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Expression patterns and immunohistochemical localization of PITX2B transcription factor in the developing mouse heart

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ABSTRACT The *Pitx2* gene is involved in the establishment of vertebrate left-right axis with an important role in subsequent heart organogenesis. Mutations in the *Pitx2* 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 PITX2 loss of function and atrial fibrillation (AF), supporting an important role of *Pitx2* not only in development but also in heart homeostasis. Three PITX2 isoforms have been described in mice: PITX2A, PITX2B, and PITX2C. During heart organogenesis, PITX2C seems to play a determinant role in left–right signalling from early somitogenesis onwards. However the participation of the PITX2A and/or PITX2B isoforms during cardiogenesis is controversial. Here we report for the first time that the *Pitx2a* and *Pitx2b* isoforms are jointly expressed with the *Pitx2c* isoform during heart development. Interestingly, in terms of relative quantification of mRNA, the *Pitx2b* and *Pitx2c* 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: *Pitx2 isoforms, heart development, mouse and ventricular septation*

The *Pitx2* gene is a member of the Bicoid-like homeobox family (Semina *et al*., 1996). During the last fifteen years several studies reported the implication of *Pitx2* in the correct establishment of vertebrate left-right axis. Left-right asymmetry is established through a molecular cascade that gives rise to *Pitx2* 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 *Pitx2* could be the molecular transducer of embryonic left–right signalling at the organ level during early stages of development. Mutations in the *Pitx2* gene have been associated with Axenfeld-Rieger syndrome, which is characterized by ocular, craniofacial, and umbilical anomalies (Semina *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 *Pitx2* gene is transcribed into three distinct isoforms in mice: *Pitx2a*, *Pitx2b*, and *Pitx2c*. *Pitx2a* and *Pitx2b* share the same promoter while *Pitx2c* uses an alternative promoter upstream of exon 4 (Gage *et al*., 1999; Schweickert *et al*., 2000). The analysis of expression patterns for the *Pitx2* 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 *Pitx2c* 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: Pitx2, pituitary homeobox 2 gene; AF, atrial fibrillation; LPM, lateral plate mesoderm; OFT, outflow tract; AS, alternative splicing.

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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 (Campione *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$^{o;\delta c\,a}$nd 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.

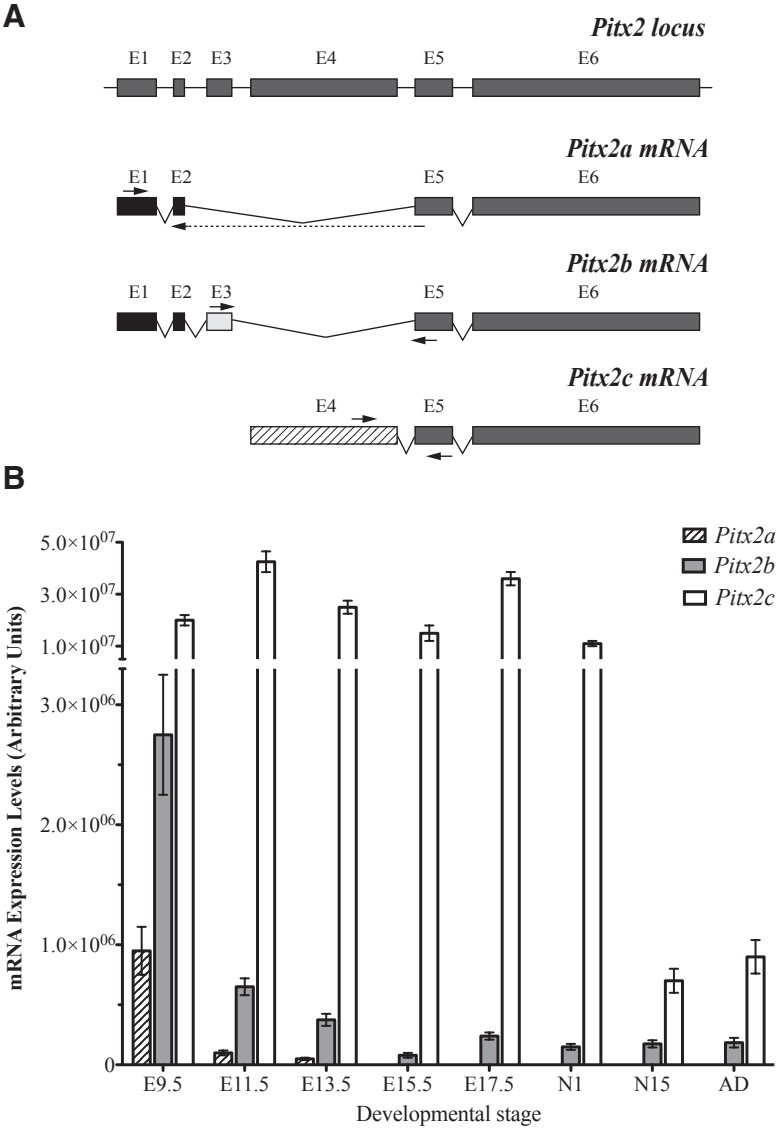

Fig. 1. *Pitx2* mRNA expression levels in cardiogenesis. (A) *Schematic representation of the strategy followed in order to measure specifically each* Pitx2 *isoform mRNA. Arrows indicate the primers annealing place used to amplify each isoform.* **(B)** Pitx2a, Pitx2b *and* Pitx2c *mRNA expression levels 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) 9.5 to 17.5; N1, 1 day neonate mouse heart; N15, 15 day neonate heart; Ad, adult mouse heart.*

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

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 anitPITX2B 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%.

Co-immunolocalization experiments with a myocardial marker (DESMIN) indicated that PITX2B protein is present not only in myocardial cells but also in cells located in endocardium and epicardium (Fig. 3A-E). In addition, myocardial, endocardial and epicardial quantitative analyses showed that the proportion of PITX2B positive cells also decreases during development in all three tissue layers, showing a maximal number of positive cells in early stages of development and decreasing as development progresses (Fig. 3E).

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

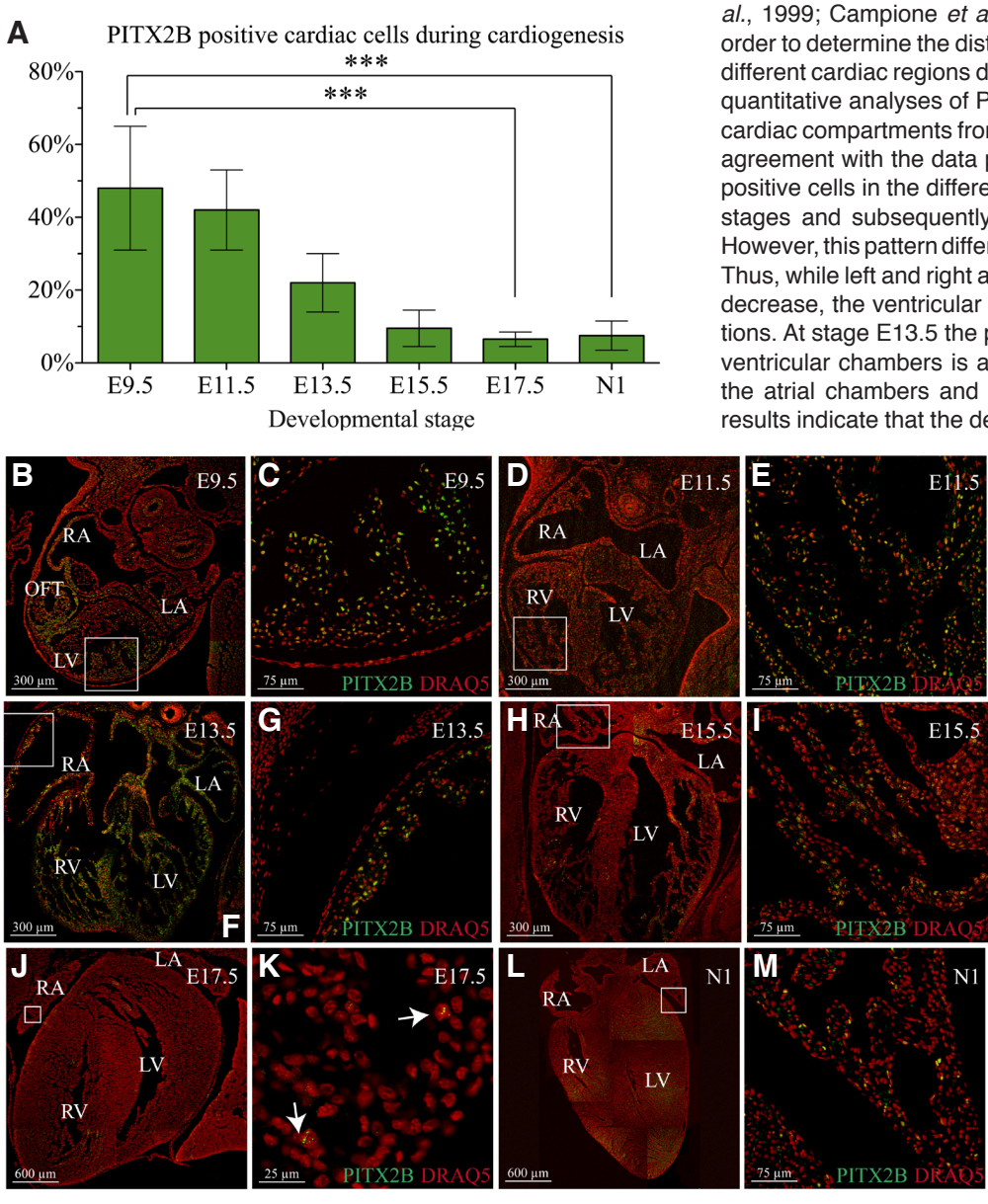

Fig. 2. PITX2B protein expression analysis during heart morphogenesis. **(A)** *Quantification of PITX2B positive cells in hearts from different stages during cardiogenesis. Embryonic day (E) 9.5 to 17.5; N1: 1 day neonate mouse heart.* **(B-M)** *Nuclear immunohistochemical stain obtained in transverse heart sections from mouse embryos in different stages of development by using specific antiPITX2B antibody. Nucleus and PITX2B stain are showed in red and green respectively.* **(B-C)** *stage E9.5;* **D-E)** *stage E11.5;* **(F-G)** *stage E13.5;* **(H-I)** *stage E15.5;* **(J-K)** *stage E17.5;* **(L-M)** *stage N1. (B, D, F, and H) show 10X transverse sections images. (J,L) Images are the result of superposition of several 10X transverse sections images. (C, E, G, I, K and M) Images show an amplification of white squares on images (B, D, F, H, J and L) respectively. White arrows indicate PITX2B positive cells at stage E17.5. RA: Right atria; LA: Left atria; RV: Right ventricle; LV: Left ventricle; OFT: Outflow tract. *** P-value <0.001.*

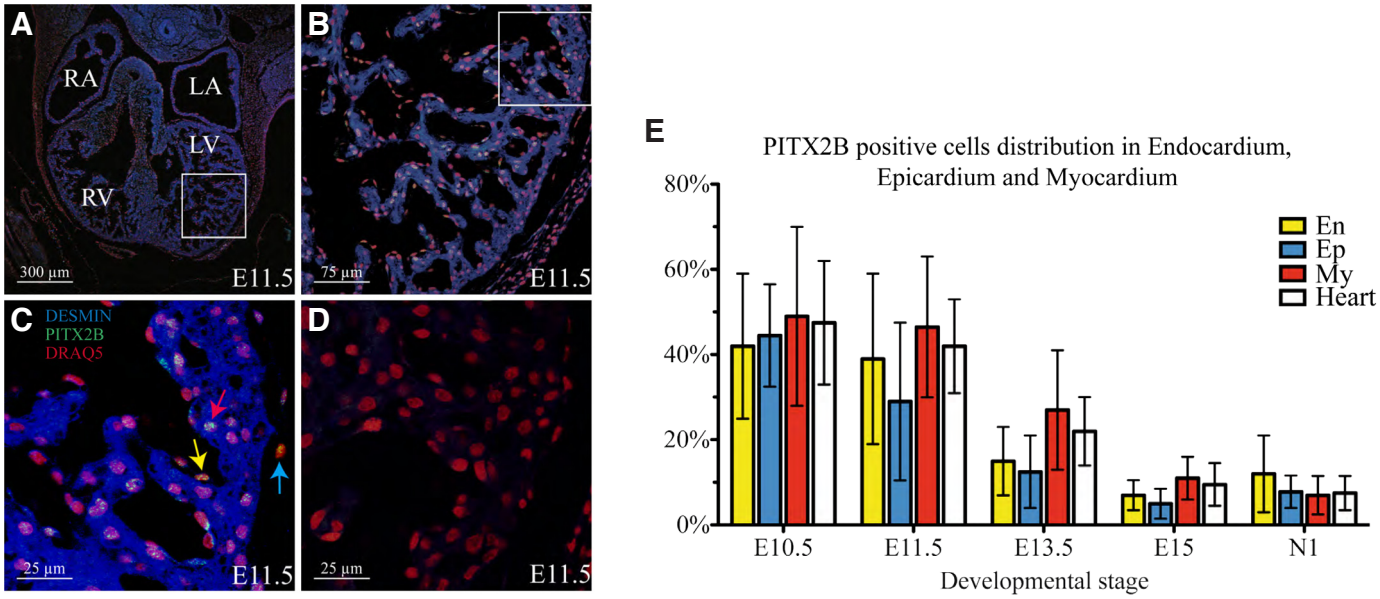

Fig. 3. Quantitative analysis of PITX2B positive cells in endocardium, myocardium and epicardium. (A-D) *Nuclear and cytoplasm immunohistochemical stain obtained in transverse sections of mouse embryos at stage E11.5 by using specific antiPITX2B and antiDESMIN antibodies respectively.* **(A)** *10X transverse section of mouse embryos heart at stage E11.5.* **(B)** *White square on image A amplified 40X.* **(C)** *White square on image B amplified 120X. Nucleus, PITX2B and DESMIN stain are showed in red, green and blue respectively. Yellow, red and blue arrows indicate PITX2B positive nucleus from endocardium, myocardium and epicardium respectively* **(D)** *Negative control.* **(E)** *Quantification of PITX2B positive cells in endocardium, epicardium and myocardium during cardiogenesis. Embryonic day (E) 10.5 to 15.5; N1: 1 day neonate mouse heart; En, endocardium; Ep, epicardium; My, myocardium; WH, Whole heart. RA: Right atria; LA: Left atria; RV: Right ventricle; LV: Left ventricle; IS: Interventricular septum.*

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 structures derived from AV canal cushions were detected (Fig. 5 C,D,I-K). Thus, while mural portions of tricuspid valves (TV), close to the right ventricular wall on Fig. 5J, presented a proportion of PITX2B positive cells similar to that estimated for whole heart at this stage (around 23%), septal portions of valves displayed a value of PITX2B positive cells at around 3% (Fig. 5 C,J). However, for the mitral valve (MV) analogues, neither presented a significantly high proportion of PITX2B positive cells, at around 3~7% for both mural and septal portions (Fig. 5 D,K). This PITX2B 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

tract (OFT) development (Kioussi *et al.*, 2002). Second, Hjalt *et al.,* (Hjalt *et al.*, 2000*)*, by using antibodies recognizing 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 (Campione *et al.*, 1999; Logan *et al.*, 1998; Ryan *et al.*, 1998; Yoshioka *et al.*, 1998). Third, analysis of the fate of *Pitx2* daughter cells in mutant embryos that only express *Pitx2a* and *Pitx2b* isoforms (*Pitx2 δabccrene*$^{o;\delta c}$ 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 *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).

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. 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 *Pix2b* during cardiogenesis. Since *Pitx2a* and *Pitx2b* share the same promoter and the mature *Pitx2a* and *Pitx2b* mRNAs are originated from a common RNA molecule that undergoes a process of alternative splicing (AS), we could speculate that posttrancriptional regulation mechanisms could balance the amount of P*itx2a* 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 *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.*,

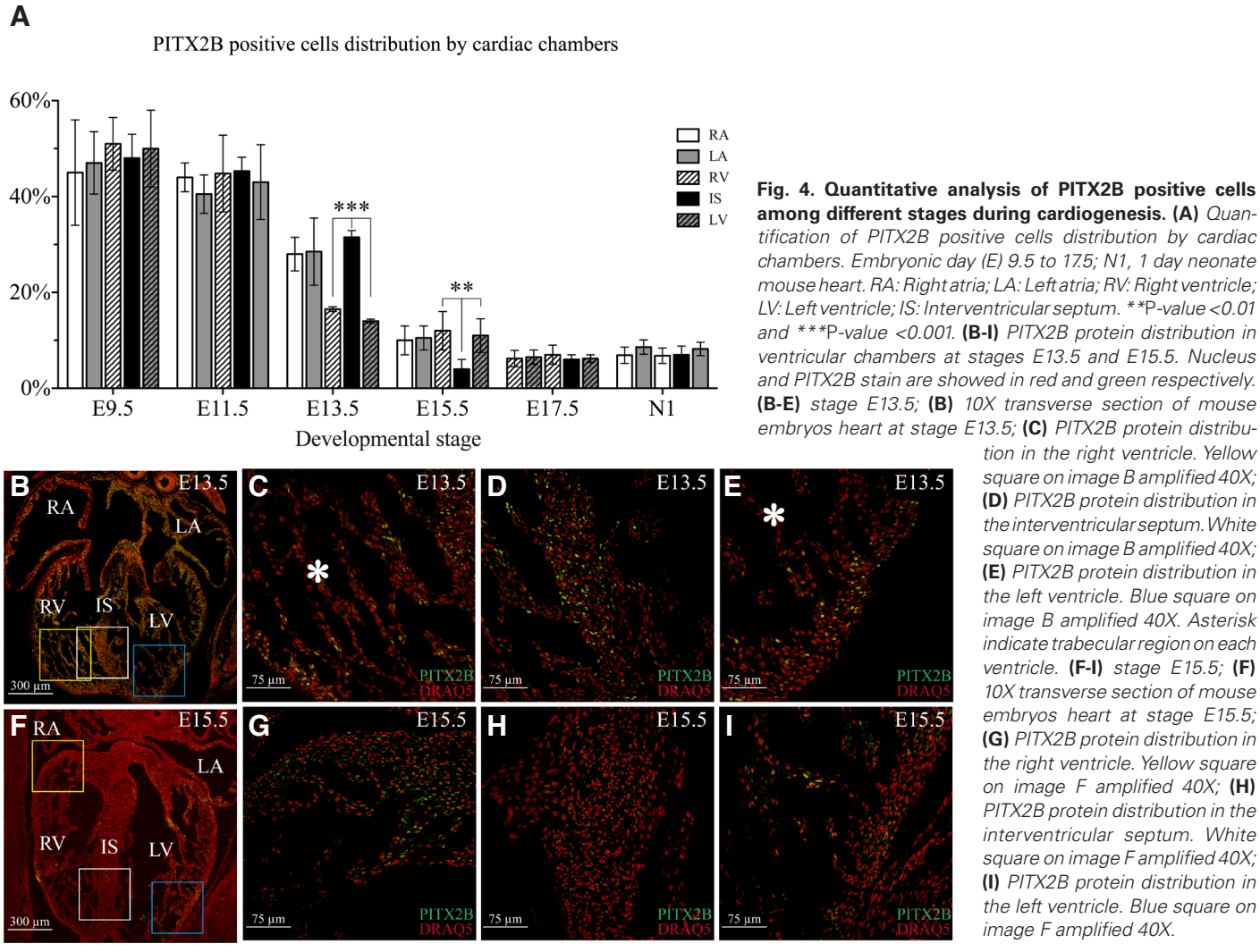

Fig. 4. Quantitative analysis of PITX2B positive cells among different stages during cardiogenesis. (A) *Quantification of PITX2B positive cells distribution by cardiac chambers. Embryonic day (E) 9.5 to 17.5; N1, 1 day neonate mouse heart. RA: Right atria; LA: Left atria; RV: Right ventricle; LV: Left ventricle; IS: Interventricular septum. **P-value <0.01 and ***P-value <0.001.* **(B-I)** *PITX2B protein distribution in ventricular chambers at stages E13.5 and E15.5. Nucleus and PITX2B stain are showed in red and green respectively.* **(B-E)** *stage E13.5;* **(B)** *10X transverse section of mouse embryos heart at stage E13.5;* **(C)** *PITX2B protein distribution in the right ventricle. Yellow square on image B amplified 40X;* **(D)** *PITX2B protein distribution in the interventricular septum. White square on image B amplified 40X;* **(E)** *PITX2B protein distribution in the left ventricle. Blue square on image B amplified 40X. Asterisk indicate trabecular region on each ventricle.* **(F-I)** *stage E15.5;* **(F)** *10X transverse section of mouse embryos heart at stage E15.5;* **(G)** *PITX2B protein distribution in the right ventricle. Yellow square on image F amplified 40X;* **(H)** *PITX2B protein distribution in the interventricular septum. White square on image F amplified 40X;* **(I)** *PITX2B protein distribution in the left ventricle. Blue square on image F amplified 40X.*

2000). Here, we report by the firs 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*-daugthers 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 linage (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

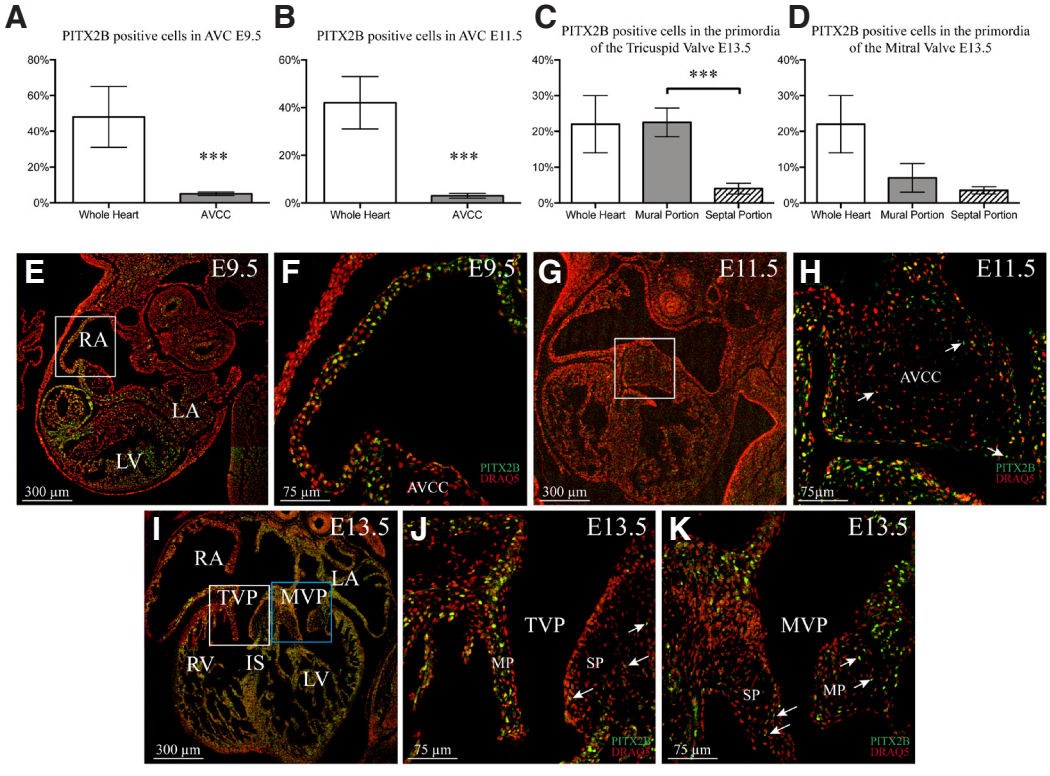

Fig. 5. Quantitative analysis of PITX2B positive cells in the AV canal structures. *Quantification of PITX2B positive cells in the AV canal structures at stage 9.5* **(A)***, stage 11.5* **(B)** *and stage 13.5,* **(C)** *Tricuspid valve primordium* **(D)** *Mitral valve primordium*). *AVCC Atrio-ventricular 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.* **(E)** *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 Mitral Valve primordia. Blue square on image I amplified 40X. AVCC: Atrio-ventricular canal cushions; TVP: Tricuspid Valve primordia; MVP: Mitral 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.*

TABLE 1

PRIMERS USED IN qRT-PCR EXPERIMENTS

Oligonucleotides	Sequence	Amplicon Size (bp)
β-actin F	5´-TGAGGAGCACCCTGTGCT-3´	144
β-actin R	5´-CCAGAGGCATACAGGGAC-3´	
Pitx2a F	5´ GAGAGCAGCAGACAGAAAC 3´	270
Pitx2a R	5´ ATCTTTCTCTAATTGCACGC 3'	
Pitx2b F	5' GGTGCAGTTCACGGACTCTC 3'	233
Pitx2b R	5' TGTCTGGGTAGCGGTTTCTC 3'	
Pitx2c F	5' CCTCACCCTTCTGTCACCAT 3'	179
Pitx2c R	5' GCCCACATCCTCATTCTTTC 3'	

during cardiogenesis.

In summary, we report that *Pitx2a* and *Pitx2b* isoforms are co-expressed 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 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 witch 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 analisys; 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.

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