. White square on image I amplified 40X. (K) PITX2B protein distribution in Mural Valve primordia. Blue square on image J amplified 40X. AVCC: Atrio-ventricular canal cushions; TVP: Tricuspid Valve primordia; MVP: Mural Valve primordia; MP: Mural portion; SP: Septal portion; RA: Right atria; LA: Left atria; RV: Right ventricle; LV: Left ventricle; IS: Interventricular septum. White arrows indicate PITX2B positive cells. ***P-value <0.001.
during cardiogenesis. In summary, we report that Pitx2a and Pitx2b isoforms are coexpressed with Pitx2c isoform during heart development. Interestingly, in terms of relative quantification of mRNA, Pitx2b and Pitx2c isoforms display similar expression profiles during cardiogenesis, decreasing with further development but maintaining its expression until adult stages. Moreover, a detailed analysis of PITX2B protein during cardiac development shows that PITX2B is dynamically expressed in the developing ventricular septum and asymmetrically expressed in the tricuspid valves primordia. This suggests a putative role for PITX2B during ventricular septation as well as in the maturation of the right portion of the atrioventricular canal.

Materials and Methods

Tissue preparation
Balb/c female mice were sacrificed and whole hearts ranging from embryonic day (E) 9.5 to E17.5 were isolated. The day of vaginal plug was taken as E0.5. Adult and neonatal hearts (1 and 15 days) were also obtained. Hearts for RNA isolation and immunohistochemistry experiments were processed as previously described (Dominguez et al., 2005).

Quantitative real time PCR (q-PCR)
Total RNA was isolated and reverse transcribed from pooled hearts at each stage (n = 5 per stage), ranging from E9.5 to E17.5 embryos, and from neonate and adult mice (n = 3 per stage) as previously described (Dominguez et al., 2005). Finally, each experimental point is represented by the average of five different pools of hearts. The primers used to detect mouse Pitx2a (NM_001042504.2), Pitx2b (NM_011098.4), Pitx2c (NM_001042502.2) and β-actin (NM_007393) gene expression (Table 1) were specifically designed by using Primer3v.0.4.0 program (available on line http://frodo.wi.mit.edu/).

Real-time PCR was performed within an iCycler PCR thermocycler (Bio-Rad, Spain) and SYBR Green detection system. Reactions were performed in 96-well plates with optical sealing tape (Bio-Rad) in 20 μl total volume containing SYBR Green Mix (Bio-Rad) and cDNA corresponding to 50 ng of total RNA. Mouse β-actin was used in parallel for each run as an internal control. Amplification conditions were: 95 °C for 5 min; 45 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s; and 72 °C for 10 min. The relative expression level of each Pitx2 isoform was calculated through Livak analysis method (Livak, Schmittgen, 2001).

Immunohistochemistry
Serial tissue sections from littermates were cut for parallel analysis. The distribution of PITX2B and DESMIN proteins were analysed by immunohistochemistry and confocal microscopy as previously described (Dominguez et al., 2005). Rabbit polyclonal antibody against mouse PITX2B (Hernández-Torres et al., 2008) and mouse monoclonal antibody against mouse DESMIN (Sigma, Spain) were incubated overnight at room temperature with a 1:50 dilution. Following rinsing, sections were incubated for 5 h with antirabbit Cy3 or antimouse Cy2 secondary antibodies (Jackson Labs, USA) diluted in TBSA-BSAT at 1:200 or 1:100 respectively. Nuclear staining was performed using DRAQ-5™ (Red Fluorescent Cell-Permeable DNA probe, Biostatus Limited).

Quantification of nucleus (total and PITX2B positive) was done as Carmona et al., (Carmona et al., 2007) indicated. This method renders images devoid of both, extranuclear staining and background, thus emphasizing the nuclear signal. Images used in this work correspond to frontal heart sections in which Left Atria, Right Atria, Interventricular Septum (when it was present), Left Ventricle and Right Ventricle structures were clearly recognizable. Each structure was isolated as an individual image for its individual analysis; the total number of nucleus as well as the number of PITX2B positive nucleus were measured. Finally, the proportion of PITX2B positive nucleus was calculated by dividing the number of PITX2B positive nucleus by the total number of nucleus counted on each image. This ratio was finally expressed as a percentage. Each experimental point is represented by the average of the analysis of five different heart images for each embryo with a total number of three embryos at each stage.

Acknowledgements
We thank Chris Doherty for reading the manuscript and the "Centro de Investigación Científico-Técnico (CICT), Universidad de Jaén" for the confocal microscopy help. This work was supported by grants of Spanish Ministry of Economy and Competitiveness, MINECO, and FEDER (BFU2013-48643-C3-2-P to F.N.) and Junta de Andalucía (BIO258, PI10-CV6521 to F.N.).

References


<table>
<thead>
<tr>
<th>PRIMERS USED IN qRT-PCR EXPERIMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligonucleotides</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>β-actin F</td>
</tr>
<tr>
<td>β-actin R</td>
</tr>
<tr>
<td>Pitx2a F</td>
</tr>
<tr>
<td>Pitx2a R</td>
</tr>
<tr>
<td>Pitx2b F</td>
</tr>
<tr>
<td>Pitx2b R</td>
</tr>
<tr>
<td>Pitx2c F</td>
</tr>
<tr>
<td>Pitx2c R</td>
</tr>
</tbody>
</table>

Trends Cardiovasc


Further Related Reading, published previously in the *Int. J. Dev. Biol.*

**Genome-wide gene expression analysis in mouse embryonic stem cells**
Juan Sainz, Fernando García-Alcalde, Armando Blanco and Ángel Concha  
*Int. J. Dev. Biol.* (2011) 55: 995-1006  
http://dx.doi.org/10.1387/ijdb.103123js

**Building the vertebrate heart - an evolutionary approach to cardiac development**
José M. Pérez-Pomares, Juan M. González-Rosa and Ramón Muñoz-Chápuli  
*Int. J. Dev. Biol.* (2009) 53: 1427-1443  
http://dx.doi.org/10.1387/ijdb.072409jp

**Heart formation and left-right asymmetry in separated right and left embryos of a newt**
Kazuhiro Takano, Yuzuru Ito, Shuichi Obata, Tautomu Oinuma, Shinji Komazaki, Hiroaki Nakamura and Makoto Asashima  
*Int. J. Dev. Biol.* (2007) 51: 265-272  
http://dx.doi.org/10.1387/ijdb.072270kt

**Xenopus nodal related-1 is indispensable only for left-right axis determination**
Ryuji Toyoizumi, Tsuyoshi Ogasawara, Shigeo Takeuchi and Kazue Mogi  
*Int. J. Dev. Biol.* (2005) 49: 923-938  
http://dx.doi.org/10.1387/ijdb.052008rt

**A P19Cl6 GFP reporter line to quantify cardiomyocyte differentiation of stem cells**
Jennifer C Moore, Rene Spijker, Anton C Martens, Teun de Boer, Martin B Rook, Marcel A G van der Heyden, Leon G Tertoolen and Christine L Mummery  
http://dx.doi.org/10.1387/ijdb.15005574

5 yr ISI Impact Factor (2013) = 2.879