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Atrial myocardium derives from the posterior region of the second heart field, which acquires left-right identity as Pitx2c is expressed

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Splanchnic mesoderm in the region described as the second heart field (SHF) is marked by *Islet1* expression in the mouse embryo. The anterior part of this region expresses a number of markers, including *Fgf10*, and the contribution of these cells to outflow tract and right ventricular myocardium has been established. We now show that the posterior region also has myocardial potential, giving rise specifically to differentiated cells of the atria. This conclusion is based on explant experiments using endogenous and transgenic markers and on DiI labelling, followed by embryo culture. Progenitor cells in the right or left posterior SHF contribute to the right or left common atrium, respectively. Explant experiments with transgenic embryos, in which the transgene marks the right atrium, show that atrial progenitor cells acquire right-left identity between the 4- and 6-somite stages, at the time when *Pitx2c* is first expressed. Manipulation of Pitx2c, by gain- and loss-of-function, shows that it represses the transgenic marker of right atrial identity. A repressive effect is also seen on the proliferation of cells in the left sinus venosus and in cultured explants from the left side of the posterior SHF. This report provides new insights into the contribution of the SHF to atrial myocardium and the effect of Pitx2c on the formation of the left atrium.

KEY WORDS: DiI labelling, Atrial myocardium, Explants, Mouse embryo, Pitx2c, Second heart field

INTRODUCTION

A major source of cardiac progenitor cells has been identified that initially lies medial to the cardiac crescent, where the first differentiated myocardial cells are present, and then is located behind the forming heart tube as the cardiac crescent fuses on the anterior-to-posterior axis. In the mouse embryo, this region of splanchnic mesoderm has been called the second heart field (SHF) (see Buckingham et al., 2005). The contribution of the rostral part of the SHF, the anterior heart field (AHF), to the arterial pole of the mouse heart is relatively well documented. This region is marked by *Fgf10* and *Fgf8* expression. A transgene, *Mlc1v-nlacZ-24*, that had integrated into the *Fgf10* locus, provided a valuable marker of these cells and their derivatives in myocardium of the outflow tract and right ventricle, which continues to be β-galactosidase (β-gal)-positive although *Fgf10* is no longer expressed (Kelly et al., 2001). Fgf8 has since been shown to play an important role in outflow tract development (Park et al., 2006; Ilagan et al., 2006). This contribution to right ventricular as well as to outflow tract myocardium was corroborated by experiments with explants from the AHF, which were demonstrated to have myocardial potential and which, by the use of marker transgenes, were shown to give rise to outflow tract and right ventricular myocardium (Zaffran et al., 2004). DiI-labelling experiments of cells in the AHF, followed by embryo culture, demonstrated a contribution to the outflow tract and right ventricular myocardium, consistent with the experiments using transgenes as markers (Kelly et al., 2001; Zaffran et al., 2004). Observations of the expression of *Islet1* [Isl1 transcription factor, LIM/homeodomain (*Isl1*)], suggested that this marks a more extensive SHF region that also contributes about two-thirds of atrial myocardial cells, based on *Islet-Cre/Rosa26* cell-tracing experiments (Cai et al., 2003). Since then, a number of genes or regulatory sequences that mark the AHF, or that show more extensive expression in the SHF, have been described. Mutation in some of these genes results in complex phenotypes that affect the venous as well as the arterial pole of the heart (Buckingham et al., 2005; Black, 2007).

Cell-lineage analysis of myocardial progenitors in the chick embryo points to differences between the location of cells in the primitive streak that contribute to arterial pole myocardium as opposed to more-posterior parts of the heart tube (Garcia-Martinez and Schoenwolf, 1993). In avian embryos, atrial markers are already detectable in the caudal part of the cardiac primordia and then in the caudal heart tube (Yutzey et al., 1994; Patwardhan et al., 2000), where progenitor cells adjacent to the sinus venosus have been shown to contribute to the growth of the tube (Arguello et al., 1975). In the mouse embryo, the cardiac crescent, where differentiating cardiomyocytes are first present, does not express atrial markers and atrial identity is only distinguishable later; atrial markers such as the atrial myosin light chain Mlc2a (Myl7 – Mouse Genome Informatics) are present throughout the tube at the 5- to 7-somite stage (Kubalak et al., 1994), whereas Mlc2v (Myl7 – Mouse Genome Informatics) is already restricted to ventricular myocardium (O'Brien et al., 1993), indicative of early transcriptional differences. Subsequently, the chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) (Pereira et

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al., 1999) and the atrial natriuretic factor ANF (also known as Nr2f2 and Nppa, respectively – Mouse Genome Informatics) (Christoffels et al., 2000), mark the atria. A retrospective clonal analysis in the mouse embryo has shown that myocardial cells in the heart tube, as it begins to loop at E8.5, derive from two distinct lineages that segregate before the first myocardial cells appear. The first lineage contributes left ventricular myocardium and to other parts of the heart with the exception of the outflow tract, whereas the second lineage shows a complementary contribution (Meilhac et al., 2004a). The myocardium of the atria is therefore formed by both lineages. This clonal analysis, which reveals the atrial contribution of the second lineage, can be correlated with observations on the SHF, which probably also contributes to the atria at the venous pole of the heart as well as to arterial pole myocardium. We have used similar approaches to those that we had employed for the AHF, namely explant experiments and DiI labelling, to examine this contribution more closely. We now demonstrate that atrial progenitors are located more caudally, in the posterior SHF (pSHF).

Left-right signalling affects cardiogenesis, as evidenced by mutant phenotypes (Logan et al., 1998; Lin et al., 1999) including right atrial isomerism (Liu et al., 2001). This is mediated in the myocardium by the Pitx2c isoform (Campione et al., 2001), which is probably also expressed in progenitor cells of the SHF (Ai et al., 2006; Nowotschin et al., 2006). We therefore investigated the role of Pitx2c in myocardial progenitor cells that contribute to the right and left atria. In order to carry out these experiments, we utilised *Mlc3f-nlacZ-2E* mice in which the transgene preferentially marks right atrial myocardium from an early stage (Kelly et al., 1995; Franco et al., 1997). We show that left-right differences, as evidenced by the myocardial potential of explants, are already present in the pSHF from the time when *Pitx2c* is first expressed in the left part of the field. Manipulation of Pitx2c expression in gain-of-function experiments, complemented by observations on *Pitx2c*$^{-/-}$ mutants, confirms its role in repressing transgene expression in explants from the left pSHF. The right side of the sinus venosus is more proliferative than the left, whereas in *Pitx2c*$^{-/-}$ mutant embryos the left sinus venosus is now more proliferative than the right. This effect is also seen in cultured explants from the SHF and is again consistent with repression by Pitx2c.

MATERIALS AND METHODS

Transgenic and mutant mice

The transgenic lines *Mlc3f-nlacZ-2E*, *Mlc3f-nlacZ-9*, *Mlc1v-nlacZ-24* have been described previously (Kelly et al., 1995; Franco et al., 1997; Kelly et al., 1998). The *Pitx2c*$^{+/-}$ mouse line is described by Liu et al. (Liu et al., 2001).

Mice were maintained on an inverted light-dark cycle to facilitate collection of embryos at the required developmental stages for explant analysis. The animals used were mainly produced in the Pasteur Institute Animal Facility, with provision of standard stocks from Janvier (Le Genest St Isle, France); the remaining animals were produced in the St George's Biological Research Facility. The care and use of laboratory animals followed the guidelines of the French Ministry of Agriculture or the UK Home Office.

X-Gal staining and whole-mount in situ hybridisation

Embryos were dissected in PBS and treated as described (Tajbakhsh and Houzelstein, 1995). Specific RNA probes used: *Islet1* (Cai et al., 2003); *Pitx2* (Campione et al., 1999).

Embryonic explant cultures

Explant culture conditions were as described (Zaffran et al., 2004). To make explants, the embryos were flattened and the number of somites counted. The cardiac crescent and the somites were used as morphological guides under the microscope. The explanted regions were lateral to the second/third somite. Explant experiments were repeated at least ten times for each transgenic line. After usually 12 or 72 hours of culture, explants were fixed in 4% paraformaldehyde, washed three times with PBS and stained with Hoechst 33258. Sections (10 μm) were prepared from frozen embryos. Treatment for fluorescent immunohistochemistry was as described (Daubas et al., 2000). The following antibodies were used, all at 1:200 dilution: polyclonal anti-β-gal (Sigma), monoclonal anti-myosin heavy chain (MF20, Developmental Studies Hybridoma Bank), monoclonal anti-phosphohistone H3 antibody (Cell Signalling) and anti-Islet1 (39.4D5, Developmental Studies Hybridoma Bank).

RT-PCR and qRT-PCR

RNA from five or ten explants at different times was extracted using the RNeasy Micro Kit (Qiagen, Cergy Pontoise, France) and cDNA was reverse-transcribed using the ThermoScript RT-PCR system (Invitrogen). The extractions and reverse transcriptions were repeated twice in independent experiments.

PCR was performed using a tenth of the reverse-transcription reaction volume, with the following program: an initial 5 minutes at 94°C; followed by 30 cycles of 45 seconds at 94°C, 45 seconds at 58-60°C (depending on the melting temperature of the primers) and 1 minute at 72°C; with a final 5 minutes at 72°C. Primers were as follows (5′-3′): *Mlc2a* fw, CAGACCTGAAGGAGACCT and *Mlc2a* rev, GTCAGCGTAA-ACAGTTGC (fragment generated of 286 bp); *Mlc2v* fw, GCCA-AGAAGCGGATAGAAGG and *Mlc2v* rev, CTGTGGTTCAG-GGCTCAGTC (499 bp) (Kubalak et al., 1994); *COUP-TFII* fw, CGCTTTTATGGACCACATACG and *COUP-TFII* rev, GTTTCGATG-GGGGTTTTACC (322 bp); *Pitx2c* fw, ACTGCATGAAAGGCCCGCTG and *Pitx2c* rev, CTTCAGGGCTGGAAGTATCG (195 bp); β-actin fw, GATGACCCAGATCATGTTTGAG and β-actin rev, GGAGCA-ATGATCTTGATCTTC (643 bp).

Quantitative (q) RT-PCR was performed as previously described (Hadchouel et al., 2000), except that PCR reactions on cDNA were performed with SYBR Green PCR Master Mix (Applied Biosystems, Courtaboeuf, France) and the quantity of each mRNA was expressed as a percentage with respect to *Gapdh* transcripts. Extractions and reverse transcriptions were repeated three times in independent experiments. Primers used were (5′-3′): *lacZ* fw, GCAGCCTGAATGGCGAAT and *lacZ* rev, CGCATCGTAACCGTGCATC (Hadchouel et al., 2000); *GFP* fw, AAGTTCATCTGCACCACCG and *GFP* rev, TCCTTGAAGAAGATG-GTGCG; *Mlc2a* rt fw, GTCAGCGTAAACAGTTGC and *Mlc2a* rt rev, GTCCGTCCCATTGAGCTTCT; *Gapdh* fw, AACGACCCCTTCATTGAC and *Gapdh* rev, TCCACGACATACTCAGCAC (Simpson et al., 2000).

Adenovirus generation

Adenoviruses were produced as described (He et al., 1998). Infection of the explants was performed after 12 hours of culture with approximately the same titre for all the viruses (10^8 plaque-forming units). This concentration was determined empirically by serial dilution on explants as the one that permitted high infection without induction of cell death. For the *Pitx2c* adenovirus, a fragment of *Pitx2c* cDNA of 956 bp (nucleotides 228 to 1184) was used.

DiI labelling

Embryos ranging from the 4- to 6-somite stages were collected, transferred to Hank's solution and injected with DiI as described (Franco et al., 2001). Embryos at the 7-somite stage were injected with DiI on one side and with DiR on the other side of the pSHF. Labelled embryos were photographed under a Leica MZ16F stereomicroscope using a Nikon Coolpix 995 digital camera. Embryos were then cultured for 40 hours in vitro (20-25 somites) in 75% rat serum, 25% T6 medium (Whittingham, 1971) with 5% CO_2, 20% O_2 and 75% N_2 in rolling bottles. After culture, DiI-labelled embryos were washed in PBS and fixed in 4% paraformaldehyde in PBS overnight. Labelled embryos were analysed with a Zeiss LSM 510 laser-scanning confocal microscope and the captured images were processed with the CS2 version of Adobe Photoshop. At 4- to 6-somites, ten embryos were injected in the left and ten in the right pSHF. At 7 somites, ten embryos were injected on both sides.

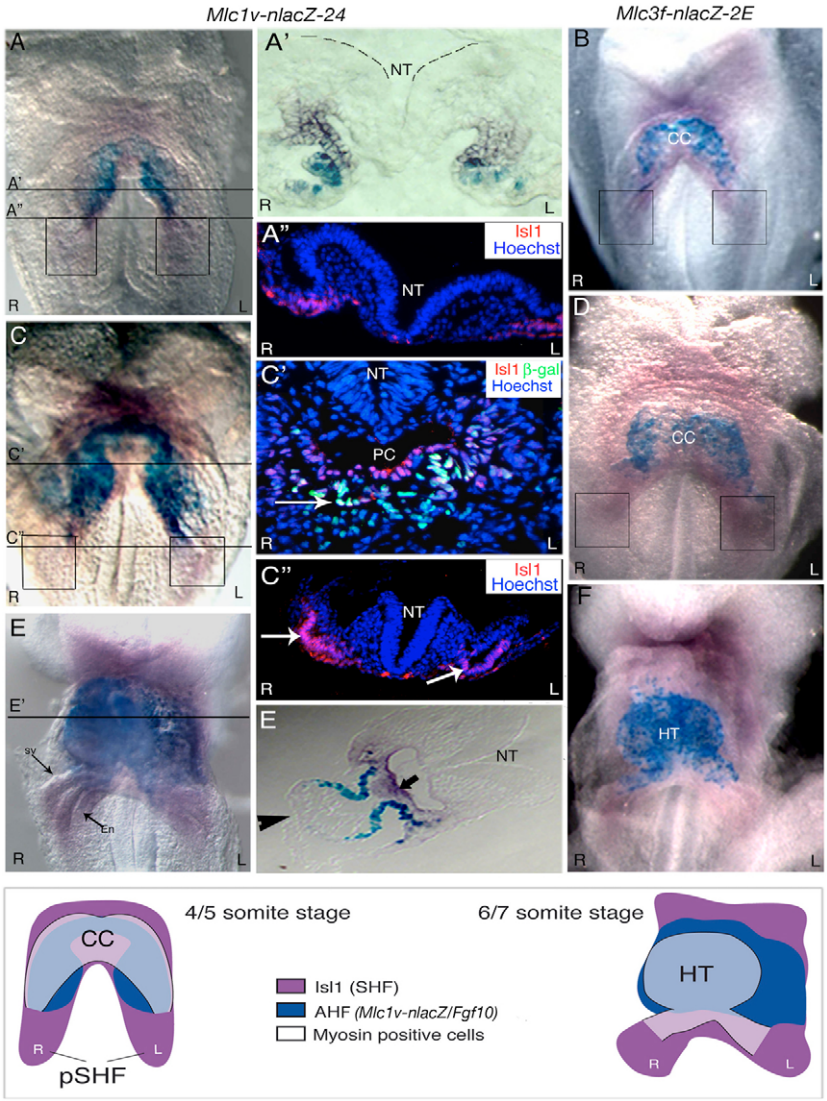

Fig. 1. X-Gal staining and *Islet1* in situ hybridisation or immunofluorescence in *Mlc1v-nlacZ-24* and *Mlc3f-nlacZ-2E* mouse embryos between the 4- and 7-somite stages. (**A-F**) X-Gal staining for β-gal (blue) and whole-mount in situ hybridisation for *Islet1* (*Isl1*) transcripts (purple) on *Mlc1v-nlacZ-24* (A,C,E) and *Mlc3f-nlacZ-2E* (B,D,F) embryos. β-gal activity in the *Mlc1v-nlacZ-24* line marks the AHF and its myocardial derivatives, whereas in the *Mlc3f-nlacZ-2E* line it marks differentiated myocardial cells. *Islet1* is transcribed in splanchnic mesoderm. Labelling by the *Islet1* probe, rostral to the heart-forming region, under the head folds, marks neurectoderm of the future central nervous system. *Islet1* is also expressed in endoderm. Embryos at the 4- (A,B), 6- (C,D) and 7- (E,F) somite stages. Lines in A,C,E indicate plane of sections in A′ and A″, C′ and C″, E′. (A″) Merge of Hoechst and Islet1 immunofluorescence. (C′,C″) Merge of Hoechst, β-gal and Islet1 immunofluorescence. The arrow in C′ points to a double-positive cell co-expressing *Mlc1v-nlacZ-24/Fgf10* and *Islet1*. PC, pharyngeal cavity. The arrows in C″ indicate the *Islet1*-positive mesoderm near the intraembryonic coelomic cavity. The arrows in E indicate the sinus venosus (sv) and the underlying endoderm (En). The arrowhead in E′ indicates the heart tube that is negative for β-gal, and the arrow points to the endoderm adjacent to the foregut, which is also positive for *Islet1* transcripts. CC, cardiac crescent; HT, heart tube; NT, neural tube. Black rectangles in A,B,C,D indicate the regions used for explants. L, left side of embryo; R, right side of embryo. Beneath is shown a schematic summary of expression of *Islet1* and the *Mlc1v-nlacZ-24* (*Fgf10*) transgene in the second heart field (SHF) and anterior heart field (AHF), respectively, and of myosin in cardiomyocytes of the cardiac crescent (CC) and heart tube (HT); p, posterior.

RESULTS

Identification of a posterior region of the second heart field where *Islet1*, but not *Fgf10*, is expressed

To examine the relative extents of the SHF and its rostral domain, the AHF, as defined by *Islet1* and *Fgf10* expression, we performed whole-mount in situ hybridisation for *Islet1* transcripts and X-Gal staining for β-gal activity on *Mlc1v-nlacZ-24* embryos, in which transgene expression is under the control of *Fgf10*, at the 4- to 7-somite stages, as the heart tube forms (Fig. 1A,C,E).

Islet1 transcripts and β-gal activity colocalised in most of the AHF, with more extensive *Islet1* expression in the pSHF. Notably, this was seen more caudally in β-gal-negative splanchnic mesoderm at the 4-somite stage (Fig. 1A-A″). At the 6-somite stage, *Islet1* transcripts were also present in the AHF and colocalised with most β-gal-positive cells (Fig. 1C,C′). However, the *Islet1* expression domain again also extended more caudally; for example, in cells (arrows in Fig. 1C″) near the intra-embryonic coelom. Strong *Islet1* expression at this and other stages was seen in neurectoderm under the head fold, prefiguring the role of *Islet1* in the central nervous system (Pfaff et al., 1996). At the 7-somite stage (Fig. 1E), *Islet1* expression continued to extend caudally beyond the sinus venosus in splanchnic mesoderm. β-gal activity was mainly concentrated behind, and rostral to, the forming heart tube (Fig. 1E′). The X-Gal staining extended into the myocardium at the arterial pole where the *Mlc1v-nlacZ-24* transgene is not transcribed, reflecting the stability of β-gal (Kelly et al., 2001). Most of the heart tube was negative for the staining (Fig. 1E′, arrowhead). *Islet1* transcripts overlapped with β-gal activity behind the tube in the AHF and also marked the endoderm adjacent to the foregut (Fig. 1E′, arrow). The location of the differentiated cardiomyocytes in relation to *Islet1* expression was examined at similar stages on whole-mount embryos of the *Mlc3f-nlacZ-2E* transgenic line in which β-gal activity marks differentiated cardiomyocytes expressing this myosin light chain gene (Fig. 1B,D,F). *Islet1* transcripts initially lay medially to the cardiomyocytes of the cardiac crescent (Fig. 1B,D) and also more caudally. Again, *Islet1* expression under the head fold, outside the cardiogenic area, was in neurectoderm. At the 7-somite stage (Fig. 1F), when the tube has fused, *Islet1* expression was still detectable behind the tube, as well as rostrally and caudally to it.

Myocardial potential of *Islet1*-expressing cells

We had previously shown that explants of the β-gal-positive region from the *Mlc1v-nlacZ-24* transgenic line, in which transgene expression marks the *Fgf10*-positive rostral domain of the SHF, the AHF, will give rise to differentiated cardiomyocytes after culture

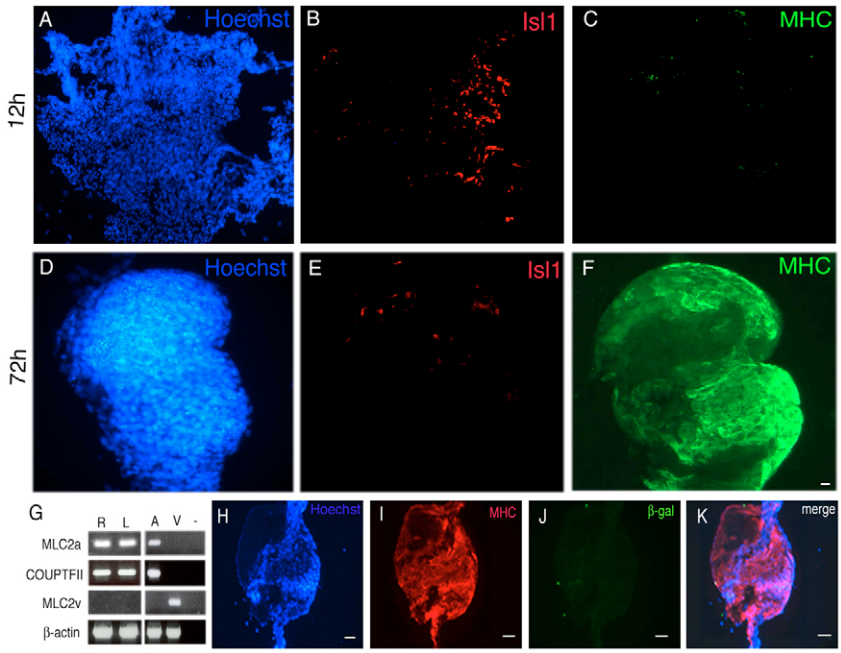

Fig. 2. Analysis of progenitor cell and myocardial markers in explants after 12 and 72 hours of culture. (**A-F**) Posterior (p) SHF explants at the 4- to 6-somite stages after 12 (A-C) and 72 (D-F) hours of culture. (A,D) Hoechst staining; (B,E) Islet1 (Isl1) immunofluorescence; (C,F) myosin heavy chain (MHC) immunofluorescence. The analysis has been repeated on five explants for each time point. (**G**) RT-PCR analysis on right (R) and left (L) explants (five pooled explants for each side) at the 4- to 6-somite stage after 72 hours of culture, with primers for *Mlc2a*, *COUP-TFII* (*Nr2f2*) and *Mlc2v* mRNAs. cDNA from the common atrium (A) or from the ventricular region (V) of five hearts from E9 embryos. Controls (–) contained RNA without reverse transcription. β-actin transcripts indicate similar quantities of RNA. (**H-K**) An explant of the pSHF from *Mlc1v-nlacZ-24* embryos at the 4- to 6-somite stage after 72 hours of culture. (H) Hoechst staining; (I) MHC immunofluorescence; (J) β-gal immunofluorescence; (K) merge of H,I,J. Scale bars: 10 μm in F for A-F; 30 μm in H-K.

(Zaffran et al., 2004). We now took explants from the caudal domain where *Islet1*, but not *Mlc1v-nlacZ-24*, is expressed, as indicated by rectangles in Fig. 1.

Initially, the 4- to 6-somite-stage explants were positive for Islet1 expression (Fig. 2B), but after 72 hours of culture we found that the number and intensity of Islet1-positive cells was reduced (Fig. 2E). We also tested myosin heavy chain expression in the explants after 12 hours of culture (Fig. 2C), but did not find myosin-positive cells, confirming that explants initially contained Islet1-expressing cells but not cardiomyocytes. After 72 hours of culture, explants were positive for myosin expression (Fig. 2F), whereas Islet1 was only weakly detectable, consistent with its expression in progenitor cells, not cardiomyocytes (Fig. 2E). These results demonstrate the myocardial potential of the caudal expression domain of *Islet1*, referred to as the posterior (p) SHF.

To check whether myocardial progenitors, present in the explants after 72 hours, gave rise to atrial or ventricular myocardial cells, we used RT-PCR to detect transcripts of atrial myosin light chain 2 (*Mlc2a*) or ventricular myosin light chain 2 (*Mlc2v*). In E8 embryos (6- to 7-somite stage), Mlc2a marks all myocardial cells, becoming restricted to the atria later, whereas Mlc2v is an early ventricular marker (Kubalak et al., 1994; O'Brien et al., 1993) and continues to be expressed only in the ventricles and temporally in the outflow tract throughout cardiac morphogenesis. Both right and left explants taken at the 4- to 6-somite stage, after 72 hours of culture, were positive for *Mlc2a* but not for *Mlc2v* (Fig. 2G), excluding the presence of ventricular-type progenitors in the explants. As another early atrial marker, we looked at transcripts of *COUP-TFII* (*Nr2f2*), which is upregulated during expansion of the common atrium by E9.0 (Pereira et al., 1999) and was also expressed in the cultured explants. We took the same posterior region from *Mlc1v-nlacZ-24* embryos at the 4- to 6-somite stage. Initially, these explants were β-gal-negative (results not shown). After 72 hours, pSHF explants from *Mlc1v-nlacZ-24* mice were positive for myosin heavy chain (Fig. 2I) but not for β-gal (Fig. 2J), confirming that myocardial progenitors, present in the explants, did not come from the AHF where this transgene is expressed, as the stable β-gal protein continues to mark the myocardial derivatives that had expressed *Mlc1v-nlacZ-24* (Kelly et al., 2001).

DiI injection in the pSHF of embryos at the 4- to 6-somite stage shows labelling of the common atrium

We next investigated the contribution of the pSHF by DiI injection in vivo, followed by embryo culture. DiI was injected into the left or right pSHF of embryos between the 4- and 6-somite stages. After 40 hours of culture (to the 20- to 25-somite stage), we analysed the embryos and found the labelling to be in the common atrium. After injection into the right pSHF, we found the labelling in the right common atrium (Fig. 3A,A′), and after injection into the left pSHF, the labelling was in the left common atrium (Fig. 3B,B′). Injections shown in Fig. 3A,B were at the 4-somite stage. A similar result was seen for 7-somite-stage embryos (Fig. 3C,C′). We never found labelling of the right or the left ventricle after dye injection in the pSHF and conclude that cells in this part of the SHF contribute to the atria and that this contribution from each side of the pSHF is apparently restricted to the corresponding side of the common atrium.

At the 4-somite stage, explants from both right and left pSHF show the same atrial myocardial potential

In *Mlc3f-nlacZ-2E* transgenic mice, β-gal is expressed mainly in the right atrium and left ventricle, with only a few β-gal-positive cells also present in the left atrium (Kelly et al., 1995). Thus, transgene expression is a marker for cardiac asymmetry in these transgenic mice. We took explants from the pSHF of *Mlc3f-nlacZ-2E* embryos at the 4-somite stage and cultured them for 72 hours, followed by analysis of expression of β-gal and myosin heavy chain. Based on RT-PCR analysis, there were no ventricular progenitors in the explants, so that *lacZ*- and myosin-positive cells represent atrial cardiomyocytes (see Fig. 2G). At the 4-somite stage, explants from both left and right pSHF gave rise to β-gal and myosin double-

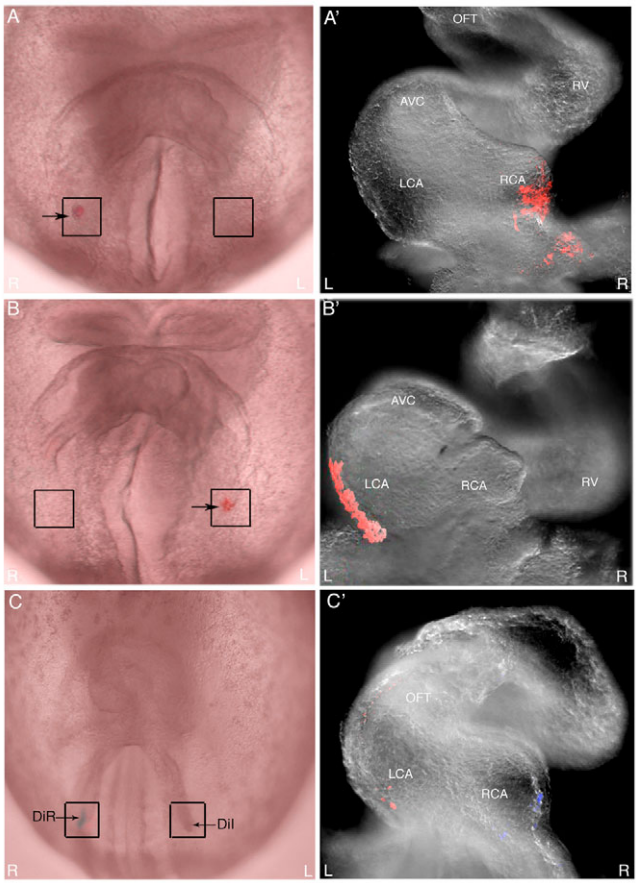

Fig. 3. DiI injection in the posterior SHF of embryos at the 4- to 6-somite stage. (**A**,**B**) DiI injection (at sites arrowed) in the right (A) and left (B) pSHF of mouse embryos at the 4-somite stage. The squares indicate the pSHF region (see also Fig. 1). (**A′**,**B′**) DiI labelling (red) in the same embryos after 40 hours of culture, focussing on the heart (dorsal view). (**C**) DiI (red) injection in the left side and DiR (blue) injection in the right side of an embryo at the 7-somite stage. (**C′**) The same embryo after 40 hours of culture (dorsal view). AVC, atrioventricular canal; LCA, left common atrium; OFT, outflow tract; RCA, right common atrium; RV, right ventricle.

positive cells (Fig. 4A-H). A quantitative analysis of atrial myocardial cells was performed both by counting β-gal and myosin double-positive cells (Fig. 4I) and by measuring the relative percentage of *lacZ* and *Mlc2a* transcripts with respect to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) transcripts in right and left explants (Fig. 4J).

At this stage (4 somites) we did not find any significant difference in the number of β-gal and myosin double-positive cells or in the proportion of *lacZ* and *Mlc2a* transcripts between right- and left-side explants.

Identification of left-right asymmetry in pSHF explants at the 6-somite stage

When explants from *Mlc3f-nlacZ-2E* embryos were taken at the 6-somite stage and cultured for 72 hours, we found a left-right difference in the number of β-gal and myosin double-positive cells, in contrast to the explants at the 4-somite stage. In left explants, the number of double-positive cells was less than in the right explants (compare Fig. 5B-D with Fig. 5F-H). In left explants, the percentage of double-positive cells was about 10%, whereas in the right explants it was about 30% (Fig. 5Q). This difference was confirmed by the relative abundance of *lacZ* and *Mlc2a* transcripts with respect to control *Gapdh* transcripts (Fig. 5R).

Similar experiments were performed with explants from the pSHF of *Mlc3f-nlacZ-9* embryos (Franco et al., 1997) (Fig. 5I-P). In these mice, the myocardial compartments of the heart are marked by β-gal, without any asymmetry. By double immunofluorescence (β-gal and myosin heavy chain) on explants after 72 hours of culture, we did not find any difference in the number of β-gal and myosin double-positive cells, providing a control for the asymmetry seen with the *Mlc3f-nlacZ-2E* mouse line (Fig. 5Q). We conclude from these experiments that the potential for expression of the *Mlc3f-nlacZ-2E* transgene, which marks the right atrium, is acquired by myocardial progenitor cells in the pSHF at the 5- to 6-somite stage.

The effect of *Pitx2c* adenoviral infection on left-right asymmetry in *Mlc3f-nlacZ-2E* explants

With the aim of manipulating left-right asymmetry in the explants, we examined the effect of ectopic Pitx2c expression because this isoform of *Pitx2* has been implicated in asymmetric development of the heart (Liu et al., 2001). Asymmetric expression of *Pitx2*, which is due to the *Pitx2c* isoform (Schweickert et al., 2000), is seen in the left SHF, including the caudal region at the 6-somite stage (see Fig. S1 in the supplementary material).

We used an adenoviral vector expressing *Pitx2c* and *GFP* (Fig. 6I-P), with an adenoviral vector expressing *GFP* alone as a control (Fig. 6A-H), to infect left and right explants from *Mlc3f-nlacZ-2E* embryos at the 5-somite stage. We chose to make explants from 5-somite embryos because this is the critical stage when *Pitx2c* is being activated. In these explants, transcripts were not detectable initially, as in vivo, but were present in left explants after 72 hours of culture (see Fig. S2 in the supplementary material). Whereas infection with the *GFP*-expressing adenovirus did not alter the left-right difference in the number of β-gal-positive cells (compare Fig. 6D with 6H), after infection with the *Pitx2c/GFP* adenovirus we found only a few β-gal-positive cells in explants from both sides (Fig. 6L,P), suggesting that Pitx2c represses the expression of the transgene. A quantitative analysis, counting the percentage of β-gal-positive cells in the total cell population, showed that there is a reduction (by about 50% in left and by about 80% in right explants) in the number of transgene-expressing cells (Fig. 6Q). By qRT-PCR for *lacZ* transcripts, we observed a similar decrease in the amount of *lacZ* mRNA in right explants infected with *Pitx2c/GFP* adenovirus, whereas infection with the *GFP* control adenovirus did not produce this effect (Fig. 6R, compare the columns indicated by the arrowheads). Reduction of the number of β-gal-positive cells could not be explained by a toxic effect of the adenovirus, because all the explants were 'beating' after 3 days of infection, and myosin expression, as detected by immunofluorescence, was comparable to controls (data not shown). Moreover, the relative abundance of *Mlc2a* transcripts was maintained, suggesting that the adenovirus effect was related to asymmetric transgene expression. The GFP infection level was measured by qRT-PCR and it was comparable (60-70%) both after infection with *GFP* (control) and *Pitx2c/GFP* (Fig. 6R). Ectopic Pitx2c therefore exerts a repressive effect on the expression of the transgene in explants from the right-hand side of the pSHF, indicating that endogenous Pitx2c in left explants reduces *Mlc3f-nlacZ-2E* transcription. In keeping with this, explants from the left and right pSHF of *Pitx2c*$^{-/-}$ mutant

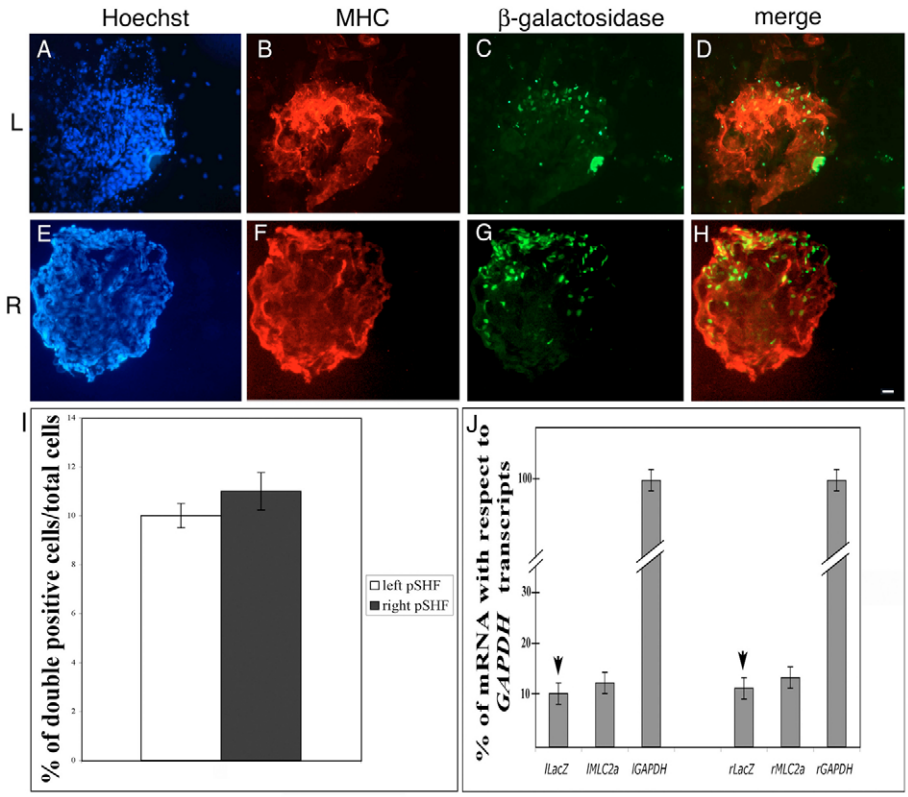

Fig. 4. Atrial progenitors are present in the posterior SHF on right and left sides of *Mlc3f-nlacZ-2E* embryos at the 4-somite stage. (**A-H**) Left (L) (A-D) and right (R) (E-H) explants of the pSHF from *Mlc3f-nlacZ-2E* mouse embryos at the 4-somite stage, after 72 hours of culture. (A,E) Hoechst staining; (B,F) myosin heavy chain (MHC) immunofluorescence; (C,G) β-gal immunofluorescence. (D) Merge of B and C; (H) merge of F and G. Scale bar: 10 μm in H for A-H. (**I**) Quantitative analysis of β-gal and MHC double-positive cells, as a percentage of the total number of cells, in 30 explants from each side of the pSHF. White, percentage in left explants; black, percentage in right explants. (**J**) qRT-PCR analysis on ten pooled explants from left (l) and right (r) sides. *lacZ* (arrowheads) and *Mlc2a* transcript abundance is expressed as a percentage of that of *Gapdh* transcripts.

embryos, at the 6-somite stage, showed an increased proportion of β-gal and myosin double-positive cells in the left explants after culture, demonstrating the loss of left-right asymmetry in *Mlc3f-nlacZ-2E* transgene expression in the absence of Pitx2c (see Fig. S3 in the supplementary material).

Since Pitx2c may affect cell proliferation (Nowotschin et al., 2006; Kioussi et al., 2002), we examined this possibility in left and right posterior domains of the SHF in wild-type embryos at the 5-, 6- and 7-somite stages, using an antibody to phosphorylated histone H3 (PHH3), which is a marker of mitosis (Cimini et al., 2003). Myocardial progenitors (and endoderm) were identified with an Islet1 antibody. We could not detect any significant differences in the mitotic frequency of Islet1-positive cells in serial sections (Fig. 7A). We also examined differentiating cells (Islet1-negative) within the right and left sinus venosus, identified on the basis of morphology, both in wild-type and in *Pitx2c*$^{-/-}$ mutant embryos (Fig. 7B-E). In the former there were 30% more mitotic cells in the right than left sinus venosus, indicating that this side of the forming atrium is more proliferative (Fig. 7A′). In the mutant, we observed the reverse result, with more mitotic cells in the left sinus venosus. This suggests that Pitx2c, which is expressed in the left sinus venosus, has a negative effect on the proliferation of differentiating cardiomyocytes. In this context, we had observed that explants from the left sinus venosus region of *Mlc3f-nlacZ-2E* embryos at the 7-somite stage tend to be smaller than right explants, after 48 hours of culture (see Fig. S4 in the supplementary material). They also had relatively fewer β-gal-positive cells, consistent with the repression of the transgene by Pitx2c expressed in the left SHF and left sinus venosus. Increased proliferation in the absence of Pitx2c was also seen in left explants of the pSHF after 72 hours of culture, when differentiated cardiomyocytes are present (Fig. 7F-J). Again, in the explant situation, proliferation was higher (Fig. 7J) and the proportion of cells in the explant expressing the transgenic marker was increased (see Fig. S3 in the supplementary material), indicating the repressive effect of Pitx2c on these two phenomena when it is present in left pSHF explants.

DISCUSSION

We have shown that the caudal domain of splanchnic mesoderm where *Islet1*, but not *Fgf10*, is expressed, forms atrial myocardium. Furthermore, left and right sides of this part of the SHF contribute to the left or right common atrium, respectively. Cardiac progenitor cells acquire left-right identity at the time when Pitx2c begins to be expressed in the left side of the field; Pitx2c represses the expression of a transgenic marker that marks right atrial myocardium. Pitx2c is also associated with reduced myocardial cell proliferation, consistent with the smaller size of the left atrium.

DiI labelling and explant experiments now demonstrate that the pSHF contains atrial and not ventricular myocardial progenitors. Indeed, we had previously shown that left ventricular cells are already present when the early heart tube fuses, indicating that they arise from the cardiac crescent, described as the first heart field (Buckingham et al., 2005), and that the AHF gives rise to right ventricular myocardium and outflow tract (Zaffran et al., 2004). Our retrospective clonal analysis had indicated a first- and second-lineage contribution to both the right ventricle and atria (Meilhac et al., 2004a). It is not clear to what extent the first lineage is represented by the cardiac crescent and the early heart tube. However, the rostral-most part of the early heart tube has right ventricular identity (Zaffran et al., 2004) and the caudal-most tube/crescent might similarly contribute to the atria although, unlike in the chick embryo (Yutzey et al., 1994; Patwardhan et al., 2000), atrial-specific markers are only detected later. The atrial

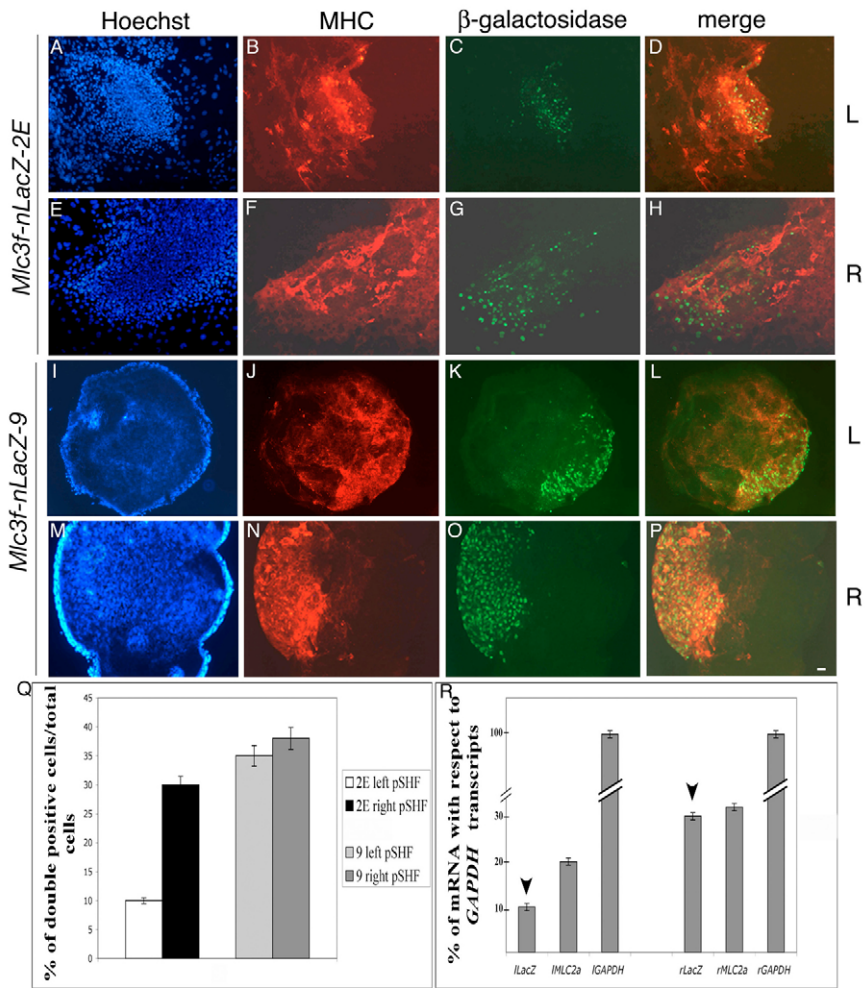

Fig. 5. Left-right asymmetry of posterior SHF explants from *Mlc3f-nlacZ-2E* embryos at the 6-somite stage. (**A-H**) Left (L) (A-D) and right (R) (E-H) explants from the pSHF of *Mlc3f-nlacZ-2E* mouse embryos at the 6-somite stage: (A,E) Hoechst staining; (B,F) myosin heavy chain (MHC) immunofluorescence; (C,G) β-gal immunofluorescence. (D) Merge of B and C; (H) merge of F and G. (**I-P**) Left (I-L) and right (M-P) explants from *Mlc3f-nlacZ*-9 embryos at the 6-somite stage. (I,M) Hoechst staining; (J,N) MHC immunofluorescence; (K,O) β-gal immunofluorescence. (L) Merge of J and K; (P) merge of N and O. Scale bar: 10 μm in P for A-P. (**Q**) The number of β-gal and MHC double-positive cells as a percentage of the total number of cells. White and black columns show the percentage in left and right pSHF explants, respectively, from *Mlc3f-nlacZ-2E* (2E) embryos at the 6-somite stage (50 explants from each side) ($P \leqslant 0.03$). Grey and dark-grey columns show the percentage in left and right explants, respectively, from *Mlc3f-nlacZ-9* (9) embryos at the same stage (25 explants for each side). (**R**) qRT-PCR on ten pooled left (l) or right (r) explants of pSHF from *Mlc3f-nlacZ-2E* embryos at the 6-somite stage after 72 hours of culture. *lacZ* (arrowheads) and *Mlc2a* transcript abundance is expressed as a percentage of that of *Gapdh* transcripts.

contribution of the Islet1-positive region of the SHF is consistent with the fate of Islet1-expressing cells, which have been demonstrated to contribute to the venous as well as the arterial pole of the heart in *Islet1-Cre/Rosa26* tracing experiments (Cai et al., 2003). The absence of *Fgf10* expression in cells with atrial potential is consistent with observations of β-gal labelling of myocardium by the *Mlc3f-nlacZ-2E* transgene, which is under *Fgf10* control and does not label the venous pole (Kelly et al., 2001). It is striking that not only myocardium, but also differentiated cells with specific chamber identity, are formed in cultured explants from different regions of the SHF. In the experiments described here, atrial identity was indicated by the expression of endogenous genes as well as of the *Mlc3f-nlacZ-2E* transgene, which is not expressed in right ventricular and outflow tract myocardium that also derive from the SHF (Zaffran et al., 2004). The formation of myocardium requires signalling molecules derived from endoderm (Harvey, 1999), present in the explants. However, whereas signals required to induce myocardial differentiation, such as Bmp4 and Fgfs, have been identified, it is not clear what signals promote atrial versus ventricular myocardium. In the SHF, genes are not uniformly expressed and atrial specification probably depends on combinations of regulatory factors peculiar to the pSHF (Buckingham et al., 2005). An example of regionalised transcriptional specificity is provided by the Tbx18-positive cells, which are mainly Islet1- and Nkx2.5-negative, unlike most of the SHF. These cells contribute to the myocardium of the sinus horns, which form the base of the venous inlet of the heart at later developmental stages, after the atria have formed (Christoffels et al., 2006).

Although we have not fully fate mapped the SHF, in this study we found that myocardial progenitors from the left or right sides of the pSHF contribute to the corresponding side of the common atrium, indicating that most cells do not migrate or mix either within the forming heart or across the SHF. This is consistent with our observations on the clustering of clonally related cells in outflow tract myocardium (Bajolle et al., 2008) or within the atria (Meilhac et al., 2004b). In the chick embryo (see Kirby, 2007), it has been shown that ventricular myocardium is derived from both left and right heart fields and our observations on the left ventricular fate of cells in the mouse cardiac crescent also show bilateral contribution to this chamber (Zaffran et al., 2004). By contrast, our observations on the left-right nature of atrial progenitors show that this identity is already prefigured, prior to formation of this part of the heart. This is not in accordance with the suggestion that both left and right atria are represented in both hearts in cardia bifida (Li et al., 2004). However, without a molecular marker it is not possible to distinguish between left-right atrial identity at these early stages, prior to morphological asymmetry.

In our study, left-right atrial identity was followed with the *Mlc3f-nlacZ-2E* transgene, which preferentially marks the right and not the left atrium and begins to show asymmetric expression in the right

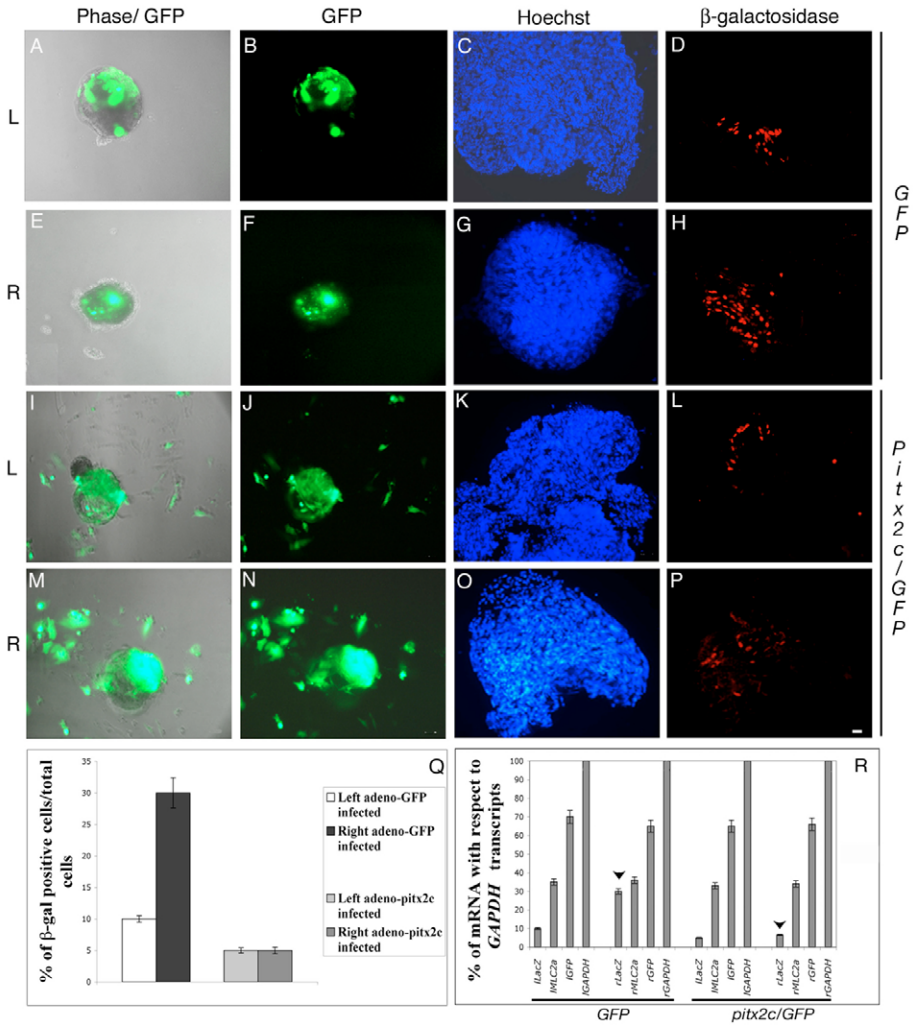

Fig. 6. Forced Pitx2c expression in posterior SHF explants from *Mlc3f-nlacZ-2E* embryos at the 5-somite stage. (**A-H**) Control *GFP* adenovirus infection on left (L) (A-D) and right (R) (E-H) explants from the pSHF of *Mlc3f-nlacZ-2E* mouse embryos. (A,E) Merge of phase-contrast and GFP fluorescence on the live explants; (B,F) GFP fluorescence on the live explants; (C,G) Hoechst staining; (D,H) β-gal immunofluorescence on explants now spread under a coverslip. (**I-P**) *Pitx2c/GFP* adenovirus infection on left (L) (I-L) and right (R) (M-P) explants of the pSHF from *Mlc3f-nlacZ-2E* embryos. (I,M) Merge of phase-contrast and GFP fluorescence on live explants; (J,N) GFP fluorescence on the live explants; (K,O) Hoechst staining; (L,P) β-gal immunofluorescence on explants now spread under a coverslip. Scale bar: 10 μm in P for A-P. (**Q**) Quantitative analysis of the percentage of β-gal-positive cells in the total cell population, after infection with the control *GFP* adenovirus (white and black; $P \leqslant 0.025$) or with the *Pitx2c/GFP*-expressing adenovirus (grey and dark grey). The *P*-value for left-GFP compared with left-Pitx2c is $\leqslant 0.01$ and for right GFP compared with right Pitx2c is $\leqslant 0.005$. White and grey, left explants; black and dark grey, right explants. The quantification was performed on ten explants for each side and for each adenovirus. (**R**) qRT-PCR analysis after *GFP* and *Pitx2c/GFP* infection on five pooled left explants (l) and five pooled right explants (r) after 72 hours of culture for each adenovirus. *lacZ* (arrowheads point to the difference in *rlacZ*), *GFP* and *Mlc2a* transcript abundance is expressed as a percentage of that of *Gapdh* transcripts.

sinus venosus from about E8.5 (Franco et al., 2001). From the 6-somite-stage, explants of left or right pSHF show asymmetric expression of the transgene in myosin-positive cells after culture. This acquisition of right versus left atrial identity correlates with the appearance of *Pitx2c* transcripts in the left pSHF. *Pitx2c* transcripts are not detectable in these explants at the 4-somite stage, whereas they are present in left explants by the 6- to 7-somite stage. In the intervening period, 5-somite explants are initially *Pitx2c*-negative, but during the culture period *Pitx2c* transcripts become detectable in left explants, indicating that nodal signalling from left lateral mesoderm (Raya and Belmonte, 2006) has implemented activation of the gene.

Pitx2c represses *Mlc3f-nlacZ-2E* transgene expression and causality is demonstrated by manipulation of Pitx2c levels. Right explant expression is reduced to similar levels to that in left explants, and indeed these levels tend to be slightly lower than normal when Pitx2c is ectopically expressed. Conversely, in *Pitx2c*$^{-/-}$ mutant embryos, the transgene is expressed at a wild-type level in right explants and at the same level in left explants. This repressive effect may be direct or indirect. In vitro experiments have indicated that Pitx2c can act as a transcriptional activator (Ganga et al., 2003). However, we have also manipulated a dominant-negative form of Pitx2c in which the DNA-binding domain is fused to the Engrailed repression domain, and obtained similar results to those described here with native Pitx2c (results not shown). There are no obvious Pitx2c target sequences on the 2 kb of 5′ flanking sequence that control the right atrial expression of the *Mlc3f-nlacZ-2E* transgene (D.G. and M.E.B., unpublished), so the effect might be indirect. In the *Pitx2c* mutant, right atrial isomerism is observed, reflected in *Mlc3f-nlacZ-2E* expression (Franco et al., 2001), consistent with the loss of repression of right atrial identity in the left atrial myocardium and its progenitors. Recently, Mommersteeg et al. (Mommersteeg et al., 2007) have proposed a role for Pitx2c in the suppression of sinoatrial node formation, which is generated as a default pathway on the right side.

Pitx2 isoforms have been associated with inhibiting (Wei and Adelstein, 2002) or promoting cell proliferation depending on the cell type (Kioussi et al., 2002). In the heart, *Pitx2* mutants showed reduced proliferation in the proximal outflow tract (Ai et al., 2006) and a proliferative role for Tbx1 through activation of *Pitx2c* has been suggested in the SHF (Nowotschin et al., 2006). Labelling by PHH3 marks cells in mitosis and the high percentage of positive cells ($\leqslant 27\%$) that we observed is consistent with very high proliferative rates in the pSHF and caudal region of the heart tube (Soufan et al., 2006). We did not detect a significant difference in mitotic cells between right and left sides of the SHF at the 6-somite stage when Pitx2c is expressed. The absence of an effect on

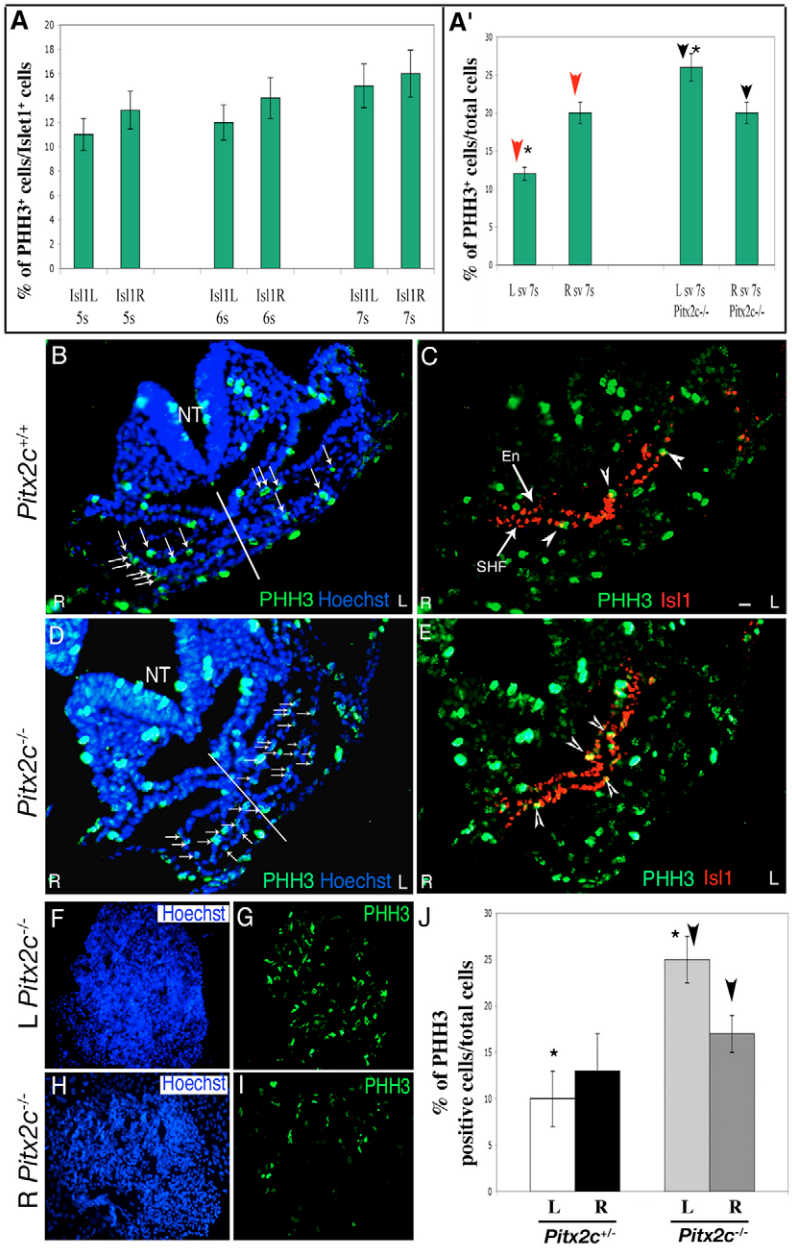

Fig. 7. Analysis of phosphohistone H3 and Islet1 double-positive cells in mouse embryos at the 5- to 7-somite stage, in left and right horns of the sinus venosus of embryos at the 7-somite stage and in 6-somite-stage posterior SHF explants. (**A**) Quantitative analysis of the percentage of proliferating cells in right (R) and left (L) sides of the SHF with respect to the total number of Islet1 (Isl1)-positive cells in this mesoderm at the 5-, 6- and 7-somite stages (s). (**A′**) Percentage of proliferating cells with respect to the total number of cells in the right and left sides of the sinus venosus (sv) at the 7-somite stage both for wild-type and for *Pitx2c*$^{-/-}$ embryos. The values are calculated from ten sections, with two embryos analysed for each stage. Red arrowheads, $P \leqslant 0.03$; black arrowheads, $P \leqslant 0.025$; asterisks, $P \leqslant 0.01$. (**B-E**) Examples of immunofluorescence analysis on a section of a wild-type (B,C) and a *Pitx2c*$^{-/-}$ (D,E) embryo at the 7-somite stage. (B,D) Merge of phosphohistone H3 (PHH3) and Hoechst staining on a wild-type (B) and on a *Pitx2c*$^{-/-}$ (D) embryo section (the arrows indicate PHH3-positive cells within the sinus venosus). NT, neural tube. (C,E) Merge of PHH3 and Islet1 immunofluorescence (the same sections as shown in B and D). The arrowheads indicate Islet1 and PHH3 double-positive cells. En, endoderm; SHF, second heart field. The white lines in B and D indicate the separation of the right (R) and left (L) sides of the sinus venosus. (**F-I**) Explants of the left (F,G) and right (H,I) pSHF from a *Pitx2c*$^{-/-}$ embryo after 72 hours of culture. (F,H) Hoechst staining; (G,I) PHH3 immunofluorescence. (**J**) Quantitative analysis of PHH3-positive cells as a percentage of the total number of cells in ten explants from left (L) and right (R) sides of *Pitx2c*$^{+/-}$ and *Pitx2c*$^{-/-}$ embryos. White, percentage in left *Pitx2c*$^{+/-}$ explants; black, percentage in right *Pitx2c*$^{+/-}$ explants; grey, percentage in left *Pitx2c*$^{-/-}$ explants; dark grey, percentage in right *Pitx2c*$^{-/-}$ explants. Arrowheads, $P \leqslant 0.03$; asterisks, $P \leqslant 0.02$.

progenitor cell proliferation is also suggested by the fact that *Islet1* expression is unchanged in the *Pitx2* mutant (Ai et al., 2006). However, we observed 30% more mitotic cells in the right than left sinus venosus. This is consistent with the larger size of explants from this part of the posterior heart tube. Moreover, in the *Pitx2c*$^{-/-}$ mutant, we observed a higher number of mitotic cells in the left sinus venosus. Therefore, a negative effect of Pitx2c on early myocardial cell proliferation might be responsible for the smaller size of the left atrium as compared with the right atrium, where pectinate trabeculation is more pronounced in the presence of Pitx2c (Liu et al., 2001). This is also indicated by findings in the chick embryo, in which the left lateral free wall of the common atrium showed a lower rate of proliferation than the right side (Thompson et al., 1990).

Interestingly, left explants of the pSHF from *Pitx2c*$^{-/-}$ mutant embryos show a significant increase in the number of mitotic cells after culture compared with the right explants. This would suggest that Pitx2c represses proliferation in differentiating explants.

The number of mitotic cells in the left sinus venosus or in cultured left pSHF explants from *Pitx2c*$^{-/-}$ embryos exceeds that on the right side. This is in keeping with preliminary observations on the left cardiac crescent before Pitx2c is expressed, where more dividing cells are present (D. Bellomo and N.A.B., unpublished). It is possible that a second left-right signalling pathway is operating to antagonise that mediated by Pitx2c and this is revealed when Pitx2c is absent. Indeed, the directionality of cardiac looping cannot be explained by the action of the Pitx2c pathway (Liu et al., 2001).

It is important to note that effects on the number of cardiomyocytes expressing the *Mlc3f-nlacZ-2E* transgene, which normally marks right atrial myocardium, are not due to differences in cell number because the results are expressed relative to the number of cells, or to *Gapdh* transcripts in the explants in the case of the gain-of-function experiments. Pitx2c therefore modulates the acquisition of atrial identity, exerting a repressive effect on the right

'default' pathway in progenitor cells from the left side of the pSHF. In addition, lower proliferation in the left sinus venosus and in cultured explants is due to Pitx2c expression, showing that it has an additional repressive effect on left atrial growth.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/6/1157/DC1

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