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The canonical Wnt signaling activator, R-spondin2, regulates craniofacial patterning and morphogenesis within the branchial arch through ectodermal–mesenchymal interaction

Yong-Ri Jin, Taryn J. Turcotte, Alison L. Crocker, Xiang Hua Han, Jeong Kyo Yoon *

Center for Molecular Medicine, Maine Medical Center Research Institute, Maine Medical Center, 81 Research Drive, Scarborough, ME 04074, USA

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ABSTRACT

R-spondins are a recently characterized family of secreted proteins that activate Wnt/β-catenin signaling. Herein, we determine *R-spondin2* (*Rspo2*) function in craniofacial development in mice. Mice lacking a functional *Rspo2* gene exhibit craniofacial abnormalities such as mandibular hypoplasia, maxillary and mandibular skeletal deformation, and cleft palate. We found that loss of the mouse *Rspo2* gene significantly disrupted Wnt/β-catenin signaling and gene expression within the first branchial arch (BA1). Rspo2, which is normally expressed in BA1 mesenchymal cells, regulates gene expression through a unique ectoderm–mesenchyme interaction loop. The Rspo2 protein, potentially in combination with ectoderm-derived Wnt ligands, up-regulates *Msx1* and *Msx2* expression within mesenchymal cells. In contrast, Rspo2 regulates expression of the *Dlx5*, *Dlx6*, and *Hand2* genes in mesenchymal cells via inducing expression of their upstream activator, Endothelin1 (Edn1), within ectodermal cells. Loss of *Rspo2* also causes increased cell apoptosis, especially within the aboral (or caudal) domain of the BA1, resulting in hypoplasia of the BA1. Severely reduced expression of Fgf8, a survival factor for mesenchymal cells, in the ectoderm of *Rspo2*$^{-/-}$ embryos is likely responsible for increased cell apoptosis. Additionally, we found that the cleft palate in *Rspo2*$^{-/-}$ mice is not associated with defects intrinsic to the palatal shelves. A possible cause of cleft palate is a delay of proper palatal shelf elevation that may result from the small mandible and a failure of lowering the tongue. Thus, our study identifies Rspo2 as a mesenchyme-derived factor that plays critical roles in regulating BA1 patterning and morphogenesis through ectodermal–mesenchymal interaction and a novel genetic factor for cleft palate.

Introduction

Craniofacial morphogenesis begins when cranial neural crest cells delaminate from the dorsal neural tube and migrate ventrolaterally to form the ectomesenchyme of the facial primordia including the frontonasal prominence and branchial arches (Kontges and Lumsden, 1996; Lumsden et al., 1991; Osumi-Yamashita et al., 1994; Serbedzija et al., 1992). In the mouse embryo, the first branchial arch (BA1) becomes apparent at E8.0–8.5 as small swellings on the side of the developing head, and the BA1 increases rapidly in size as cranial neural crest cells migrate into and proliferate within the arch (Kaufman, 1992). While neural crest-derived cells are localized immediately subadjacent to the covering surface ectoderm, myogenic cells derived from the cranial paraxial mesoderm are more centrally located (Trainor and Tam, 1995; Trainor et al., 1994). The BA1 further divides into maxillary and mandibular components to give rise to most skeletal elements of the jaw, teeth, lateral skull wall, palate, and middle ear, as well as part of the tongue and other soft tissue derivatives (Chai et al., 2000; Chai and Maxson, 2006; Helms and Schneider, 2003).

Previous experimental embryological studies suggest that development of the BA1 mesenchyme is controlled by ectoderm-derived signals that are responsible for cell proliferation, survival, patterning, and differentiation (Cobourne and Sharpe, 2003; Francis-West et al., 1994; Helms et al., 2005; Richman and Tickle, 1989; Trainor and Krumlauf, 2001). Bone morphogenetic proteins (Bmps), fibroblast growth factors (Fgfs), and endothelin1 (Edn1) include the signaling molecules expressed in the ectoderm of BA1 with a specific spatial pattern (Chai and Maxson, 2006; Francis-West et al., 1998; Francis-West et al., 1994; Kurihara et al., 1994; Trumpp et al., 1999; Tucker et al., 1999a; Tucker et al., 1998). These signal cues trigger positive or negative regulation of a diverse set of genes encoding transcription factors, such as Dlx and Msx homeobox factors, in mesenchymal cells that control patterning and morphogenesis of BA1-derived skeletal structures (Depew et al., 2002; Depew et al., 2005; Satokata et al., 2000; Satokata and Maas, 1994).

Strong canonical Wnt/β-catenin signaling activity is detected within various craniofacial regions including the BA1 (Brugmann

* Corresponding author.
E-mail address: yoonje@mmc.org (J.K. Yoon).

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et al., 2007) in canonical Wnt signaling reporter transgenic mice such as *TopGAL* (DasGupta and Fuchs, 1999) or *BATGAL* (Maretto et al., 2003). In the BA1, Wnt/β-catenin signaling activity is visible as early as E8.5 and then is specifically detected within most of the maxillary BA1 and a medial portion of the mandibular BA1 through E9.5–E11.5 (Brugmann et al., 2007). In support of Wnt/β-catenin signaling in BA1 patterning and morphogenesis, genetic deletion of the mouse *Lrp6* gene encoding the co-receptor of canonical Wnt/β-catenin signaling resulted in a systematic failure of proper development of craniofacial structures (Pinson et al., 2000; Song et al., 2009). This failure in the *Lrp6* mutant is associated with severe ablation of Wnt/β-catenin signaling activity and disrupted regulation of *Msx1* and *Msx2* gene expression in craniofacial primordial structures (Song et al., 2009). Several Wnt ligand genes such as *Wnt3*, *Wnt5a*, and *Wnt9b* are expressed in developing mouse craniofacial structures (Geetha-Loganathan et al., 2009; Summerhurst et al., 2008) and also are associated with cleft palate/lip phenotypes in human and mice (Blanton et al., 2004; Chiquet et al., 2008; He et al., 2008; Juriloff et al., 2006; Niemann et al., 2004).

R-spondins (Rspo) are a novel class of cysteine-rich secreted proteins that are structurally unrelated to the Wnt proteins and activate the β-catenin signaling pathway (Binnerts et al., 2007; Kamata et al., 2004; Kazanskaya et al., 2004; Kim et al., 2005a; Kim et al., 2008; Nam et al., 2006; Wei et al., 2007). We found that *Rspo2*, a member of the *Rspo* gene family, is expressed in mesenchymal cells of BA1, BA2, and the nasal processes in mouse embryos (Nam et al., 2007b). Mice lacking a functional *Rspo2* gene developed various developmental defects including cleft palate (Aoki et al., 2008; Nam et al., 2007a; Yamada et al., 2009).

In this study, we report a systematic analysis of craniofacial development in *Rspo2* null mice. We found that Rspo2 is a key regulator of canonical Wnt/β-catenin signaling and downstream gene expression in both ectoderm and mesenchyme in the BA1. Ex vivo gain-of-function studies revealed a unique role of Rspo2 as a mesenchyme-derived regulatory factor, in the signaling interaction between ectoderm and mesenchyme of the BA1.

Materials and methods

Animals

Mice carrying the *Rspo2* null (*Rspo2*$^{-/-}$) allele was previously described (Nam et al., 2007a) and maintained on a mixed C57BL/6×129 background. A second mutant allele of the *Rspo2* (*Rspo2*$^{\Delta ZN}$) gene in which the *LacZ* and *neo* gene cassettes were removed by Flp-dependent recombination was generated as described in supplementary materials (Fig. S1). *Rosa26*$^{+/Flp}$ and *TopGAL* mice were obtained from The Jackson Laboratory (Bar Harbor, ME). *Lrp6*$^{+/-}$ mice (Pinson et al., 2000) were kindly provided by Dr. William Skarnes (Sanger Institute, Cambridge, U.K.) and maintained on a mixed C57BL/6×C3H background. The *Rspo2* null, *Rspo2*$^{\Delta ZN}$, and *Lrp6* null alleles were genotyped by polymerase chain reaction (PCR) as described (Nam et al., 2007a; Pinson et al., 2000). Genotyping of *TopGAL* and *Rosa26*^{Flp} mice were performed according to protocols available from The Jackson Laboratory. To activate Wnt/β-catenin signaling in embryos in utero, 30 μl of 1 M LiCl solution was intraperitoneally injected into pregnant females three times at 8.5, 9.5, and 10.0 dpc (day post coitum). A 1 M NaCl solution was used as control. Mice were housed in a pathogen-free air barrier facility, and animal handling and procedures were approved by the Maine Medical Center Institutional Animal Care and Use Committee.

Skeletal preparation, β-galactosidase staining, and whole-mount in situ hybridization

Skeletal preparations of E18.5 fetuses and whole-mount in situ hybridization were performed as described (Nam et al., 2007a). To visualize expression of the *LacZ* gene encoding β-galactosidase (β-gal), freshly collected embryos were fixed with 0.2% glutaraldehyde for 15 min at room temperature and stained with X-Gal substrate (Invitrogen, Carlsbad, CA) overnight at 37 °C. The stained embryos were processed as cryosections. Fetuses/embryos and the cryosections were photographed under StemiSV6 stereomicroscope (Zeiss, Germany) and Axioskop microscope (Zeiss, Germany) using AxioCam digital camera (Zeiss, Germany).

Cell apoptosis and proliferation

For 5-Bromo-2′-deoxy-uridine (BrdU) labeling, pregnant females were injected intraperitoneally with 100 μl of BrdU solution (Zymed Laboratories, South San Francisco, CA) per 10 g of body weight, and the embryos were harvested 30 min after BrdU injection. Embryos were processed for paraffin-embedded sections (5 μm) by standard protocol, and immunohistochemical detection of BrdU was performed using a BrdU detection kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instruction.

TdT-mediated dUTP nick-end labeling (TUNEL) assay, and phospho-histoneH3 and activated caspase3 immunostaining procedures were performed on cryosections (10 μm). TUNEL assay was performed using an in situ cell death detection kit (Roche Applied Science) according to the manufacturer's instruction. Anti-phospho-histoneH3 (1:100 dilution), anti-cleaved caspase3 (1:100 dilution), and anti-β-catenin (1:100 dilution) antibodies were obtained from Cell Signaling Technology (Beverly, MA). The processed sections were appropriately counterstained with hematoxylin/eosin or 4′,6-diamidino-2-phenylindole (DAPI).

Western blot and a real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analyses

The first branchial arches were dissected from wild type and *Rspo2*$^{-/-}$ embryos at E10.5 and homogenized in a hypertonic lysis buffer containing 20 mM HEPES (pH 7.4), 50 mM NaF, 0.2 mM $MgCl_2$, 20% glycerol, 0.1% Triton X-100, 1 mM PMSF, 0.2 mM sodium orthovanadate and 1× protease inhibitor cocktail set V (EMD Chemicals, Gibbstown NJ). Tissue lysate was mixed with sucrose solution (0.25 M sucrose, 1 mM EDTA) and centrifuged at 20,000*g* relative centrifugal force (RCF) for 60 min at 4 °C to prepare a cytosolic fraction. Antibodies against β-catenin (1:1000 dilution, BD Pharmigen) and β-tubulin (for loading control, 1:1000 dilution, Santa Cruz Biotechnology) were used in western blotting. Signals were developed by using the Pierce Super Signal West Dura kit (Thermo Scientific/Pierce, Rockford IL) and quantified using Image J software.

Total RNA was isolated from the branchial arch dissections and explants by using Trizol reagent (Sigma-Aldrich, St Louis MO). Two microgram of total RNA was used in cDNA synthesis. The cDNA equivalent to 40 ng total RNA was used for qRT-PCR. DNA sequences of the primers used in qRT-PCR are listed in Table S1 in supplementary materials.

Palatal shelf and branchial arch explant culture

Palatal shelf explant culture was performed as described previously (Jin and Ding, 2006). Briefly, mouse palatal shelves at E13.5–E14.0 were dissected out in cold α-MEM containing 25 mM HEPES. The dissected palatal shelves were paired and placed in an organ culture plate containing α-MEM medium with 0.1 μg/ml ascorbic acid and cultured at 37 °C with 5% CO_2 for up to 48 hours.

Branchial arch explant culture was performed as a hanging drop culture. Branchial arch explants were isolated from E9.5–E10.5 embryos. Ectoderm and mesenchyme explants were prepared from branchial arch explants by enzymatic digestion with dispase (2.4 U/