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Smad4 is required to regulate the fate of cranial neural crest cells

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Abstract

Smad4 is the central mediator for TGF-β/BMP signals, which are involved in regulating cranial neural crest (CNC) cell formation, migration, proliferation and fate determination. It is unclear whether TGF-β/BMP signals utilize Smad-dependent or -independent pathways to control the development of CNC cells. To investigate the functional significance of Smad4 in regulating CNC cells, we generated mice with neural crest specific inactivation of the Smad4 gene. Our study shows that Smad4 is not required for the migration of CNC cells, but is required in neural crest cells for the development of the cardiac outflow tract. Smad4 is essential in mediating BMP signaling in the CNC-derived ectomesenchyme during early stages of tooth development because conditional inactivation of Smad4 in neural crest derived cells results in incisor and molar development arrested at the dental lamina stage. Furthermore, Smad-mediated TGF-β/BMP signaling controls the homeobox gene patterning of oral/aboral and proximal/distal domains within the first branchial arch. At the cellular level, a Smad4-mediated downstream target gene(s) is required for the survival of CNC cells in the proximal domain of the first branchial arch. Smad4 mutant mice show underdevelopment of the first branchial arch and midline fusion defects. Taken together, our data show that TGF-β/BMP signals rely on Smad-dependent pathways in the ectomesenchyme to mediate epithelial–mesenchymal interactions that control craniofacial organogenesis.

Keywords: TGF-β; Smad4; Tooth; Cranial neural crest cells

Introduction

The TGF-β superfamily is a large group of extracellular growth factors mediating a wide range of biological activities, including cell proliferation, differentiation, extracellular matrix formation, and induction of homeobox genes, suggesting that TGF-β signaling is important for pattern formation during embryogenesis (Chai et al., 2003). At the cell surface, the TGF-β ligand binds a transmembrane receptor serine/threonine kinase complex, consisting of a type I and a type II receptor, and induces transphosphorylation of the GS segments in the type I receptor by the type II receptor kinases (Derynck and Zhang, 2003). Most mammalian cells express different members of this receptor family, some of which may be shared by different TGF-β ligands (Massague, 2000).

The activated type I receptor phosphorylates Smad proteins in the cytoplasm. The type I receptors for TGF-β, activin, nodal and myostatin (ALK 4, 5 and 7) phosphorylate Smads 2 and 3, whereas the BMP and AMH type I receptors (ALK 1, 2, 3, and 6) phosphorylate Smads 1, 5 and 8 (Massague and Gomis, 2006). These receptor-activated Smads (R-Smads) dissociate from the type I receptor and then oligomerize with a common partner, Smad4 (DPC4). Activated Smad complexes move into the nucleus, where they regulate transcription of target genes (Derynck and Zhang, 2003). Although ubiquitously involved in Smad-mediated transcription, Smad4 is not essential for TGF-β responses: some TGF-β responses occur in the absence of Smad4 and some Smad4-deficient cell lines have a limited responsiveness to TGF-β. TGF-β has been shown to activate other mediators such as the mitogen-activated protein kinases

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(MAPKs), ERK, Jun N-terminal kinase (JNK), p38, PI3K kinases, PP2A phosphatases and Rho family members (Derynck and Zhang, 2003). Moreover, some genetic studies suggest that certain Smad-dependent effects may not require Smad4 (Sirard et al., 1998; Wisotzkey et al., 1998; Subramanian et al., 2004).

Neural crest cells are a pluripotent cell population generated transiently along almost the entire vertebrate axis at the interface between the surface ectoderm and the neural plate of the embryo, a region that is referred to as the neural plate border. During this induction process, neuroepithelial cells undergo an epithelial to mesenchymal transformation at which point they delaminate and begin to emigrate from the neural tube. Murine neural crest cell formation and migration commences at approximately the 4–5 somite stage in the region of the caudal midbrain and rostral hindbrain and proceeds simultaneously as a wave rostrally towards the forebrain and caudally towards the tail. Murine cranial neural crest cells contribute to the formation of the condensed dental mesenchyme, dental papilla, odontoblasts dentine matrix, pulp, cementum, periodontal ligaments, chondrocytes in Meckel's cartilage, mandible, the articulating disk of the temporomandibular joint and the branchial arch nerve ganglia (Chai et al., 2000; Trainor, 2005).

During early mouse craniofacial development, TGF-β subtypes are expressed in cranial neural crest-derived mesenchyme during critical epithelial–mesenchymal interactions related to the formation of various organs (Lumsden, 1984; Hall, 1992; Lumsden and Krumlauf, 1996). Although *Smad4* is not always essential for TGF-β responses, it is a central mediator of TGF-β signals. Unfortunately, targeted inactivation of *Smad4* results in early embryonic lethality in mice, making it difficult to investigate the function of Smad4. These *Smad4* mutant mice are arrested at E7.5–E8.5 by growth retardation, an abnormal visceral endoderm and the failure of mesoderm formation (Sirard et al., 1998).

To understand the roles of Smad4-mediated TGF-β signaling in neural crest cells, we specifically deleted the *Smad4* gene using the *Wnt1-Cre* recombination system. The development of embryos lacking *Smad4* in neural crest cells is arrested at E11.5–E12.5 likely due to heart failure. We first detected defects in *Wnt1-Cre;Smad4*$^{fl/fl}$ embryos at E10.5. These phenotypes included under-development of the first branchial arch and failure of fusion not only in the middle of the frontonasal process but also in the middle of the mandibular process. Defects in lateral development of the first branchial arch were more severe than those in anterior–posterior development in mutant embryos. Tooth development was also affected and arrested at the dental lamina stage. We also found alterations in ectomesenchyme patterning and increased numbers of apoptotic cells in the first branchial arch in *Wnt1-Cre;Smad4*$^{fl/fl}$ embryos. Our results suggest that *Smad4* plays a critical role in cranial neural crest (CNC) development.

Materials and methods

Generation of Wnt1-Cre;R26R and Wnt1-Cre;Smad4$^{fl/fl}$ *mutant*

The *Wnt1-Cre* transgenic line (Danielian et al., 1998), *ROSA26* conditional reporter (*R26R*) transgene (Soriano, 1999) and conditional *Smad4/Dpc4* allele (Yang et al., 2002) have been previously described. Mating *Wnt1-Cre* with *R26R* mice generated *Wnt1-Cre;R26R* mice in which neural crest derived cell was permanently marked with β-galactosidase expression during embryogenesis (Chai et al., 2000). *Wnt1-Cre;Smad4*$^{fl/+}$ male mice were crossed with *Smad4*$^{fl/fl}$ female mice to generate *Wnt1-Cre;Smad4*$^{fl/fl}$ embryos in which *Smad4* was deleted in neural crest cells. *K14-Cre;Smad4*$^{fl/fl}$ mutant was also generated by crossing *K14-Cre;Smad4*$^{fl/+}$ male mice and *Smad4*$^{fl/fl}$ female mice.

Kidney capsule transplantation

The mandibular process of the first branchial arch at E10.5 was dissected from embryos. The explants were placed on Millipore filters supported by metal grid in a tissue culture dish and cultured for 1 day during genotyping. The host mouse was anesthetized using pentobarbital (0.5 mg/10 g body weight) and the explants were grafted under the kidney capsule according to standard procedure. Six days and 4 weeks after transplantation, the host mice were sacrificed and the grafts were processed for histological analysis. All procedures were done according to IACUC approved protocols.

Whole mount X-gal staining

Whole embryos (E10.5) were stained for β-galactosidase activity according to the standard procedures. Embryos were fixed for 1 h on ice in 4% paraformaldehyde in PBS (phosphate buffered saline) and washed in PBS. Embryos were stained several hours at 37 °C using X-gal staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.4% X-gal in PBS) and washed in PBS.

Organ culture of wild type and Wnt1-Cre;Smad4$^{fl/fl}$ *mutant mandibular process of the 1st branchial arch explants*

Wild type and *Wnt1-Cre;Smad4*$^{fl/fl}$ mutant mandibular process of the first branchial arch from E10.5 embryos was microdissected and cultured in serumless, chemically defined medium. After 2 days culture, the explants were fixed in 4% paraformaldehyde in PBS, processed for paraffin sections, and stained with hematoxylin and eosin.

Analysis of cell proliferation and apoptosis

BrdU (5-bromo-2′-deoxyuridine, Sigma) solution was injected intraperitonially with 100 μg/g body weight in E10.5 and E11.5 pregnant mice. One hour after injection, mice were sacrificed and embryos were fixed in 4% paraformaldehyde in PBS and processed. Detection of BrdU labeled cells was performed by using a BrdU Labeling and Detection kit by following manufacturer's protocol (Zymed). TUNEL assay was performed using In Situ Cell Death Detection (fluorescent) kit (Roche Molecular Biochemicals) by following the manufacturer's protocol.

Immunohistochemistry

Sectioned immunohistochemistry was done with an Immunostaining Kit (Zymed) according to manufacturer's directions. Anti-Smad4 (Santa Cruz Biotechnology) antibody was used for this experiment. The slides were counterstained with Hematoxylin. Whole mount immunohistochemistry was performed according to Mark et al. (1993), using the 2H3 anti-neurofilament monoclonal antibody (1:500, Developmental Studies Hybridoma Bank).

Whole-mount in situ hybridization and histological analysis

Whole-mount in situ hybridization was performed using standard procedures. Embryos were harvested and fixed in 4% paraformaldehyde overnight at 4 °C. Digoxigenin (Roche)-labeled anti-sense RNA probes were used for this experiment. For histological analysis, embryos were fixed in 4% paraformaldehyde and processed into serial paraffin sections using routine procedures and stained with Hematoxylin and Eosin.