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Smad signaling in the neural crest regulates cardiac outflow tract remodeling through cell autonomous and non-cell autonomous effects

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Abstract

Neural crest cells (NCCs) are indispensable for the development of the cardiac outflow tract (OFT). Here, we show that mice lacking *Smad4* in NCCs have persistent truncus arteriosus (PTA), severe OFT cushion hypoplasia, defective OFT elongation, and mispositioning of the OFT. Cardiac NCCs lacking *Smad4* have increased apoptosis, apparently due to decreased *Msx1/2* expression. This contributes to the reduction of NCCs in the OFT. Unexpectedly, mutants have MF20-expressing cardiomyocytes in the splanchnic mesoderm within the second heart field (SHF). This may result from abnormal differentiation or defective recruitment of differentiating SHF cells into OFT. Alterations in Bmp4, Sema3C, and PlexinA2 signals in the mutant OFT, SHF, and NCCs, disrupt the communications among different cell populations. Such disruptions can further affect the recruitment of NCCs into the OFT mesenchyme, causing severe OFT cushion hypoplasia and OFT septation failure. Furthermore, these NCCs have drastically reduced levels of *Id*s and *MT1-MMP*, affecting the positioning and remodeling of the OFT. Thus, Smad-signaling in cardiac NCCs has cell autonomous effects on their survival and non-cell autonomous effects on coordinating the movement of multiple cell lineages in the positioning and the remodeling of the OFT.

Keywords: Smad; Neural crest; Outflow tract; Secondary heart field; Congenital heart diseases

Introduction

Nearly 1% of live births in humans have congenital heart diseases (CHDs) with about a third of these CHDs affecting the outflow tract (OFT) and its derivatives (Thom et al., 2006). Many of these defects result from abnormalities in the incorporation of distinct cellular lineages into the cardiac outflow tract, which requires precise timing and cell–cell communications. Cardiac development initiates with progenitor cells in the two bilaterally regions of the lateral mesoderm, namely the primary or first heart field (FHF) (Srivastava, 2006). Recent studies have provided convincing evidence that a second heart field (SHF), comprised of cells in pharyngeal and splanchnic mesoderm anterior and medial to the FHF, contributes myocardium to the OFT and right ventricle (Abu-Issa et al., 2004; Black, 2007; Eisenberg and Markwald, 2004; Kelly, 2005; Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). The recruitment of the SHF mesoderm into the OFT and right ventricle requires precise control of gene expression and interactions among different cell lineages (Black, 2007; Kelly, 2005).

The involvement of cardiac neural crest (CNC), a subgroup of neural crest cells (NCCs), in OFT development has been well documented by CNC ablation studies in chick as well as by genetic studies in mice and other model organisms (Brown and Baldwin, 2006; Hutson and Kirby, 2007; Stoller and Epstein,

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2005). After populating pharyngeal arch (PA) 3, 4, and 6, NCCs migrate into the OFT as early as E9.5 in mice (Jiang et al., 2000), and mediate the remodeling of the OFT into the pulmoõnary trunk and the ascending aorta. NCC ablation or the deletion of genes in NCCs results in a spectrum of defects, including pharyngeal arch patterning defects, double outlet of right ventricle, tetralogy of Fallot, and persistent truncus arteriosus (PTA) in which the aorticopulmonary septum fails to form (Brown and Baldwin, 2006; Hutson and Kirby, 2007; Stoller and Epstein, 2005). In addition, cell ablation studies in chick have shown that CNC is necessary for the addition of the myocardium from the SHF to the OFT and the caudal movement of the OFT (Waldo et al., 2001, 2005a; Yelbuz et al., 2002).

Smad transcription factors are at the core of the transcriptional responses in the transforming growth factor β (Tgfβ) signaling pathway. Tgfβ superfamily members are structurally related secreted cytokines that include Tgfβ isoforms, activins, bone morphogenetic proteins (BMPs), and others. The binding of ligands to their receptors leads to the phosphorylation of the receptor-regulated Smads (R-Smads). The phosphorylated R-Smads complex with the common Smad, Smad4, before translocating into the nucleus to regulate the transcription of the target genes (Massague et al., 2005). Signals from different Tgfβ ligands and receptors diverge and converge on different sets of R-Smads, producing distinct and sometimes opposing outcomes. The Tgfβ pathway is one of the most versatile cytokine signaling pathways in metazoans, regulating biological processes from cell division to the patterning of the organism (Massague et al., 2005). Previous studies have shown that a spectrum of OFT and pharyngeal arch artery (PAA) defects can result from germline deletions and NCC-specific inactivation of a number of Tgfβ superfamily ligands and receptors (Choudhary et al., 2006; Gaussin et al., 2005; Kaartinen et al., 2004; Kim et al., 2001; Ma et al., 2005; Molin et al., 2004; Sanford et al., 1997; Stottmann et al., 2004; van Wijk et al., 2007; Wang et al., 2006; Wurdak et al., 2005). Recent advances have revealed that besides the kinase activities of the Tgfβ type I receptors, other kinases, such as MAPK, CDK, CamK II, and GRK2, can also phosphorylate Smads (Massague et al., 2005; Xu, 2006). In addition, Smad-independent Tgfβ responses have been reported in *Smad*-deficient cell lines and animal models (Massague et al., 2005; Xu, 2006). Signal transduction from Tgfβ ligands and receptors to Smads is complicated and nonlinear. Thus, to better understand the mechanism by which NCCs regulate cardiac development, it is necessary to investigate the precise role of Smad signaling in this context in addition to the studies of the Tgfβ ligands and receptors.

In this study, we have found that the absence of *Smad4* in NCCs causes a wide spectrum of OFT defects, including OFT cushion hypoplasia, OFT septation defect, OFT elongation defect, and OFT alignment defect. We have observed increased apoptosis in the mutant cardiac NCCs, suggesting an indispensable cell autonomous role of Smad signaling in NCC survival. Furthermore, mice with NCCs lacking *Smad4* have alterations in the expression of *Bmp4*, *Sema3C*, and *PlexinA2* and other molecules in the OFT myocardium, SHF mesoderm, or NCCs, reflecting disrupted communications among these cell lineages. These defects lead to disruptions in NCC recruitment to OFT cushion, contributing to the observed OFT cushion hypoplasia. We have also observed abnormal presence of MF20-expressing cardiomyocytes in the splanchnic mesoderm within the SHF and a concurrent failure in the OFT caudal movement. The ectopic presence of MF20-expressing cells in the SHF may be a result of defective recruitment of mesodermal cells from the SHF to OFT myocardium, or abnormal differentiation due to the altered signaling between the *Smad4*-deficient NCCs and the SHF mesodermal cells. Our data also show that cardiac NCCs lacking *Smad4* have greatly reduced expression of *Inhibitor of differentiation* (*Id*) genes and *Membrane type-1 matrix metalloproteinase* (*MT1-MMP*), both of which are critical for tissue remodeling. Thus the reduction of *Ids* and *MT1-MMP* may provide the basis for the failure of OFT caudal movement in the mutants that involves extensive tissue remodeling. This study reveals both a direct role of Smad signaling on NCC survival and indirect effects, through communications with other cell lineages, in orchestrating gene expression and the integration of multiple cell lineages for the remodeling of the OFT.

Materials and methods

Mouse (Mus musculus) strains and sample collection

The generation of the floxed-*Smad4* allele was described previously (Yang et al., 2002). Mice carrying this floxed-*Smad4* allele were crossed with the *Wnt1Cre* transgenic mice to produce *Wnt1Cre;Smad4*$^{floxed/floxed}$ embryos that would have homozygous deletion of *Smad4* in NCCs. *Wnt1Cre;Smad4*$^{floxed/floxed}$ embryos are designated as mutants in this study. Their littermates with no homozygous deletion of *Smad4* in any cells are considered controls. To fate map the NCCs, the *Rosa26RLacZ* transgene was introduced into the *Wnt1Cre;floxed-Smad4* mice. Direct comparison was made between littermates. All experiments were repeated at least three times.

Histological analysis

For histological analyses, embryos were fixed with 4% paraformaldehyde and embedded in paraffin. Sections of 7 μm were collected and stained following standard protocol. For immunohistochemistry, sections were stained with a rabbit polyclonal anti-beta galactosidase antibody (MP Biomedical, 7A6, 1:1000) and a mouse monoclonal anti-MF20 (Developmental study hybridoma bank, 1:50). Appropriate AlexaFlour488 or 555-conjugated secondary antibodies (Molecular Probe, 1:1000) were used to detect the corresponding primary antibodies. Whole-mount immunostaining was carried out with an antibody for Pecam-1 (BD Pharmingen, CD31, 1:50) as described (Graef et al., 2001). 5-Bromo-4-chloro-3-indolyl-D-galactoside (Xgal) whole-mount staining of embryos was performed as described (Chang et al., 2004).

Proliferation and apoptosis

BrdU was injected (i.p.) into pregnant mice 1.5 h before embryo harvest and was detected by a mouse monoclonal anti-BrdU antibody (Developmental study hybridoma bank, 1:200). Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) analysis was performed on paraffin-embedded sections by using the ApopTag plus peroxidase *in situ* apoptosis detection kit (Roche, Nutley, NJ). Proliferation index is presented as the average number of BrdU positive cells per 100 cells counted. NCC proliferation index was determined by counting about 200 NCCs in the PA-OFT region for each sample ($n=6$ for each group). About 30–60 cells were counted in distal region of OFT myocardium in each mouse for OFT myocardium proliferation index ($n=9$ for each group). Exactly 30 cells in splanchnic mesoderm caudal to the OFT