sonic hedgehog is required in pulmonary endoderm for atrial septation

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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 Gli1CreERT2, 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

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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>tm1(Sto)</sup>] patched<sup>lacZ</sup> (B6;129-Pitch<sup>tm1(Sto)</sup>) and conditional knockout smo<sup>med</sup> (also known as Smo<sup>med</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 CreER<sup>T2</sup> 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 CreER<sup>T2</sup> without toxicity, as measured by embryonic size and viability (data not shown). As CreER<sup>T2</sup> 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<sub>3</sub>Fe(CN)<sub>6</sub>, 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 2 mM MgCl<sub>2</sub>, 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 maeic acid buffer (MAB; 0.1 M maeic 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 for genotyping. Embryos were fixed for 1 hour in 4% paraformaldehyde and stained with X-gal staining solution (5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 2 mM MgCl<sub>2</sub>, 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.

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, TGGGTTCTTA; Gli1 reverse, CACTTGCTTGGGTTCCTTA; β-actin forward, TAAAGCCAACCGTGAA-AAGATG; β-actin reverse, ACCGCTGTGCCAATAATGTATG.

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-Isaa 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 µl 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

β-Galactosidase positive cells were counted on all sections with a DMP in WT specimens and R26R<sup>Smo<sup>Gli1-CreERT2</sup></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 β-galactosidase positive cells. Mean numbers of β-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 Me<sup>f2</sup>-AHC-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 (GFIM) (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-receiving 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 CreER<sup>T2</sup> 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 R26<sup>R</sup> (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. β-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.
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 β-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 β-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 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 β-galactosidase positive cells than the pulmonary trunk (Fig. 2A-C). Few β-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<sup>R<sub>Cre</sub></sup>-<sup>Gli1-CreERT2</sup> 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 (Godeeirs 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<sup>CreERT2</sup> 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<sup>Gli1-CreERT2</sup> 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, β-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 and dorsal cells in the atrioventricular endocardial cushion (Fig. 3E). Some marked cells were also observed in dorsal locations in 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 β-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 β-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 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<sup>CreERT2</sup> was used to knock out a
loxP-flanked conditional allele of the obligate Hh receptor smoothened (Smo<sup>B</sup>) (van den Heuvel and Ingham, 1996; Long et al., 2001). Smoothened 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>B</sup>) were 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 sepsation 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 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 smoothened, and by extension Hh signaling, is required in the SHF prior to E10.5 for atrial sepsation 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

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Fig. 4. Migration of Hedgehog-marked cells into the pulmonary trunk. (A-E) Hedgehog-receiving lineage marked by TM administration at E7.5 and E8.5 in R26<sup>Gli1-CreERT2</sup> embryos and analyzed at E8.5 (A), E9.5 (B), E10.5 (C), E11.5 (D) and E12.5 (E). β-Galactosidase positive cells were identified by whole-mount (left, 4x magnification) or section (middle and right; 4x and 10x 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.

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>B</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>B</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>B</sup> littermates (E). AS, atrial septum.
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). SmoGli1-CreERT2 (TM administered at E7.5 and E8.5) and Shh–/– mutants were compared with littermate controls (SmoGli1-CreERT2 and Shh+/+, 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–/– mutant embryos before E10.5. The posterior SHF was analyzed following BrdU treatment 5 and 2.5 hours prior to dissection. No discernible difference in the number of proliferating cells was observed in the posterior SHF of Shh–/– 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;SmoGli1-CreERT2 mutant embryos (n=3), the majority of marked cells were localized to the atrial free wall (28% DMP versus 72% atrial free wall; P=5.18×10^{-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=7.83×10^{-11}) and less likely to contribute to the atrial free wall (28% DMP versus 72% free wall; P=7.83×10^{-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 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-CreERT2 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-smoM2Gli1-CreERT2 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 Nkx2-5Cre (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-smoM2Nkx2-5Cre caused lethality by E13.5

We simultaneously activated lacZ expression and inactivated smoothened in R26R;SmoGli1-CreERT2 (TM administered at E7.5 and E8.5) embryos. Hh responsive cells were analyzed in mutant R26R;SmoGli1-CreERT2 embryos (Fig. 6B,D) and in littermate control (R26R;SmoGli1-CreERT2+/+) 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;SmoGli1-CreERT2 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;SmoGli1-CreERT2 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;SmoGli1-CreERT2 (TM administered at E7.5 and E8.5) embryos and in littermate controls. The total number of marked embryos. We simultaneously activated lacZ expression and inactivated smoothened in R26R;SmoGli1-CreERT2 (TM administered at E7.5 and E8.5) embryos. Hh responsive cells were analyzed in mutant R26R;SmoGli1-CreERT2 embryos (Fig. 6B,D) and in littermate control (R26R;SmoGli1-CreERT2+/+) 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;SmoGli1-CreERT2 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;SmoGli1-CreERT2 mutant embryos.

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Hedgehog-dependent 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,D), 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. Nkx2-5-Cre 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\textsuperscript{Nkx2-5-Cre} 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. Nkx2-5-Cre was used to selectively remove a conditional allele of Shh in pulmonary endoderm. Shh\textsuperscript{Nkx2-5-Cre} embryos demonstrated reproducible atrial septal defects at E13.5 (3/3), whereas littermate Shh\textsuperscript{+/–} 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...
Atrial cardiomyocytes was inferred from retrospective clonal analysis (Meilhac et al., 2004) and from fate map experiments using Isl1Cre (Cai et al., 2003) and Mef2Cre (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 implicate 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. Our observations support 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, Nks2.3 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 (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.
Septation.

SHF might underlie early events in the evolution of cardiac primordial respiratory structures to splanchnic mesoderm of the body. We speculate that cardiac septal structures and ancestral cardiac septal structures observed in basal tetrapods are orthologous to the earliest progenitors in mice, the atrial septum in the inflow and the pulmonary trunk in the outflow tract, are orthologous to the earliest progenitors in mice, the atrial septum in the inflow and the pulmonary trunk in the outflow tract. The structures populated by Hh-induced cardiac signaling 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 (Goddereis et al., 2007), based on the phenocopy of the cardiovascular phenotype of Shh−/− embryos, including both outflow tract and atrioventricular septal defects, by conditional ablation of Shh with Nkx2.5-Cre in concert with the overlap in expression of Shh and Nkx2.5 in the pharyngeal endoderm. Here we demonstrated that ablation of Shh with the pulmonary endoderm-specific Cre driver Nkx2.5-Cre results in atrial, but not outflow tract, septal defects (Fig. 8K,L and data not shown). As the only overlapping domain of expression between Nkx2.5-Cre 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−/−Nkx2.5-Cre embryos with our conclusion that the lungs are the responsible signaling source, we demonstrated that Nkx2.5-Cre 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−Nkx2.5-Cre 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.

**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 (Goddereis et al., 2007), based on the phenocopy of the cardiovascular phenotype of Shh−/− embryos, including both outflow tract and atrioventricular septal defects, by conditional ablation of Shh with Nkx2.5-Cre in concert with the overlap in expression of Shh and Nkx2.5 in the pharyngeal endoderm. Here we demonstrated that ablation of Shh with the pulmonary endoderm-specific Cre driver Nkx2.5-Cre results in atrial, but not outflow tract, septal defects (Fig. 8K,L and data not shown). As the only overlapping domain of expression between Nkx2.5-Cre 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−/−Nkx2.5-Cre embryos with our conclusion that the lungs are the responsible signaling source, we demonstrated that Nkx2.5-Cre 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−Nkx2.5-Cre 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.

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**Supplementary material**

Supplementary material available online at [http://dev.biologists.org/cgi/content/full/136/10/1761/DC1](http://dev.biologists.org/cgi/content/full/136/10/1761/DC1)

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