Intracardiac septation requires hedgehog-dependent cellular contributions from outside the heart

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Septation of the mammalian heart into four chambers requires the orchestration of multiple tissue progenitors. Abnormalities in this process can result in potentially fatal atrioventricular septation defects (AVSD). The contribution of extracardiac cells to atrial septation has recently been recognized. Here, we use a genetic marker and novel magnetic resonance microscopy techniques to demonstrate the origins of the dorsal mesenchymal protrusion in the dorsal mesocardium, and its substantial contribution to atrioventricular septation. We explore the functional significance of this tissue to atrioventricular septation through study of the previously uncharacterized AVSD phenotype of Shh−/− mutant mouse embryos. We demonstrate that Shh signaling is required within the dorsal mesocardium for its contribution to the atria. Failure of this addition results in severe AVSD. These studies demonstrate that AVSD can result from a primary defect in dorsal mesocardium, providing a new paradigm for the understanding of human AVSD.

KEY WORDS: Sonic hedgehog, Atrioventricular septal defect, Intracardiac septation, Heart development, Morphogenesis, Mouse

INTRODUCTION
The internal division of the heart tube into four chambers is a complex developmental process of high relevance to human congenital anomalies. Atrioventricular (AV) septation includes separation of the left and right atria from ventricles, and formation of the mitral and tricuspid valves (Lamers and Moorman, 2002). AV septation defects (AVSDs) occur in over 1 in 2800 live births, with highly variable presentation (Hoffman, 1995; Hoffman and Kaplan, 2002). The three defects most common to AVSD are atrial septum primum defect, membranous ventricular septum defect, and abnormal or missing AV valve septal leaflets. The combined effect is juncture among all four chambers of the heart, resulting in mixing of freshly oxygenated and oxygen-depleted blood.

Our understanding of the process of AV septation comes in part from study of the mouse embryo. At embryonic day (E) 9.0, the interface between the atria and ventricles becomes specified as the AV canal, and formation of endocardial cushions begins. Two AV cushions form as a result of epithelial to mesenchymal transition and subsequent proliferation. The cushions themselves undergo extensive remodeling to contribute to the septal leaflets of the mitral and tricuspid valves, as well as to a large region of the membranous ventricular septum. In addition to AV cushions, the AV septal complex also includes outflow tract (OFT) endocardial cushions, the muscular ventricular septum and components of the atrial septa (reviewed by Lamers and Moorman, 2002).

The complicated process of atrial septation and its impact on AV septation is an emergent body of research. Recent work has focused on the extracardiac contribution to atrial septation by the dorsal mesocardium (DM), the region of the splanchnic mesoderm ventral to the endodermal tube and dorsal to the heart that maintains contact with the venous pole of the heart tube throughout development. Starting at E10.5, mesenchymal cells from the DM enter the atria at the site of pulmonary vein formation (Webb et al., 1998). The primary atrial septum, a myocardial extension of the dorsal atrial wall that becomes capped by mesenchyme, is already present. Webb et al. (Webb et al., 1998) re-examined via histology the contribution of DM to an atrial structure identified originally by Wilhelm His as the spina vestibuli (His, 1880), and suggested that this extracardiac tissue has a significant contribution to atrial septation. Wessels and colleagues found a similar ‘dorsal mesenchymal protrusion’ (DMP) in human embryos, composed of mesenchyme entering the heart between the pulmonary ridges at the site of future pulmonary vein formation (Wessels et al., 2000). These authors suggested that this tissue contributes to the mesenchyme capping the primary atrial septum, and helps to close the primary atrial foramen by filling the space between the primary atrial septum and AV cushions. Studies of transgenic markers of gene expression in mesenchymal cells have provided further support for a discrete contribution from the DM to the septal complex (Mommersteeg et al., 2006; Snarr et al., 2007a). Here we refer to this structure descriptively as the DMP.

The identification of the DMP as a source of cells contributing to atrial septation raises many questions. For example, what is the structural contribution of the DMP to atrial septation, and more broadly to AV septation? Does the DMP functionally interact with other components of the AV complex? What genetic pathways are important for DMP development? Can defective DMP development result in AVSD? To address these questions, we use a multi-technique approach. We report a genetic marker for the DM, and use novel imaging techniques to address its morphogenesis and ultimate contribution to AV septation. We then use this knowledge to address the functional relevance of the DMP to the pathogenesis of an uncharacterized mouse model of AVSD, demonstrating a crucial role for hedgehog signaling in this process.

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MATERIALS AND METHODS

Mouse lines and generation of conditional mutants

Mice were maintained on an outbred ICR genetic background. Shh+/Cre produces a fusion protein of Cre with human estrogen receptor targeted to the Shh locus (Shhtm2cre/ESR1)Cjt (Harfe et al., 2004). Descriptions of remaining mouse lines used here can be found in Goddeeris et al. (Goddeeris et al., 2007). Noon of the day of vaginal plug detection was defined as embryonic day (E) 0.5. Embryos were genotyped for Cre (Meyers et al., 1999) and for the Smo wild-type and flox alleles (Zhang et al., 2001). In all figures, wild-type (WT) refers to littermates that are either Cre(+) and flox/+.

Immunohistochemistry and β-galactosidase detection

Histological preparation of embryos followed standard procedures (Goddeeris et al., 2007; Hogan et al., 1994). Section immunohistochemistry was conducted on sagittal paraffin sections (8 μm) incubated with rat anti-PECAM-1 (1:250; Pharmingen, C5673355) or rabbit anti-phosphorylated histone H3 antibody (1:250; Upstate Biotechnology). Anti-phosphorylated histone H3 signal was amplified with biotinylated anti-rabbit (Vector Labs). Detection was with Cy3 conjugated streptavidin (Vector Labs, 1:250) and Cy5 conjugated anti-rabbit (Jackson Immuno, 1:250). Nucleus detection was with Syto13 (Invitrogen, 1:1000) in PBT. Undiluted MF-20 supernatant (Developmental Studies Hybidioma Bank) was used with methanol-fixed embryos and detected with rhodamine red-conjugated goat anti-mouse IgG, Fcγ subclass 2b specific secondary (Jackson Immunoresearch) followed by paraffin sectioning and nuclei detection with Syto13. Lysotracker Red cell death analysis was as described (Abu-Issa et al., 2002; Zucker et al., 1999). Whole-mount in situ hybridization with digoxigenin-labeled riboprobes was as described (Neubuser et al., 1997). The Shh riboprobe has been previously reported (Echelard et al., 1993).

Confocal microscopy was performed on a Zeiss LSM 510 META. Images were prepared in Adobe Photoshop 7.0.1. Control and mutant images were treated identically and represent the data set as a whole. Manual mesenchyme cell counts were analyzed by Student’s t-test. Volume was measured using ImageJ Freeware (1.37v, NIH).

Transwell explant cultures

Dorsal mesocardium was dissected from E10.5 ICR embryos and cultured on type 1 collagen and placed in a 24-well plate. Each bottom well was filled with 600 μL DMEM/1% FCS. Pooled explants were dissociated with trypsin, washed and resuspended in 500 μL OPTI-MEM/1% FCS. 15,000 cells in 100 μL OPTI-MEM/1% FCS were added to each transwell insert (Costar 3422) coated with type 1 collagen and placed in a 24-well plate. Each bottom well was filled with 600 μL DMEM/10% FCS. In some wells, 10 μM cyclopamine was added. Control cells were treated with DMSO, or cultured in media alone. Cells were incubated at 37°C for 48 hours. Cells on the underside of the transwell insert membrane were fixed and stained with Gills #1 Hematoxylin and Eosin.

MRM

Embryos were imaged at E14. Contrast procedures and imaging were as described (Petiet et al., 2007), using a vertical bore 9.4T magnet interfaced to a GE EXCITE console, modified for MRM through an intermediate stage in the radiofrequency (rf) chain. Images were acquired using 3D rf refocused spin warp encoding with extended dynamic range (Johnson et al., 2007) yielding 3D image arrays of 512x512x1024 with isotropic spatial resolution of 19.5 μm³. Acquisition time was 4 hours. Hearts were color-labeled and segmented using VoxPort Software by MRPath (Durham, NC) and a Matlab routine. Volume-rendered images were generated using VGStudio Max by Volume Graphics GmbH (Germany).

RESULTS

We have previously demonstrated that sonic hedgehog (Shh) signaling is essential for the contribution of the anterior heart field (AHF) to the developing OFT (Goddeeris et al., 2007). The AHF includes a splanchnic mesodermal region that contributes to the arterial pole of the primary heart tube to form much of the OFT and right ventricle (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). Intriguingly, expression of AHF markers also has been described in or near the developing atria, supporting the view that this domain contributes to both the arterial and venous poles of the primary heart tube (Cai et al., 2003; Huynh et al., 2007; Kelly et al., 2001; Mommersteeg et al., 2006; Snarr et al., 2007b). In the course of our previous studies, we noted similar atrial expression with the Cre transgenic line Mef2C-AHF-Cre (Verzi et al., 2005) using the Cre recombinator reporter R26R (Soriano, 1999), which indicates where recombination has occurred via β-galactosidase (β-gal) expression. The atrial expression pattern includes regions of the atrial septum thought to be of extracardiac mesenchymal origin, specifically the DMP. As little is understood about the nature of this tissue, we sought to address its origin and contributions to atrial septation using Mef2C-AHF-Cre as a genetic tool.

We crossed Mef2C-AHF-Cre with R26R in a series of pseudo-lineage trace experiments to determine the extent to which this cell population contributes to AV septation in later stages of heart development. By E10.5, this Cre line leads to β-gal expression in the splanchnic mesoderm, which is continuous with the DM (Fig. 1A,B). Mef2C-AHF-Cre was also observed in the medial dorsal myocardial wall of the atria continuous with the splanchnic mesoderm. By E11.5, R26R expressing mesenchymal cells continuous with the DM are seen to protrude into the atria at the future point of pulmonary vein attachment. This DMP expression domain extends to the inferior AV cushion, and comprises part of the mesenchymal cap that extends to the superior AV cushion (Fig. 1C). This pattern indicates that Mef2C-AHF-Cre is either expressed in the DMP and mesenchymal cap, or that these tissues are derived from tissue that once expressed Cre, such as the DM.

In embryos from E12.5-E16.5, cells derived from the Mef2C-AHF-Cre expression domain constitute much of the tissue continuous with the AV cushion-derived central mesenchymal mass, as well as much of the primary atrial septum (Fig. 1D-F). Cre expression is specific to this midline tissue and largely excludes AV cushion-derived mesenchyme. At E14.5, labeled cells were observed continuous with the primary atrial septum, forming a wedge over the fused AV cushion-derived tissue. By this stage, the AV cushion tissue has redistributed to form the septal leaflets of the mitral and tricuspid valves. In summary, histological analysis suggests that the primary atrial septum is partially derived from Mef2C-AHF-Cre-positive cells.

Using magnetic resonance microscopy (MRM) of E14.5 embryos in comparison to Mef2C-AHF-Cre; R26R embryos, we generated three-dimensional (3D) renderings of the Mef2C-AHF-Cre lineage contribution to the atrial septum. Heart structures were color-labeled from the MRM slices according to the pattern observed in closely matched Mef2C-AHF-Cre; R26R sections (Fig. 1G). These heart structures were isolated and recompiled to generate a 3D structure (Petiet et al., 2007). Multiple views suggest that these labeled cells contribute much of the atrial septal complex and present a large interface with the remodeling AV cushions (Fig. 1H). Near the dorsal atrial wall, cells derived from the Mef2C-AHF-Cre domain occupy a greater volume than the remaining component of the primary atrial septum. Together, these data confirm that the DMP is derived from the DM, and show that the DMP in turn gives rise to much of the tissue mass in the atrial septal complex.

Hh responsive DMP is deficient in Shh+/– mutant embryos

As the pathogenic mechanisms of many AVSDs are unclear, we wished to determine whether abnormal DMP development could be a causal defect. Besides OFT phenotypes, Shh+/– mutant
embryos have ventricular and atrial septal defects (Washington Smoak et al., 2005), as well as incompletely-penetrant AVSD with a single AV valve (Fig. 2A,B). Most Shh−/− mutants have rudimentary septal AV valve leaflets, though some lack even these. The causes of the large atrial septation defect observed in Shh−/− mutants have not been addressed, but the phenotype is consistent with the absence of tissue from the AV septal complex. Recent data indicate that the ventral pharyngeal endoderm (PE) is the source of Shh required for normal OFT development (Goddeeris et al., 2007), but there are no overt domains of Shh expression within the heart to explain a role in AV septation. We therefore investigated this apparent contradiction.

First, we further assessed the potential Shh source relevant for AV septation. There are no detectable areas of Shh expression within the OFT, ventricles or atria, as assayed by whole-mount in situ hybridization, RT-PCR or Shher-Cre; R26R analysis from E9.5 to E11.5 (Fig. 2C) (Goddeeris et al., 2007). However, the ventral PE expresses Shh and is juxtaposed to the dorsal mesocardium during DMP formation (Fig. 2D). Consistent with this observation, we have
previously demonstrated that loss of Shh from the PE results in AVSD (Goddeeris et al., 2007). The lack of overt Shh expression within the heart itself and the presence of AVSD in embryos lacking endodermal Shh led us to hypothesize that Shh produced in the PE acts on the DM, which in turn forms the DMP and contributes to atrial septation. Shh is one of three mammalian hedgehog (Hh) ligands, the signal transduction of which is positively affected in responsive cells by a receptor, smoothened (Smo). It is negatively regulated by patched 1 (Ptch1), a positive transcriptional target widely used to report Hh signaling activity (Ingham and McMahon, 2001). Ptch1lacZ activity appears to be restricted to the developing atria and distal OFT myocardium (Fig. 2E,F) (Washington Smoak et al., 2005). Atrial expression is first observed at E10.5 in the DM near the pulmonary pit (data not shown). At later stages (E11.5-E13.5), weak expression is observed in the DMP within the atria (Fig. 2E,F). This Ptch1 domain is Shh dependent, as Shh–/– embryos lack Ptch1lacZ expression within the atria (Fig. 2G,H; see Fig. S1 in the supplementary material). Thus, the DMP is the only component of the AV septation complex with detectable, Shh-dependent, Ptch1lacZ expression.

Early development of the AV cushions does not require Shh signaling

Classical models for the etiology of AVSD have focused on early abnormalities in AV cushion development. To see whether these pertain to Shh–/– mutants, we conducted histological analysis at early stages in AV formation (E9.5-E11.5), a hallmark of which is epithelial to mesenchymal transition (EMT) of endocardial cells to populate the cardiac jelly.

The AV cushions appeared normal in size and morphology at E9.5. Appropriate numbers of mesenchymal cells were present in the cushion cardiac jelly (data not shown), suggesting that AV cushion specification and the initial events of EMT proceed normally in the AV canal of Shh–/– embryos. To assess the subsequent extent of EMT, we performed section immunohistochemistry on Shh–/– mutants and wild-type littermates at E10.5 and E11.5 (Fig. 3A,B). Cushion mesenchymal cells were assessed by location and lack of the endothelial marker PECAM1, and counted in serial sections through a given embryo. No significant difference in total cell number was found in either E10.5 (Fig. 3C) or E11.5 (data not shown). Cell proliferation was assayed by immunodetection of phosphorylated histone H3. No difference was observed in E10.5 AV cushion proliferation (data not shown) or total AV cushion volume (Fig. 3D) between wild type and Shh–/– mutants. Together, these data indicate early stages of AV cushion formation and growth are normal despite loss of Shh.

By E14.5, AV cushion structure is abnormal in Shh mutants (Fig. 2B); thus, it is possible that disrupted Hh signaling during later stages of AV cushion development might play an important role in generating AVSD. On the other hand, our present analysis, combined with the observation that the DM and DMP are Shh responsive, suggests that loss of the DMP may be part of the etiology of Shh–/– AVSD. Such considerations suggested three hypotheses: (1) that defective Hh signaling in the AV cushions causes defects not only in this tissue but in AV septation in general; (2) loss of Hh signaling in the developing DMP alone is sufficient to recapitulate the full AVSD; or (3) defective signaling in both DMP and AV cushion development lead to the Shh–/– AVSD phenotype. To distinguish between these possibilities, we used tissue-specific gene ablation to disrupt Hh signaling.

Neither endocardium nor myocardium requires direct Hh signaling for AV septation

To test whether direct Hh signaling to the developing AV cushions is required for normal AV septation, we ablated the Smo receptor, and thus Hh receptiveness, from the two primary tissues of the AV cushions: the myocardium, the endocardium, or both, in combination, then analyzed mutant embryos for AVSD. To test necessity of Hh signaling in the myocardium, we generated mutants of the genotype Tnt-Cre; Smoflox/–. Tnt-Cre is expressed throughout the differentiated myocardium of the heart but not in the endocardium (Jiao et al., 2003) (Fig. 4A,A’). All mutants sectioned (7/7) had normal AV septation (Fig. 4B). Thus, loss of Hh signaling to the myocardium fails to recapitulate the Shh–/– AVSD phenotype. Interestingly, R26R analysis of Tnt-Cre expression at E11.5 and later stages revealed Tnt-Cre expression at the leading edge of the DMP (data not shown), consistent with observations that this mesenchyme becomes myocardial after entry into the atria (Soufan et al., 2004). As Tnt-Cre; Smoflox/– mutants have no atrial septation defects, we conclude that Hh signaling to the DMP is not required after differentiation into myocardium.

We next tested whether the endocardium requires a direct Hh signal. We ablated Smo from the endocardium using Tie2-Cre (Fig. 4C,C’) (Koni et al., 2001). As the AV cushion mesenchyme is
expression in any of these mutant classes, further suggesting that the normal AV septation. Notably, the DM does not demonstrate nor the combination of the two require direct Hh signaling for results indicate that neither AV cushion myocardium, endocardium and therefore generated a double signaling to both tissues was necessary to induce the defect. We its derivatives do not require Hh signaling for normal AV septation.

Given these results, we could not rule out the possibility that loss of signaling to both tissues was necessary to induce the defect. We therefore generated a double Cre transgenic line using both TnT-Cre and Tie2-Cre. These embryos have the combined expression pattern of TnT-Cre and Tie2-Cre as detected by R26R (Fig. 4E,E'). By section analysis, we found normal AV septation in TnT-Cre;Tie2-Cre; Smo

\[\text{results in normal AV septation (E18.5).} \]

Hedgehog in atrioventricular septation.

\[\text{Mef2C-AHF-Cre; Smo}^{\text{lox}}\text{-mutants recapitulate Shh}^{\text{−/+}}\text{intracardiac septation defects} \]

To test whether Hh signaling is required within the DM for heart septation, we generated Mef2C-AHF-Cre; Smo

\[\text{mutants observed (15/15) had a large atrial septation defect as well as a pronounced VSD at the level of the OFT-ventricle connection. The atrial septation defect is consistent with loss of the DMP (Fig. 5A-D). Typically, the muscular primary atrial septum was observed along the dorsal-most atrial wall (data not shown). In addition to atrial septation defects, most Mef2C-AHF-Cre; Smo

\[\text{mutants (11/15) have abnormally short, rounded septal AV valve leaflets. In most cases, the central mass of mesenchyme affixed to the muscular ventricular septum appeared much larger and more rounded than in control embryos (Fig. 5B,D). This mass is probably comprised entirely of AV cushion-derived tissue, as it is not β-gal} \]
positive in Mef2C-AHF-Cre; Smo\textsuperscript{fllox−/−} R26R embryos (Fig. 5B). The most severe forms of AVSD found at low penetrance in Shh\textsuperscript{−/−} mutants, i.e. lack of all septal leaflets of the AV valves and the central mesenchymal mass, were not observed in Mef2C-AHF-Cre; Smo\textsuperscript{fllox−/−} mutants. These results indicate that the DM requires Hh signaling for intracardiac septation and that loss of receptiveness within this domain can largely recapitulate the AV septation defects observed in Shh\textsuperscript{−/−} embryos.

To better characterize the Mef2C-AHF-Cre; Smo\textsuperscript{fllox−/−} mutant class, we used MRM imaging in comparison with stage-matched, E14.5 lineage traced embryo sections to generate color-labeled heart renderings. These structures confirmed the absence of the DMP and subsequent large atrial septation defect in these mutants (Fig. 5E-F'). Complete sets of MRM serial slices of Mef2C-AHF-Cre; Smo\textsuperscript{fllox−/−}, Shh\textsuperscript{−/−}, and wild-type controls are available for reference online (http://www.civm.duhs.duke.edu/dev01/index.html).

Taken together, our tissue-specific ablations indicate that the DMP is essential for intracardiac septation. Hh signaling is necessary within the DM for DMP morphogenesis and contribution to the septal complex. Moreover, absence of the DMP contribution has an indirect effect on the morphology of AV cushions.

**Loss of Hh signaling results in abnormal DM differentiation and migration**

To understand the early etiology of the AVSD in Mef2C-AHF-Cre; Smo\textsuperscript{fllox−/−} embryos, we characterized the distribution of β-gal positive cells in Mef2C-AHF-Cre; Smo\textsuperscript{fllox−/−}; R26R and control embryos. At E10.5, no differences in the total amount or location of β-gal positive cells were observed (Fig. 6A,B). However, far fewer mesenchymal cells were observed within the atria of mutant embryos at E11.5 relative to wild-type littermates (Fig. 6C,D). At E10.5 and E11.5 there appeared to be an increase in midline β-gal positive atrial myocardial cells. However, increased Mef2C-AHF-Cre contribution to the atrial myocardium was not detectable at E14.5 and later stages (Fig. 5A,B and data not shown). These data indicate that although an adequate population of DM cells is present at E10.5 in mutants, it is not capable of DMP formation.

Next we analyzed whether loss of Hh signaling in the DM affects proliferation, cell death, differentiation or migration. Analysis of proliferation in Mef2C-AHF-Cre; Smo\textsuperscript{fllox−/−} embryos was assessed via anti-phosphorylated histone H3 antibody detection; no differences were observed in the DM (data not shown). Cell death was assayed using the LysoTracker Red fluorescent probe, a lysosomal marker, at E10.5 and E11.5. No differences in cell death within the DM or atria were observed comparing Mef2C-AHF-Cre; Smo\textsuperscript{fllox−/−} embryos with control littermates (Fig. 6E-H). Together, these data suggest that loss of DMP tissue in Mef2C-AHF-Cre; Smo\textsuperscript{fllox−/−} embryos is not the result of decreased proliferation or increased cell death within the DM at these stages.

As discussed here and previously (Soufan et al., 2004; Snarr et al., 2007b), a region of the DMP undergoes differentiation into myocardium after its formation. One possible explanation for abnormal DMP development is abnormal control of differentiation. We addressed this possibility by testing for the presence of striated muscle specific myosin using the myofilament-20 (MF-20) antibody in E11.5 embryos (Bader et al., 1982). We observed the presence of MF-20 positive cells within the splanchic mesoderm and DM of Mef2C-AHF-Cre; Smo\textsuperscript{fllox−/−} mutants (Fig. 6J'). MF-20 positive cells were never observed outside of the heart in the body wall in wild-type controls (Fig. 6I-J'). Therefore, inappropriate differentiation may contribute to the loss of DMP formation in these mutants.

Another mechanism for the DMP defect might be a reduction in the ability of DM cells to move into the atria, for example by compromised migration. To address this possibility, we employed a transwell migration assay (see Materials and methods). Explanted DM (and closely adherent tissues) from E10.5 wild-type embryos was assayed for migratory properties. Three migration culture conditions were tested using the same starting population of DM

![Fig. 6. Deficient Hh activity results in abnormal DM development and reduced cell motility. Mef2C-AHF-Cre; Smo\textsuperscript{fllox−/−}; R26R mutant embryos and controls are compared. β-galactosidase staining at E10.5 (A,B) demonstrates relatively normal DM levels in mutant (B). Staining at E11.5 (C,D) demonstrates loss of DMP (asterisk) while some atrial myocardial and PAS staining is maintained. (E-H) Lysotracker Red cell death analysis demonstrates no detectable increase in mutant embryos (F,H) when compared with controls (E,G) at E10.5 or E11.5. (I,J) Sagittal sections stained for MF-20 (red) and nuclei (green) demonstrate normal myocardial staining in the DMP and absence of MF-20 staining in DM at E11.5 wild type (I) compared with abnormal MF-20 staining within the DM of Mef2C-AHF-Cre; Smo\textsuperscript{fllox−/−} embryos (J). MF-20 channel only (I,J) shows ectopic staining. (K) Significantly decreased DM migration is observed with 10 μM cyclopamine treatment compared with media alone or DMSO controls. A, atria; IAVC, inferior atrioventricular cushion; PAS, primary atrial septum; RV, right ventricle; SAVC, superior atrioventricular cushion. *P=0.02 (media versus cyclopamine), *P=0.001 (DMSO versus cyclopamine).
in DMP development culminates in the most severe forms of AVSD. A V cushion rightward expansion. Together, these data imply that cells with abnormal A V cushion shape or distribution (data not shown) were not observed relative to normal OFT and right ventricle length. We observed a 37% reduction in migration with cyclopamine treatment compared with the DMSO control treatment ($P=0.001$, $n=3$, Fig. 6K). Therefore, in addition to inappropriate differentiation of DM cells, another cellular cause of the defect may be a reduction in their migratory capability.

**Abnormal AV cushion rightward expansion and DMP development in Shh$^{−/−}$ mutants**

Relative to Mef2C-AHF-Cre; Smo$^{flx}$ embryos, Shh$^{−/−}$ mutants tend to have more severe cases of AVSD, exemplified by the complete lack of septal AV valves and a central mesenchymal mass. Accordingly, we analyzed Shh$^{−/−}$ mutants for additional AV complex defects. Although differences in cell number were not found, abnormalities in AV cushion shape were detectable by section analysis in Shh$^{−/−}$ mutants. At E11.5, a pronounced shape change was observed in both the superior and inferior cushions in some embryos. The inferior cushion appears less rounded than in wild-type embryos while the superior cushion is elongated or stretched. We were able to demonstrate this shift graphically by plotting the cushion surface area of consecutive sagittal sections (see Fig. S2 in the supplementary material). This shape change reduces the amount of superior AV cushion tissue present at the midline of the heart, where the cushions interact with the DMP and primary atrial septum, implying a role for Hh in AV cushion rightward expansion. However, this role must be indirect, as this phenotype was not observed in the simultaneous conditional ablations of Smo in the myocardium and endocardium. We suggest that this abnormal AV cushion distribution in Shh$^{−/−}$ mutants is the result of OFT and right ventricle shortening (Washington Smoak et al., 2005), causing general disrupted heart curvature. Consistent with this, Mef2C-AHF-Cre; Smo$^{flx/−}$ embryos (which have relatively normal OFT and right ventricle length) were not observed to have abnormal AV cushion shape or distribution (data not shown), suggesting normal OFT lengthening is required for proper AV cushion rightward expansion. Together, these data imply that a combination of defective AV cushion rightward expansion and DMP development culminates in the most severe forms of AVSD in Shh$^{−/−}$ mutants.

**DISCUSSION**

Conventional understanding of the etiology of AVSDs has largely focused on abnormal development of the AV cushions. However, new findings suggest that AVSDs seen in perinatal humans are more complex in their origins. Previous work has demonstrated that deficient DMP development occurs in mouse (Webb et al., 1999; Snarr et al., 2007b; Wirrig et al., 2007) and human (Blom et al., 2003) fetuses with AVSD. As such individuals exhibit malformations in other septal structures, the specific role of DMP defects in generating these malformations has been unclear. Here, we show that the DMP is crucial for atrial and AV valve development. We demonstrate that AVSD can arise from a primary defect in the DMP, rather than in the AV cushions. These studies further support a new paradigm for understanding the pathogenic mechanisms of AVSD (Snarr et al., 2007b).

**Mef2C-AHF-Cre activity marks extracardiac contributions to the primary atrial septum**

Recently, several groups have recognized the late extra-cardiac contribution of the DM to the atria (Mommersteeg et al., 2006; Webb et al., 1998; Wessels et al., 2000; Snarr et al., 2007a; Snarr et al., 2007b). These studies found that continuous with the DM, the DMP expands into the atria and wedges to close the gap formed between the primary atrial septum and the AV cushion mesenchyme. However, owing to a lack of adequate markers for this population, direct confirmation of these observations and assessment of DMP contribution to later atrial development has been difficult. We demonstrate that Mef2C-AHF-Cre is a faithful marker for the DM and its contribution to atrial septation.

From our pseudo-lineage trace and MRM analysis, we confirm that DM enters the developing atria at the site of pulmonary vein formation. The resulting mesenchymal mass, the DMP, elongates to fuse with the inferior AV cushion. Mef2C-AHF-Cre cells make up a region of the ‘mesenchymal cap’ of the primary atrial septum, which directly fuses with the superior AV cushion (Fig. 7A). These findings are consistent with previous models (Lammers and Moorman, 2002; Wessels et al., 2000). Loss of Hh signaling affects both the DMP and mesenchymal cap of the primary atrial septum, consistent with a common origin of these tissues.

The specificity of Mef2C-AHF-Cre allowed for the analysis of DMP contributions to later stages of atrial development. As visualized by MRM, the DMP maintains a large and clearly demarcated interface with the tissue derived from the AV cushions. We observed Mef2C-AHF-Cre; R26R expressing cells in the dorsal-medial myocardium of the atria. We suggest that splanchnic mesodermal derivatives distinct from the DMP add to the atria during the same time frame as the DMP. However, in contrast to the
DMP, these cells do not directly require Hh signaling, as they are still present in Mef2C-AHF-Cre; Smo\textsuperscript{flox/–} mutants. Alternatively, these cells may independently express the Mef2C-AHF-Cre transgene at this point in development.

The DMP is an outgrowth into the atrial lumen from the DM, in turn composed largely of mesenchyme ventral to the foregut. Given that Mef2C-AHF-Cre activity results in recombined cells in both the DMP and splanchnic mesoderm, it is conceivable that these tissues arise from a common precursor. We note that DMP labeling by cells from the Mef2C-AHF-Cre expression domain is probably not unique to this particular AHF-Cre driver, as similar atrial expression has been reported in studies of other mouse genes expressed in the AHF, such as Fgf10, Isl1 and Tbx1 (Cai et al., 2003; Huynh et al., 2007; Kelly et al., 2001; Mommersteeg et al., 2006; Snarr et al., 2007b).

**AVSD can result directly from DMP defects**

To address whether the DMP is necessary for atrial septation and to further explore its role in AV septation, we identified a mouse mutant in which this structure is defective. Shh\textsuperscript{–/–} mutants suffer from a severe atrial septal defect and AVSD, and Hh responsiveness occurs endogenously in the DM (Fig. 7B). Results reported here and previously (Goddeeris et al., 2007) imply that Shh signaling from the foregut endoderm is required for normal AV septation, as it is the only relevant domain of Shh expression. This relatively distant ligand source elicits its effect on AV septation by being juxtaposed to the DM prior to its contribution to the AV complex. We demonstrate that Ptc\textsuperscript{1lacZ} is expressed within the DM and that Cre-mediated loss of Hh receptiveness within the Mef2C-AHF-Cre expression domain is sufficient to recapitulate the consequences of absent Shh ligand on septal development (Fig. 7D).

Little is understood concerning the mechanism by which the DM enters the atria, and several possibilities might underlie the mechanism underlying the DMP defect. First, Shh produced by foregut endoderm may signal directly to the DM to elevate levels of proliferation, thus providing a sufficient population of cells to form the DMP. Although we were unable to observe proliferation differences in Mef2C-AHF-Cre; Smo\textsuperscript{flox/–} mutant DM, such differences may occur at time points not studied here. Alternatively, Shh may promote cell shape changes or the coordinated migration of DM cells into the atria, resulting in an actual ‘protrusion’ into the lumen. Equally plausible is that a combination of both migration and proliferation is responsible. In tissue culture assays, we found a reduction of over 35% in migrating DM cells after 24 hours of Hh inhibition. Perhaps a prolonged reduction of Hh signaling (as in the mutants in vivo) would increase the level of migration inhibition, accounting for the severe DMP deficiency. Finally, we detected DM cells that express the myofibril marker MF-20 within the body wall of E11.5 Mef2C-AHF-Cre; Smo\textsuperscript{flox/–} mutants. Normally, these cells should only express such markers after they have entered the atria. Based on this surprising result, we suggest two possible explanations. DM cells may require Hh signaling to repress actively the expression of myocardial differentiation genes, and loss of this signaling results in premature differentiation of cells en route to the atrial compartment. Alternatively, the DM we observed in mutants may be differentiating in the normal time frame, but in an inappropriate location owing to a cell-motility defect.

A surprising finding is the role of the DMP in AV cushion maturation. The AV valves are completely derived from tissues unrelated to the DMP and theoretically should not be affected (de Lange et al., 2004). Nevertheless, discrete loss of the DMP results in severe defects in AV valve formation. Shh\textsuperscript{–/–} and Mef2C-AHF-Cre; Smo\textsuperscript{flox/–} mutant embryos each had abnormal AV valve development, for which several possibilities exist. The DMP may provide remodeling signals to the AV cushion mesenchyme that are crucial to AV valve formation. Alternatively, the DMP may provide a physical force, pressing the AV mesenchyme into its proper conformation. We observed the central AV mesenchymal mass to be abnormally large and rounded atop the muscular septum in multiple Mef2C-AHF-Cre; Smo\textsuperscript{flox/–} mutants, consistent with both models. If loss of the DMP results in loss of a secondary signal from the DMP to the AV cushions, this secondary signal is unlikely to be a Hh ligand, as we were unable to detect any Ptc\textsuperscript{1lacZ} activity within the AV cushions. Furthermore, myocardial and endocardial loss of Smo does not affect AV septation (Fig. 7C).

**OFF defects can increase the severity of AVSDs**

Mef2C-AHF-Cre; Smo\textsuperscript{flox/–} mutant AVSDs are often less severe than those of Shh\textsuperscript{–/–} embryos. This may be due to the AV cushion expansion defect we observed in some Shh\textsuperscript{–/–} embryos at E11.5. In these embryos the AV cushions do not expand correctly or evenly to the right. As schematized in Fig. 7, the major cardiac phenotypic difference between these two classes of embryos is that Shh\textsuperscript{–/–} embryos have a much shorter OFF and smaller right ventricle (Goddeeris et al., 2007; Washington Smoak et al., 2005). Webb et al. (Webb et al., 1999) linked a similar AV cushion expansion defect in Ts16 trisomic mouse mutants with abnormalities in the curvature of the heart tube. Shh\textsuperscript{–/–} mutants have significant shortening of the right ventricle and OFF, which subsequently results in decreased outer curvature. Thus, we propose that more severe aspects of AVSD in Shh\textsuperscript{–/–} embryos are the combined result of DMP developmental failure and abnormal AV cushion rightward expansion, in turn related to severe OFF and right ventricle shortening. Consistent with this connection, another class of mutants with severe AVSD, Nkx2.5\textsuperscript{Cre}; Shh\textsuperscript{flox/–}, has both abnormal AV cushion shape and OFF shortening (data not shown) (Goddeeris et al., 2007). Mef2C-AHF-Cre; Smo\textsuperscript{flox/–} mutants, which possess relatively normal OFF and right ventricle lengthening, do not have these more severe defects.

Mef2C-AHF-Cre; Smo\textsuperscript{flox/–} mutant intracardiac defects resemble human AVSD

Mouse models that directly impact AV cushion development typically result in embryonic lethality, limiting their clinical relevance to human AVSD. In our study of Mef2C-AHF-Cre; Smo\textsuperscript{flox/–} and Shh\textsuperscript{–/–} mutant AV septation defects, we found that the range of severity and constellation of AVSDs observed are consistent with those found in humans. For example, individuals with Down syndrome (Trisomy 21) have remarkably similar types of AVSD that are associated with defects in DMP expansion (Blom et al., 2003). AVSD is also observed in the Ts16 Down syndrome mouse model, in which the DMP is deficient (Webb et al., 1999; Snarr et al., 2007). Here, we demonstrate that AVSD can be the direct result of defective DMP development in mice. A broader analysis of defective DMP development in humans with AVSD is necessary to better understand the basis of these serious birth defects.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/10/1887/DC1

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