R-spondin 2 is required for normal laryngeal-tracheal, lung and limb morphogenesis

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Herein, we demonstrate that Lrp6-mediated R-spondin 2 signaling through the canonical Wnt pathway is required for normal morphogenesis of the respiratory tract and limbs. We show that the footless insertional mutation creates a severe hypomorphic R-spondin 2 allele (Rspo2Tg). The predicted protein encoded by Rspo2Tg neither bound the cell surface nor activated the canonical Wnt signaling reporter TOPFLASH. Rspo2 activation of TOPFLASH was dependent upon the second EGF-like repeat of Lrp6. Rspo2Tg/RgTg mice had severe malformations of laryngeal-tracheal cartilages, limbs and palate, and lung hypoplasia consistent with sites of Rspo2 expression. Rspo2Tg/RgTg lung defects were associated with reduced branching, a reduction in TOPGAL reporter activity, and reduced expression of the downstream Wnt target Irx3. Interbreeding the Rspo2Tg and Lrp6– alleles resulted in more severe defects consisting of marked lung hypoplasia and absence of tracheal-bronchial rings, laryngeal structures and all limb skeletal elements.

KEY WORDS: R-spondin 2, Lrp6, Lung, Larynx, Trachea, Limb, Development, Wnt signaling, Sp8, Mouse

INTRODUCTION

The canonical Wnt signaling pathway is regulated by the binding of Wnt ligands with a complex of one of 10 frizzled (Fzd) transmembrane receptors and low density lipoprotein receptor related protein 5 or 6 (Lrp) resulting in stabilization of intracellular β-catenin (for a review, see Logan and Nusse, 2004). β-Catenin translocates to the nucleus and interacts with lymphoid enhancer factor (Le)/T-cell factor (Tcf) transcription factors to alter gene transcription. Lrp6-mediated activation of the canonical Wnt signaling pathway also occurs in response to a new family of ligands: the R-spondins (Rspo) (Kazanskaya et al., 2004; Nam et al., 2006; Wei et al., 2007).

Disruption of the Wnt signaling pathway alters normal development of the lung and limb. Murine lung development begins at E9.5 as the foregut endoderm invaginates into the surrounding mesenchyme. Lung morphogenesis is dependent upon mesenchymal-epithelial interactions that promote branching of lung tubules. Primary branching forms the two bronchi, whereas asymmetric secondary branching, which occurs between E10 and E11.5, defines the number of airways and lobulation. Continued proximodistal branching generates the conducting airways that lead to the alveoli in the mature lung. High levels of Wnt signaling, indicated by the TOPGAL reporter, occur in the epithelium and mesenchyme adjacent to the proximal airways between E10.5 and E12.5 (De Langhe et al., 2005). The early epithelium expresses Wnt7b and Lrp6, whereas the mesenchyme expresses Wnt2a (De Langhe et al., 2005; Wang et al., 2005). Both tissues express Wnt11, Wnt5a and β-catenin (Li et al., 2002; Tebar et al., 2001; Weidenfeld et al., 2002). Wnt5a−/− fetuses exhibit truncation of the trachea, overexpansion of distal airways and disrupted lung maturation (Li et al., 2002). Lung hypoplasia and defects in pulmonary vessel smooth muscles were observed in Wnt7b−/− mice (Shu et al., 2002). Removal of β-catenin expression in the respiratory epithelium resulted in a failure of distal airway formation (Mucenski et al., 2003).

Canonical Wnt signaling is also required for normal limb formation. Cells within the emerging limb bud ventral ectoderm will form the apical ectodermal ridge (AER) (Bell et al., 2003b). The mature AER is localized at the limb bud apex and required for proximodistal elongation of the limb. The absence of Wnt3 or β-catenin expression in murine AER precursor cells disrupts AER formation, resulting in distal limb truncations (Barrow et al., 2003). The AER also fails to form in embryos that lack Lef and Tcf (Galceran et al., 1999). Lrp6 deletion causes limb abnormalities attributed to AER deficiencies (Pinson et al., 2000).

Reminiscent of Lrp6−−, the hindlimbs of fetuses homozygous for the footless mutation lack either the entire paw (autopod) or the posterior digits and, frequently, the fibula (Bell et al., 2003a). footless homozygotes also lack right forelimb digits 1 and/or 2. Distal telephalanges and fingernails were missing on formed digits. These malformations were previously correlated with regional deficiencies in AER formation at E10.5. Homozygous footless progeny had cleft palate and died of unknown causes at birth.

Herein, we tested the hypothesis that integration of the footless transgene affected a component of the Wnt signaling pathway critical for formation of limbs and the respiratory tract. Transgene integration disrupted the R-spondin 2 (Rspo2) gene, creating a mutant allele: Rspo2Tg. We present data that Rspo2Tg/Tg fetuses possess laryngeal-tracheal cartilage malformations and lung hypoplasia. The limb and lung phenotypes were exacerbated by the absence of Lrp6.

MATERIALS AND METHODS

Mouse models and analysis

Animal protocols were approved by the Institutional Animal Care and Use Committee in accordance with NIH guidelines. Detection of a vaginal plug was defined as E0.5. Lrp6-deficient animals were provided by Dr W. Skarnes (Wellcome Trust Sanger Institute). TOPGAL mice were provided by Dr E. Fuchs (The Rockefeller University). For Cre activation, animals

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were fed food containing doxycycline as described (Mucenski et al.; 2003). E18.5 fetal crown-rump lengths, weights and body circumference under the forelimbs were recorded. Lung, trachea and laryngeal tissues were dissected and weighed prior to fixation. For visualization of cartilage and bone, alcohol (95%) fixed, skinned fetuses and trachea were stained with Alizarin Red and Alcian Blue, and cleared in graded 2% KOH:glycerol solutions (100:0-0:100). Paraformaldehyde (4%) fixed tissue was embedded in paraffin wax. Genomic DNA from the fetal visceral was used for genotyping.

**Gene and cDNA characterization**

RT-PCR and Northern blot analyses were performed on E11 embryo total RNA. RNA was reversed transcribed using Oligo dT and Superscript II (Invitrogen) and amplified with the Rs2p primer pairs 5’-GGGGGT-GTTCGAAAACTTTC-3’ and 5’-AATCCGGGCTTGTTGCTCAG-3’ (818 bp product spanning exons 1-3); 5’-CATTACGGGTATTAACCCGAG-3’ and 5’-GGGGCGACCTCTCATCGAC-3’ (spanning exons 3-6); and 5’-GGGGGTGTCGAAAACTTTC-3’ with the SV40 primer AGGTAGTTTGTCAATTATG-3’ (spanning exon 1 into transgene). Products were subcloned and sequence verified. The 818 bp product was hybridized to northern blots.

**Alkaline phosphatase-Rspo2 fusion constructs and cell lines**

The Rs2p2 fusion PCR was amplified using a common NheI flanked 5’ primer 5’-GGGGGTGTCGAAAACTTTC-3’ or 5’-TGTCGGCAAACTTTTTC-3’ with either 5’-AGTGAATCCACCTCCTGTATCGTGG-3’ or 5’-CCGGAGATCTTACCTCATACATCGTGG-3’ or 5’-CCGGAGATCTTACCTCATACATCGTGG-3’ (spanning exons 3-6); and 5’-GGGGGTGTCGAAAACTTTC-3’ with the SV40 primer AGGTAGTTTGTCAATTATG-3’ (spanning exon 1 into transgene). Products were subcloned and sequence verified. The 818 bp product was hybridized to northern blots.

**Alkaline phosphatase activity assays**

AP assay reagents A and S (GenHunter) were used according to the manufacturer instructions to quantitate AP activity in conditioned media, eluates, or cell lysates. For cell binding assays, subconfluent cultures of HEK293T, MLE15 or HeLa cells were rinsed in Hanks Balanced Salt Solution (HBSS), incubated with conditioned media containing equivalent AP activity units for 1.5 hours at room temperature, rinsed five times in HBSS and lysed in 0.5 ml of AP lysis buffer (GenHunter). In some experiments, heparin (Sigma) was added with the media. Lysates were heated at 65°C for 15 minutes to inactivate endogenous AP activity. Average activity from triplicate wells is presented.

**Heparin binding assay**

Conditioned media were absorbed to 300 µl of heparin agarose (Sigma) at 4°C with rocking for 48 hours. The agarose was pelleted by centrifugation and washed by sequential resuspensions in 50 mM Tris (pH 8.0) with the indicated amount of NaCl. AP activity present in each eluate was presented.

**Luciferase assays**

HEK293T cells (~1.5x10⁶/well) were co-transfected with TOPFLASH (Upstate), PRL-TK (Promega) and Lrp6 plasmids using Fugene 6 (Roche). Conditioned medium containing equivalent levels of AP activity was added 24 hours post-transfection and incubated 24 hours prior to cell lysis. Firefly luciferase and Renilla luciferase activity were determined using the Promega Dual Luciferase assay. Each assay was performed in triplicate and all experiments were performed at least twice.

**β-Gal assays**

E11.5 lung buds from 46-54 somite embryos were dissected and frozen at −80 until genotyped. Each lung was resuspended in 25 µl of lysis reagent. Two 5 µl aliquots were incubated with Galacton-Star substrate (Applied Biosystems) for 90 minutes and light emission read in a Monolight 3010 luminometer. The amount of protein present in each lysate was determined in duplicate using the Bio-Rad protein reagent according to the manufacturer’s directions. For each lung, β-galactosidase activity was normalized to total protein.

For whole-mount visualization, lungs were fixed for 1 hour, rinsed three times in PBS, and developed for 5 hours in 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 2 mM MgCl2, 0.01% sodium deoxycholate, 0.2% NP-40 and 1 mg/ml X-Gal.

**Lung organ culture**

Dissected E11.5 lung buds were placed onto Nucleopore Trach-etched 8 µm membranes (Whatman) in wells containing 0.5 ml of DMEM containing 10% FCS, 2 mM glucose, 100 units/ml penicillin/streptomycin and 0.5 ml of the indicated conditioned medium. Explants were incubated at 37°C with 5% CO2 and photographed every 24 hours.

**Western blots**

Samples were boiled in Laemmli buffer, electrophoresed under denaturing conditions and blotted onto nitrocellulose. Rs2p2-conditioned media were concentrated using Centricon 30 centrifugal filter units (Millipore). Rs2p2AP fusion proteins were detected using a rabbit polyclonal antibody to human placental AP (1:2000) (GenHunter). Lrp6 constructs were transfected into HEK293T cells and cell lysates generated 48 hours later in the presence of protease inhibitor cocktail (Sigma). The Lrp6-flag epitope was detected using monoclonal M2 anti-Flag antibody (Sigma) (1:10,000), an anti-rabbit IgG peroxidase conjugate (Sigma) (1:10,000) and ECL western blotting detection reagent (GE Healthcare).

**Immunohistochemistry**

Paraflin wax-embedded tissue was sectioned at 5 µm, deparaffinized in graded ethanol, subjected to antigen retrieval, and endogenous peroxidase activity was quenched in methanol and H2O2 for 15 minutes. Antibodies requiring antigen retrieval were FoxJ1 (using 0.1 M citrate buffer, pH 6.0 and heat) and Pecam1 (15 minutes trypsin digestion at 37°C). Biotinylated secondary antibodies (1:200, Vector Laboratories) were detected using avidin-biotin-peroxidase detection system (ABC reagent, Vector Laboratories, Burlingame, CA). Sections were counterstained with Nuclear Fast Red. As a negative control, primary antibody was omitted on some slides. Primary antibodies used were rabbit anti-FoxJ1 (1:5000; from Dr Robert Costa), mouse anti-α-smooth muscle actin (1:10,000; monoclonal 1a4, Sigma), rabbit anti-proSP-C and rabbit anti-CCSP (1:1000, Seven Hills Bioreagents), rat anti-Pecam-1 (1:500; monoclonal CD-31, Pharmigen), and hamster anti-TI-alpha (1:500; Clone 8.1, Iowa Developmental Studies Hybridoma Bank).

**RESULTS**

**Identification of the site of insertional mutagenesis**

Previous studies determined that the footless transgene insertional mutation was attributable to a single integration site on mouse chromosome 15 (Bell et al., 2003a). Using genomic fragments immediately flanking the integration site, overlapping clones were isolated and characterized from a genomic 129S/SvJ library. NCBI
Fig. 1. *Rspo2* is disrupted in the transgene insertional mutation *footless*. (A) *Rspo2* gene. Untranslated exons are shown in black; arrows indicate the location of PCR primers. (B) Northern blot of E11.0 embryo total RNA. Arrow indicates full-length transcript, and arrowhead indicates alternatively spliced transcript. (C) RT-PCR of E11.0 embryonic cDNA. The primers for exons 3-6 span the transgene integration site detected only small amounts of product in *Rspo2* transgenic embryos.

Blast analysis of sequenced clones localized the transgene integration site to the third intron of the *R-spondin 2* (*Rspo2*) gene (Kazanskaya et al., 2004; Lowther et al., 2005; Nam et al., 2006), denoted the *Rspo2* allele. The entire *Rspo2* gene uses six exons and spans ~150 kb (Fig. 1A). No other transcripts have been mapped to this genomic region. EST analysis and whole-mount in situ hybridization assays confirmed that the full-length transcript spans an ~1.6 kb 5′ untranslated region and includes sequences present within NM_172815, EST BB707197 and EST AK011587.

Although transgene integration did not result in excision of any exons, we evaluated *Rspo2* expression by northern blot analysis, RT-PCR and whole-mount in situ hybridization assays. Northern blot analysis of total RNA detected an ~4.5 kb band in *Rspo2* transgenic RNA. No other transcripts have been mapped to this genomic region. EST analysis and whole-mount in situ hybridization assays confirmed that the full-length transcript contains an ~1.6 kb 5′ untranslated region and includes sequences present within NM_172815, EST BB707197 and EST AK011587.

secretion of Rspo2 and activation of canonical Wnt signaling

The murine *R-spondin* gene family consists of four structurally similar members located on distinct chromosomes. *Rspo2* is a 243 amino acid protein comprising a 21 amino acid signal peptide followed by a cysteine-rich region (amino acids 40-80 encoded by exon 3), a furin-like domain (amino acids 89-144 encoded by exon 4), a thrombospondin type 1 (TSP1) domain (amino acids 148-203 encoded by exon 5) and a C-terminal highly charged region (amino acids 207-243 encoded by exon 6) (Fig. 2A). Alkaline phosphatase (AP) was fused to the C-terminus of either full-length *Rspo2* (*Rspo2AP*) or domain deletion mutants. To create the truncated protein encoded by *Rspo2*Δ148-243AP, amino acids 96-243 (Δ96-243AP) were deleted. Δ148-243AP removed the TSP1 and highly charged domains of *Rspo2*. Δ212-243AP removed the highly charged C-terminus. Stable HEK293T cell lines expressing each construct secreted AP into the media (data not shown). Western blot analysis confirmed the presence of correctly sized *Rspo2*AP fusion proteins (Fig. 2B) demonstrating that all of the *Rspo2*AP fusion proteins were secreted.

The TSP1 domain of *Rspo2* is closely related to the single TSP1 domain in minid and fifth TSP1 domain in F-spondin, both previously shown to directly interact with heparin (Feinstein et al., 1999; Tzarfaty-Majar et al., 2001). TSP1 domains identify other heparin-binding signaling molecules, including HB-GAM and midkine, form a heparin binding β-sheet structure (Kilpelainen et al., 2000). The basic C-terminal domain of *Rspo2* (Fig. 2A) is similar to that found in growth factors that interact with extracellular matrix proteins on cell surfaces (Houck et al., 1992; LaRochelle et al., 1991). The ability of *Rspo2* to interact with the cell surface was evaluated by absorbing conditioned media containing *Rspo2AP* fusion proteins to HEK293T, HeLa and MLE15 cells. Both the full-length and Δ212-243AP fusion proteins bound the cell surface of all cell types with greatest binding detected with *Rspo2AP* (Fig. 2C, data not shown). No cell-surface binding was observed with media containing AP, Δ96-243AP or Δ148-243AP. *Rspo2AP* and Δ212-243AP cell-surface interactions were dose-dependently displaced with heparin (see Fig. S1 in the supplementary material). To confirm direct interactions between *Rspo2* fusion proteins and heparin, conditioned media were absorbed to heparin-agarose. The absorbed agarose was sequentially washed with buffers containing increasing amounts of NaCl, and AP activity was evaluated in the eluates. AP, *Rspo2*Δ96-243AP and *Rspo2*Δ148-243AP were poorly retained by the heparin agarose. Consistent with the cell binding assays, *Rspo2AP* exhibited a higher affinity for heparin agarose than did *Rspo2*Δ212-243AP (Fig. 2D). These data indicate that *Rspo2* interacts with cell surface heparin sulfate proteoglycans, that the TSP1 domain is critical for this interaction and that amino acids 212-243 of *Rspo2* potentiate heparin binding. Homologous domains in *mRspo3* are required for *Rspo3* binding to heparin sulfates (Nam et al., 2006).

Rspo family members activate the canonical Wnt signaling pathway by interacting with Lrp6 (Kazanskaya et al., 2004; Nam et al., 2006; Wei et al., 2007). To evaluate the biological activity of the *Rspo2-AP* fusion proteins and to further define the domains required for interaction with Lrp6, HEK293T cells were transfected with the luciferase Wnt signaling reporter TOPFLASH and pRL-TK (Renilla luciferase under control of the HSV-Tk promoter), and exposed to the indicated conditioned media. TOPFLASH reporter activation was observed in the presence of *Rspo2AP*, Δ212-243AP and, to a lesser extent, Δ148-243AP, but was not induced by Δ96-243AP (Fig. 2E). Thus, the truncated *Rspo2* product encoded by the *Rspo2Δ8* allele would not be able to activate the canonical Wnt signaling pathway.
Rspo2 and Lrp6 interactions
In HEK293T cells expressing Lrp6, Rspo2 increased Wnt signaling approximately sevenfold (Fig. 2F). The extracellular domain of Lrp6 contains 4 EGF-like domains separated by multiple LDL repeats. To identify the regions within the extracellular domain of Lrp6 that interact with Rspo2, Flag-tagged Lrp6 and deletion mutants lacking either EGF-like domain 2 (EGF2) or 3 and 4 (EGF3-4) were co-transfected with TOPFLASH and pRL-TK into HEK293T cells and then exposed to Rspo2AP or AP conditioned media for 24 hours. TOPFLASH expression was highest in the presence of Rspo2AP and full-length Lrp6. Although EGF3-4 increased TOPFLASH activity in the presence of Rspo2AP, EGF2 was not active (Fig. 2F). Western blot analysis detected appropriately sized Lrp6 proteins in the transfected cells (Fig. 2F insert). Therefore, Rspo2 activation of canonical Wnt signaling is dependent on the second EGF-like domain of Lrp6. The reduction in the level of TOPFLASH activation observed by EGF3-4 suggests that these domains may also be involved in Rspo2 signaling.

Embryonic expression of Rspo2
Rspo2 expression in the developing mouse embryo was evaluated at midgestation by in situ hybridization. Antisense riboprobes to various regions of the cDNA resulted in identical expression patterns. Rspo2 was first observed in the pre-forebrain neural tissue in E8 embryos with 7-10 pairs of somites (data not shown). By E9.5, expression was present within the dorsal forebrain at the forebrain/midbrain junction, the isthmus, ventral ectoderm of the emerging forelimb bud and lung bud (Fig. 3A). Through E10 and E11, Rspo2 mRNA persisted in the forebrain, isthmus, limb ectoderm and lung, and was detected in the branchial arches, genital ridge and limb mesenchyme (Fig. 3B,D). At E10, a transient stripe of expression was detected at the ventral base of the hindlimb bud (Fig. 3B). Expression of Rspo2 persisted in the lung bud mesenchyme through E14 and was detected as discrete domains within the laryngeal region from E11.5-15 (Fig. 3F-H, data not shown). Other sites of expression included the cortical hem of the telencephalon (E14), nasal pits and tooth mesenchyme (E15) (Fig. 3E, data not shown). These observations reveal additional sites of expression and are in agreement with previous reports (Kazanskaya et al., 2004; Nam et al., 2007a).

Sp8 is required for expression of Rspo2 in the AER
Morphogenesis of the AER is dependent upon expression of the transcription factor Sp8 in the ventral ectoderm of the emerging limb bud (Bell et al., 2003b). Cells initially fated to form the AER are present in the ventral ectoderm of Sp8−/− hindlimb buds, express a variety of AER markers, but fail to form a mature AER (Bell et al., 2003b). Rspo2 expression was assessed in Sp8−/− embryos since Rspo2GFP/EGFP embryos exhibit a disruption in AER maturation (Bell et al., 2003a) and Rspo2 is coordinately expressed with Sp8 in the forming and mature AER cells (Fig. 3A,B,D,J,K). Rspo2 mRNA was not detected in limb buds of E10 Sp8−/− embryos, although expression was present in the telencephalon (Fig. 3C). Real-time RT-PCR determined that the Rspo2 mRNA level in E10.5 Sp8−/−
hindlimb ectoderm was reduced eightfold compared with controls (Fig. 3I). The AER markers Bmp4 and Dlx2 were reduced only 1.5- to twofold (Fig. 3I, data not shown). As the hindlimb AER matured between stages 1 (Fig. 3B) and 4 (Fig. 3D), Rspo2 mRNA increased approximately fourfold. Expression of Bmp4 and Dlx2 was unchanged (Fig. 3I, data not shown). By contrast, Sp8 expression was detectable in the fore and hindlimb AERs of Rspo2+/Tg and Rspo2Tg/Tg embryos (Fig. 3I,K). As previously described (Bell et al., 2003a), AER deficiencies are not readily detectable in E10.5 Rspo2Tg/Tg embryos with fewer than ~35 somites. Like other AER markers, disruptions in Sp8 expression were observed along the posterior hindlimb and anterior forelimb margins of Rspo2+/Tg embryos (Fig. 3K). Notably, Sp8 expression within the AER containing areas of the Rspo2+/Tg fore and hindlimbs appeared weaker. Nam et al. recently reported a similar limb phenotype in fetuses homozygous for a gene targeted Rspo2 allele and also observed a decrease in Sp8 expression (Nam et al., 2007b). These observations suggest that Rspo2 expression is dependent on Sp8 within the AER precursors and support previous observations indicating that continued Sp8 expression is regulated by canonical Wnt signaling (Bell et al., 2003b; Kawakami et al., 2004).

Developmental roles of Rspo2

To test whether Rspo2 activation of the canonical Wnt signaling pathway was influenced by Lrp6 in vivo, the limb phenotypes were assessed in progeny with varying doses of each allele. The data presented in Table S1 in the supplementary material indicates the frequency of missing skeletal elements observed by genotype. Rspo2+/Tg;Lrp6+/– fetuses exhibited a 38% incidence of right forelimb ectodactyly 1 and/or 2 (Fig. 4A) (Bell et al., 2003a). Forelimb ectodactyly 5 was a characteristic of Rspo2+/Tg;Lrp6+/– fetuses (99%) (Pinson et al., 2000) and was also observed in the right forelimb of Rspo2+/Tg;Lrp6+/– animals (~20%) (Fig. 4B,D; see Table S1 in the supplementary material). An increase in the number of progeny missing digits and with malformations of more proximal skeletal elements was observed in Rspo2+/Tg;Lrp6+/– and Rspo2+/Tg;Lrp6–/– mice (Fig. 4C,E). Rspo2+/Tg;Lrp6–/– fetuses lacked all forelimb skeletal elements and had scapular malformations (Fig. 4F).

Severe malformations of the hindlimb, including loss of the autopod and fibula were observed in Rspo2+/Tg fetuses (Fig. 4G) (Bell et al., 2003a). Similarly, Lrp6 homozygotes lacked the autopod and fibula (100% penetrant) and possessed malformations of the tibia (52%) and femur (24%) (Fig. 4H) (Pinson et al., 2000). Rspo2+/Tg;Lrp6–/– mice exhibited a higher incidence of tibia and femur defects (Fig. 4I). Reciprocally, Rspo2+/Tg;Lrp6–/– fetuses consistently lacked both autopods and all or most of the tibia and fibula. Additionally, an increase in the severity of femur and pelvic girdle malformations was observed (compare Fig. 4K with 4H). No hindlimb structures and only rudiments of the pelvic girdle were observed in Rspo2+/Tg;Lrp6–/– progeny (Fig. 4L). A few Rspo2+/Tg;Lrp6–/– fetuses lacked digits (Fig. 4I).

Rspo2 and Lrp6 influence laryngeal-tracheal morphogenesis

Malformations in laryngeal and tracheal cartilages of E18 Rspo2+/Tg fetuses were observed and exacerbated by the progressive loss of Lrp6 alleles. The laryngeal region consists of the hyoid bone and thyroid, arytenoid and cricoid cartilages. In Rspo2+/Tg or +/Tg animals, regardless of Lrp6 genotype, the arytenoid cartilages floated freely above the cricoid cartilage. These cartilages were usually fused to the top of the cricoid in Rspo2+/Tg;Lrp6–/– and Rspo2+/Tg;Lrp6+/– fetuses (Fig. 5B-D; see Table S2 in the supplementary material). The cricoid normally forms a thickened ring with a dorsal region of cartilage that extends caudally. The cricoid ring was not formed in Rspo2+/Tg fetuses and the dorsal aspect of the cricoid was absent (Fig. 5A-C). In Rspo2+/Tg;Lrp6–/– and Rspo2+/Tg;Lrp6+/– progeny, 12-15 tracheal rings were evenly spaced with one or two bifurcated rings. Defects in cartilage ring formation varied from normal to bifurcated or consisted of abnormal cartilaginous nodules within the
trachea and mainstem bronchi in Rspo2^{Tg/Tg};Lrp6^{+/+} and Rspo2^{Tg/Tg};Lrp6^{+/-} mice (Fig. 5B,C). A severe phenotype was observed in Rspo2^{Tg/Tg};Lrp6^{+/-} fetuses; only rudiments of the thyroid cartilage were present and tracheal rings were absent (Fig. 5D). Laryngeal and tracheal cartilages were normal in Rspo2^{+/+};Lrp6^{--} and Rspo2^{+/Tg};Lrp6^{--} fetuses (Fig. 5E). Supporting the hypothesis that Rspo2 signals to the airway epithelium and acts through the canonical Wnt signaling pathway, tracheal ring defects were observed in doxycycline-exposed Tg(SFPTC-rtTA^{5Jaw+};Tg(TetO7-CMVcre)^{H9252-cat} mice (Mucenski et al., 2003) in which H9252-catenin was conditionally deleted from the embryonic airway epithelium (Fig. 5F, see Table S3 in the supplementary material).

Rspo2 and Lrp6 influence lung growth and morphogenesis

Rspo2^{Tg/Tg} mice die immediately after birth. As Rspo2 mRNA was observed in the lung bud mesenchyme, lung morphogenesis was assessed in Rspo2^{Tg/Tg} embryos. Fetal weights and crown-rump lengths varied by genotype, owing to the limb malformations and presence of spina bifida in Lrp6^{--} fetuses. Therefore, lung weights were normalized to fetal body circumferences, Table 1. As noted for the laryngeal and tracheal cartilages, lung size was normal in Rspo2^{+/+};Lrp6^{--} and Rspo2^{+/Tg};Lrp6^{--} progeny. An ~50% or more reduction in normalized lung weight was observed in Rspo2^{Tg/Tg};Lrp6^{+/-}, Rspo2^{Tg/Tg};Lrp6^{--}, Rspo2^{Tg/Tg};Lrp6^{+/-} and Rspo2^{Tg/Tg};Lrp6^{+/+} fetuses, all statistically significant at P < 0.05. Although the right lung lobes of Rspo2^{Tg/Tg} mice were frequently fused proximally, the overall lung structure and was not substantially altered. Immunohistochemical staining for specific cell types using antibodies to pro-surfactant protein C (type II epithelial cells), FoxJ1 (ciliated epithelial cells), Pecam1 (vascular endothelial cells), T1-alpha (type I epithelial cells), alpha-smooth muscle actin (smooth muscle cells) and Clara cell secretory protein (nonciliated bronchiolar cells) indicated that the expected differentiation of the various pulmonary cell types had occurred in lungs of all genotypes (Fig. 5G-N; see Fig. S2 in the supplementary material, data not shown). Thus, the hypoplasia observed in Rspo2^{Tg/Tg};Lrp6^{+/-} and Rspo2^{Tg/Tg};Lrp6^{--} animals did not correlate with the absence of a specific population of differentiated cells in the E18.5 lung.

To determine the timing of lung malformations, lung buds were dissected from E11.5-15.5 embryos. As early as E11.5, the number of epithelial branches was reduced in Rspo2^{Tg/Tg} mice and these defects persisted throughout embryogenesis, as highlighted by Sox9 staining at E13 (Fig. 6; Fig. 7A,E-G,H). Lung buds isolated from E11.5 Rspo2^{Tg/Tg} and nonmutant littermates were cultured in the presence of conditioned media containing either AP or Rspo2/H9004212-243AP, and terminal epithelial tips were counted after 3 days in organ culture. Confirming a defect in branching morphogenesis, the branching of Rspo2^{Tg/Tg} lung buds in AP medium was significantly reduced compared with controls, P < 0.001 by two-tailed Student’s t-test (compare Fig. 6A with 6C). This defect was substantially rescued by culturing Rspo2^{Tg/Tg} lung buds in media containing Rspo2 H9004212-243AP, P < 0.001 (Fig. 6A,B). Within individual experiments, similar levels of branching were observed in Rspo2^{+/+} and Rspo2^{+/Tg} lung buds exposed to Rspo2 H9004212-243AP or AP (Fig. 6C,D).
To determine whether *Rspo2* deficiency influenced canonical Wnt signaling during embryogenesis, the TOPGAL reporter transgene (DasGupta and Fuchs, 1999) was mated into the *Rspo2Tg* line. In E11.5 *Rspo2Tg/Tg;TOPGAL+/−* lung buds, the normal pattern of TOPGAL activity was disrupted; staining was decreased at the distal tips of the branching epithelium (compare Fig. 7A with 7B,C,E). A statistically significant difference in the amount of TOPGAL signaling was found by assaying protein concentration and /H9252-galactosidase activity in individual E11.5 wild-type and *Rspo2Tg/Tg* lung buds (*P*<0.00002 by two-tailed Student’s *t*-test of unequal variance) (Fig. 7D). Real-time RT-PCR of E11.5 lung cDNA samples evaluated the expression of the Wnt downstream target genes *Lef1* and *Irx3* (Braun et al., 2003; Hovanes et al., 2001), both expressed by the lung epithelium (De Langhe et al., 2005; Houweling et al., 2001). *Fgf10* expression was also examined owing to its known role in branching morphogenesis (Bellusci et al., 1997). *Irx3* expression was decreased threefold in *Rspo2Tg/Tg* lung buds, consistent with its proposed role in branching morphogenesis (van Tuyl et al., 2006) (Fig. 7F). *Lef1* and *Fgf10* expression varied between the two *Rspo2Tg/Tg* samples but were decreased less than 1.5-fold. These observations are consistent with the concept that *Rspo2* is involved in activation of canonical Wnt signaling in the embryonic lung.

Taken together, these observations support the concept that *Rspo2* is required for normal branching morphogenesis and growth of the embryonic lung, but is not required for cephalo-caudal patterning of the respiratory tract or differentiation of epithelial and mesenchymal cell types.

### DISCUSSION

We have shown that the insertional mutation *footless* creates a severe mutation in the *Rspo2* gene. In vitro, *Rspo2*-AP activated canonical Wnt signaling while *Rspo2* deficiency reduced Wnt signaling in the embryonic respiratory tract, as indicated by in vivo TOPGAL expression. The *Rspo2* allele caused severe malformations of the hindlimb, larynx, trachea, bronchi and lungs that were exacerbated by reduction in Lrp6.

### Table 1. E18.5 fetal lung weights

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of litters</th>
<th>Number of fetuses</th>
<th>Average lung weight (g.a.e.m.)</th>
<th>Average body circumference (cm.a.e.m.)</th>
<th>Average lung weight/body circumference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rspo2+/+,Lrp6+/+</td>
<td>12</td>
<td>17</td>
<td>0.038±0.0003</td>
<td>2.43±0.0104</td>
<td>0.0157±0.0001</td>
</tr>
<tr>
<td>Rspo2+/+,Lrp6+/-</td>
<td>8</td>
<td>12</td>
<td>0.040±0.0005</td>
<td>2.46±0.0067</td>
<td>0.0160±0.0001</td>
</tr>
<tr>
<td>Rspo2+/+,Lrp6–/–</td>
<td>11</td>
<td>20</td>
<td>0.036±0.0002</td>
<td>2.39±0.0067</td>
<td>0.0149±0.0001</td>
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<tr>
<td>Rspo2+/+,Lrp6+/+</td>
<td>9</td>
<td>11</td>
<td>0.028±0.0007</td>
<td>2.20±0.0167</td>
<td>0.0125±0.0002</td>
</tr>
<tr>
<td>Rspo2+/+,Lrp6+/-</td>
<td>11</td>
<td>16</td>
<td>0.022±0.0003</td>
<td>2.52±0.0090</td>
<td>0.0085±0.0001*</td>
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<tr>
<td>Rspo2+/+,Lrp6–/–</td>
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<td>13</td>
<td>0.016±0.0004</td>
<td>2.18±0.0250</td>
<td>0.0074±0.0002*</td>
</tr>
<tr>
<td>Rspo2+/+,Lrp6+/+</td>
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<td>2</td>
<td>0.002±0.0007</td>
<td>2.10±0.0707</td>
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<tr>
<td>Rspo2+/+,Lrp6+/-</td>
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<td>19</td>
<td>0.041±0.0003</td>
<td>2.51±0.0087</td>
<td>0.0163±0.0001</td>
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<tr>
<td>Rspo2+/+,Lrp6–/–</td>
<td>6</td>
<td>10</td>
<td>0.032±0.0003</td>
<td>2.25±0.0158</td>
<td>0.0141±0.0002</td>
</tr>
</tbody>
</table>

*Statistically significant by one-way ANOVA, *P*<0.05.
In vitro evidence indicates that Rspo binding to Lrp6 activates canonical Wnt signaling. In the present studies, Rspo2 increased TOPFLASH activity in response to increasing levels of cellular Lrp6, consistent with previous studies (Kazanskaya et al., 2004; Nam et al., 2006). Rspo1 and Rspo3 have been co-immunoprecipitated with the extracellular domain of Lrp6 (Nam et al., 2006; Wei et al., 2007). Herein, we observed that the second Lrp6 EGF-like repeat was required for Rspo2 activation of canonical Wnt signaling, whereas some signaling was retained in the absence of EGF-like repeats 3 and 4. Prior studies using human Lrp6 constructs demonstrated that EGF-like domains 1 and 2 were required for binding to Wnts and Wise (Itasaki et al., 2003; Mao et al., 2001). As the cellular response to Wnts is increased in the presence of Rspo2, it is unclear whether Rspo2 competes with Wnts for binding to the EGF 1 and 2 domains on the same Lrp6 molecule or whether a Wnt ligand binds to one EGF domain and Rspo2 to the other to activate signaling. The higher affinity of Rspo proteins for Lrp6, when compared with frizzled proteins, may indicate that they bind in a larger complex, with Lrp6-Rspo2 interacting with a Wnt-frizzled (Wei et al., 2007). Alternatively, the effects of ligand binding to frizzled or Lrp6 receptors may activate shared downstream targets independently.

Morphogenetic defects caused by deletion of Rspo2 or Lrp6 alone were exacerbated by decreasing the dosage of the other gene. These findings support the concept that Rspo2 and Lrp6 function in a shared/overlapping pathway affecting formation of the limb and respiratory tract. Rspo2Tg/Tg;Lrp6–/– fetuses lacked appendages. Defects in the pelvic and shoulder girdles of Rspo2Tg/Tg;Lrp6–/– mice are probably related to the absence of Rspo2 expression in the most proximal mesenchyme of the emerging limb bud, as formation of these structures is independent of AER formation (Sun et al., 2002). By contrast, the progressive loss of distal limb structures in Rspo2Tg/Tg;Lrp6+/– and Rspo2+/+;Lrp6–/– and Rspo2+/+;Lrp6–/– is probably related to the progressive loss of AER. Both Lrp6 and Rspo2 mutants are known to affect AER morphogenesis (Bell et al., 2003a; Pinson et al., 2000) and the AER is required for outgrowth of the underlying mesenchyme. The importance of Rspo2 for normal AER morphogenesis is supported by the observations that Rspo2 expression increased as the AER matured and that it was dramatically decreased in Sp8+/– limb buds that fail to form an AER (Bell et al., 2003b). The highest levels of canonical Wnt signaling colocalize in the pre and mature AER cells where Rspo2 is expressed (Barrow et al., 2003; Maretto et al., 2003). Notably, Wnt3 and Lrp6 both are expressed throughout the limb. 

**Fig. 6. Branching defects in Rspo2Tg/Tg lungs.** Organ culture of E11.5 lungs in the presence of conditioned media containing either AP or Rspo2 Δ212-243AP. (A) Rspo2 Δ212-243AP partially rescued the branching defect observed in Rspo2Tg/Tg lung buds. (B) Counted epithelial tips of mutant lung buds in culture. (C, D) Rspo2+/+ and Rspo2Tg/Tg lung buds grew similarly in culture in response to either AP or Rspo2 Δ212-243AP. (D) Number of distal tips of individual lung buds observed within a representative experiment.

**Fig. 7. Rspo2Tg/Tg lungs exhibit reduced Wnt signaling.** The Rspo2Tg allele was crossed into the Wnt signaling reporter TOPGAL line and β-galactosidase activity examined in whole-mount lung specimens at E11.5: Rspo2+/- (A), Rspo2Tg/Tg (B, C, E). A, B and E are littermates. White arrows indicate high levels of Wnt signaling activity in the distal tips of wild-type lungs compared with the lower level in the distal tips of mutants (arrowheads). (D) Quantitation of β-galactosidase activity in E11.5 lung buds. (F) Semi-quantitative real-time RT-PCR of E11.5 cDNA samples, indicating that Irx3 is reduced threefold. (G, H) Sox9 is expressed in a normal proximal distal pattern in Rspo2+/- (G) and Rspo2Tg/Tg (H) lungs.
ectoderm, and, together with β-catenin, are required for normal AER morphogenesis (Barrow et al., 2003; Pinson et al., 2000). We propose a model in which Rspo2 in the AER enhances canonical Wnt signaling to a level necessary for normal limb morphogenesis. This process is tightly regulated as the AER simultaneously expresses the Wnt signaling antagonist Dickkopf 1 (Dkk1) (Mukhopadhyay et al., 2001). An overactive AER is present in Dkk1 mutants, resulting in limbs with extra digits. Notably, in the absence of Dkk1, reduction of either Lrp5 or Lrp6 expression lowers the incidence of limb malformations (MacDonald et al., 2004).

The morphogenetic defects present in Rspo2^{+/Tg} included severe malformations of the larynx, trachea, bronchi and lung. At E11.5, Rspo2^{+/Tg} fetuses were approximately half the size of littermate lungs but generally retained normal structure and cephalo-caudal patterning of differentiated cell types. Laryngeal and tracheal-bronchial ring abnormalities were observed in all Rspo2^{+/Tg} fetuses and included malformation of the cricoid ring and arytenoids within the larynx, and absent or malformed cartilage rings in the trachea and bronchi. Lrp6 heterozygosity did not increase the severity of these Rspo2^{+/Tg} malformations, suggesting that other Rspos or Wnts can compensate in the absence of Rspo2. The lack of tracheal and laryngeal defects in Rspo2^{+/-},Lrp5^{-/-} and Rspo2^{+/-};Lrp6^{-/-} fetuses suggests that in the absence of Lrp6, Rspo2 may signal through Lrp5. Like Lrp6, Lrp5 can mediate canonical Wnt signaling but, surprisingly, loss of Lrp5 is compatible with life (Holmen et al., 2004; Mikels and Nusse, 2006). The dramatic effect of the Rspo2 mutation on lung growth and laryngeal-tracheal-bronchial morphogenesis observed in Rspo2^{+/-};Lrp6^{-/-} mice indicates a critical requirement for early Lrp6-mediated Rspo2 signaling. The finding that deletion of β-catenin from the embryonic respiratory epithelium also resulted in tracheal ring malformations supports a role of Rspo2-mediated activation of the canonical Wnt signaling pathway in tracheal cartilage morphogenesis.

During early lung formation, canonical Wnt signaling is highest in the distal lung epithelium and immediately adjacent mesenchyme (De Langhe et al., 2005). At E11.5, a reduction in expression of the Wnt signaling reporter TOPGAL was observed in distal epithelial tips of Rspo2^{+/-} lung buds with a defect in epithelial branching. As Rspo2 is expressed in the distal tip mesenchyme, we propose that paracrine Rspo2 signaling occurs in the lung. In support of this concept, the highest levels of Lrp6 and Lrp5 expression are observed in the respiratory epithelium that also expresses Wnt7b, Wnt5a, Wnt11, Fzd8 and Fzd10 (De Langhe et al., 2005; Li et al., 2002). Wnt2a, Wnt5a, Wnt11, Fzd1, Fzd4 and Fzd7 are expressed in the lung mesenchyme (Li et al., 2002; Wang et al., 2005; Weidenfeld et al., 2002). In vitro studies demonstrated R-spondin activation of the canonical Wnt pathway through interactions with Lrp6 and Fzd1, Fzd4, Fzd5 and Fzd8, as well as potentiation of Wnt1 and Wnt3a signaling (Kazanskaya et al., 2004; Nam et al., 2006; Wei et al., 2007; Weidenfeld et al., 2002). Whether Rspo2 acts independently or potentiates the canonical signaling activity of Wnt7b, Wnt5a, Wnt2 or Wnt11 in the lung remains to be determined (Mikels and Nusse, 2006). Wnt7b signaling is thought to be dependent on interactions with Lrp5, Fzd1 and Fzd10 (Wang et al., 2005). The early branching defects and lung hypoplasia observed in Rspo2^{+/-} embryos, were similar to those observed in Wnt7b mutants. However, epithelial cell differentiation proceeded normally in Rspo2^{+/-} mice, whereas Wnt7b^{−/−} mice exhibited a deficiency in type I cell differentiation (Shu et al., 2002).

The distinct combination of tracheal malformations and lung hypoplasia observed in Rspo2^{+/-} mice is reminiscent of other genetic mutations. Retinoic acid receptor (RAR) α/β2−/− animals exhibit lung hypoplasia attributable to a delay in branching morphogenesis that was detectable as early as E11.5 (Mendelsohn et al., 1994). Laryngeal and tracheal defects in Rara−/−Rarβ2−/− fetuses also included failure of the cricoid to fuse dorsally, fusion of the arytenoids to the cricoid, and abnormally shaped cartilage rings. By contrast, the foreshortening of the trachea seen in Rara−/−Rarβ2−/− was not observed in Rspo2^{+/-} mice. Abnormally formed tracheal-bronchial rings were also observed after conditional deletion of Shh in the respiratory epithelium (Miller et al., 2004). By contrast, the tracheal cartilage abnormalities found in Wnt5a and TNF receptor-associated factor 4 (Traf4)-null mice are distinct. In Wnt5a^{−/−} mice, tracheal length was shorter but the tracheal rings were normal (Li et al., 2002). The upper three to six cartilage rings below the cricoid were consistently either frontally disrupted or fused in Traf4^{−/−} fetuses (Regnier et al., 2002; Shiels et al., 2000). The role of Rspo2 in this developmental process is currently unclear.

In summary, the footless insertion mutation disrupts the Rspo2 gene, causing severe malformations in the lung, larynx, trachea, bronchi, limb and palate. The restricted sites of Rspo2 expression correspond with the sites of malformation. The effects of the Rspo2 mutation on morphogenesis is distinct from those associated with mutations of other R-spondin family members. Deletion of Rspo3 resulted in embryo lethality at E10 that was attributed to impaired placentation (Aoki et al., 2007). Rspo1 mutations were associated with XX sex reversal, palmoplantar hyperkeratosis and a predisposition to squamous cell carcinoma (Parma et al., 2006). Rspo4 mutations result in anonychia (Blaydon et al., 2006), consistent with the finding that Rspo4 is selectively expressed in the nail bed. The present studies indicate that Rspo2 and Lrp6 interact to activate canonical Wnt signaling affecting limb and laryngeal-tracheal cartilages, and lung morphogenesis.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/6/1049/DC1

References


