Independent requirements for Hedgehog signaling by both the anterior heart field and neural crest cells for outflow tract development

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Cardiac outflow tract (OFT) septation is crucial to the formation of the aortic and pulmonary arteries. Defects in the formation of the OFT can result in serious congenital heart defects. Two cell populations, the anterior heart field (AHF) and cardiac neural crest cells (CNCCs), are crucial for OFT development and septation. In this study, we use a series of tissue-specific genetic manipulations to define the crucial role of the Hedgehog pathway in these two fields of cells during OFT development. These data indicate that endodermally-produced SHH ligand is crucial for several distinct processes, all of which are required for normal OFT septation. First, SHH is required for CNCCs to survive and populate the OFT cushions. Second, SHH mediates signaling to myocardial cells derived from the AHF to complete septation after cushion formation. Finally, endodermal SHH signaling is required in an autocrine manner for the survival of the pharyngeal endoderm, which probably produces a secondary signal required for AHF survival and for OFT lengthening. Disruption of any of these steps can result in a single OFT phenotype.

KEY WORDS: Anterior heart field (AHF), Neural crest, Shh, Outflow tract, Congenital heart defect, Hedgehog, Cre, loxP, Septation, Mouse

INTRODUCTION

The morphogenesis of the outflow tract (OFT) of the heart is a complex process that is disrupted in the majority of serious congenital heart defects. The OFT begins as an endothelial cell-lined channel surrounded by cardiac muscle. Cells are added to lengthen and to ultimately septate the OFT into the aortic and pulmonary arteries. There are two distinct cell populations that contribute to the OFT after the formation of the initial heart tube – the cardiac neural crest cells (CNCCs) and the anterior heart field (AHF). An important question in heart development is which signaling pathways coordinate the development of CNCCs with the AHF?

Neural crest cells (NCCs) are multi-potential and migrate to populate numerous structures in the embryo. CNCCs are a distinct subpopulation of neural crest originating from the post-otic rhombencephalon to the third somite and migrating into the third, fourth and sixth pharyngeal arches and into the OFT of the heart (Ferguson and Graham, 2004; Hutson and Kirby, 2003; Kirby and Stewart, 1983). CNCCs migrate in close proximity to both the AHF and the pharyngeal endoderm (reviewed in Harvey, 2002). CNCCs are crucial for two separate OFT-related processes: for the control of normal myocardial differentiation, and OFT septation, via population of the OFT endocardial cushions and for the formation of the aorticopulmonary septum (Kirby et al., 1983; Waldo et al., 1999).

The AHF in mouse includes the early pharyngeal core arch mesoderm and splanchnic mesoderm, which overlies the ventral pharyngeal endoderm and can be identified by distinct markers within the primary heart field as early as the cardiac-crescent stage (Cai et al., 2003; Ilagan et al., 2006; Kelly et al., 2001). AHF cells contribute to definitive OFT myocardium as well as to the right ventricle and to some endocardium (Kelly and Buckingham, 2002; Noden, 1991; Verzi et al., 2005; Ward et al., 2005). Previous data have suggested that AHF and CNCCs may be interdependent, because ablation of CNCCs results in changes in OFT length, whereas loss of Fgfr8 can negatively impact both CNCC and AHF development (Hutson and Kirby, 2003; Ilagan et al., 2006; Park et al., 2006; Waldo et al., 1999; Yelbuz et al., 2002).

One signaling pathway that is crucial for heart development is that of Hedgehog (Hh). The Hh ligand sonic hedgehog (Shh) is required for OFT development, because Shh+/− mutants have a single OFT. These defects seem to be the result of both CNCC and AHF defects (Washington Smoak et al., 2005). Although Shh is not overtly expressed at early stages within the developing heart, it is expressed in the ventricle neural tube and ventral pharyngeal endoderm, and could therefore directly affect CNCC and AHF cell development.

Hh signaling from the early endoderm has been implicated as being important in initial myocyte specification. Mouse mutants lacking the obligate Hh receptor smoothened (Smo) exhibit downregulated expression of Nkx2.5, whereas loss of the inhibitor patched homolog 1 (Pch1) results in the upregulation of this gene (Zhang et al., 2001). Recently, more interest has focused on the role of the pharyngeal endoderm in later heart remodeling events (Brown et al., 2004; Garg et al., 2001; Hu et al., 2004; Ilagan et al., 2006; Xu et al., 2004). The OFT defects observed in Shh+/− mice and the Shh expression detected within the pharyngeal endoderm strongly suggest a role for this SHH-signal source in OFT development.

Here, we use conditional gene ablation to generate several tissue-specific Hh pathway mutants to identify the crucial source of SHH and its target tissues during OFT development. These data demonstrate the first known pharyngeal endodermal signal directly required by both the AHF and CNCC fields for OFT morphogenesis, and dissect the relationship between these two fields.
MATERIALS AND METHODS

Mouse lines

All mice used in this study were maintained on an outbred genetic background. Nkx2.5Cre+ is a Cre insert into the Nkx2.5 locus, rendering it a null allele (Moses et al., 2001). ShhCre+ (Shhfllox/Cre+) is a targeted gene disruption resulting in a null allele (Chiang et al., 1996). The Shhfllox (Shhfllox/Cre Jax #004293) contains loxP sites surrounding exon 2. This allele maintains wild-type expression prior to recombination (Levis et al., 2001). The Smo+/- targeted allele (Smo+/- Jax #004288) contains loxP sites surrounding the first exon (Long et al., 2001; Zhang et al., 2001). A Smo- (null) allele was generated by crossing the Smo+/- allele to b-actin-Cre (gift from M. Lewandoski, NCI, MD), an allele with germline Cre expression, and subsequently crossing out the Cre allele by backcrossing to ICR (Institute for Cancer Research outbred CD-1, Harlan Sprague Dawley). SmoLOX [Gt(Rosa)26Sor+/-SmoATF/SmoATF Jax #005130] is a targeted allele whereby a constitutively active Smo-EYFP fusion gene was inserted into the Rosa26 locus, expression of which is blocked by a loxP-flanked STOP fragment (Jeong et al., 2004). The Pch1lacZ (Pch1FlvJax Jax #003081) line is a null allele because the lacZ insert replaces exons 1 and 2 (Goodrich et al., 1997). Mef2C-AHFe-Cre, a transgenic line using Mef2C promoter elements, was kindly provided by B. Black (UCSF, CA) (Verzi et al., 2005). Wnt1-Cre [Tg(Wnt1-cre)1Rth Jax #003829] uses an enhancer of the Wnt1 gene to express Cre in early NCCs (Danielian et al., 1998), whereas P0-Cre [Tg(P0-Cre)1Ky] expresses Cre in migratory NCC precursors and is driven using promoter elements of protein P0 (Yamauchi et al., 1999). The endothelial b-galactosidase marker Tie2-lacZ (Tg(TIE2-lacZ)182Sato/J Jax #002856) uses an endothelial-cell-specific Tie2 promoter element (Schlaeger et al., 1997). Tropomodulin-FCre (TnT-Cre), a myocardial-specific Cre, uses rat TnT promoter elements to control the expression of Cre and was a gift provided by K. Jiao (University of Alabama at Birmingham, AL). Tie2-Cre [Tg(Tek-cre)12Pth Jax #004128] expresses Cre under the control of the tyrosine kinase Tek (Koni et al., 2001). The Cre reporter allele R26R [Gt(Rosa)26Sortm10Cre Jax #00347]) concomitantly expresses lacZ in cells after Cre recombination (Soriano, 1999). Fgf8loxZ is a ‘knock-in’ allele into the Fgf8 locus (Iagan et al., 2006). In all figures, wild-type (WT) refers to littermates that are either Cre+ and flox/+ heterozygous, or flox/flox but Cre–, unless otherwise specified.

Generation of Cre-mediated Shh- and Smo-null mutants

Nkx2.5Cre+/-; Shhfllox females were mated with Shhfllox males to generate Nkx2.5Cre+/-; Shhfllox mutant embryos. Additional alleles (Pch1loxZ or Tie2-lacZ) were carried by the male in the generation of Nkx2.5Cre+/- mutants (see Results). Embryos were genotyped for Cre (Meyers et al., 1998), and for the presence of the Shhfllox and wild-type alleles (Levis et al., 2001). Smo-null mutant embryos were generated via similar mating schemes, except that Pch1loxZ or Tic2-lacZ alleles were carried by Smo-/- females. Embryos were typed for Cre and for the Smo wild-type allele (Zhang et al., 2001). Embryonic day (E) is defined as E0.5 on day of vaginal plug.

Dissection, imaging and b-galactosidase staining

Embryos were dissected in either di-ethyl-pyruvionate (DEPC)-treated phosphate-buffered saline (PBS) or PBS containing 0.1% Triton X-100 (PBT). Fixation was in 4% paraformaldehyde (PFA) overnight at 4°C. Mutants and controls at E11.5 and earlier were somite-count matched to wild-type littermates. For detection of b-galactosidase activity (b-gal), embryos were fixed for 5 minutes (E9.5) or 10 minutes (E10.5 and older) at room temperature in 2% formaldehyde/0.2% glutaraldehyde in PBS with 0.02% NP40, and were then stained overnight at 37°C in standard X-gal stain or Bluo-Gal stain (5-Bromo-3-Indolyl-b-D-Galactopyranoside, Sigma B2904). Bluo-Gal provides a deeper blue color than the standard stain, allowing for better visualization during section analysis. Control and mutants were treated with the same stain in all cases. Embryos were then either cleared with glycerol or were embedded in paraffin and sectioned using standard procedures (Hogan, 1994).

In situ hybridization

Whole-mount in situ hybridization with Digoxigenin-labeled antisense riboprobes has previously been described (Neubauer et al., 1997). All riboprobes have been previously reported: Ap2a (Tcfap2a – Mouse Genome Informatics) (Mitchell et al., 1991), Crabp1 (Stoner and Gudas, 1989), Shh (Echelard et al., 1993), Tbx1 (Chapman et al., 1996) and Fgfl (Crossley and Martin, 1995).

Immunohistochemistry and cell death analysis

Whole-mount immunohistochemistry was performed as previously described (Washington Smoak et al., 2005). Primary antibodies were used at the following concentrations: 2H3 (1:3 supernatant; Developmental Studies Hybridoma Bank and developed by T. Jessel and J. Dodd, Columbia University, New York, NY), PECAM-1 (1:250; Pharmingen, CN537555) and AP2a (1:4; 3B5 supernatant, Developmental Studies Hybridoma Bank and developed by T. Williams, University of Colorado, Denver, CO). Cell proliferation was determined using anti-phosphorylated histone H3 antibody (1:1000; Upstate Biotechnology). Cell death analysis was performed using Lysotracker Red, a lysosomal marker previously shown to indicate cell death (Zucker et al., 1999) as described by Abu-Issa et al. (Abu-Issa et al., 2002). All in situ and immunohistochemistry results are from at least three mutants and three controls.

Confocal analysis and image handling

Confocal microscopy was performed on a Zeiss LSM 510 META. Images were prepared in Adobe Photoshop 7.0.1. Control and mutant embryos were treated identically and are representative of the data set as a whole.

RESULTS

Deletion of Shh using Nkx2.5Cre results in loss of pharyngeal endoderm Hh signaling activity

Complete loss of Shh results in multiple developmental abnormalities, including cardiovascular defects such as a single OVF vessel (Washington Smoak et al., 2005). Given the wide range of functions for SHH, it is possible that the cardiovascular defects are due to the abnormal development of the embryo as a whole. Equally plausible is that SHH signaling has a direct function on OVT development. Described signaling sources of SHH that may impact early morphogenesis of the heart include the pharyngeal endoderm, notochord and ventral floorplate (Fig. 1A,C) (Moore-Scott and Manley, 2005). Both the AHF and the CNCCs are in juxtaposition to the pharyngeal endoderm adjacent to the OVT (Harvey, 2002). Additionally, expression of the downstream target Pch1 demonstrates high levels of Hh activity in the pharyngeal endoderm and overlying mesenchyme (Fig. 1E,‘). We therefore sought to test the hypothesis that the pharyngeal endodermal domains of Shh act directly on the AHF and CNCCs during OVT development.

To test this hypothesis, we genetically ablated Shh in the Nkx2.5Cre domain and compared the resulting phenotypes to those observed in Shh–/- mutants. Nkx2.5Cre is expressed throughout the heart tube, pharyngeal endoderm, AHF and first arch ectoderm (Fig. 1B and see Fig. S1 in the supplementary material). The pharyngeal endoderm is the only apparent region of overlap between Nkx2.5Cre and Shh expression (Fig. 1A,B and our unpublished observations). This suggests that Nkx2.5Cre-mediated deletion results in loss of Shh expression specifically within the pharyngeal endoderm.

To confirm that this strategy effectively and specifically ablates pharyngeal endoderm expression of Shh, we detected Shh mRNA via whole-mount in situ hybridization (Fig. 1C,D). Whereas other expression domains remained intact, we found a specific loss of Shh mRNA within the pharyngeal endoderm. Moreover, Nkx2.5Cre-/-; Shhflflo/+; Pch1lacZ+ triple-mutant embryos exhibited a specific loss of Hh activity, as demonstrated by the absence of Pch1lacZ expression in the pharyngeal...
endoderm and arches (Fig. 1E-F). Combined, these results highlight Nkx2.5Cre/+; Shhflox/– as a possible endodermal-specific deletion of Shh.

**Loss of Shh in the pharyngeal endoderm recapitulates cardiac defects observed in Shh–/– embryos**

Similar to Shh–/– mutants, near-term Nkx2.5Cre/+; Shhflox/– double mutants had single, unseptated OFT vessels (9/10) and reduced right ventricles, leaving their hearts with a small, rounded appearance (Fig. 1G,H). Abnormal arch-artery patterning was also present (7/10), with mutants exhibiting no brachiocephalic artery, a shortened distance between the left common carotid and left subclavian artery, and other defects. This constellation of defects is similar to those seen in Shh–/– mutants, representing fourth and sixth arch-artery defects (Fig. 1 and data not shown).

Histological analysis of four near-term Nkx2.5Cre/+; Shh flox/– double mutants confirmed the presence of a single OFT. Additionally, a complete atrioventricular septal defect was observed in each mutant (Fig. 1G,H). These defects are consistent with histological analysis of Shh–/– embryos (Washington Smoak et al., 2005). These data demonstrate that loss of Shh function within the Nkx2.5Cre domain is sufficient to recapitulate the OFT, arch-artery and intra-cardiac defects observed in Shh–/– mutants. Finally, when we ablated Shh from the AHF (the major non-endodermal domain of Nkx2.5Cre) using Mef2C-AHF-Cre (Verzi et al., 2005), we detected no OFT defects (data not shown), supporting our model that endodermal SHH is necessary for OFT development.

**Abnormal early OFT and right ventricle development in Nkx2.5Cre/+; Shhflox/– mutant embryos**

We next examined Nkx2.5Cre/+; Shhflox/– mutant embryos at earlier stages to determine the cause and timing of the OFT defects. Lengths of both the OFT and right ventricle were compared between mutant E10.5 embryos and littermates. Mutants displayed obvious OFT and right ventricle shortening (Fig. 2A-D,G). The OFT and right ventricle were reduced, on average, by 20 and 15%, respectively (Fig. 2A-D, black bars, Fig. 2G), whereas the left ventricle was not statistically different in size (Fig. 2A,B, white bar). These data imply a role for endodermally-derived SHH in the elongation of the OFT and, therefore, in AHF development.

At E10.5, the OFT conotruncal cushions were already distinct bulges populated by CNCCs. We carried out fluorescent-confocal imaging for the endothelial marker PECAM-1 (Fig. 2E,F), and histological analysis (Fig. 2E′,F′) of Nkx2.5Cre/+; Shhflox/– mutant and control embryos. While mesenchyme was present, distinct OFT cushions were poorly formed and small, suggesting a deficit.
in CNCCs (Fig. 2E–F’). Finally, the aorticopulmonary septum within the aortic sac failed to form by E10.5 (data not shown). These data imply a role for endodermal SHH in CNCC development as well as in AHF development.

**Increased pharyngeal cell death in Nkx2.5Cre/+; Shhfllox/– embryos**

To determine why the AHF and CNCCs are deficient in these mutant embryos, we tested for increased cell death and/or reduced cellular proliferation. Lysotracker Red fluorescent probe demonstrated increased levels of cell death at E9.5 and E10.5 in all Nkx2.5Cre/+; Shhfllox/– embryos tested, compared with controls (Fig. 2H–K’). High levels of cell death were observed in the splanchnic mesoderm and/or CNCCs located ventral to the pharyngeal endoderm and posterior to the OFT (putative AHF cells) (Fig. 2H,1,1,1,K’). Interestingly, an increase in cell death was also observed in the pharyngeal arches, with particularly high levels specifically in the presumptive core arch mesoderm, a tissue identified as a component of the AHF at earlier stages, but which, at these stages, is a precursor to craniofacial muscle groups (Fig. 2H’,I’,J,J’). Minimal levels of cell death were observed within the OFT itself at E10.5 (data not shown). Finally, increased levels of cell death were observed within the pharyngeal endoderm (Fig. 2I’,K’,K’), indicating not only a survival requirement for SHH in the AHF and NCC, but in the pharyngeal endoderm as well.

Cell-proliferation studies at E9.5 using an antibody against phosphorylated histone H3 revealed no difference in the number of cells in mitosis within the splanchnic mesoderm or in the pharyngeal endoderm compared to controls (data not shown). Therefore, the overall increase in cell death in the AHF is probably the main factor contributing to the shortened OFT–right-ventricle phenotype observed in the mutants.

**Arch-artery development requires endodermal Shh expression**

Shh−/− mutants have defective NCC migration, resulting in the abnormal development of many NCC derived structures (Washington Smoak et al., 2005). As a possible explanation for the CNCC deficits in Shh−/− embryos, loss of ventral neural tube expression of Shh could be the sole factor affecting NCC migration. Alternatively, the early requirement for Shh in the neural tube of the embryo may be masking
a direct or indirect role for \( Shh \) in the pharyngeal endoderm. Therefore, we examined the development of several NCC derivatives in \( Nkx2.5^{Cre+}; Shh^{flox/−} \) mutant embryos. The NCC-derived cranial neurofilaments of \( Shh^{−/−} \) embryos were highly disorganized at E10.5 as observed using the 2H3 neurofilament antibody (Fig. 3C). However, we did not detect significant differences in neurofilament organization between wild-type and \( Nkx2.5^{Cre+}; Shh^{flox/−} \) embryos (Fig. 3A,B), although minor defects in cranial nerve patterning could not be ruled out.

Another important role for NCCs is to support arch-artery development. Using two methods, endothelial cell detection via the \( Tie2-lacZ \) allele (Fig. 3D-F) and via India-ink injections at E10.5 (data not shown), we detected fourth and sixth arch-artery defects consistent with the terminal arch-artery pattern defects described for \( Nkx2.5^{Cre+}; Shh^{flox/−} \) mutant embryos and similar to \( Shh^{−/−} \) embryos (Fig. 3D-F). \( Tie2-lacZ \)-positive cells were observed in mutant embryos where the fourth arch-artery should form, but a well-formed artery, patent to ink, was not observed (data not shown). These results indicate that \( Shh \) expression within the \( Nkx2.5^{Cre+} \) domain is required for arch-artery patterning but not for grossly normal neuroganglia development.

A contributing factor to the OFT and pharyngeal arch defects observed in \( Shh^{−/−} \) embryos is the abnormal early NCC migration and subsequent increased levels of NCC death. We examined multiple NCC markers in \( Nkx2.5^{Cre+}; Shh^{flox/−} \) mutants to determine whether NCC migration is altered. Whole-mount in situ analysis of \( AP2α \) protein did reveal subtle migratory disorganization of the neural crest in both post-otic streams of \( Nkx2.5^{Cre+}; Shh^{flox/−} \) mutants, but this was minor compared with the defects seen in \( Shh^{−/−} \) embryos (Fig. 3M-O). These data indicate that neural tube and/or notochord \( Shh \), but not pharyngeal endoderm \( Shh \), expression is most probably required for normal early NCC migration; by contrast, endodermal \( Shh \) is required for pharyngeal survival of NCCs.

NCCs require endogenous Hh signaling for OFT septation

Loss of endodermal \( Shh \) results in abnormal AHF and CNCC development. As stated earlier, previous studies have suggested a possible interaction between these two fields of cells. To determine whether endodermal \( Shh \) is acting directly or indirectly on these two populations of cells, we performed further tissue-specific loss-of-function studies. The obligate Hh receptor \( Smo \) is expressed throughout the developing embryo (Zhang et al., 2001). Loss of \( Smo \) from a responding cell results in the complete inactivation of all Hh signaling pathways (Zhang et al., 2001). \( Smo \) homozygous null mutants die at approximately E9.5 with a variety of defects, including defective cardiac tube formation (Zhang et al., 2001). To address whether CNCCs directly respond to Hh signaling during cardiovascular development, we conditionally ablated \( Smo \) using the NCC-specific Cre recombinase \( Wnt1-Cre \).

Although previous studies have characterized the striking craniofacial defects of \( Wnt1-Cre; Smo^{flox/−} \) mutant embryos, cardiac defects were not described (Jeong et al., 2004). We determined that \( Wnt1-Cre; Smo^{flox/−} \)-embryos survive to term with single OFT septation defects (17/23) and arch-artery defects. Remaining mutant embryos had partial septation of the OFT, resulting in either a hypoplastic pulmonary artery with an aberrant origin (4/23) or a complete separation of a transposed aorta and hypoplastic pulmonary artery (2/23) (Fig. 4A,B and data not shown). Neither of these phenotypes were observed in \( Nkx2.5^{Cre+}; Shh^{flox/−} \)–/−embryos is the abnormal early NCC migration, although minor defects in cranial nerve patterning could not be ruled out.

Reduced number and abnormal pattern of CNCCs within the OFT

To follow CNCCs, we crossed the Cre recombinase reporter \( R26R \) with \( Smo^{flox/−} \) females to generate \( Wnt1-Cre; Smo^{flox/−}; R26R \) mutant embryos. This method affords a pseudo-‘NCC-lineage trace’ by
marking NCCs with Cre recombination. In wild-type embryos, CNCCs enter the OFT organized into two opposing streams that spiral along the length of the OFT. We analyzed NCC migration in Wnt1-Cre; Smo<sup>fl</sup> mutants at E9.5, E10.5 and E11.5 by detection of β-galactosidase activity (β-gal) (Fig. 4C-D and data not shown). The overall number of CNCCs reaching the OFT in mutant embryos appeared to be moderately reduced (compare number of blue cells in Fig. 4C and D). In Wnt1-Cre; Smo<sup>fl</sup>-mutants, there is decreased activity within the pharyngeal arches (bracket, F) and dorsal to the aortic sac (arrow in F). (G,H) Constitutive activation of the Hh pathway within NCCs using Smo<sup>OEX</sup> also results in a single OFT (H versus G). (I-L) Proximal/distal section analysis of Wnt1-Cre; Smo<sup>OEX</sup> embryos at E10.5 (I,J) and E11.5 (L,L′) reveals abnormal localization and the compaction of cells within the OFT (J,J′), and that, despite the presence of cushions, septation is not taking place (L,L′) when compared with controls at E10.5 (J,J′) and E11.5 (K,K′). In all panels, arrows and arrowheads mark abnormal and normal findings, respectively, in mutants as compared with control embryos. Ao, aorta; Pa, pulmonary artery; AS, aortic sac; AHF, anterior heart field; A, atrium; V, ventricle.

Histological analysis at E10.5 revealed a reduction in CNCCs dorsal to the aortic sac and ventral to the pharyngeal endoderm, consistent with the patterns of cell death that were also observed in these mutants (data not shown). This CNCC population also contributed to the development of the aorticopulmonary septum within the aortic sac. While present in Wnt1-Cre; Smo<sup>fl</sup> embryos at E10.5, this structure was sparsely populated with CNCCs and was smaller than in somite-matched wild-type embryos (data not shown). These data indicate that CNCC contribution to the aorticopulmonary septum and OFT cushions requires direct Hh signaling.

Previously published data demonstrate that NCCs abnormally cross the midline ventral to the neural tube in Shh<sup>−/−</sup> embryos (Washington Smoak et al., 2005). This abnormal early NCC migration was not observed in Wnt1-Cre; Smo<sup>fl</sup>-embryos (data not shown). Together, these data implicate a non-cell-autonomous repulsive effect of Hh signaling on early NCC migration and support a cell-autonomous effect for Hh signaling on pharyngeal NCCs.

**A subpopulation of CNCCs respond to Hh signaling**

Previous study of Shh<sup>−/−</sup> mutants demonstrated that a majority of NCCs apparently do not express Ptc<sup>lacZ</sup> (Washington Smoak et al., 2005). By contrast, our phenotypic analysis of the NCC genetic
ablation of Smo indicates that CNCCs are responding directly to Hh signaling. To investigate this apparent contradiction, we crossed the Ptc1fluor females to generate mutants of the genotype Wnt1-Cre; SmoOEX. This genetic strategy enables us to detect Hh responsiveness in a mutant background.

Embryos collected at E10.5 and stained for lacZ expression revealed a Hh-responsive CNCC sub-population dorsal to the aortic sac and ventral to the pharyngeal endoderm at the axial level of the OFT. In wild-type embryos, these cells had high levels of Ptc1fluor activity, which was reduced in Wnt1-Cre; SmoOEX mutant embryos (Fig. 4E-F, arrowhead). Histological analysis confirmed that cells are still present in this region in mutant embryos despite the loss of Ptc1fluor activity (Fig. 4E’.F’, arrows). This population was adjacent to splanchnic mesoderm, which remained positive for Ptc1fluor activity (Fig. 4E’.F’, arrowhead) in both wild-type and mutant embryos. In addition, there was a decrease in the number of Ptc1fluor-positive cells immediately dorsal to the aortic sac, consistent with the loss of some NCCs (Fig. 4E’.F’, brackets). As predicted by our Cre reporter expression, Wnt1-Cre elimination of Smo did not affect OFT myocardial, pharyngeal core arch or endodermal Ptc1fluor expression (Fig. 4E’.F’ and data not shown).

Finally, to confirm that the phenotype was due to loss of Smo from NCCs and not from other tissues, we used an additional NCC-specific Cre allele, P0-Cre (Yamauchi et al., 1999). P0-Cre elimination of Smo resulted in identical cardiac defects (data not shown), confirming that Smo is required in NCCs for OFT development.

**Localized Hh signaling to CNCCs is not required for cushion formation, but is necessary for OFT septation**

Hh signaling directly to the CNCCs appears crucial for CNCC survival and population of the OFT. It is unclear whether endodermal Hh signaling is directly organizing CNCCs into forming opposing conotruncal OFT cushions before they enter the OFT, or whether the two lines of OFT myocardial Hh activity are necessary to guide the CNCCs within the OFT, as hypothesized in our previous study (Washington Smoak et al., 2005). To help address this, we used an inducible, constitutively activated Smo transgene (SmoOEX) in combination with the NCC-specific Cre allele Wnt1-Cre. This cross results in continuous activation of the Hh signaling pathway in all NCCs. Wnt1-Cre; SmoOEX embryos have previously been described as having striking craniofacial abnormalities that are consistent with NCC defects (Jeong et al., 2004). We found that surviving E15.5 mutants also had a single, patent OFT vessel (Fig. 4G,H). However, well-developed OFT cushions were observed at E10.5. The CNCC-derived cushion mesenchyme was more compact, probably due to differences in cell morphology, although an increased quantity of CNCCs could not be ruled out (Fig. 4I-J).

In addition, more CNCCs were observed in the cardiac jelly, where the endocardium and myocardium are usually in close proximity (Fig. 4I’.J’, arrows). Early analysis showed that approximately half of these mutants died at approximately E11.5 and that the OFT was not patent to ink (data not shown). This abnormal localization could result in the obstruction of the developing OFT. In addition, section analyses of surviving E11.5 Wnt1-Cre; SmoOEX embryos clearly demonstrated a lack of cushion fusion in the distal OFT (Fig. 4K’.L’), whereas the more proximal OFT cushions were in closer approximation to each other (Fig. 4K.L). Together, these data demonstrate that CNCCs can populate the OFT cushions in the presence of continuous Hh signaling, but that later septation events are inhibited by such signaling. This result also supports the hypothesis that direct localized Hh signaling is required for normal CNCC localization within the OFT but not for population of the conotruncal cushions in general.

**The AHF directly requires intact Hh signaling for OFT septation but not for OFT lengthening**

The OFT-shortening defects and increased cell death in Shh+/− and Nkx2.5Cre+/−; Shhlox/− embryos suggest that SHH has a role in AHF contribution to OFT development. Although the data presented above clearly demonstrates a direct requirement on CNCCs, SHH produced in the overlying pharyngeal endoderm may also influence the AHF either directly or indirectly. To determine whether Hh signaling directly to the AHF is required for normal OFT development, we conditionally ablated Smo from the AHF using Me2C-AHF-Cre (referred to here as AHF-Cre) (Verzi et al., 2005). AHF-Cre is expressed in the splanchnic and core arch mesoderm, but is not expressed within the pharyngeal endoderm (see Fig. S1 in the supplementary material).

AHF-Cre; SmoOEX−/− mutant embryos also had a single OFT (14/16) and abnormal arch-artery patterning (4/16) (see Table 1, Fig. 5A,B). Surprisingly, unlike Nkx2.5Cre+/−; Shhlox/− mutants, we did not detect the same early defects of OFT lengthening in embryos lacking Smo specifically within the AHF (Fig. 5C-D’ and data not shown). OFT cushions appeared relatively normal, although a slightly reduced size could not be ruled out, as detected by both Tie2-lacZ β-gal staining and histological section analysis at E10.5 in AHF-Cre; Tie2-lacZ; SmoOEX embryos (Fig. 5C’,D’,E,F). Consistent with these findings and in contrast to the observed increase in cell apoptosis observed in Nkx2.5Cre+/−; Shhlox/− embryos, there was no appreciable difference in cell death between wild-type and AHF-Cre; SmoOEX−/− embryos at E10.5 (data not shown). Section analysis at E12.5 demonstrated that, although OFT cushions were present in AHF-Cre; Tie2-LacZ; SmoOEX−/− mutants, no OFT septation had occurred (Fig. 5G-H’).

To test the specificity of Hh signaling and where it was absent in these AHF-Cre knockouts, we also examined AHF-Cre; SmoOEX−/−; Ptc1fluor mutants. Loss of Ptc1fluor expression was detected in AHF

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<th>Cre used (gene deleted)</th>
<th>Domain of expression</th>
<th>Single OFT at E18.5</th>
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<th>Abnormal arch arteries at E10.5/E18.5</th>
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<td>Nkx2.5Cre (Shh or Smo)</td>
<td>PE, AHF, specified myocardium, endocardium</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Wnt1-Cre (Smo)</td>
<td>NCCs</td>
<td>+</td>
<td>–</td>
<td>+/-</td>
<td>–</td>
<td>+</td>
<td>?+</td>
</tr>
<tr>
<td>Me2C-Cre (Smo)</td>
<td>AHF</td>
<td>+</td>
<td>–</td>
<td>+/-</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TnT-Cre (Smo)</td>
<td>Specified myocardium</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Tie2-Cre (Smo)</td>
<td>Endocardium</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

+, present; –, not present; ND, no data; ?, possibly; PE, pharyngeal endoderm; AHF, anterior heart field; NCC, neural crest cell.
cells only, specifically those that are continuous with the wall of the OFT (Fig. 5I,J and data not shown). Together, these data indicate that, although there is a similar single-OFT phenotype and also a direct role for Hh signaling via \textit{Smo} in AHF cells, Hh is required in AHF-derived cells for a later septation step and not for AHF survival or OFT lengthening.

Finally, although E18.5 mutants showed defects in arch-artery patterning, arch-artery development appeared normal in \textit{AHF-Cre; Tie2-\lacZ; Smoflox/–} mutant embryos at E10.5 (8/8, data not shown).

Therefore, Hh signaling within the AHF is not required for early arch-artery development, but has a later role in arch-artery patterning/remodeling. These data also indicate that some of the defects observed in \textit{Nkx2.5\cre/+; Shh\floox/–} embryos cannot be explained by AHF or CNCC deficiencies alone.

The endoderm requires Hh signaling cell-autonomously for OFT lengthening

To determine whether cell-autonomous \textit{Smo} elimination from NCCs could also affect AHF development, we examined OFT lengthening. OFT length was indistinguishable between \textit{Wnt1-Cre; Smoflox/–} mutant embryos and somite-matched wild-type littermates when compared at E10.5 (data not shown). This indicates that the reduction of OFT length observed in both \textit{Shh\floox/–} and in \textit{Nkx2.5\cre/+; Shh\floox/–} embryos is not due to a direct cell-autonomous effect on either CNCCs or on the AHF.

What tissue is responsible? SHH can diffuse unknown distances and, therefore, several candidate tissues remain. In order to address this question, we first tested whether cells in the \textit{Nkx2.5} domain itself require \textit{Smo} cell-autonomously for OFT lengthening. To do this, we generated \textit{Nkx2.5\cre/+; Smoflox/–} mutants (see Materials and methods). Informatively, these mutants have a single OFT and an OFT shortening similar to \textit{Shh\floox/–} embryos, indicating either that a non-NCC and non-AHF cell population within the \textit{Nkx2.5} domain requires Hh signaling for OFT lengthening, or that a combination of AHF and NCC Hh signaling is required for OFT lengthening.

We have identified three significant differences between \textit{Nkx2.5\cre} and \textit{AHF-Cre} expression that may account for why \textit{Nkx2.5\cre/+; Smoflox/–} mutants, but not \textit{AHF-Cre; Smoflox/–} mutants, have a greatly shortened OFT. Besides the AHF, \textit{Nkx2.5\cre} is expressed in the pharyngeal endoderm, primary heart field and endocardium (a small population of endocardium appears positive in \textit{Nkx2.5\cre; R26R} embryos that is not positive in \textit{AHF-Cre; R26R} embryos) (see Fig. S1 in the supplementary material; data not shown). To test which of these three populations of cells is required for AHF survival and OFT lengthening, we generated two additional classes of \textit{Smo} mutants.

The first is a myocardial-specific (\textit{TnT-Cre}) mutant, in order to rule out the primary heart field. These mutants have normal OFT septation and length and a normal OFT phenotype at birth.
Similarly, mRNA levels for signal? to produce a second signal for OFT lengthening, what is that question that if SHH signals via Smo in the pharyngeal endoderm survival and OFT lengthening. These data therefore raise the fashion between E9.5 and E10.5 and that it is required for AHF pharyngeal endoderm signals to the endoderm in an autocrine lengthening (Table 1). We propose that SHH produced from myocardium or the endocardium alone does not affect OFT experiments demonstrate that deletion of Smo
in the supplementary material). Together, these genetic band was detected in some samples at these stages (see Fig. S2D)
low levels of expression because, at 35 cycles of RT-PCR, a faint
expression could be observed in Shh–/– embryos at E9.5. By wholemount in situ hybridization, we found that E9.5 Shh–/– embryos had Tbx1 endodermal expression patterns consistent with wild-type littermates (Fig. 6C). These data, combined with previous studies, suggest that SHH is not required for pharyngeal endodermal Tbx1 induction.

Two potential ligands expressed in the pharyngeal endoderm and implicated in OFT lengthening are Fgf8 and Bmp4. Fgf8 is expressed both within the AHF and in the pharyngeal endoderm, and has been implicated in AHF and NCC development (Ilagan et al., 2006; Park et al., 2006). We examined the in situ expression of Fgf8 in Nkx2.5Cre+; Shhflox–/– mutant embryos and Shh–/– mutant embryos at E10.5, and compared them to E10.5 controls (Fig. 6D-F): no significant expression differences were detected. To confirm this finding, we also examined the expression of Fgf8lacZ (Ilagan et al., 2006) in Shh–/– embryos and detected no significant differences in pharyngeal endoderm expression (Fig. 6G,H). However, there was a reduced number of β-galactosidase-positive cells in the developing OFT and right ventricle of Shh–/– mutants, which we attributed to the

The second class was an endocardial knockout using Tie2-Cre. Again, these mutants did not have OFT septation or shortening defects (see Fig. S2A-C in the supplementary material; data not shown).

Finally, even though we cannot detect Shh expression within the developing heart by mRNA in situ analysis, we tested whether low levels of SHH may be produced within the heart. We generated RNA from whole hearts between E9.5 and E12.5, and performed reverse transcriptase (RT)-PCR for Shh. We were unable to detect Shh expression at 30 cycles [we could not rule out low levels of expression because, at 35 cycles of RT-PCR, a faint band was detected in some samples at these stages (see Fig. S2D in the supplementary material)]. Together, these genetic experiments demonstrate that deletion of Smo in either the myocardium or the endocardium alone does not affect OFT lengthening (Table 1). We propose that SHH produced from pharyngeal endoderm signals to the endoderm in an autocrine fashion between E9.5 and E10.5 and that it is required for AHF survival and OFT lengthening. These data therefore raise the question that if SHH signals via SMO in the pharyngeal endoderm to produce a second signal for OFT lengthening, what is that signal?

\[ \text{Tbx1 is induced in both Shh}^{–/–} \text{ and Nkx2.5}^{\text{Cre}+} \text{, Shhflox– embryo}
\]

Tbx1 is a transcription factor within the DiGeorge syndrome critical-deletion region. Several studies have suggested that Tbx1 is regulated by SHH via forkhead box (Fox) transcription factors within the pharyngeal arches (Garg et al., 2001; Yamagishi et al., 2003). In situ hybridization was performed to determine whether Tbx1 is downregulated in Nkx2.5Cre+; Shhflox– mutant embryos at E9.5, prior to the appearance of the mutant pharyngeal arch phenotype. No differences in expression pattern or intensity of pharyngeal endoderm expression were observed between mutant and wild-type littermates (Fig. 6A,B). As original Tbx1 in situ studies focused on Shh–/– embryos at E10.5 and later stages (Garg et al., 2001), we sought to determine whether a difference in Tbx1 expression could be observed in Shh–/– embryos at E9.5. By whole-mount in situ hybridization, we found that E9.5 Shh–/– embryos had Tbx1 endodermal expression patterns consistent with wild-type littermates (Fig. 6C). These data, combined with previous studies, suggest that SHH is not required for pharyngeal endodermal Tbx1 induction.

Fig. 6. Endodermal Tbx1, Fgf8 and Bmp4 expressions appear normal in mutants. (A-C) In situ analysis for Tbx1 at E9.5 in Nkx2.5Cre+; Shhflox– (B) and Shh–/– (C) mutants appears normal within the pharyngeal endoderm when compared to control (A). (D-F) Similarly, mRNA levels for Fgf8 (D-F) and Bmp4 (I,J) also appear normal. (G-H) Fgf8lacZ expression is also grossly normal in Shh–/– pharyngeal endoderm (H) and OFT/right ventricle (H') when compared to controls (G,G').

Fig. 7. Model of Hh signaling during OFT development. Pharyngeal-derived (PE) SHH performs three main roles. First (1), SHH acts as a direct survival factor to CNCCs. Second (2), in AHF-derived myocardium, SHH acts via Smo to provide a patterning signal that is required for an unknown function in completing septation between E10.5 and E12.5. Third (3), SHH is a direct survival factor for the pharyngeal endoderm, and its loss results in the absence of a secondary signal ("X") necessary for AHF survival and for the lengthening of the OFT. Disruption of any of these processes either alone or in combination can result in a single-OFT phenotype, via different mechanisms. RV, right ventricle.
reduction of these tissues and not to a loss of expression (Fig. 6G’/H’). Finally, we did not detect a significant loss of Bmp4 expression within the pharyngeal endoderm (Fig. 6I,J). If there is a secondary endodermal signal induced by SHH for OFT lengthening, it is not likely to be mediated by transcriptional changes in either Fgf8 or Bmp4. In addition, these data demonstrate that the pharyngeal endoderm is specified and fairly normal at these stages in Shh−/− embryos, as evinced by the relatively normal expression of Bmp4, Fgf8 and Tbx1.

**DISCUSSION**

Previous studies have implicated Hh signaling in various aspects of heart development, from the early induction of cardiac progenitors and left-right axis determination to its direct roles in cardiac morphogenesis. This work indicates a direct role for endodermal SHH signaling on OFT and arch-artery patterning. Moreover, it demonstrates that the AHF, CNCCs and the pharyngeal endoderm all require Hh signaling directly. Previous work on OFT development has primarily focused on the signaling and genetic requirements of the AHF and CNCCs independently; here, we identify a common signaling pathway that affects both fields directly in their contribution to a common process, OFT septation. Additionally, this work confirms a role for AHF development in OFT septation separate from its previously defined role in OFT lengthening.

Endodermal Shh is required for cardiac development

This work reveals SHH as a key signaling molecule that mediates a long-suspected role for endodermal tissues in late cardiac formation (Fig. 7). In addition to the previously appreciated role for Shh in foregut development (Litingtung et al., 1998), these data are unique in demonstrating a direct requirement in heart development. Using Nkx2.5Cre to conditionally ablate Shh, we recapitulated the cardiac defects observed in Shh−/− embryos. Although the only recognized domain of Shh expression overlapping with Nkx2.5Cre is the pharyngeal endoderm, we cannot rule out additional, uncharacterized domains that may contribute to cardiac development, particularly within the developing atria. We are unable to detect significant Shh expression by RT-PCR within the heart between E9.5 and E10.5 (the timeframe in which the AHF and CNCCs appear to be dying), supporting our contention that the pharyngeal endoderm itself is the primary source of SHH. Eliminating Shh with the myocardial-specific TnT-Cre or in the AHF domain using AHF-Cre results in no OFT defects, supporting the idea that the endoderm is the crucial SHH signaling source. The development and use of an early, endodermal-specific Cre transgene would be useful to confirm this finding.

Hh signaling within the endoderm is required for AHF development

Previous work on Shh−/− mutants implicated the AHF as a direct target of SHH signaling. Although we confirmed that the AHF does require the signal in a cell-autonomous manner, we were surprised to find that it is required for OFT septation but not for OFT elongation. These data indicate that direct signaling to the AHF is not required for its survival or for OFT lengthening. Instead, we hypothesize that the pharyngeal endoderm itself has a requirement for SMO in an autocrine fashion, and that AHF survival is dependent on an unknown secondary signal from the endoderm. Neither Fgf8 nor Bmp4 appear to be this signal, because their expression does not appear to be altered in Shh−/− mutants.

In support of this hypothesis, we generated mutant mice of the genotype Nkx2.5Cre+; SmoLox/−, in which the AHF, the primary heart field and the pharyngeal endoderm all lack Hh receptiveness. These embryos also displayed increased levels of AHF cell death and significant OFT and right ventricle shortening (data not shown). Consistent with our results, the OFT shortening resulting from Isl1-Cre; Smo−/− embryos appears much milder compared with Shh−/− embryos (Lin et al., 2006). These authors report that Isl1-Cre is restricted to cardiac precursors and that Shh expression within pharyngeal endoderm is maintained. Any differences between our findings and those of Lin et al. could be due to different expression domains for Mef2C-AHF-Cre (AHF-Cre) and Isl1-Cre, or to the fact that Isl1-Cre is a heterozygous null. Additionally, the images provided for this particular line (Yang et al., 2006) suggest that Isl1-Cre includes tissues other than AHF cells (left ventricle, for example). Using the same Isl1-Cre as first reported by Cai et al. (Cai et al., 2003), we found that this transgenic line has incomplete and inconsistent expression within the AHF, which may explain why Mef2C-AHF-Cre; SmoLox/− embryos have differences in phenotype compared with the Isl1-Cre; SmoLox/− embryos. We therefore conclude that the AHF does not require Smo for the majority of OFT lengthening or for AHF survival. Rather, it appears that expression of Smo within the AHF is required for a latter stage of septation, between E10.5 and E12.5.

So what is the direct role of Smo within the AHF domain on OFT septation? Based on our analysis, we favor three possibilities. First, endocardial or myocardial Smo may be required for cushion fusion (Fig. 5G-H’ and data not shown). Second, Smo may be required for the process of OFT myocardialization. Another possible role is in maintaining the proper cushion positioning within the developing OFT (Fig. 5). Loss of Smo from the developing AHF results in loss of Pch1lacZ expression in the OFT myocardium (Fig. 5J). We previously hypothesized that this Pch1 expression may be required for the guidance of CNCCs (Washington Smoak et al., 2005), and it was also implicated in this role by Lin et al. (Lin et al., 2006). We do not favor this last possibility because we were unable to demonstrate consistent abnormal positioning of the CNCCs/cushions as seen in our reported results for Shh−/− embryos. In any case, the requirement of the AHF for Hh signaling occurs prior to the addition of this field to the OFT, because ablation of Smo from the myocardium of the OFT (TnT-Cre; SmoLox/−) did not recapitulate the septation defect. Together, these data implicate both an indirect (endodermally elicited survival) and a direct (late septation) role for pharyngeal endoderm SHH signaling on the derivatives of the AHF.

**CNCCs, but not other NCCs, directly require endodermal SHH for survival**

Another surprising result in these studies is that CNCCs require SHH directly for their survival. Our previous work indicated little, if any, expression of the downstream target Pch1lacZ in CNCCs. Our Wnt1-Cre; SmoLox/− results clearly indicate that, contrary to our earlier interpretations, there is a cell-autonomous requirement for Smo within CNCCs. Loss of Smo resulted in the loss of CNCCs, with reduced OFT cushions and septation defects later in development. This requirement for endodermally-derived SHH did not extend to other distal NCCs, such as cranial nerve or dorsal root ganglia derivatives, because these structures were relatively normal when compared with Shh−/− embryos. Presumably, these structures are patterned by the midline neural expression of Shh. Analysis of Pch1lacZ expression in Wnt1-Cre; SmoLox/− embryos found a small region of CNCCs that were negative for lacZ just ventral to the endoderm and near the aortic sac. Most probably, the loss of
signaling to this and earlier populations of CNCCs is responsible for the observed reduction in OFT cushions and for aortico-pulmonary septum defects. However, we cannot rule out additional regions of Hh signaling to the CNCCs that are not detectable using the Pchh<sup>1<sup>heterozygous</sup></sup> allele.

Previous work in chick has suggested that NCC ablation can affect OFT lengthening (Hutson et al., 2006; Yelbuz et al., 2002). However, we did not detect a significant change in OFT length or in AHF cell death in Wnt1-<sup>Cre</sup>; Smo<sup>heterozygous</sup> embryos, indicating that, if NCCs do influence OFT lengthening in the mouse, it is not via a Hh-dependent pathway. However, changes in CNCCs alone can result in late OFT defects that are independent of OFT lengthening defects. At this time, we cannot rule out that combinatorial loss of Smo from both the AHF and CNCCs would result in a shortened OFT.

**Similar phenotype, different mechanism?**

Prior work investigating the OFT defects of Shh<sup>−/−</sup> mutant mice, and the conditional ablation studies described here, illustrate deficits in both the AHF and CNCCs due to a loss of Hh signaling. Additionally, ablation of Smo from either the AHF or from the CNCCs also results in abnormal septation. Although most near-term mutants of both types demonstrate completely unseptated OFTs, several ‘escapers’ were observed that indicated that the etiologies of the septation defect are not identical in these mutant classes. Several embryos of the genotype Wnt1-<sup>Cre</sup>; Smo<sup>heterozygous</sup> were found to have partially-septated OFTs with hypoplastic pulmonary arteries, whereas several AHF-<sup>Cre</sup>; Smo<sup>heterozygous</sup> mutants demonstrated a septated OFT with a severely hypoplastic aorta (data not shown). Although early (E10.5) defects are detected in the cushions of Wnt1-<sup>Cre</sup>; Smo<sup>heterozygous</sup> embryos, OFT development was fairly normal in E10.5 AHF-<sup>Cre</sup>; Smo<sup>heterozygous</sup> mutants. These data are consistent with the idea that failure to sepaate the OFT results from early defects in OFT cushion formation, aortico-pulmonary septum development, late cushion fusion and, possibly, OFT lengthening, or some combination thereof.

In summary, we uncovered multiple roles for endodermal Hh in OFT development. Use of conditional genetics allowed us to reveal the different effects of signal loss to the AHF, to CNCCs and, by process of elimination, to the endoderm. Differing genetic perturbations resulted in similar term defects, stressing the process of elimination, to the endoderm. Differing genetic perturbations resulted in similar term defects, stressing the process of elimination, to the endoderm. Differing genetic perturbations resulted in similar term defects, stressing the process of elimination, to the endoderm.


