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Sonic hedgehog is required for cardiac outflow tract and neural crest cell development

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Abstract

The Hedgehog signaling pathway is critical for a significant number of developmental patterning events. In this study, we focus on the defects in pharyngeal arch and cardiovascular patterning present in *Sonic hedgehog* (*Shh*) null mouse embryos. Our data indicate that, in the absence of *Shh*, there is general failure of the pharyngeal arch development leading to cardiac and craniofacial defects. The cardiac phenotype results from arch artery and outflow tract patterning defects, as well as abnormal development of migratory neural crest cells (NCCs). The constellation of cardiovascular defects resembles a severe form of the human birth defect syndrome tetralogy of Fallot with complete pulmonary artery atresia. Previous studies have demonstrated a role for *Shh* in NCC survival and proliferation at later stages of development. Our data suggest that SHH signaling does not act directly on NCCs as a survival factor, but rather acts to restrict the domains that NCCs can populate during early stages (e8.5–10.5) of cardiovascular and craniofacial development.

Keywords: Cardiac; Neural crest; Migration; Pulmonary atresia; Tetralogy of Fallot; Outflow tract; Septation; Anterior heart field; Bmp4; Repulsion

Introduction

Shh is one of three mammalian gene homologues of the extracellular signaling molecule, Hedgehog, first described as a segment polarity gene product in *Drosophila* (Nusslein-Volhard and Wieschaus, 1980). Previous loss-of-function studies in the mouse have demonstrated that *Shh* is required for patterning of several organs such as the nervous system, limbs, lungs, and foregut (Chiang et al., 1996; Litingtung et al., 1998, 2002). Mouse embryos that are homozygous null for *Shh* ($Shh^{-/-}$) are also known to have pharyngeal arch and cardiac defects, but the etiology of these defects has yet to be described in detail (Izraeli et al., 1999; Kim et al., 2001; Meyers and Martin, 1999; Tsukui et al., 1999).

NCCs are ecto-mesenchymal cells that migrate from the junction of the surface ectoderm and neuroectoderm along the dorsal neural tube to populate numerous structures throughout the embryo. Cranial NCCs arise from the forebrain and hindbrain regions to populate craniofacial structures, as well as pharyngeal arches one through three, giving rise to the maxilla, mandible, and other structures of the neck and face (reviewed in Le Douarin and Dupin, 2003; Santagati and Rijli, 2003). Cardiac NCCs populate the third, fourth, and sixth pharyngeal arches, as well as the outflow tract (OT) of the heart and contribute to the smooth muscle of arch arteries in both chick (Kuratani and Kirby, 1992; Nishibatake et al., 1987) and mouse (Jiang et al., 2000). Ablation and genetic studies have shown that NCC production, delamination, directed migration, and survival

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are critical for cardiovascular patterning (reviewed in Gitler et al., 2002; Hutson and Kirby, 2003; Maschhoff and Baldwin, 2000). Recent evidence suggests that SHH function is required for NCC survival in chick (Ahlgren and Bronner-Fraser, 1999; Ahlgren et al., 2002) and mouse (Jeong et al., 2004). In this last study, a direct requirement for Hedgehog signals in the development of murine craniofacial NCCs was demonstrated by genetically removing the *Smoothened (Smo)* receptor from migratory NCCs. However, this effect on NCCs appeared after mid-gestation and was not as severe as that demonstrated by functional blocking studies in chick embryos or by the phenotype of $Shh^{-/-}$ mouse embryos, suggesting an additional earlier cell autonomous role for *Shh* in NCC development.

In this study, we sought to characterize the early (e9.5–11.5) cardiovascular and pharyngeal arch development of $Shh^{-/-}$ mouse embryos to better understand how *Shh* expression affects NCC development and these tissues. Our data indicate that *Shh* is required for both NCC and pharyngeal endoderm survival and support *Shh* as a candidate gene defective in patients with tetralogy of Fallot, as well as a possible modifying locus for the 22q11deletion syndromes. Furthermore, mislocalization of NCC derivatives in $Shh^{-/-}$ embryos supports a model where SHH signaling is required for NCC guidance cues.

Materials and methods

Experimental animals

Mouse strains were maintained on an outbred ICR background unless otherwise indicated. This background was chosen because of the multiple transgenes used and the late-term survival with consistent cardiovascular defects. In addition, cardiac looping reversals were absent in $Shh^{-/-}$ embryos on this outbred background, simplifying the analysis of cardiac development at later stages and consistent with previous studies (Izraeli et al., 1999; Tsukui et al., 1999). $Shh^{-/-}$ embryos were generated by intercrossing $Shh^{+/-}$ (Shh^{tm1Chg}) males and females (Chiang et al., 1996). Noon on the day of vaginal plug was designated embryonic (e) day 0.5. More accurate staging was then determined by somite number. Genotyping was by phenotype (late stages) or PCR (early stages) as described (Chiang et al., 1996). All embryos were dissected in either DEPC (diethylpolycarbonate)-treated phosphate-buffered saline (PBS) or PBS with 0.1%Triton X-100 (PBT). Fixation was at 4°C overnight in 4% paraformaldehyde (PFA) unless otherwise specified.

Cardiovascular system visualization in $Shh^{-/-}$ embryos

$Shh^{+/-}$ males also transgenic for the *Tie2-LacZ* transgene (Tg(TIE2-*lac*Z)182Sato/J), in which *LacZ* is controlled by a *Tie2* endothelial-cell-specific promoter element (Schlaeger et al., 1997), were crossed to $Shh^{+/-}$ females to visualize endothelial structures in $Shh^{-/-}$ embryos. The chest wall/pericardial sac was opened by blunt dissection to better expose the heart and vessels. Embryos were then fixed and washed. β-galactosidase (β-Gal) activity was detected by X-Gal staining. Following staining, embryos were washed, post fixed (4% PFA), and either cleared in 50% glycerol in PBS for whole mount visualization or embedded in paraffin for sectioning using standard procedures (Hogan, 1994).

Generation of $Shh^{-/-}$;P0-Cre;R26R embryos

In order to lineage trace NCCs in $Shh^{-/-}$ embryos, we generated studs that were $Shh^{+/-}$ and transgenic for *P0-Cre* (tg(P0-Cre)1Ky), which expresses the Cre transgene in migratory NCC precursors (Abu-Issa et al., 2002; Yamauchi et al., 1999). These transgenic studs were crossed with $Shh^{+/-}$ female mice that were also transgenic for the CRE reporter, *R26R* ($Gt(Rosa)26Sor^{tm1Sor}$), which irreversibly expresses *LacZ* in cells that have undergone recombination by the CRE protein (Soriano, 1999). This method allowed us to effectively compare lineage-marked NCCs in $Shh^{-/-}$ and control embryos. *Wnt1-Cre* (Tg(Wnt1-cre)11Rth) males (Danielian et al., 1998) were crossed to R26R females to compare to *P0-Cre* pattern. Embryos were fixed, stained for β-Gal activity, and cleared as described above.

$Ptch1^{LacZ}$

$Ptch1^{LacZ}$, ($Ptch^{tm1Mps}$) with *LacZ* inserted into the *Ptch1* locus, is heterozygous null for *Ptch1* function (Goodrich et al., 1997). Embryos expressing $Ptch1^{LacZ}$ were generated on $Shh^{+/-}$ and $Shh^{-/-}$ backgrounds by generating $Shh^{+/-}$;$Ptch1^{LacZ/+}$ males that were crossed to $Shh^{+/-}$ females.

In situ hybridization

Whole-mount mRNA in situ hybridization was performed as described previously (Neubuser et al., 1997) using digoxygenin-labeled antisense riboprobes constructed from linearized plasmids. Riboprobes were constructed for *Pax1*, *Ap2α*, *CrabP1*, *Bmp4*, *Sox9*, and *Twist.* Embryos were examined at stages indicated within text and figures. All in situ hybridization results were confirmed in at least three $Shh^{-/-}$ mutant embryos and three control embryos that were treated identically.

Whole-mount immunohistochemistry

Immunohistochemistry (IHC) was performed on whole embryos that were fixed and washed in PBS with 0.1%Triton X-100 (PBT). Embryos were placed in blocking solution (BS = 3% bovine serum albumin and 10% sheep or horse serum in PBT) for 1 h then incubated in primary antibody in BS at 4°C overnight. Control embryos were incubated similarly but not exposed to primary antibody.