

# sonic hedgehog is required in pulmonary endoderm for atrial septation

Andrew D. Hoffmann<sup>1</sup>, Michael A. Peterson<sup>1</sup>, Joshua M. Friedland-Little<sup>1</sup>, Stuart A. Anderson<sup>2</sup> and Ivan P. Moskowitz<sup>1,\*</sup>

The genesis of the septal structures of the mammalian heart is central to understanding the ontogeny of congenital heart disease and the evolution of cardiac organogenesis. We found that Hedgehog (Hh) signaling marked a subset of cardiac progenitors specific to the atrial septum and the pulmonary trunk in the mouse. Using genetic inducible fate mapping with *Gli1*<sup>CreERT2</sup>, we marked Hh-receiving progenitors in anterior and posterior second heart field splanchnic mesoderm between E8 and E10. In the inflow tract, Hh-receiving progenitors migrated from the posterior second heart field through the dorsal mesocardium to form the atrial septum, including both the primary atrial septum and dorsal mesenchymal protrusion (DMP). In the outflow tract, Hh-receiving progenitors migrated from the anterior second heart field to populate the pulmonary trunk. Abrogation of Hh signaling during atrial septal progenitor specification resulted in atrial and atrioventricular septal defects and hypoplasia of the developing DMP. Hedgehog signaling appeared necessary and sufficient for atrial septal progenitor fate: Hh-receiving cells rendered unresponsive to the Hh ligand migrated into the atrium in normal numbers but populated the atrial free wall rather than the atrial septum. Conversely, constitutive activation of Hh signaling caused inappropriate enlargement of the atrial septum. The close proximity of posterior second heart field cardiac progenitors to pulmonary endoderm suggested a pulmonary source for the Hh ligand. We found that *Shh* is required in the pulmonary endoderm for atrial septation. Therefore, Hh signaling from distinct pulmonary and pharyngeal endoderm is required for inflow and outflow septation, respectively. These data suggest a model in which respiratory endoderm patterns the morphogenesis of cardiac structural components required for efficient cardiopulmonary circulation.

**KEY WORDS:** Hedgehog, Heart, Organogenesis, Cardiac progenitor, Second heart field, Atrial septum, Mouse

## INTRODUCTION

The comprehensive description of cardiac organogenesis must elucidate the sequential steps required for the specialization of the cardiac progenitor cell lineage into the distinct components that form the final organ. Considerable effort is ongoing to define the factors required for specification of cardiac progenitors from unspecified mesoderm within the early embryo. FGF, BMP, Wnt and Hedgehog (Hh) signaling pathways have all recently been implicated in the specification of early embryonic cardiac progenitor fate (Solloway and Robertson, 1999; Reifers et al., 2000; Klaus et al., 2007; Ueno et al., 2007; Thomas et al., 2008). However, the subsequent steps linking cardiac progenitor specification to cardiac morphogenesis are less well described. The degree to which cardiac progenitors are subspecified to unique lineages, the cues that specify fates among cardiac precursor cells, and whether lineage specialization occurs in progenitors or later during formal cardiac morphogenesis remain open questions. Here we investigate the relationship between cardiac progenitor subspecification and atrial septum morphogenesis.

The cellular origin of the atrial septal structures is of considerable clinical, developmental and evolutionary interest. The morphogenesis of the atrial septum occurs in the mouse between E10 and E13 (reviewed by Anderson et al., 2003). This process includes the coordinated development of two distinct physical septa, the primary atrial septum (PAS) and the dorsal mesenchymal protrusion (DMP; also known as the spina vestibuli or vestibular

spine) (Snarr et al., 2007a). The fusion of the mesenchymal cap of the PAS, the DMP and mesenchyme of the atrioventricular canal endocardial cushions closes the primary atrial ostium. Recent studies have highlighted a requirement for the DMP in atrioventricular septation (Tasaka et al., 1996; Webb et al., 1998; Wessels et al., 2000; Kim et al., 2001; Blom et al., 2003; Mommersteeg et al., 2006; Snarr et al., 2007a; Goddeeris et al., 2008), and an extracardiac origin of the DMP from the posterior second heart field (SHF) has also been inferred (Mommersteeg et al., 2006; Snarr et al., 2007b; Goddeeris et al., 2008).

Atrial septal defects (ASDs) are a common class of congenital heart defect in humans (Hoffman, 1995). Several cardiogenic transcription factors have been implicated in human ASDs; haploinsufficiency of *Gata4*, *Nkx2-5* or *Tbx5* causes human and murine ASDs (Lyons et al., 1995; Basson et al., 1997; Kuo et al., 1997; Li et al., 1997; Schott et al., 1998; Bruneau et al., 2001; Garg et al., 2003). Each is expressed in the atria during atrial septation (Molkentin et al., 1997; Kasahara et al., 1998; Bruneau et al., 1999), engendering a paradigm for atrial septation in which these transcription factors are involved in establishing intracardiac positional information during atrial septum morphogenesis (Bruneau, 2002). Recent studies have also demonstrated a role for Hh signaling in atrial and outflow tract septation within the SHF (Jacob and Lum, 2007; Washington Smoak et al., 2005; Lin et al., 2006; Goddeeris et al., 2007; Goddeeris et al., 2008).

Here we report the identification of Hh-induced atrial septum and pulmonary trunk progenitors in the SHF. Genetic inducible fate mapping (Joyner and Zervas, 2006) demonstrates that Hh-receiving cells generate both the primary atrial septum and the DMP. Hh signaling marks atrial septal progenitors between E8 and E10, several days prior to atrial septum morphogenesis. Hh signaling also marks pulmonary trunk progenitors to a greater degree than aortic progenitors during this period. Marking of atrial septum progenitors

<sup>1</sup>Departments of Pediatrics and Pathology, University of Chicago, Chicago, IL 60637, USA. <sup>2</sup>Department of Psychiatry, Weill Medical College of Cornell University, New York, NY 10065, USA.

\*Author for correspondence (e-mail: imoskowitz@uchicago.edu)

by Hh signaling in the second heart field, their migration into the atria and their participation in atrial septum morphogenesis is demonstrated. Removal of Hh responsiveness during atrial septum progenitor specification results in both atrial and atrioventricular septal defects. Loss- and gain-of-function studies suggest that Hh signaling acts to specify atrial septum from non-septum atrial progenitors. Removal of *Shh* from pulmonary endoderm causes atrioventricular septal defects, implicating the lung as the source of the Hh signal required for atrial septation. These observations have implications for the pathogenesis of atrial septal defects and for the evolution of cardiopulmonary circulation.

## MATERIALS AND METHODS

### Mouse lines

The *Gli1<sup>CreERT2</sup>* line was obtained from the Joyner laboratory (Sloan Kettering Institute, New York, NY, USA). The *Nkx2-1<sup>Cre</sup>* line was obtained from the Anderson laboratory (Cornell Medical Center, New York, NY, USA). *R26R* [*Gt(ROSA)26Sor<sup>tm1Sor</sup>*], *Patched1-lacZ* (B6;129-*Ptch1<sup>tm1Mps</sup>/J*) and conditional knockout smoothened (*Smo<sup>fl</sup>*, also known as *Smo<sup>tm2Ame</sup>*) mice were obtained from The Jackson Laboratory and genotyping was performed as described (www.jax.org). All mouse experiments were performed in a mixed B6/129/SvEv background. All experiments involving mice were carried out according to a protocol reviewed and approved by the Institutional Animal Care and Use Committee of the University of Chicago, in compliance with the USA Public Health Service Policy on Humane Care and Use of Laboratory Animals.

### Tamoxifen administration

Activation of *CreERT2* was accomplished by oral gavage with 2 mg Tamoxifen (TM) per dose in corn oil to pregnant dams. Dose titration was performed to achieve optimal activation of *CreERT2* without toxicity, as measured by embryonic size and viability (data not shown). As *CreERT2* activation by TM induction is mosaic (Ahn and Joyner, 2004a; Ahn and Joyner, 2004b), serial section analysis of six hearts from each time point was used for analysis of *R26R<sup>Gli1-CreERT2</sup>* embryos.

### Embryo dissection and X-gal staining

Embryos were dissected from maternal tissue samples and tail samples were taken for genotyping. Embryos were fixed for 1 hour in 4% paraformaldehyde and stained with X-gal staining solution (5 mM  $K_3Fe(CN)_6$ , 5 mM  $K_4Fe(CN)_6$ , 2 mM  $MgCl_2$ , 0.02% NP-40, 0.01% deoxycholate, 0.1% X-gal in PBS) when appropriate or fixed in 10% neutral buffered formalin. Embryos older than E10.5 were dissected prior to staining. If X-gal stain was present, slides were counterstained with 50% Eosin for 1 second. If no X-gal stain was present, the slides were stained with Hematoxylin and Eosin.

### In situ hybridization

In situ hybridization was performed using digoxigenin-labeled probes. The protocol was as described in Biris et al. (Biris et al., 2007) with the following changes: embryos were washed for 1 hour six times in maelic acid buffer (MAB; 0.1 M maelic acid pH 7.5, 0.15 M NaCl, 0.1% Tween-20 and 0.002 M levamisole) followed by a 16 hour overnight wash at room temperature. Color reactions were allowed to develop overnight and images were taken prior to storage in 80% glycerol. Probes for *Gli1* and *Shh* were a kind gift from Dr Elizabeth Grove (University of Chicago, Chicago, IL, USA).

### RT-PCR

RNA was isolated from three embryos per genotype and extracted using Trizol (Invitrogen). RT-PCR was performed using a OneStep RT-PCR Kit (Qiagen). Reactions were hot-started at 50°C for 30 minutes for reverse transcription and heated to 95°C for 15 minutes; then cycled from 95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute for 20 cycles; followed by a final extension for 10 minutes at 72°C. Primer sequences were: *Gli1* forward, TGCCTATAGCCAGTGTCTC; *Gli1* reverse, CATCTGCT-TGGGGTTCCTTA;  $\beta$ -actin forward, TAAGGCCAACCGTGAA-AAGATGAC;  $\beta$ -actin reverse, ACCGCTCGTTGCCAATAGTGATG.

### Cell death

Whole-mount cell death analysis was performed using the vital lysosomal dye LysoTracker Red (Invitrogen), previously shown to be an accurate marker of cell death (Zucker et al., 1999; Abu-Issa et al., 2002; Goddeeris et al., 2007). Embryos were imaged with an IX70 Olympus Fluoview 200 laser scanning confocal microscope with a 10× (NA 0.3) dry objective. Images were prepared with ImageJ software (NIH) and Adobe Photoshop 10.0.1.

### Cell proliferation

Pregnant mice at E9.5 and E10.5 were given intraperitoneal injections of 150  $\mu$ l BrdU solution (Zymed, 00-0103) 5 and 2.5 hours prior to embryo harvesting. After embryo dissection and sectioning was performed as described above, proliferation was assessed by marking BrdU-labeled cells using a kit from Zymed (93-3943).

### Statistical methods

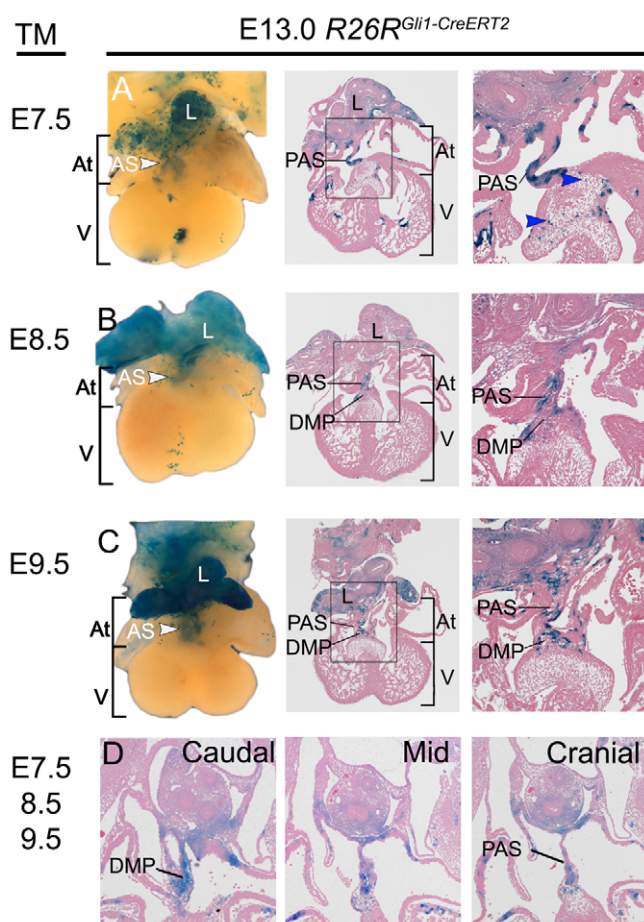
$\beta$ -Galactosidase positive cells were counted on all sections with a DMP in WT specimens and *R26R;Smo<sup>Gli1-CreERT2</sup>* specimens. Cells were classified as either having atrial free wall or DMP location and were counted, and their sum was used as the total number of atrial  $\beta$ -galactosidase positive cells. Mean numbers of  $\beta$ -galactosidase positive cells were compared using the Student's *t*-test.

## RESULTS

### Hh signaling marks atrial septum and pulmonary trunk progenitors

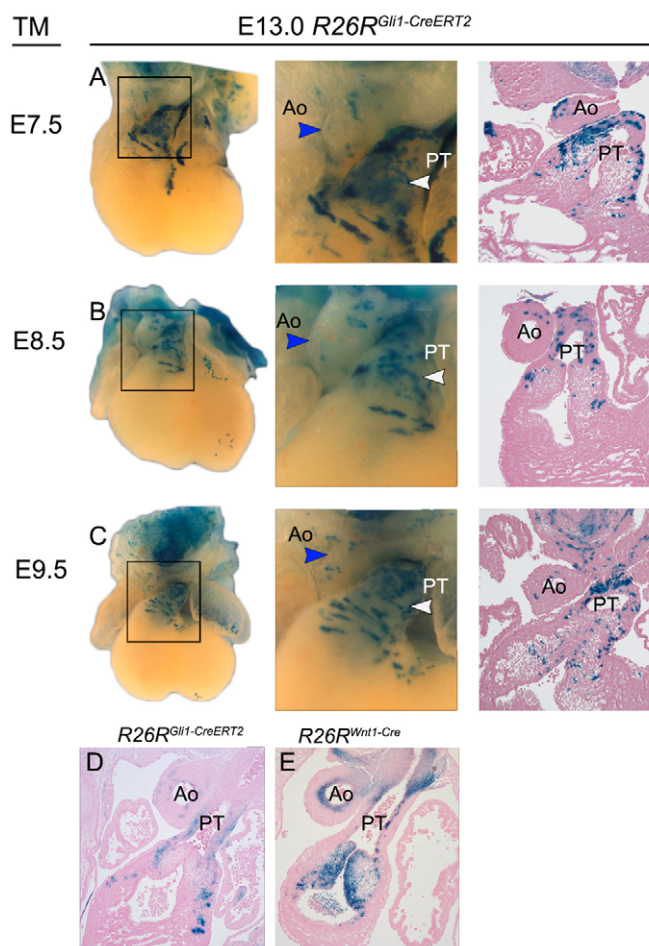
Previous work has shown that Hh signaling is required for atrial and outflow tract septation (Washington Smoak et al., 2005; Goddeeris et al., 2007; Goddeeris et al., 2008). Although activation of the Hh pathway is not observed within the developing heart (Goddeeris et al., 2007; Goddeeris et al., 2008), tissue-specific ablation of Hh signaling within the anterior heart field using the transgenic line *Mef2c-AHF-Cre* has suggested an anterior heart field role for Hh signaling in atrial septation (Goddeeris et al., 2008). We hypothesized that non-cardiac Hh-receiving cells contribute directly to cardiac septal structures. We tested this hypothesis in mice using genetic inducible fate mapping (GIFM) (Joyner and Zervas, 2006) to mark cells that receive Hh signaling early during early cardiac morphogenesis and then evaluate their location at the completion of cardiac septation. Hh-responding cells were marked in space and time using a Tamoxifen (TM)-inducible *Cre* recombinase expressed from the *Gli1* locus (*Gli1<sup>CreERT2</sup>*), a transcriptional target of Hh signaling (Ahn and Joyner, 2004a). TM administration activates *CreERT2* recombinase activity within 8 to 12 hours of treatment, for up to 24 hours (Ahn and Joyner, 2004a; Ahn and Joyner, 2004b; Joyner and Zervas, 2006; Zervas et al., 2004). Using the *Cre*-inducible *lacZ* reporter *R26R* (Soriano, 1999) in concert with *Gli1<sup>CreERT2</sup>*, cells and the progeny of cells that receive Hh signaling and TM simultaneously are labeled by constitutive *lacZ* expression.  $\beta$ -galactosidase activity in *R26R<sup>Gli1-CreERT2</sup>* embryos administered with TM at E7.5 or E8.5 and analyzed 24 hours later recapitulated the endogenous *Gli1* expression pattern (data not shown).

Atrial septal structures were specifically marked at E13.0 in *R26R<sup>Gli1-CreERT2</sup>* embryos administered with TM at E7.5, E8.5 or E9.5 (Fig. 1A,B and C, respectively). Hh-receiving cells contributed to structures that included the primary atrial septum (PAS) and the dorsal mesenchymal protrusion (DMP) (Fig. 1A-C and data not shown). The cumulative contribution of Hh-receiving cells to the atrial septum was determined in *R26R<sup>Gli1-CreERT2</sup>* embryos administered with TM at E7.5, 8.5 and 9.5 (Fig. 1D). The complete mesenchymal core of the DMP, the core of the primary atrial septum and the mesenchymal cap of the primary atrial septum were marked



**Fig. 1. Hedgehog-receiving cells mark the atrial septum.** (A–C) Hh-receiving lineage is marked in  $R26R^{Gli1-CreERT2}$  embryos by a single dose of Tamoxifen (TM) at E7.5 (A), E8.5 (B) or E9.5 (C), and analyzed at E13.0.  $\beta$ -Galactosidase positive cells identified in whole-mount (left, 4 $\times$  magnification) and cross-section (middle and right; 4 $\times$  and 10 $\times$  magnification, respectively) histology. In the cardiac inflow, marked cells contributed to the primary atrial septum (PAS in A–D) from embryos administered with TM at E7.5 to E9.5, the dorsal mesenchymal protrusion (DMP, right and middle) from embryos administered with TM at E8.5 to E9.5, and the atrioventricular canal endocardial cushions (blue arrowheads in A, right) from embryos administered with TM at E7.5. Marked cells are absent from the heart in embryos administered with TM at E10.5. (D) Daily administration of TM at E7.5, E8.5 and E9.5 to  $R26R^{Gli1-CreERT2}$  embryos demonstrates that the entire PAS and DMP are populated by Hh-receiving cells, evident in caudal, mid and cranial transverse sections of an E13.0 embryo. At, atria; AS, atrial septum; L, lungs; V, ventricle.

by E13.0. Marking of atrial septum structures was specific and consistent, in contrast, the atrial free wall, atrial appendages and ventricular chambers were only occasionally marked by small groups of  $\beta$ -galactosidase positive cells, with no consistent pattern between specimens (Fig. 1 and data not shown). Small numbers of Hh-receiving cells also contributed to the dorsal superior and inferior endocardial cushions in embryos administered with TM at E7.5 (Fig. 1A; blue arrowheads). Few  $\beta$ -galactosidase positive cells were observed in the atrial septum from embryos administered with TM earlier, at E6.5, or later, at E10.5 (data not shown). Consistent with recent studies (Thomas et al., 2008) TM at E6.5 marked some



**Fig. 2. Hedgehog-receiving cells mark the pulmonary trunk.** (A–C) Hh-receiving lineage marked in  $R26R^{Gli1-CreERT2}$  embryos by a single dose of TM at E7.5 (A), E8.5 (B) or E9.5 (C), and analyzed at E13.0.  $\beta$ -Galactosidase positive cells identified in whole-mount (left and middle; 4 $\times$  and 10 $\times$  magnification, respectively) and cross-section (right, 10 $\times$  magnification) histology. In the outflow tract, marked cells contribute specifically to the pulmonary trunk (white arrowheads and PT in A–E) and pulmonary trunk endocardial cushion at each time point. Few marked cells are observed in the aorta (middle, blue arrowheads; right, 'Ao'). (D,E) Comparison of E13.5 outflow tracts marked by  $\beta$ -galactosidase expression in  $R26R^{Wnt1-Cre}$  (D) and  $R26R^{Gli1-CreERT2}$  (E) mice. Cells that receive a Hh signal, marked by  $Gli1-CreERT2$ , primarily populate the outer edge of the outflow tract vessels, whereas cells originating from the neural crest, marked by  $Wnt1-Cre$ , primarily populate the inner wall of the outflow tract.  $Gli1-CreERT2$  and  $Wnt1-Cre$  expressing cells appear to populate complementary domains that may overlap slightly, but not broadly.

atrial and ventricular myocytes (data not shown). These data demonstrate that Hh signaling marks atrial septum progenitors between E8.0 and E10.5.

The pulmonary trunk was also marked at E13.0 in  $R26R^{Gli1-CreERT2}$  embryos administered with TM at E7.5, E8.5 or E9.5 (Fig. 2A,B and C, respectively). Myocardial cells of the right ventricular conus and smooth muscle cells of the proximal pulmonary trunk ('PT' in Fig. 2) were labeled. This pattern is reminiscent of the *lacZ* expression pattern observed in the pulmonary trunk of transgenic mouse line ( $y96-Myf5-nlacZ-16$ ) (Bajolle et al., 2006). In  $R26R^{Gli1-CreERT2}$  marked embryos, the aorta



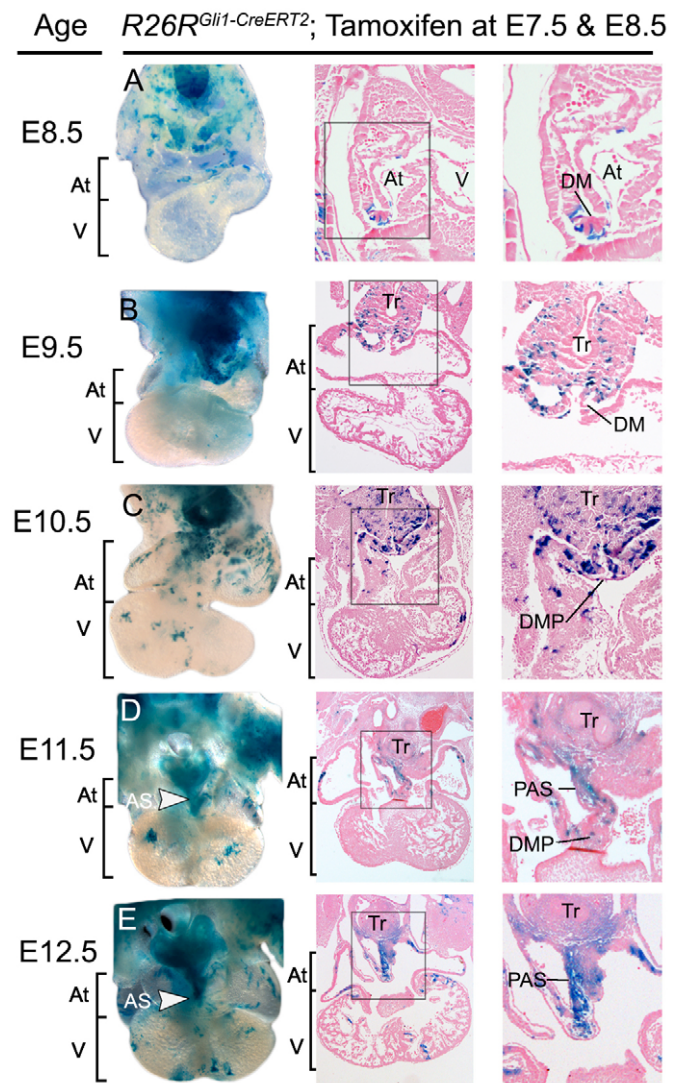
and aortic trunk ('Ao' in Fig. 2) showed far fewer  $\beta$ -galactosidase positive cells than the pulmonary trunk (Fig. 2A-C). Few  $\beta$ -galactosidase positive cells were observed in the outflow tract from embryos administered with TM earlier, at E6.5, or later, at E10.5 (data not shown). Comparison with neural crest-derived cells marked in  $R26R^{Wnt1-Cre}$  embryos revealed domains of labeling that appeared non-overlapping, suggesting that Hh signaling at the times analyzed marked non-neural crest derivatives (Fig. 2D,E). These data demonstrate that Hh signaling marks pulmonary trunk progenitors between E8.0 and E10.5.

### Hh-receiving cells migrate from the second heart field into the atrial septum and pulmonary trunk

Previous studies have demonstrated that Hh pathway activation appears to be absent from the developing heart (Goddeeris et al., 2007; Goddeeris et al., 2008), implying that atrial septum and pulmonary trunk progenitors migrate into the heart after receiving Hh signaling elsewhere. The conclusion that Hh-receiving cells are marked outside the heart by  $Gli1^{CreERT2}$  relied on exclusion of  $Gli1$  expression from the heart. We evaluated  $Gli1$  expression by in situ hybridization and semi-quantitative RT-PCR. By both methods,  $Gli1$  expression was excluded from the heart at E9 and E10, and was confined to the splanchnic mesoderm and neural tube in the axial planes that included the heart (see Fig. S1 in the supplementary material).

To test the hypothesis that Hh-receiving cardiac progenitors were labeled in SHF mesoderm and subsequently migrated into the heart, the location of Hh-receiving cells was followed by time course analysis. Hh-receiving cardiac progenitors were marked by TM administration to  $R26R^{Gli1-CreERT2}$  embryos at E7.5 and E8.5. The location of marked progenitors was evaluated at daily intervals from E8.5 to E12.5 (Figs 3 and 4). At E8.5,  $\beta$ -galactosidase expressing cells marked splanchnic mesoderm and dorsal mesocardium adjacent to the common atrium (Fig. 3A). At E9.5, marked Hh-receiving cells had migrated ventrally to begin populating the dorsal wall of the common atrium (Fig. 3B). At E10.5, a large expansion of Hh-receiving cells had populated the dorsal mesenchymal protrusion and the dorsal wall of the common atrium (Fig. 3C). A population of marked cells extended from the splanchnic mesoderm adjacent to the pulmonary endoderm through the dorsal mesocardium into the DMP. At E11.5, cells in the Hh-receiving population had migrated into central positions within the primary atrial septum and DMP (Fig. 3D). At E12.5, Hh-marked cells formed the primary atrial septum, DMP and dorsal cells in the atrioventricular endocardial cushion (Fig. 3E). Some marked cells were also observed in dorsal locations within the inferior and superior atrioventricular canal endocardial cushions at E11.5 and E12.5 (data not shown). These observations suggest that Hh-receiving cardiac progenitors migrate between E9.5 and E11.5 from posterior SHF splanchnic mesoderm into the atrial septum.

In the cardiac outflow tract, few  $\beta$ -galactosidase expressing cells were identified at E8.5 (Fig. 4A). At E9.5, a few Hh-receiving cells had entered the outflow tract (Fig. 4B). At E10.5, cells in the anterior Hh-receiving population were present in the medial, presumptive pulmonary, region of the outflow tract (Fig. 4C). A direct continuum of cells from the pharyngeal mesoderm into the outflow tract was observed. At E11.5, the medial common cardiac outflow tract was well populated by  $\beta$ -galactosidase expressing cells (Fig. 4D). Outflow tract endocardial cushions were also populated by significant numbers of marked cells (Fig. 4D, right column). At E12.5, Hh-marked cells contributed significantly to the pulmonary trunk primordium, including the pulmonary artery wall and endocardial cushion (Fig. 4E). The continuum of cells from Hh-receiving regions in the



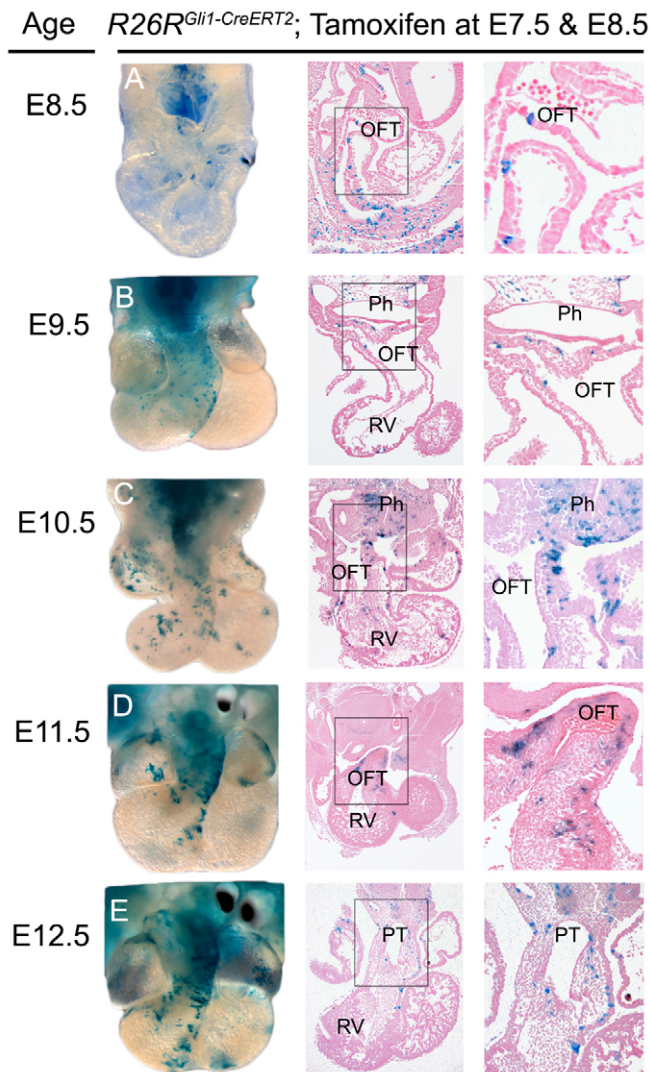
**Fig. 3. Migration of Hedgehog-marked cells into the atrial septum.** (A-E) Hh-receiving lineage marked by TM administration at E7.5 and E8.5 in  $R26R^{Gli1-CreERT2}$  embryos and analyzed at E8.5 (A), E9.5 (B), E10.5 (C), E11.5 (D) and E12.5 (E).  $\beta$ -Galactosidase positive cells were identified in the cardiac inflow by whole-mount (left, 4 $\times$  magnification) or section (middle and right; 4 $\times$  and 10 $\times$  magnification, respectively) histology. At E8.5, marked cells are observed in the dorsal mesocardium (DM). At E9.5 and E11.5, a population of marked cells extends from second heart field splanchnic mesoderm through the DM and dorsal mesenchymal protrusion (DMP) into the atria (B-D, right). At E11.5 and E12.5, the migrating Hh-marked lineage populates the primary atrial septum (PAS in D,E) and DMP (D). At, atria; AS, atrial septum; V, ventricles; Tr, pulmonary endoderm.

pulmonary and pharyngeal mesoderm was no longer observed, suggesting that active migration had ceased. These observations suggest that Hh-receiving cardiac progenitors migrate between E9.5 and E11.5 from pharyngeal mesoderm into the pulmonary artery.

### Hh signaling is required in atrial septum progenitors

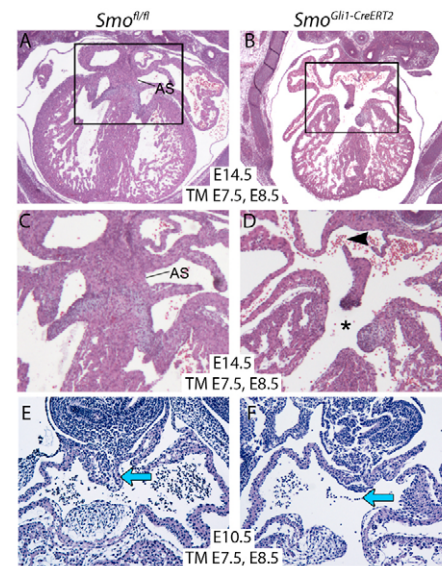
We predicted that Hh signaling would be required in atrial septum progenitors for atrial septation. To abrogate Hh signal transduction in atrial septum progenitors,  $Gli1^{CreERT2}$  was used to knock out a





**Fig. 4. Migration of Hedgehog-marked cells into the pulmonary trunk.** (A-E) Hh-receiving lineage marked by TM administration at E7.5 and E8.5 in *R26R<sup>Gli1-CreERT2</sup>* embryos and analyzed at E8.5 (A), E9.5 (B), E10.5 (C), E11.5 (D) and E12.5 (E).  $\beta$ -Galactosidase positive cells were identified by whole-mount (left, 4 $\times$  magnification) or section (middle and right; 4 $\times$  and 10 $\times$  magnification, respectively) histology. At E8.5 and E9.5, marked cells are primarily outside of the heart, in the mesoderm surrounding the pharyngeal endoderm (Ph). By E10.5 and E11.5, marked cells populate the outflow tract, with a continuous population of marked cells from the pharyngeal endoderm to the outflow tract present at E10.5, and a larger proportion concentrating to the pulmonary side of the single outflow tract at E11.5. At E12.5, separate pulmonary and systemic trunks have formed and the marked cells primarily populate the pulmonary trunk. OFT, outflow tract; RV, right ventricle; PA, pulmonary artery; PT, pulmonary trunk.

*loxP*-flanked conditional allele of the obligate Hh receptor smoothed (*Smo<sup>fl/fl</sup>*) (van den Heuvel and Ingham, 1996; Long et al., 2001). Smoothed is required for all Hh signal transduction, and *Smo* knockout mice exhibit the complete inactivation of all Hh signaling pathways, as evidenced by the total absence of *Ptc1-lacZ* expression (Zhang et al., 2001). Hh responsiveness was thereby disrupted in cells receiving TM and Hh simultaneously. *Smo<sup>Gli1-CreERT2</sup>* embryos and littermate controls (*Smo<sup>fl/fl</sup>*) were



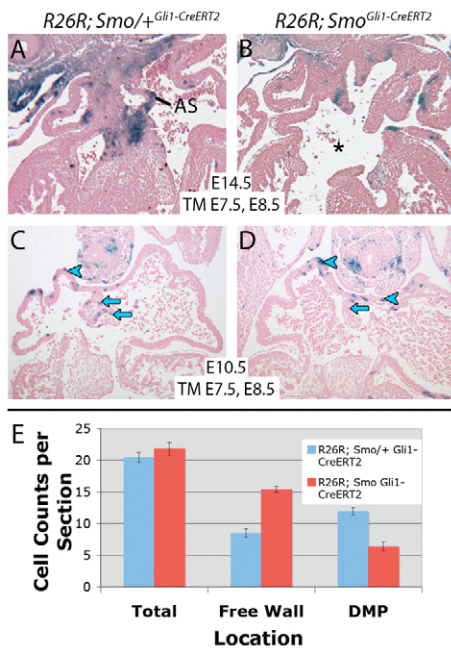
**Fig. 5. Atrial and atrioventricular septal defects in *Gli1CreERT2* conditional *Smo* mutants.** (A-D) E14.5 cardiac anatomy by cross-section histology in control *Smo<sup>fl/fl</sup>* (A, C) and mutant *Smo<sup>Gli1-CreERT2</sup>* (B, D) embryos administered with TM at E7.5 and E8.5. Atrial septal (D, arrowhead) and atrioventricular canal (D, asterisk) defects are present in *Smo<sup>Gli1-CreERT2</sup>* but not *Smo<sup>fl/fl</sup>* embryos. C and D correspond to boxes in A and B, respectively. (E, F) *Smo<sup>Gli1-CreERT2</sup>* embryos (F) at E10.5 show hypoplastic dorsal mesenchymal protrusions (DMP, arrow) as compared with wild-type *Smo<sup>fl/fl</sup>* littermates (E). AS, atrial septum.

administered with TM at E7.5 and E8.5 and evaluated at E13.5. Mutant *Smo<sup>Gli1-CreERT2</sup>* embryos demonstrated atrial and atrioventricular septal defects (5/6; Fig. 5B,D). All five affected animals demonstrated atrioventricular septal defects and three out of five also possessed a common atrium. Each littermate control demonstrated normal cardiac septation (6/6,  $P=0.01$ ; Fig. 5A,C). Hh signaling is therefore required in atrial septum progenitors for atrial septation. Neither *Smo<sup>Gli1-CreERT2</sup>* embryos (TM administered at E7.5 and E8.5) nor littermate controls demonstrated outflow tract septation defects (data not shown). This observation suggests that either the timing of the requirement for Hh signaling in atrial and outflow tract progenitors is different or that atrial septation is more sensitive to partial abrogation of Hh signaling than outflow tract septation.

To define the embryonic age at which atrial septation is first disrupted in Hh signaling mutant embryos, we performed a morphological evaluation of the developing atrial septum by time course analysis. At E9.5, no discernable atrial septal structures are present, and *Smo<sup>Gli1-CreERT2</sup>* embryos (TM administered at E7.5 and E8.5) and littermate controls were indistinguishable (data not shown). However, by E10.5, the DMP was hypoplastic or absent in *Smo<sup>Gli1-CreERT2</sup>* embryos (4/4) compared with littermate controls (0/5) (Fig. 5E,F). Therefore smoothed, and by extension Hh signaling, is required in the SHF prior to E10.5 for atrial septation to occur.

### Hh signaling specifies septum from non-septum atrial progenitors

We hypothesized that Hh signaling specifically altered the fate of atrial septal progenitors. We therefore marked Hh-receiving cells and analyzed their location in wild-type and Hh signaling mutant



**Fig. 6. Atrial septum versus atrial free wall distribution is dependent on Hedgehog signaling.** (A-E) *R26R; Smo<sup>Gli1-CreERT2</sup>* embryos (B,D) and littermate controls (A,C) were administered with TM at E7.5 and E8.5, and dissected at E14.5 and E10.5. (A,B) E14.5 *R26R; Smo<sup>Gli1-CreERT2</sup>* embryos have severe atrial septal defects (asterisk), corresponding to the absence of  $\beta$ -galactosidase marked cells that are observed in wild-type controls. (C,D) E10.5 *R26R; Smo<sup>Gli1-CreERT2</sup>* embryos demonstrate a hypoplastic DMP compared with littermate controls (arrows) and an increased number of  $\beta$ -galactosidase positive cells in the atrial free wall (arrowheads). (E) E10.5 *R26R; Smo<sup>Gli1-CreERT2</sup>* embryos have significantly fewer  $\beta$ -galactosidase positive cells in the DMP and significantly more  $\beta$ -galactosidase positive cells in the atrial free wall when compared with littermate controls. AS, atrial septum.

embryos. We simultaneously activated *lacZ* expression and inactivated *smoothed* in *R26R; Smo<sup>Gli1-CreERT2</sup>* (TM administered at E7.5 and E8.5) embryos. Hh responsive cells were analyzed in mutant *R26R; Smo<sup>Gli1-CreERT2</sup>* embryos (Fig. 6B,D) and in littermate control (*R26R; Smo<sup>Gli1-CreERT2</sup>/+*) embryos. All mutant embryos demonstrated atrioventricular septal defects (3/3) compared with littermate controls showing normal morphology (0/5;  $P=0.02$ ; Fig. 6A,C). Hh-receiving cells failed to populate the atrial septum in *R26R; Smo<sup>Gli1-CreERT2</sup>* embryos, leading to an atrioventricular septal defect at E13.5 (Fig. 6B, asterisk) and a hypoplastic DMP at E10.5 (Fig. 6D, arrow), in contrast to the normal Hh-marked atrial lineages observed in littermate controls. Although the removal of Hh signaling from atrial septal progenitors caused atrial septal defects, we observed that numerous Hh-marked cells were nevertheless present in the atria of *R26R; Smo<sup>Gli1-CreERT2</sup>* mutant embryos.

Qualitative analysis of Hh-marked cells could not discern whether fewer Hh-receiving cells populated mutant atria, implying a migration defect, or whether Hh-receiving cells populated mutant atria normally but specifically failed to form the atrial septum, implying normal cell migration into the atria but a specific defect in atrial septum formation. To distinguish between these possibilities, we performed quantitative analysis of the location of Hh-receiving cells in Hh signaling mutant and littermate control embryos. Hh-marked cells were analyzed at E10.5 in mutant *R26R; Smo<sup>Gli1-CreERT2</sup>* (TM administered at E7.5 and E8.5) embryos and in littermate controls. The total number of marked

cells in the atria was quantified (Fig. 6E). No difference in the total number of Hh-marked cells present in the atria of mutant or control embryos was discerned (Fig. 6). This finding suggests that Hh signaling is not required for the migration of cardiac progenitors from the SHF into the atrial primordium.

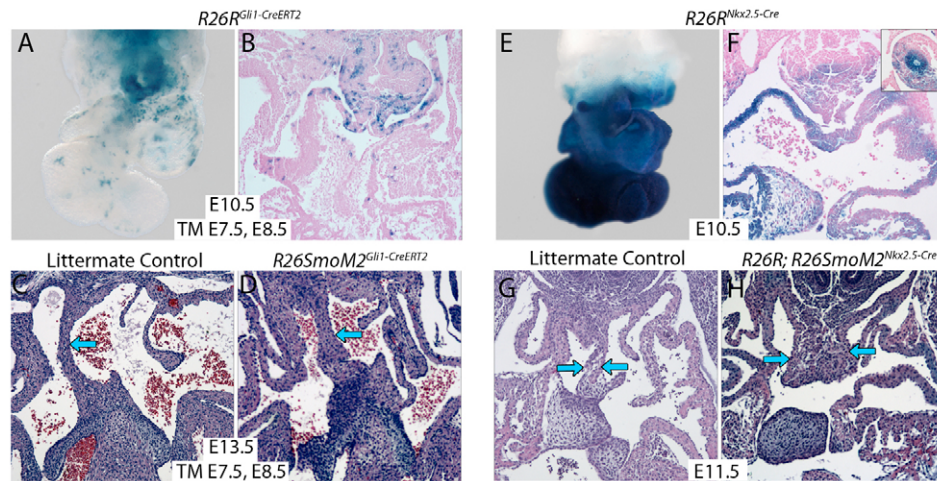
Having established that Hh signaling did not affect the total number of atrial cells, we hypothesized that neither cell death nor proliferation would be altered in Hh signaling mutants. We assessed cell death in Hh pathway mutants using LysoTracker Red, a vital lysosomal dye that has been shown to accurately mark cell death in whole mouse embryos (Zucker et al., 1999). *Smo<sup>Gli1-CreERT2</sup>* (TM administered at E7.5 and E8.5) and *Shh<sup>-/-</sup>* mutants were compared with littermate controls (*Smo<sup>+/+</sup>* and *Shh<sup>+/+</sup>*, respectively). No difference in the prevalence of LysoTracker fluorescence was observed in the posterior SHF and DMP at E9.0 or E10.0, indicating that Hh signaling does not affect SHF cell survival (see Fig. S2A,B in the supplementary material and data not shown).

We next assessed proliferation in the posterior SHF in Hh signaling mutants. Proliferation was analyzed using BrdU incorporation in wild-type and *Shh<sup>-/-</sup>* mutant embryos before E10.5. The posterior SHF was analyzed following BrdU treatment 5 and 2.5 hours prior to dissection. No discernable difference in the number of proliferating cells was observed in the posterior SHF of *Shh<sup>-/-</sup>* and wild-type littermate control embryos (see Fig. S2C-F in the supplementary material). Together, these findings suggest that neither cell survival, nor proliferation, nor migration of cardiac progenitors into the atrium are abnormal in Hh signaling mutants.

We hypothesized that Hh signaling might be necessary to specify atrial septum from non-septum progenitor fate. We analyzed the distribution of Hh-marked atrial cardiac progenitors in control and Hh signaling mutant embryos. In control embryos ( $n=3$ ), the majority of Hh signaling marked cells were localized to the primordial atrial septum, the DMP (58% DMP versus 42% atrial free wall;  $P=0.002$ ). However, in *R26R; Smo<sup>Gli1-CreERT2</sup>* mutant embryos ( $n=3$ ), the majority of marked cells were localized to the atrial free wall (28% DMP versus 72% atrial free wall;  $P=5.18 \times 10^{-9}$ ). Thus, a significant reduction in the number of Hh-receiving cells contributing to the atrial septum was observed. Abrogation of Hh signaling caused marked cells to be significantly less likely to contribute to the atrial septum (58% DMP versus 28% DMP;  $P=7.83 \times 10^{-11}$ ), and more likely to contribute to the atrial free wall (42% free wall versus 72% free wall;  $P=7.83 \times 10^{-11}$ ). These results suggest that Hh signaling is required for the specification of a subset of atrial progenitors specific for the atrial septum.

We next hypothesized that Hh signaling might be sufficient for specifying atrial septum progenitor fate in SHF atrial progenitors. We predicted that activating the Hh pathway in an expanded domain in the posterior SHF might cause specification of too many septal progenitors, at the expense of non-septum atrial progenitors. To test this prediction, we used a conditional Cre-dependent, constitutively active allele of *Smo*, *R26-smoM2* (Jeong et al., 2004), in conjunction with two Cre drivers with different SHF expression patterns. When *Gli1<sup>CreERT2</sup>* was used to constitutively activate *Smo* in SHF cells that typically receive Hh signaling, no phenotypic consequences were observed compared to wild-type embryos (Fig. 7A,B). *R26-smoM2<sup>Gli1-CreERT2</sup>* embryos (TM administered at E7.5 and E8.5) had normal viability and demonstrated normal cardiac morphology, including the atrial septum (Fig. 7C,D). By contrast, when *Nkx2-5<sup>Cre</sup>* (Moses et al., 2001) was used to constitutively activate Hh signaling in a broader domain of cardiac progenitors, including within the SHF (Fig. 7E,F), embryonic lethality and severe atrial septal defects were observed. *R26-smoM2<sup>Nkx2.5-Cre</sup>* caused lethality by E13.5





**Fig. 7. Expanded activation of Hedgehog signaling causes aberrant atrial septum development.** (A-H) Constitutive activation of the Hh pathway using the Cre-activated smoothened-M2 allele (*R26-smoM2*) in cells that normally receive Hh signal has no effect on atrial septation, whereas activating the Hh pathway in a broader domain results in a hypercellular atrial septum. Whole-mount (A,E) and cross-section (B-D,F-G) histology. (A,B) *Gli1-CreERT2* expression domain demonstrated with the *R26R* reporter. (C,D) Constitutive activation of the Hh pathway in the *Gli1-CreERT2* expression domain has no effect on the size or overall structure of the atrial septum compared with littermate controls (arrows). (E,F) *Nkx2-5<sup>Cre</sup>* expression domain demonstrated with the *R26R* reporter. Inset in F depicts *Nkx2-5<sup>Cre</sup>* expression in pulmonary endoderm. (G,H) Constitutive activation of the Hh pathway in the broader *Nkx2-5<sup>Cre</sup>* domain results in an enlarged atrial septum when compared with wild-type littermates (arrows).

(12/12). *R26-smoM2<sup>Nkx2.5-Cre</sup>* embryos demonstrated specific abnormalities of the atria and atrial septum compared with *R26-smoM2* littermates at E11.5. The DMP of mutant embryos was significantly enlarged, filling the dorsal cavity of the common atrium (Fig. 7G,H). The morphology of the ventricles appeared normal (Fig. 7G,H). These results suggest that expanded second heart field Hh signaling specifies too many atrial septum progenitors.

### ***Shh* is required in pulmonary endoderm for atrial septation**

We attempted to identify and locate the Hh signal to posterior SHF cardiac progenitors. The location of Hh-responsive cells in the SHF was determined by analyzing the expression of *Patched1*, a Hh-responsive locus (Goodrich et al., 1997), during atrial septal progenitor specification at E8.5, E9.5 and E10.5. Two regions of strong Hh responsiveness could be discriminated along the anterior-posterior axis of the SHF splanchnic mesoderm, particularly at E9.5 (Fig. 8C,D) and E10.5 (Fig. 8E,F). A previously described anterior (cranial) center was observed within the pharyngeal mesoderm ('Ph' in Fig. 8C,E), adjacent to the cardiac outflow tract. A distinct posterior (caudal) center was also present, located within the splanchnic mesoderm of the pulmonary primordium, adjacent to tracheal pulmonary endoderm ('Tr' in Fig. 8C-F). These observations raise the possibility that pulmonary endoderm, a known source of *Shh* expression (Litingtung et al., 1998), is the source of the Hh signal received by posterior SHF cardiac precursors.

We tested the possibility that *Shh* is required in pulmonary endoderm for atrial septation. *Nkx2-1<sup>Cre</sup>* drives *Cre* expression very early in pulmonary endoderm, concomitant with atrial septal progenitor specification (Lazzaro et al., 1991). Comparison of *Shh* expression by in situ hybridization (Fig. 8G,H) and *Cre* activity in *R26R<sup>Nkx2.1-Cre</sup>* embryos demonstrated overlap in the pulmonary endoderm at E9.0 and 10.0 (Fig. 8I,J and data not shown), the only location of overlap in the thorax or abdomen. *Nkx2-1<sup>Cre</sup>* was used to selectively remove a conditional allele of *Shh* in pulmonary endoderm. *Shh<sup>Nkx2.1-Cre</sup>* embryos demonstrated reproducible atrial

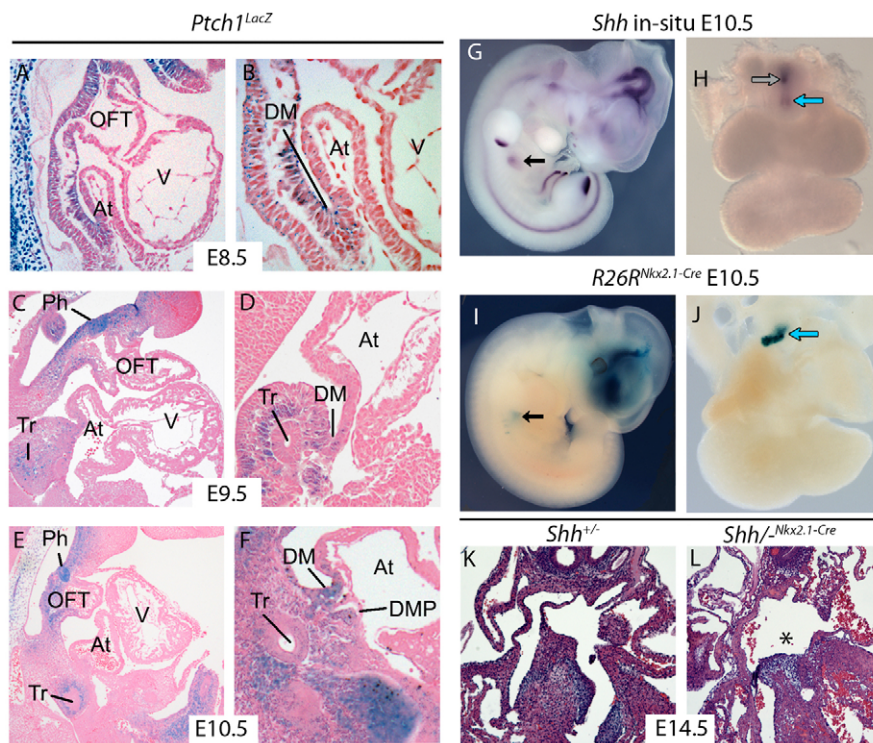
septal defects at E13.5 (3/3), whereas littermate *Shh<sup>+/-</sup>* control embryos were morphologically normal (0/5;  $P < 0.01$ ) (Fig. 8K,L). We conclude that *Shh* expression in the pulmonary endoderm is required for atrioventricular septation.

### **DISCUSSION**

We demonstrate that Hh signaling differentiates atrial septum and pulmonary trunk cardiac progenitors from other cardiac progenitors in the second heart field. We observe the migration of Hh-receiving progenitors and their progeny from the posterior and anterior SHF into the atrial septum and pulmonary trunk, respectively, with remarkable specificity. Our data suggest that Hh signaling is required for the specification of atrial septum progenitor fate in a subset of atrial progenitors. This specification event appears to be induced by *Shh* expression in the pulmonary endoderm, implicating the developing lungs as the source of instructive cues required for atrial septation. We propose a model in which molecular differences among cardiac progenitors predict subsequent roles of their differentiated progeny in cardiac morphogenesis. Receipt of Hh signaling between E8 and E10 defines a unique origin for cells of the atrial septum and establishes a new fate in the posterior second heart field, the atrial septum progenitor. Our studies engender a novel paradigm for atrial septation; atrial septum and non-septum cardiomyocytes are distinguished at the level of progenitor cell specification, rather than by positional information acquired later within the developing atrium. These findings define the cellular origin and molecular requirements for the myocardium of the atrial septum and have implications for the ontogeny of atrial septal defects and the evolution of cardiac septation.

### **The atrial septum progenitor: a subclass of second heart field cardiac progenitor**

The description of the Hh-dependent lineage of atrial septal progenitors extends our understanding of SHF cardiac progenitor cell specification. Posterior SHF contributions to the atrium have been recently documented in detail. The existence of SHF-derived



**Fig. 8. *Shh* is required in pulmonary endoderm for atrial septation.** (A-F) *Ptch<sup>LacZ</sup>* demonstrates Hh signal responsiveness outside of the heart in two axial domains; one adjacent to the pharyngeal endoderm (Ph) and one adjacent to the pulmonary endoderm (Tr). This localization is visible at E8.5 (A,B); E9.5 (C,D); and E10.5 (E,F). (G,H) In situ hybridization against *Shh* in an E10.5 embryo demonstrates *Shh* expression in the pulmonary endoderm (blue and black arrows) and esophagus (gray arrow) during early atrial septum formation. No expression is observed in the heart. (I,J) *Nkx2-1<sup>Cre</sup>* and *Shh* expression overlap specifically in the pulmonary endoderm tissues (blue and black arrows). Removal of *Shh* expression from the pulmonary endoderm using *Nkx2-1<sup>Cre</sup>* and conditional loxP-flanked *Shh* (*Shh<sup>Nkx2.1-Cre</sup>*) results in atrial septal defects (L, asterisk), which is absent from control littermates (K).

atrial cardiomyocytes was inferred from retrospective clonal analysis (Meilhac et al., 2004) and from fate map experiments using *Isl1<sup>Cre</sup>* (Cai et al., 2003) and *Mef2c<sup>Cre</sup>* (Goddeeris et al., 2008). Recent prospective lineage tracing experiments have directly demonstrated that posterior SHF derivatives include left and right atrial cardiomyocytes (Galli et al., 2008). It was noted that differentiated cells with specific chamber identity are formed from different regions of the posterior SHF, implying distinct left/right fate among posterior SHF progenitors. Our results extend this paradigm of predetermination within the posterior SHF to include septum versus non-septum atrial progenitor fates. These observations imply considerable molecular prepatternning within cardiac progenitors and suggest that the molecular logic governing atrial morphogenesis, including atrial septation, is firmly established within posterior SHF cardiac progenitors long before formal atrial septum morphogenesis begins. Whether molecular prepatternning of cardiac progenitors predicts cardiac morphogenesis as a general principle remains an important open question.

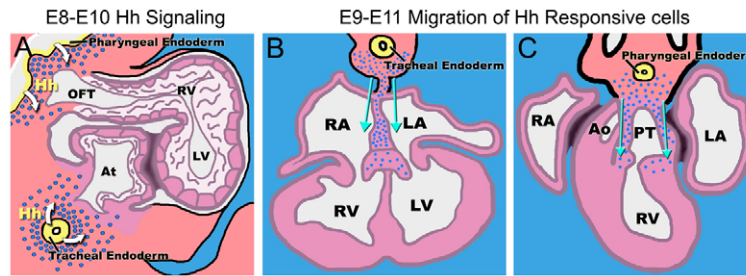
We hypothesize that Hh signaling acts directly to specify atrial septum progenitor fate in a subset of SHF atrial progenitors. Other possible roles for Hh signaling, including effects on proliferation or survival of posterior SHF cells, were not observed by us or others (see Fig. S2 in the supplementary material) (Goddeeris et al., 2008). Our in vivo quantitative analysis also suggested that Hh signaling is dispensable for migration of atrial progenitors from the SHF into the atrium (Fig. 6). We can not rule out the possibility that Hh signaling is required for proper intra-atrial migration of the septal progenitor cells. A role for Hh signaling in SHF cell migration was observed in vitro by Goddeeris et al. (Goddeeris et al., 2008); and might pertain to the behavior of septal progenitors within the atrium. Our experimental approach also required recombination of conditional *Smo* alleles in a Hh-receiving cell. Therefore, a caveat to our conclusions is the possibility that earlier or more complete Hh abrogation could uncover effects on atrial septum progenitor proliferation, cell survival or migration from the SHF into the atrium.

### Atrial septum progenitor specification and congenital heart disease

Anomalies of atrial septation are a major class of human congenital heart disease. Deficiency of the primary atrial septum (PAS) causes atrial septal defects of the secundum type. Recent work has implicated maldevelopment of the DMP as a cause of atrioventricular septal defects, including atrial septal defects of the primum type, in mice and humans (Wessels et al., 2000; Snarr et al., 2007a; Snarr et al., 2007b; Goddeeris et al., 2008). Here we demonstrate that a common molecular pathway marks progenitors of both the PAS and the DMP and is required for their development. This observation suggests the possibility of a mechanistic link between at least some primum and secundum atrial septal defects. The presence of both primum and secundum atrial septal defects segregating in human families haploinsufficient for either *Tbx5* or *Gata4* supports a potential causal association.

To what degree is atrial septum morphogenesis patterned within the posterior SHF rather than within the developing atria? The cardiogenic transcription factors *Tbx5*, *Nkx2-5* and *Gata4* are all implicated in human atrial septal defects and are expressed in atrial myocardium during formal atrial septum morphogenesis (Molkentin et al., 1997; Kasahara et al., 1998; Bruneau et al., 1999). These observations have engendered a paradigm describing roles for these transcription factors in generating intracardiac positional information. Intriguingly, each transcription factor is also expressed in the posterior SHF during atrial septal progenitor specification (Molkentin et al., 1997; Kasahara et al., 1998; Bruneau et al., 1999). These observations raise the possibility that these transcription factors might play a role in atrial septum progenitor specification. An expanded paradigm for the requirement of these important cardiac transcription factors in atrial septation and human atrial septal defects should include possible roles in both atrial septum progenitors and atrial cardiomyocytes until further work establishes their true role.





**Fig. 9. Specification of cardiac progenitors by Hedgehog signaling.** (A–C) Hh signaling from pulmonary endoderm and pharyngeal endoderm specifies cardiac progenitors in the posterior and anterior second heart field splanchnic mesoderm, respectively (A). Required Hh signaling occurs between E8 and E10, during heart tube and looping stages. Physical continuity between the developing atria and the posterior SHF Hh signaling, and between the outflow tract and the anterior SHF Hh signaling, is present via the dorsal mesocardium. Hh-induced cardiac progenitors migrate from the pulmonary mesoderm into the atrial septum (B), and from the pharyngeal mesoderm into the developing pulmonary trunk (C). Ao, aorta; At, atrium; LA, left atrium; RA, right atrium; LV, left ventricle; RV, right ventricle; OFT, outflow tract; PT, pulmonary trunk.

### Respiratory endoderm patterns cardiac morphogenesis: implications for the evolution of cardiac septation

This work identified the pulmonary endoderm as a unique source of cardiac induction. Classical and molecular studies have established the role of the anterior definitive endoderm in the early induction of cardiac mesoderm (reviewed by Foley et al., 2006). These studies also imply a requirement for endoderm in later stages of cardiogenesis, including promotion of cardiomyocyte differentiation and morphogenesis. Recent studies have inferred a direct role for *Shh* in the pharyngeal endoderm for cardiac septation (Goddeeris et al., 2007), based on the phenocopy of the cardiovascular phenotype of *Shh*<sup>−/−</sup> embryos, including both outflow tract and atrioventricular septal defects, by conditional ablation of *Shh* with *Nkx2-5*<sup>Cre</sup>, in concert with the overlap in expression of *Shh* and *Nkx2-5*<sup>Cre</sup> in the pharyngeal endoderm. Here we demonstrated that ablation of *Shh* with the pulmonary endoderm-specific Cre driver *Nkx2-1*<sup>Cre</sup> results in atrial, but not outflow tract, septal defects (Fig. 8K,L and data not shown). As the only overlapping domain of expression between *Nkx2-1*<sup>Cre</sup> and *Shh* outside of the head was limited to the pulmonary endoderm, we concluded that pulmonary *Shh* is required for atrial septation. Reconciling the presence of atrial septal defects in *Shh*<sup>Nkx2.5-Cre</sup> embryos with our conclusion that the lungs are the responsible signaling source, we demonstrated that *Nkx2-5*<sup>Cre</sup> is also expressed in the pulmonary endoderm, including the mainstem and branching trachea (inset, Fig. 7F). No outflow tract septation defects were observed in *Shh*<sup>Nkx2.5-Cre</sup> mutant embryos, demonstrating specificity of pulmonary *Shh* for inflow tract septation and implying that two respiratory endodermal sources of *Shh* are required for cardiac septation: pharyngeal endoderm for outflow tract septation and pulmonary endoderm for atrial septation.

Thus, the respiratory primordium appears to pattern cardiac septation of the inflow and outflow tracts, specifying development of cardiac structures vital for efficient cardiopulmonary circulation (Fig. 9). The structures populated by Hh-induced cardiac progenitors in mice, the atrial septum in the inflow and the pulmonary trunk in the outflow tract, are orthologous to the earliest ancestral cardiac septal structures observed in basal tetrapods (Icardo et al., 2005). We speculate that cardiac septal structures and the respiratory apparatus coevolved and that Hh signaling from primordial respiratory structures to splanchnic mesoderm of the SHF might underlie early events in the evolution of cardiac septation.

The authors thank A. Joyner for providing the *Gli1-CreER*<sup>T2</sup> line and A. Imamoto for providing *Wnt1-Cre;R26R* embryos; and C. Micchelli, M. Nobrega, E. Ferguson, J. Martin, E. McNally and members of the Moskowitz laboratory for critical reading of the manuscript. This study was funded by the NIH (NHLBI-HL078180), the March of Dimes and the American Heart Association. Deposited in PMC for release after 12 months.

#### Supplementary material

Supplementary material available online at  
<http://dev.biologists.org/cgi/content/full/136/10/1761/DC1>

#### References

- Abu-Issa, R., Smyth, G., Smoak, I., Yamamura, K. and Meyers, E. N. (2002). Fgf8 is required for pharyngeal arch and cardiac development in the mouse. *Development* **129**, 4613–4625.
- Ahn, S. and Joyner, A. L. (2004a). Dynamic changes in the response of cells to positive hedgehog signaling during mouse limb patterning. *Cell* **118**, 505–516.
- Ahn, S. and Joyner, A. L. (2004b). In vivo analysis of quiescent adult neural stem cells responding to Sonic hedgehog. *Nature* **437**, 894–897.
- Anderson, R. H., Webb, S., Brown, N. A., Lamers, W. and Moorman, A. (2003). Development of the heart: (2) Septation of the atria and ventricles. *Heart* **89**, 949–958.
- Bajolle, F., Zaffran, S., Kelly, R. G., Hadchouel, J., Bonnet, D., Brown, N. A. and Buckingham, M. E. (2006). Rotation of the myocardial wall of the outflow tract is implicated in the normal positioning of the great arteries. *Circ. Res.* **98**, 421–428.
- Basson, C. T., Bachinsky, D. R., Lin, R. C., Levi, T., Elkins, J. A., Soultz, J., Grayzel, D., Kroumpouzou, E., Traill, T. A., Leblanc-Straceski, J. et al. (1997). Mutations in human TBX5 cause limb and cardiac malformation in Holt-Oram syndrome. *Nat. Genet.* **15**, 30–35.
- Biris, K. K., Dunty, W. C., Jr and Yamaguchi, T. P. (2007). Mouse *Ripply2* is downstream of Wnt3a and is dynamically expressed during somitogenesis. *Dev. Dyn.* **236**, 3167–3172.
- Blom, N. A., Ottenkamp, J., Wenink, A. G. and Gittenberger-de Groot, A. C. (2003). Deficiency of the vestibular spine in atrioventricular septal defects in human fetuses with down syndrome. *Am. J. Cardiol.* **91**, 180–184.
- Bruneau, B. G., Logan, M., Davis, N., Levi, T., Tabin, C. J., Seidman, J. G. and Seidman, C. E. (1999). Chamber-specific cardiac expression of Tbx5 and heart defects in Holt-Oram syndrome. *Dev. Biol.* **211**, 100–108.
- Bruneau, B. G., Nemer, G., Schmitt, J. P., Charron, F., Robitaille, L., Caron, S., Conner, D. A., Gessler, M., Nemer, M., Seidman, C. E. et al. (2001). A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. *Cell* **106**, 709–721.
- Bruneau, B. G. (2002). Transcriptional regulation of vertebrate cardiac morphogenesis. *Circ. Res.* **90**, 509–519.
- Cai, C. L., Liang, X., Shi, Y., Chu, P. H., Pfaff, S. L., Chen, J. and Evans, S. (2003). Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. *Dev. Cell.* **5**, 877–889.
- Foley, A. C., Gupta, R. W., Guzzo, R. M., Korol, O. and Mercola, M. (2006). Embryonic heart induction. *Ann. New York Acad. Sci.* **1080**, 85–96.
- Galli, D., Domínguez, J. N., Zaffran, S., Munk, A., Brown, N. A. and Buckingham, M. E. (2008). Atrial myocardium derives from the posterior region of the second heart field, which acquires left-right identity as Ptx2c is expressed. *Development* **35**, 1157–1167.

- Garg, V., Kathiriyai, I. S., Barnes, R., Schluterman, M. K., King, I. N., Butler, C. A., Rothrock, C. R., Eapen, R. S., Hirayama-Yamada, K., Joo, K. et al. (2003). GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. *Nature* **424**, 443-447.
- Goddeeris, M. M., Schwartz, R., Klingensmith, J. and Meyers, E. N. (2007). Independent requirements for Hedgehog signaling by both the anterior heart field and neural crest cells for outflow tract development. *Development* **134**, 1593-1604.
- Goddeeris, M. M., Rho, S., Petiet, A., Davenport, C. L., Johnson, G. A., Meyers, E. N. and Klingensmith, J. (2008). Intracardiac septation requires hedgehog-dependent cellular contributions from outside the heart. *Development* **135**, 1887-1895.
- Goodrich, L. V., Milenkovi, L., Higgins, K. M. and Scott, M. P. (1997). Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science* **277**, 1109-1113.
- Hoffman, J. I. E. (1995). Incidence of congenital heart disease: I. Postnatal incidence. *Pediatr. Cardiol.* **16**, 103-113.
- Icardo, J. M., Ojeda, J. L., Colvee, E., Tota, B., Wong, W. P. and Ip, Y. K. (2005). Heart inflow tract of the African lungfish *Protopterus dolloi*. *J. Morphol.* **263**, 30-38.
- Jacob, L. and Lum, L. (2007). Deconstructing the hedgehog pathway in development and disease. *Science* **318**, 66-68.
- Jeong, J., Mao J., Tenzen T., Kottmann, A. H. and McMahon A. P. (2004). Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia. *Genes Dev.* **18**, 937-951.
- Joyner, A. L. and Zervas, M. (2006). Genetic inducible fate mapping in mouse: establishing genetic lineages and defining genetic neuroanatomy in the nervous system. *Dev. Dyn.* **235**, 2376-2385.
- Kasahara, H., Bartunkova, S., Schinke, M., Tanaka, M. and Izumo, S. (1998). Cardiac and extracardiac expression of Csx/Nkx2.5 homeodomain protein. *Circ. Res.* **82**, 936-946.
- Kim, J. S., Virágh, S., Moorman A. F., Anderson, R. H. and Lamers, W. H. (2001). Development of the myocardium of the atrioventricular canal and the vestibular spine in the human heart. *Circ. Res.* **88**, 395-402.
- Klaus, A., Saga, Y., Taketo, M. M., Tzahor, E. and Birchmeier, W. (2007). Distinct roles of Wnt/beta-catenin and Bmp signaling during early cardiogenesis. *Proc. Natl. Acad. Sci. USA* **104**, 18531-18536.
- Kuo, C. T., Morrissey, E. E., Anandappa, R., Sigrist, K., Lu, M. M., Parmacek, M. S., Soudais, C. and Leiden, J. M. (1997). GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev.* **11**, 1048-1060.
- Lazzaro, D., Price, M., de Felice, M. and Di Lauro, R. (1991). The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted areas of the foetal brain. *Development* **113**, 1093-1104.
- Li, Q. Y., Newbury-Ecob, R. A., Terrett, J. A., Wilson, D. I., Curtis, A. R., Yi, C. H., Gebuhr, T., Bullen, P. J., Robson, S. C., Strachan, T. et al. (1997). Holt-Oram syndrome is caused by mutations in TBX5, a member of the Brachyury (T) gene family. *Nat. Genet.* **15**, 21-29.
- Lin, L., Bu, L., Cai, C. L., Zhang, X. and Evans, S. (2006). Isl1 is upstream of sonic hedgehog in a pathway required for cardiac morphogenesis. *Dev. Biol.* **295**, 756-763.
- Litingtung, Y., Lei, L., Westphal, H. and Chiang, C. (1998). Sonic hedgehog is essential to foregut development. *Nat. Genet.* **20**, 58-61.
- Long, F., Zhang, X. M., Karp, S., Yang, Y. and McMahon, A. P. (2001). Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation. *Development* **128**, 5099-5108.
- Lyons, I., Parsons, L. M., Hartley, L., Li, R., Andrews, J. E., Robb, L. and Harvey, R. P. (1995). Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. *Genes Dev.* **9**, 1654-1666.
- Meilhac, S. M., Esner, M., Kelly, R. G., Nicolas, J. F. and Buckingham, M. E. (2004). The clonal origin of myocardial cells in different regions of the embryonic mouse heart. *Dev. Cell* **6**, 685-698.
- Molkentin, J. D., Lin, Q., Duncan, S. A. and Olson, E. N. (1997). Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev.* **11**, 1061-1072.
- Mommersteeg, M. T., Soufan, A. T., de Lange, F. J., van den Hoff, M. J., Anderson, R. H., Christoffels, V. M. and Moorman, A. F. (2006). Two distinct pools of mesenchyme contribute to the development of the atrial septum. *Circ. Res.* **4**, 351-353.
- Moses, K. A., DeMayo, F., Braun, R. M., Reecy, J. L. and Schwartz, R. J. (2001). Embryonic expression of an Nkx2-5/Cre gene using ROSA26 reporter mice. *Genesis* **31**, 176-180.
- Reifers, F., Walsh, E. C., Léger, S., Stanier, D. Y. and Brand, M. (2000). Induction and differentiation of the zebrafish heart requires fibroblast growth factor 8 (fgf8/acerebellar). *Development* **127**, 225-235.
- Schott, J. J., Benson, D. W., Basson, C. T., Pease, W., Silberbach, G. M., Moak, J. P., Maron, B. J., Seidman, C. E. and Seidman, J. G. (1998). Congenital heart disease caused by mutations in the transcription factor NKX2-5. *Science* **281**, 108-111.
- Snarr, B. S., Wirrig, E. E., Phelps, A. L., Trusk, T. C. and Wessels, A. (2007a). A spatiotemporal evaluation of the contribution of the dorsal mesenchymal protrusion to cardiac development. *Dev. Dyn.* **236**, 1287-1294.
- Snarr, B. S., O'Neal, J. L., Chintalapudi, M. R., Wirrig, E. E., Phelps, A. L., Kubalak, S. W. and Wessels, A. (2007b). Isl1 expression at the venous pole identifies a novel role for the second heart field in cardiac development. *Circ. Res.* **101**, 971-974.
- Solloway, M. J. and Robertson, E. J. (1999). Early embryonic lethality in Bmp5/Bmp7 double mutant mice suggests functional redundancy within the 60A subgroup. *Development* **126**, 1753-1768.
- Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.
- Tasaka, H., Krug, E. L. and Markwald, R. R. (1996). Origin of the pulmonary venous orifice in the mouse and its relation to the morphogenesis of the sinus venosus, extracardiac mesenchyme (spina vestibuli), and atrium. *Anat. Rec.* **246**, 107-113.
- Thomas, N. A., Koudijs, M., van Eeden, F. J., Joyner, A. L. and Yelon, D. (2008). Hedgehog signaling plays a cell-autonomous role in maximizing cardiac developmental potential. *Development* **135**, 3789-3799.
- Ueno, S., Weidinger, G., Osugi, T., Kohn, A. D., Golob, J. L., Pabon, L., Reinecke, H., Moon, R. T. and Murry, C. E. (2007). Biphasic role for Wnt/beta-catenin signaling in cardiac specification in zebrafish and embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **104**, 9685-9690.
- van den Heuvel, M. and Ingham, P. W. (1996). Smoothed encodes a receptor-like serpentine protein required for hedgehog signalling. *Nature* **382**, 547-551.
- Washington Smoak, I., Byrd, N. A., Abu-Issa, R., Goddeeris, M. M., Anderson, R., Morris, J., Yamamura, K., Klingensmith, J. and Meyers, E. N. (2005). Sonic hedgehog is required for cardiac outflow tract and neural crest cell development. *Dev. Biol.* **283**, 357-372.
- Webb, S., Brown, N. A. and Anderson, R. H. (1998). Formation of the atrioventricular septal structures in the normal mouse. *Circ. Res.* **82**, 645-656.
- Wessels, A., Anderson, R. H., Markwald, R. R., Webb, S., Brown, N. A., Virágh, S., Moorman A. F. and Lamers, W. H. (2000). Atrial development in the human heart: an immunohistochemical study with emphasis on the role of mesenchymal tissues. *Anat. Rec.* **259**, 288-300.
- Zervas, M., Millet, S., Ahn, S. and Joyner A. L. (2004). Cell behaviours and genetic lineages of the mesencephalon and rhombomere 1. *Neuron* **43**, 345-357.
- Zhang, X. M., Ramalho-Santos, M. and McMahon, A. P. (2001). Smoothed mutants reveal redundant roles for Shh and Ihh signaling including regulation of LR asymmetry by the mouse node. *Cell* **105**, 781-792.
- Zucker, R. M., Hunter E. S., III and Rogers J. M. (1999). Apoptosis and morphology in mouse embryos by confocal laser scanning microscopy. *Methods* **18**, 473-480.