Signaling through BMP receptors promotes respiratory identity in the foregut via repression of Sox2

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SUMMARY
The mammalian foregut gives rise to the dorsally located esophagus and stomach and the ventrally located trachea and lung. Proper patterning and morphogenesis of the common foregut tube and its derived organs is essential for viability of the organism at birth. Here, we show that conditional inactivation of BMP type I receptor genes Bmpr1a and Bmpr1b (Bmpr1a;b) in the ventral endoderm leads to tracheal agenesis and ectopic primary bronchi. Molecular analyses of these mutants reveal a reduction of ventral endoderm marker NKX2-1 and an expansion of dorsal markers SOX2 and P63 into the prospective trachea and primary bronchi. Subsequent genetic experiments show that activation of canonical WNT signaling, previously shown to induce ectopic respiratory fate in otherwise wild-type mice, is incapable of promoting respiratory fate in the absence of Bmpr1a;b. Furthermore, we find that inactivation of Sox2 in Bmpr1a;b mutants does not suppress ectopic lung budding but does rescue trachea formation and NKX2-1 expression. Together, our data suggest that signaling through BMPRA1;B performs at least two roles in early respiratory development: first, it promotes tracheal formation through repression of Sox2; and second, it restricts the site of lung bud initiation.

KEY WORDS: BMP, Sox2, Foregut, Mouse, Trachea, Esophagus

INTRODUCTION
Although ultimately performing very distinct functions, the mammalian respiratory (trachea/lung) and anterior digestive (esophagus/stomach) systems originate from a common foregut tube (Cardoso and Lu, 2006; Warburton et al., 2000). For these two structures to develop correctly, a multitude of events must occur, including specification and maintenance of the two lineages, proliferation and differentiation of the progenitor populations, and morphological rearrangement of the foregut tissue to produce two distinct tracts. Whereas much research has focused on development of the respiratory and digestive systems after their separation, less is known regarding the control of their establishment.

The first morphological distinction between the respiratory and digestive lineages is evident at E9.75 in the mouse, when a pair of ventral pulmonary evaginations arise from the foregut endoderm (Cardoso and Lu, 2006; Morrisey and Hogan, 2010; Que et al., 2006). Shortly after evagination of the primary lung buds (beginning at ~E10.0), the common foregut tube anterior to the lung buds is partitioned into a ventral trachea and a dorsal tube (Cardoso and Lu, 2006; Warburton et al., 2000). For these two structures to develop correctly, a multitude of events must occur.

Molecularly, the respiratory lineage can be first identified by expression of Nkx2-1 in the ventral foregut endoderm as early as embryonic day (E)8.25 (Lazzaro et al., 1991; Serls et al., 2005). Complementary to the Nkx2-1 expression pattern, high levels of Sox2 expression mark the dorsal foregut endoderm (Que et al., 2007). Nkx2-1 and Sox2 encode transcription factors that inhibit each other’s expression, and in turn delineate opposing identities in the foregut. These genes are also necessary for proper formation of the trachea and esophagus, respectively (Minoo et al., 1999; Que et al., 2007). After separation of the trachea and the esophagus, Nkx2-1 expression remains restricted to the trachea, while Sox2 is expressed more highly in the esophagus. Concurrent with this, P63, which is initially expressed in the entire foregut endoderm, shifts its domain to be more highly expressed in the esophagus (Que et al., 2007).

The embryonic foregut is a hub for secreted signals. Signaling pathways that have been implicated in foregut patterning and/or morphogenesis include sonic hedgehog (SHH) (Litingting et al., 1998), fibroblast growth factor (FGF) (Que et al., 2007; Serls et al., 2005), WNT (Goss et al., 2009; Harris-Johnson et al., 2009), transforming growth factor β (TGFβ) (Chen et al., 2007) and bone morphogenetic protein (BMP) pathways (Li et al., 2007; Li et al., 2008; Que et al., 2006). In this study, we focus on the role of BMP signaling in foregut patterning and morphogenesis. Upon BMP ligand binding, type I and type II receptors form heteromultimers, allowing the type II receptor to phosphorylate the type I receptor (Wrama et al., 1994; Mishina et al., 2002). The type I receptors phosphorylate downstream targets, including SMAD1, SMAD5 and SMAD8 (pSMAD1/5/8) (Heldin et al., 1997; Whitman, 1998), which then bind to SMAD4 and regulate transcription of downstream targets.

Data from expression and functional studies suggest a role for BMP signaling in early development of the respiratory system. Bmp4 is expressed in the mesenchyme surrounding the ventral foregut prior to and during emergence of the lung buds (Li et al., 2008; Que et al., 2006; Weaver et al., 1999; Belluscio et al., 1996; Weaver et al., 2003). This expression pattern contrasts with that of BMP antagonist noggin (Nog), which is expressed in the dorsal endoderm (Li et al., 2007; Que et al., 2006). Nog mutant mice frequently display esophageal atresia/tracheoesophageal fistula (EA/TEF), which is rescued in a Bmp4+/− (Que et al., 2006) or Bmp7+/− genetic background (Li et al., 2007). In addition, conditional inactivation of Bmp4 in the foregut endoderm and

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surrounding mesenchyme results in tracheal atresia (TA) and lung hypoplasia (Li et al., 2008). Analyses of these Bmp4 mutants led to the conclusion that the primary requirement for BMP signaling is to promote cell proliferation, but not respiratory specification. NOG and BMPs are secreted molecules, therefore it is not clear whether they are primarily required in the endoderm or surrounding mesenchyme for proper development of the anterior foregut.

In this study, we investigate the role of two principal BMP receptors, BMP receptors 1A (BMPR1A; ALK3) and 1B (BMPR1B; ALK6) in the foregut endoderm during establishment of respiratory and digestive lineages. We show that signaling through BMPR1A and BMPR1B (BMPR1A;B) is required to maintain respiratory identity of the prospective trachea. We also find that ectopic activation of canonical WNT signaling, previously shown to be necessary and sufficient to promote respiratory fate in an otherwise wild-type background (Goss et al., 2009; Harris-Johnson et al., 2009), does not rescue the loss of respiratory identity in Bmpr1a;b mice. By inactivating Sox2 from the ventral endoderm of Bmpr1a;b mice, we show that signaling through BMPR1A;B promotes respiratory identity via repression of Sox2. Last, we find that signaling through BMPR1A;B regulates the location and number of lung buds that develop from the ventral foregut. Collectively, our data indicate that BMPR1A;B function in respiratory specification and morphogenesis, two distinct aspects of respiratory lineage initiation.

MATERIALS AND METHODS

Generation of mutant embryos and whole-mount in situ hybridization

The mutant alleles used are: Shh<sup>+1EGFPVEGF-C</sup> (Shh<sup>+</sup>) (Harfe et al., 2004), Bmpr1atm2.1Bhr (Mishina et al., 2002), Bmpr1atm2.2Bhe (Bmpr1a<sup>C</sup>) (Mishina et al., 2002), Bmpr1btm1Kml (Bmpr1b<sup>C</sup>) (Yi et al., 2000), Fgfr2tm1Dor (Fgfr2<sup>C</sup>) allele (Yu et al., 2003), Ctnnb1tm1Mmt (β-catenin<sup>C</sup>) (Harada et al., 1999), Ctnnb1tm2Kem (β-catenin<sup>C</sup>) (Brault et al., 2001) and Sox2<sup>2μluc</sup>/Shu (Sox2<sup>C</sup>) (Favaro et al., 2009). Littermates were used as controls.

Embryos were fixed in 4% PFA at 4°C for 2 hours to overnight, then dehydrated in methanol and stored, equilibrated in 30% sucrose and frozen in OCT for cryosectioning, or embedded in paraffin. Whole-mount in situ hybridization was performed as previously described (Neubuser et al., 1997).

Immunofluorescent and immunohistochemistry staining

For immunofluorescence, frozen sections (10 μm) were stained using a standard protocol, and mounted in Vectashield (Vector Laboratories). Paraaffin sections (7 μm) were dewaxed and rehydrated, heated to boiling in 10 mM citrate buffer for 15 minutes, then stained using a standard protocol. For immunofluorescence, antigen detection was performed with DAB kit (Vector Laboratories).

Primary antibodies used were mouse anti-NKX2-1 (also termed anti-TTF1, B6G7G3/1, Neomarkers, 1:100 dilution), rabbit anti-SOX2 (Novus Biologicals, 1:4000 dilution), mouse anti-β3 (Santa Cruz Biotechnology, 1:100 dilution), rabbit anti-caspase 3 (Cell Signaling Technology, 1:500 dilution), rabbit anti-phospho-SMAD1/5/8 (Cell Signaling, 1:1200 dilution), rabbit anti-E-cadherin (Cell Signaling, 1:200 dilution), goat anti-BMPR1a (Santa Cruz, 1:50 dilution), mouse anti-MSX1/2 (4G1s, Developmental Studies Hybridoma Bank, 1:10 dilution), rabbit anti-smooth muscle myosin (Biomedical Tech, 1:100 dilution) and mouse anti-BrdU (Ab-2, Calbiochem, 1:50 dilution). Secondary antibodies used were Cy3-conjugated goat anti-mouse, FITC-conjugated goat anti-rabbit, DyLight 488-conjugated donkey anti-goat and HRP-conjugated goat anti-rabbit (Jackson ImmunoResearch, 1:400 dilution).

Cell proliferation assay

Pregnant females received an intraperitoneal injection of 100 μg BrdU (Sigma) per gram of bodyweight 1 hour prior to sacrifice. The percentage BrdU<sup>C</sup> nuclei between control (three embryos, n=9 sections) and Bmpr1a;b (three embryos, n=9 sections) was compared using Student’s t-test. Results are reported as mean±s.e.m., and were considered statistically significant at P≤0.05. Similar statistical methods are used to analyze and present real-time RT-PCR and luciferase assay data as described below.

Real-time RT-PCR

Total RNA was extracted from three control and three mutant pooled samples of two or three foreguts each using Trizol (Invitrogen). RNA was reverse-transcribed using Superscript-III first-strand synthesis system (Invitrogen). cDNA was PCR amplified and quantified using SYBRgreen (Applied Biosystems). Primers used were: Nkrx2-1 (5'-CGCGTTACCGAGGACACAC-3' and 5'-CCCATGCACCTCATATTCACT-3'), Axin2 (5'-AGGACACGTCGCAAAAAG-3' and 5'-CCTCGTAGCAGGAGGACT-3') and β-actin (5'-CGGCCAGGCTCATCTAATTTTC-3' and 5'-GCCACAGGATTCCATACCAAAG-3'). Two technical replicates were performed. Expression values were normalized using β-actin and compared using Student’s t-test.

Luciferase reporter assay

To make the luciferase reporter Sox2<sup>2μluc</sup>, the –500 to +16 bp region of the Sox2 promoter was amplified using the following primers: 5'-TGATCAGCTACACAGGAGCCGCGGAT-3' and 5'-TGATCAAGCTTTTGGAACAGTTAAGACAAAACT-3', and the fragment was cloned into pC12 Basic (Promega). To make the reporter Sox2<sup>2μluc</sup>, in which substitution mutations were placed in the 5' and 3'-most putative SMAD4-binding motifs, the following primers were used: 5'-TGATCAGCTACACAGGAGCCGCGGAT-3' and 5'-TGATCAAAGCTTTTGACAAAGTTAATCTTCAACCAT-3'. The plasmid pBmpr1a<sup>ca</sup> is composed of the coding sequence of a constitutively active form of Bmpr1a cloned into pC1 vector (Promega).

HepG2 cells were transfected with reporter (200 ng) and either empty vector or pBmpr1a<sup>ca</sup> (600 ng) using Lipofectamine 2000 (Invitrogen). Plasmid encoding Renilla luciferase (10 ng) was used as transfection control. Cells were analyzed using Dual-Luciferase Reporter Assay System (Promega). pBmpr1a<sup>ca</sup>-induced reporter activity was compared with that when empty vector was transfected. Results were compared using Student’s t-test.

RESULTS

Inactivation of Bmpr1a and Bmpr1b in foregut endoderm causes failed establishment of distinct trachea versus esophagus and supernumerary buds

To investigate the requirement for Bmprs in trachea/ lung initiation, we inactivated the two principal BMP receptors, Bmpr1a and Bmpr1b, in the ventral foregut endoderm. This was achieved by conditionally inactivating Bmpr1a using Shh<sup>+</sup>, and combining these alleles with a null allele of Bmpr1b to generate Shh<sup>−/−</sup>; Bmpr1a<sup>−/−</sup>; Bmpr1b<sup>−/−</sup> (hereafter termed Bmpr1a;b) embryos. Consistent with previous findings (Harris et al., 2006; Harris-Johnson et al., 2009), we show that recombination activity in Shh<sup>+</sup> mice is first detected at E9.0, and is widespread in the ventral foregut endoderm by E9.5 (see Fig. S1A in the supplementary material), prior to lung budding and separation of trachea/esophagus. BMPR1A staining was greatly reduced in Shh<sup>−/−</sup>; Bmpr1a<sup>−/−</sup>; Bmpr1b<sup>−/−</sup> embryos at E9.5 (see Fig. S1B,C in the supplementary material). Consistent with this result, our quantitative RT-PCR (qRT-PCR) analysis at a later time point (E12.0) in lung confirmed that Cre activity leads to efficient recombination of Bmpr1a in endoderm (see Fig. S1D in the supplementary material). We next assayed for levels of pSMAD1/5/8 and BMP targets MSX1/2 in control and Bmpr1a;b embryos to determine whether BMP signaling was abrogated in mutant endoderm. At E9.5, pSMAD1/5/8 and MSX1/2 staining was greatly reduced in the ventral foregut endoderm of Bmpr1a;b; embryos relative to control samples (Fig. 1A-D), indicating that BMP signaling is effectively abrogated from the foregut endoderm of Bmpr1a;b mutants.
Bmpr1a;b mutants died at birth with respiratory distress, while ShhCre;Bmpr1a mutants often survived for 1 or 2 days after birth, then died of undetermined causes. Bmpr1b mutants were viable but sterile. To investigate the cause of respiratory distress in Bmpr1a;b mutants, we compared the morphology of the early respiratory system among the various mutants. At E11.5, the foreguts of embryos with at least one functional copy of Bmpr1a or Bmpr1b were indistinguishable from wild-type foreguts (data not shown). However, Bmpr1a;b embryos exhibited two clear defects in the foregut region (Fig. 1E-H). First, in control embryos by E11.5, the foregut anterior to the lung buds has separated into two distinct tubes: a ventral trachea and a dorsal esophagus (Fig. 1E,G). In foregut anterior to the lung buds has separated into two distinct foregut region (Fig. 1E-H). First, in control embryos by E11.5, the mutants, such as two distinct foregut tubes was also observed in other BMP pathway Nog et al., 2007; Que et al., 2006). However, unlike the phenotype of Nog mutants, where this defect coincides with failed separation of the notochord from the foregut endoderm (Li et al., 2007; Que et al., 2006). However, unlike the phenotype of Nog mutant embryos, the notochord of Bmpr1a;b embryos was properly separated from the foregut (see Fig. S2A,B in the supplementary material). Second, in control embryos, only a single tube was present anterior to the lung buds (Fig. 1F,H). Failed formation of two distinct foregut tubes was also observed in other BMP pathway mutants, such as Nog mutants, where this defect coincides with failed separation of the notochord from the foregut endoderm (Li et al., 2007; Que et al., 2006). However, unlike the phenotype of Nog mutant embryos, the notochord of Bmpr1a;b embryos was properly separated from the foregut (see Fig. S2A,B in the supplementary material). Second, in control embryos, only a single tube was present anterior to the lung buds (Fig. 1F,H). Failed formation of two distinct foregut tubes was also observed in other BMP pathway mutants, such as Nog mutants, where this defect coincides with failed separation of the notochord from the foregut endoderm. To test this possibility, we addressed whether there is an increase in FGF signaling activity in Bmpr1a;b mutants by examining the expression of Spry2 and Etv5, two transcriptional readouts of FGF signaling (Fig. 1I; data not shown) (Chambers et al., 2000; Firnberg and Neubuser, 2002). We found that expression of neither Spry2 nor Etv5 was noticeably altered in the tips of the buds of Bmpr1a;b mutants relative to control, suggesting that core FGF signaling activity is not increased in Bmpr1a;b mutants.

This result does not preclude the possibility that FGF signaling may act genetically downstream of BMPRI1A;B to control bud formation. To test this possibility, we conditionally inactivated the receptor Fgfr2 in the Bmpr1a;b mutant background (generating ShhCre;Bmpr1a;d14;Bmpr1b;c14;Fgfr2flo/flo hereafter termed Bmpr1a;b:Fgfr2). Deletion of Fgfr2 suppressed the formation of ectopic buds in Bmpr1a;b:Fgfr2 embryos, indicating that formation of ectopic buds is dependent on FGF signaling (Fig. 1K-N).

**Bmpr1a;b mutants display trachea agenesis**

Next, we sought to characterize the identity of the single tube located anterior to the lung buds in Bmpr1a;b embryos (Fig. 2A-H). In agreement with previous reports (Que et al., 2006; Que et al., 2007), we found that at E11.5 in control embryos, the trachea stained positive for NKX2-1, negative for P63 and positive for SOX2 at a low level (Fig. 2A,C,E). Conversely, the esophagus of control embryos stained negative for NKX2-1, positive for P63 and positive for SOX2 at a high level. In Bmpr1a;b embryos, the single tube stained negative for high level. In Bmpr1a;b embryos, the single tube stained negative for NKX2-1, positive for P63 and positive for SOX2 at a low level (Fig. 2A,C,E). Conversely, the esophagus of control embryos stained negative for NKX2-1, positive for P63 and positive for SOX2 at a high level. In Bmpr1a;b embryos, the single tube stained negative for NKX2-1, positive for P63 and positive for SOX2 at a low level (Fig. 2A,C,E). Conversely, the esophagus of control embryos stained negative for NKX2-1, positive for P63 and positive for SOX2 at a high level. In Bmpr1a;b embryos, the single tube stained negative for NKX2-1, positive for P63 and positive for SOX2 at a low level (Fig. 2A,C,E). Conversely, the esophagus of control embryos stained negative for NKX2-1, positive for P63 and positive for SOX2 at a high level. In Bmpr1a;b embryos, the single tube stained negative for NKX2-1, positive for P63 and positive for SOX2 at a low level (Fig. 2A,C,E). Conversely, the esophagus of control embryos stained negative for NKX2-1, positive for P63 and positive for SOX2 at a high level. In Bmpr1a;b embryos, the single tube stained negative for NKX2-1, positive for P63 and positive for SOX2 at a low level (Fig. 2A,C,E). Conversely, the esophagus of control embryos stained negative for NKX2-1, positive for P63 and positive for SOX2 at a high level. In Bmpr1a;b embryos, the single tube stained negative for NKX2-1, positive for P63 and positive for SOX2 at a low level (Fig. 2A,C,E). Conversely, the esophagus of control embryos stained negative for NKX2-1, positive for P63 and positive for SOX2 at a high level. In Bmpr1a;b embryos, the single tube stained negative for NKX2-1, positive for P63 and positive for SOX2 at a low level (Fig. 2A,C,E). Conversely, the esophagus of control embryos stained negative for NKX2-1, positive for P63 and positive for SOX2 at a high level. In Bmpr1a;b embryos, the single tube stained negative for NKX2-1, positive for P63 and positive for SOX2 at a low level (Fig. 2A,C,E). Conversely, the esophagus of control embryos stained negative for NKX2-1, positive for P63 and positive for SOX2 at a high level. In Bmpr1a;b embryos, the single tube stained negative for NKX2-1, positive for P63 and positive for SOX2 at a low level (Fig. 2A,C,E). Conversely, the esophagus of control embryos stained negative for NKX2-1, positive for P63 and positive for SOX2 at a high level. In Bmpr1a;b embryos, the single tube stained negative for NKX2-1, positive for P63 and positive for SOX2 at a low level (Fig. 2A,C,E). Conversely, the esophagus of control embryos stained negative for NKX2-1, positive for P63 and positive for SOX2 at a high level.
NKX2-1, positive for P63 and positive for SOX2 at a high level (Fig. 2B,D,F). At E17.5, this single foregut tube remained negative for NKX2-1, and was surrounded by a continuous ring of smooth muscle, similar to the esophagus of control embryos (Fig. 2G,H). These results indicate that the single tube present in Bmpr1a;b embryos displays esophageal characteristics. We conclude that signaling through BMPR1A;B in the foregut endoderm is essential for formation of the trachea, and that Bmpr1a;b mutant embryos exhibit tracheal agenesis.

**Tracheal agenesis in Bmpr1a;b embryos results from a cell fate change in the ventral foregut endoderm**

To determine the mechanism underlying the tracheal agenesis defect, we investigated whether specification of the respiratory fate ever occurred in the prospective trachea in the mutant. We examined the expression of Nkx2-1, the earliest known marker of the respiratory lineage, by whole-mount in situ hybridization at indicated stages. Broken lines in C and D indicate approximate plane of section for J-O. Arrows in D and H indicate ectopic buds. Bifurcation of bronchi is labeled with an arrowhead in E and F. (I) qRT-PCR results of Nkx2-1 expression in anterior foregut at E10.5. Results are mean ± s.e.m. (J-O) Antibody-stained transverse sections of E9.5 embryos anterior to prospective primary bronchi. Dorsal is towards the top. Scale bars: 40 μm.

Fig. 3. Loss of trachea in Bmpr1a;b embryos results from foregut patterning defects. (A-H) Ventral view of foregut stained for Nkx2-1 expression by whole-mount in situ hybridization at indicated stages. Broken lines in C and D indicate approximate plane of section for J-O. Arrows in D and H indicate ectopic buds. Bifurcation of bronchi is labeled with an arrowhead in E and F. (I) qRT-PCR results of Nkx2-1 expression of anterior foregut at E10.5. Results are mean ± s.e.m. (J-O) Antibody-stained transverse sections of E9.5 embryos anterior to prospective primary bronchi. Dorsal is towards the top. Scale bars: 40 μm.
analysis. At E9.5, we observed no ectopic cell death in Bmpr1a;b foregut compared with control (see Fig. S2C,D in the supplementary material; data not shown). We next assayed for cell proliferation in the presumptive tracheal region by comparing the rate of BrdU-incorporation between control and Bmpr1a;b embryos. We observed no statistically significant differences in rates of BrdU incorporation in the endoderm or surrounding mesenchyme (see Fig. S2E-G in the supplementary material). From these data, we conclude that signaling through BMPR1A;B in the ventral foregut endoderm is not required for proper cell proliferation or survival at E9.5.

To determine whether changes in cell fate may account for reduced Nkx2-1 expression in the mutant, we addressed whether ventral endoderm cells have switched to express different cell lineage markers (Fig. 3J-O). In control embryos at E9.5, Nkx2-1 was expressed in the ventral foregut, whereas Sox2, the esophagus marker, was expressed most highly in the dorsal foregut (Fig. 3J,L,N). In Bmpr1a;b embryos, however, concomitant with the reduction of NKX2-1, the domain of SOX2 expression was greatly expanded (Fig. 3K,M,O). These data suggest that tracheal agenesis in Bmpr1a;b embryos is caused by a failure to maintain respiratory fate in the anterior ventral foregut.

**Signaling through BMPR1A;B is required for normal lung development**

We next addressed whether the change in cell fate observed in the tracheal region of the foregut extends into the main bronchi in Bmpr1a;b embryos. We examined marker expression in the prospective lung region at 28 somites (E9.75). Although evaginations of the primary lung buds from the foregut tube are apparent at this stage, the morphology of the foregut in Bmpr1a;b embryos is still similar compared with control embryos. In control embryos, cells in the entire ventral endoderm including nascent buds express NKX2-1, but not SOX2 (Fig. 4A,C,E). In Bmpr1a;b mutants, cells at the tips of the nascent buds also express NKX2-1 and not SOX2, but cells located at the ventral midline in between the buds express SOX2 but not NKX2-1 (Fig. 4B,D,F). To determine whether patterning defects persist in the lungs of Bmpr1a;b embryos at later stages, we examined marker expression at E11.5. By whole-mount immunostaining, we found that NKX2-1 is expressed throughout the respiratory lineage of control embryos, whereas high levels of SOX2 are restricted to the esophagus (Fig. 4G). In Bmpr1a;b lungs, however, the expression of NKX2-1 is restricted to the distal tips of the lung buds (Fig. 4H), consistent with our result from whole-mount in...
situ hybridization (see Fig. 3D,F,H). Strikingly, in the mutant, high SOX2 localization has spread from the entire common anterior foregut tube into a portion of the primary bronchi. To confirm this observation from whole-mount study, we examined protein localization in transverse sections. We found that NKX2-1 was present in the primary bronchi of control embryos, but was largely absent from the medial region of the primary bronchi of Bmpr1a;b embryos (Fig. 4I,J). Conversely, although P63 localization is restricted to the esophagus of control embryos, it is present in the primary bronchi of Bmpr1a;b embryos (Fig. 4K,L). At E13.5, branching morphogenesis was reduced in Bmpr1a;b lungs relative to control (Fig. 4M,N). By E17.5, however, the primary bronchi of Bmpr1a;b mutants expressed NKX2-1 and showed patterning of smooth muscle and cartilage similar to control (Fig. 4O-R), suggesting a recovery of respiratory fate. Differentiation in the bronchiolar and distal epithelium was abnormal, however, as the expression of secretoglobin 1a1 (Scgb1a1) and surfactant protein C (SpC) was reduced in Bmpr1a;b embryos relative to control (Fig. 4S-X). By contrast, at a gross morphological level at this stage, the lungs of Shhcre;Bmpr1a or Bmpr1b single mutant embryos were indistinguishable from controls (data not shown). Taken together, these data suggest that signaling through BMPR1A;B is transiently required for maintaining normal respiratory fate of the main bronchi, and is required for normal branching and differentiation of the respiratory epithelium.

Reduction in respiratory fate persists in Bmpr1a;b embryos following constitutive activation of WNT signaling

Recent data indicate that canonical WNT signaling is necessary and sufficient to promote respiratory fate in the foregut endoderm (Goss et al., 2009; Harris-Johnson et al., 2009). We therefore sought to determine the functional relationship between WNT and BMP signaling during foregut cell fate specification. We addressed whether ectopic activation of WNT might bypass the requirement for BMPR by introducing a conditional gain-of-function allele of β-catenin (β-catact, also termed Ctnnb1tm1Mmt) (Harada et al., 1999) into Bmpr1a;b background, generating Shhcre/+; Bmpr1a fl/–; Bmpr1b–/–; β-catact/+ (Bmpr1a;b; β-catact) mice. In β-catact mice, we first confirmed activation of WNT signaling by assaying for the expression of Axin2, which is positively regulated by WNT activity. We found by whole-mount in situ hybridization and qRT-PCR that, at E9.5 and E10.5, Axin2 was upregulated in the foregut endoderm in β-catact and Bmpr1a;b; β-catact embryos compared with control (Fig. 5A-H,Q, Bmpr1a;b; β-catact 1.59±0.13, β-catact 1.52±0.19 versus control 1±0.13, n=3 each, P<0.05).

We next compared expression of Nkx2-1 among control, Bmpr1a;b; β-catact, and Bmpr1a;b; β-catact embryos at E10.5 (Fig. 5I-P). We found that consistent with previous reports (Goss et al., 2009; Harris-Johnson et al., 2009), in β-catact embryos, expression of activated β-catenin led to ectopic expression of Nkx2-1 in the ventral stomach (Fig. 5K). This was not observed in Bmpr1a;b; β-
cat^mut^ embryos (Fig. 5L). Furthermore, in the presumptive tracheal region, we found that NKX2-1 remains expressed in the ventral foregut endoderm of control and β-cat^mut^ but is largely reduced in Bmpr1a;b or Bmpr1a;b;β-cat^mut^ embryos (Fig. 5M-P). Interestingly, in both Bmpr1a;b and Bmpr1a;b;β-cat^mut^ embryos, the few NKX2-1-expressing cells that remain in the ventral foregut localize to ectopic buds (Fig. 5N,P). From these data, we conclude that in the Bmpr1a;b mutant background, forced activation of WNT signaling no longer induces respiratory fate ectopically, nor can it rescue respiratory fate in the presumptive tracheal region.

The above data suggest that WNT signaling does not operate genetically downstream of BMP to promote respiratory fate. To address whether BMP signaling may be genetically downstream of WNT in the ventral foregut, we examined the expression of MSX1/2 in Shhcre;Bmpr1afl/–;Bmpr1b–/–;Sox2fl/fl mutants (hereafter termed Bmpr1a;b;Sox2^mut^) embryos where WNT signaling is inactivated (Fig. 5R,S) (Goss et al., 2009; Harris-Johnson et al., 2009). Expression of MSX1/2 was similar to that displayed by Shhcre;Bmpr1afl/–;Bmpr1b–/–;Sox2fl/fl (E11.5) embryos. Non-nuclear red signal is background staining detected in E11.5 frozen sections. (K,L) Sox2 expression in E11.0 foreguts assayed by whole-mount in situ hybridization. Sox2 is upregulated in Bmpr1a;b mutant lungs (filled arrow in L) relative to control (open arrow in K). (M) Diagram of 500 bp upstream regulatory region of Sox2. Putative SMAD1/5/8- and SMAD4-binding sites are indicated with arrows and asterisks, respectively. (N,O) Graphs of luciferase activity of reporters, when co-transfected with Bmpr1a^mut^ expression constructs, relative to luciferase activity of the respective reporter when co-transfected with empty vector. Results are mean + s.e.m. Scale bar: 40 μm.

Inactivation of Sox2 rescues tracheal formation and NKX2-1 expression in Bmpr1a;b mutant background

The tracheal agenesis phenotype displayed by Bmpr1a;b embryos is similar to that displayed by Nkx2-1 mutant mice (Minoo et al., 1999), and is the converse of that displayed by Sox2 hypomorphic mice (Que et al., 2007). In both Nkx2-1 and Sox2 mutants, a single endoderm-derived tube is present anterior to the lung buds. However, in Nkx2-1 mutant mice, the tube has an esophageal phenotype (Minoo et al., 1999), whereas in Sox2 mutant mice, the tube has a tracheal phenotype (Que et al., 2007). It was also shown that Nkx2-1 and SOX2 antagonize each other’s expression in the developing foregut (Que et al., 2007). These comparisons raise the issue of whether signaling through BMPR1A;B promotes respiratory fate directly through positive regulation of Nkx2-1, or indirectly through negative regulation of Sox2. To distinguish between these possibilities, we inactivated Sox2 in the ventral foregut of Bmpr1a;b embryos by generating Shhcre;Bmpr1a^mut^;Bmpr1b^mut^;Sox2^mut^ embryos (Bmpr1a;b;Sox2^mut^) and examined the morphology of the foregut (Fig. 6A-D). At E11.5, Bmpr1a;b;Sox2 embryos displayed ectopic buds (Fig. 6B), similar to Bmpr1a;b mutants (Fig. 1F). However, unlike Bmpr1a;b mutants where only one common foregut tube is present, Bmpr1a;b;Sox2 embryos exhibited a separate trachea and esophagus, similar to control (Fig. 6C,D).

To determine whether inactivation of Sox2 also rescued respiratory fate in the ventral foregut of Bmpr1a;b;Sox2 embryos, we examined the expression of NKX2-1 and SOX2 in transverse sections (Fig. 6E,F,I,J). We found that expression of NKX2-1 was partially rescued in the ventral endoderm of Bmpr1a;b;Sox2 embryos at E10.5, and this rescue is more apparent by E11.5 (compare Fig. 6FJ with Fig. 5N and Fig. 2B,F). To ensure that the rescue of NKX2-1 expression and tracheal formation was not
simply due to incomplete inactivation of Bmpr1a in Bmpr1a;b;Sox2 embryos, we compared the expression of BMPRI1A between control and Bmpr1a;b;Sox2 mutants (Fig. 6G,H). Similar to Bmpr1a mutants (see Fig. S1C in the supplementary material), we found that BMPRI1A protein was absent in Bmpr1a;b;Sox2 mutants (Fig. 6H). From these data we conclude that inactivation of Sox2 rescues trachea formation and NKX2-1 expression in the Bmpr1a;b mutant background.

**BMP represses Sox2 promoter activity in vitro**

We next investigated the mechanism by which signaling through BMPRIA;B inhibited SOX2 expression in the ventral foregut. We compared the expression pattern of Sox2 in the developing foregut of control and Bmpr1a;b mutants at E11.0 by whole-mount in situ hybridization, and found that Sox2 transcripts were more readily detected in mutant foregut, especially in the primary buds (Fig. 6K,L). This suggests that signaling through BMPRIA;B either inhibits transcription of Sox2 or the stability of Sox2 transcripts.

We then addressed the possibility that BMP signaling acts through downstream transcription factors, such as SMADs to directly repress Sox2 transcription (Heldin et al., 1997; Whitman, 1998). In human ES cells, BMP signaling does not require protein synthesis to repress Sox2, suggesting that BMP may directly regulate Sox2 (Greber et al., 2008). Consistent with this possibility, we found three sequences matching the SMAD4-binding motifs (CAGA and GTCT) (Wong et al., 1999) and three sequences matching SMAD1-binding motifs (AGGAAG and GCCCGC) (Alvarez Martinez et al., 2002; Mandel et al., 2010) within 500 bp of the transcriptional start site of Sox2 (Fig. 6M). To determine whether BMP signaling can regulate the activity of the Sox2 promoter, we performed luciferase reporter assays in cultured cells, in which we activated BMP pathway through transfection of an in vitro promoter in vitro. This is mediated, at least in part, through SMAD4-binding sites.

**DISCUSSION**

In this study, we provide genetic evidence that signaling through BMPRIA;B within the mouse foregut endoderm plays two distinct roles in the establishment of the respiratory lineage (Fig. 7). First, it inhibits digestive fate and promotes respiratory fate in the prospective trachea and primary bronchi. Second, it restricts the site of primary bud formation to the posterior region of the respiratory lineage. We note that the trachea agenesis phenotype resembles human birth defects that are highly lethal. In addition, a similar supernumerary bronchi phenotype in humans is associated with frequent respiratory infections (Brunner and van Bokhoven, 2005; Desir and Ghaye, 2009; Evans, 1990). Our findings therefore provide mechanistic insights into the etiology of these diseases.

**Combined function of BMPRIA;B is required in the endoderm to maintain respiratory fate in prospective trachea**

Although recent studies have shown that inactivating Bmpr1a disrupts later stages of lung development (Eblaghie et al., 2006; Sun et al., 2008), this study is the first to show that Bmpr1a and Bmpr1b together play essential roles during early stages of respiratory formation. Furthermore, our findings define a clear requirement for these receptors in the foregut endoderm. It remains possible that BMP signaling also plays a role within the surrounding mesenchyme.

Data from Bmpr1a;b mutants indicate that signaling through these receptors is required for maintaining respiratory fate in the ventral foregut. This mechanism is distinct from the proposed requirement for BMP signaling in cell proliferation based on data from Bmp4 conditional mutants (Li et al., 2008), although both mutants exhibit tracheal agenesis. These differences may result from divergences in the timing and domain of BMP signaling inactivation. For example, in the Bmp4 mutant, inactivation of BMP signaling occurs not just in the endoderm, but also in the surrounding mesenchyme. Functional redundancy from other BMP ligands may also contribute to the phenotypic differences between the two mutants. For example, Bmp7 may partially compensate for the loss of Bmp4 in the Bmp4 mutant (Li et al., 2007).

Although respiratory fate is not maintained in Bmpr1a;b mutants, it is initially induced. Bmp4 is expressed in the mesenchyme surrounding the prospective lung buds as early as E8.5 (Danesh et al., 2009; Li et al., 2008; Que et al., 2006; Weaver et al., 1999). The transient expression of Nkx2-1 in the proximal respiratory region of Bmpr1a;b mutants may be due to residual BMPRIA following Cre-mediated recombination, or may reflect a primary requirement for Bmpr1a;b in the maintenance, but not induction, of respiratory fate.

It is interesting to note that in the Bmpr1a;b mutant, while the prospective trachea never regains respiratory fate, the prospective lung endoderm undergoes more dynamic changes in fate. Ectopic buds express Nkx2-1 when they are first initiating (Fig. 3D, Fig. 5N), but only buds that have grown into the lung mesenchyme maintain respiratory fate at E11.5 (Fig. 4H). In addition, the proximal region of the bronchi, while having a digestive fate at E11.5 (Fig. 4H), has gained respiratory fate by E17.5 (Fig. 4P). One possible explanation is that the fate of lung endoderm remains labile after initiation of lung morphogenesis. In the absence of BMP signaling, prospective respiratory cells may rely on other signals from the lung mesenchyme. Alternatively, the apparent recovery of respiratory fate in the proximal bronchi could be due to proliferation and migration of Nkx2-1-positive cells from the distal lung epithelium.

Although this study focuses on respiratory initiation, in regard to later stages of lung development, it is surprising that three different conditional Bmpr1a single mutants differ in their phenotypes (this work) (Eblaghie et al., 2006; Sun et al., 2008). The lack of gross lung defect in our Shhcre;Bmpr1a mutant is not likely to be due to incomplete Cre-mediated excision, as BMPRIA is absent from E9.5 onwards (see Fig. S1C in the supplementary material). Further work is needed to resolve the causes for the different phenotypes. However, it is worth noting that conclusions drawn from late lung phenotypes exhibited by the Bmpr1a;b mutant in this study are consistent with previous conclusions drawn from the other Bmpr1a single mutants regarding the general role of BMP in later lung development.
Signal pathway relationships in the developing foregut endoderm

Given the multiple signaling pathways that are active in the foregut endoderm, the binary cell fate choices and simple morphogenesis events, this tissue offers an excellent setting with which to study pathway relationships. Data from Bmpr1a;b mutants suggest that these receptors are essential to maintain the respiratory fate of trachea but not distal lung buds. One possible signal that promotes respiratory fate in lung is FGF10 (Fig. 7A). It is expressed at highest levels in the distal lung mesenchyme and is capable of inducing expression of Nkx2-1 and downregulating expression of Sox2 and P63 (Que et al., 2007). Complementary to the requirement for BMP signaling, FGF10 signaling is essential for the formation of the lung buds, but not trachea (Min et al., 1998; Sekine et al., 1999). This molecular difference is consistent with the finding that trachea and lung progenitors are set apart as two independent populations much earlier than the first sign of respiratory development (Perl et al., 2002).

Recent studies provide evidence suggesting that WNT/β-catenin signaling promotes respiratory fate throughout the entire respiratory lineage (Goss et al., 2009; Harris-Johnson et al., 2009). Thus, WNT/β-catenin and BMP are both required to maintain respiratory fate of the prospective trachea. We find that ectopic activation of β-catenin fails to promote respiratory fate in the absence of BMP signaling, different from its capability in the presence of Bmpr1a;b (Goss et al., 2009; Harris-Johnson et al., 2009). Together with our finding that BMP signaling remains active in β-catenmut embryos, these data favor the model that WNT and BMP function in parallel to promote respiratory fate (Fig. 7A).

Bmpr1a;b maintain respiratory fate through repression of Sox2

Because Nkx2-1 and SOX2 antagonize each other’s expression, signaling through BMPR1A;B may promote respiratory identity in an instructive fashion by inducing Nkx2-1, thereby repressing Sox2. Alternatively, it could promote respiratory fate in a permissive fashion by repressing Sox2, thereby allowing expression of Nkx2-1. Strikingly, inactivation of Sox2 in a Bmpr1a;b background rescues both Nkx2-1 expression and tracheal formation, suggesting that signaling through BMPR1A;B functions to allow tracheal formation primarily through repression of Sox2 (Fig. 7A). In accordance with this, we found that BMP signaling repressed Sox2 promoter activity in vitro, but that this repression was attenuated when putative SMAD4-binding sites were mutated. This result from the foregut is reminiscent of BMP inhibition of Sox2 in the neural plate (Linker and Stern, 2004; Stern, 2006; Steventon et al., 2009). However, in the developing mouse lungs placode and taste papillae, BMP signaling promotes Sox2 (Beites et al., 2009; Furuta and Hogan, 1998; Rajagopal et al., 2009). These findings indicate that the effects of BMP on Sox2 transcription are influenced by the specific developmental context. As recent work has begun to elucidate cis-regulatory elements required for Sox2 expression (Takemoto et al., 2006; Uchikawa et al., 2003), it will be important to determine which elements drive Sox2 expression in the foregut, and the suite of transcription factors that bind them.

Bmpr1a;b are required to restrict the number of primary buds

Not only do lung buds form in Bmpr1a;b mutants, extra buds are observed. Interestingly, inactivation of Bmpr1a in the kidney leads to a similar phenotype, suggesting that BMP signaling may restrict budding in multiple branching organs (Hartwig et al., 2008). In both cases, later branching events are reduced, suggesting that BMP signaling may have different roles during budding and branching morphogenesis. Previous results indicate that BMP4 inhibits the ability of FGF10 to promote proliferation and migration of lung endoderm, although it does not affect the ability of FGF10 to induce expression of a Bmp4lacZ reporter allele (Weaver et al., 2000). This suggests that BMP antagonizes a subset of downstream responses to FGF10, rather than antagonizing core BMP signaling pathway (Fig. 7B). Consistent with these results, we did not observe an overt increase in FGF activity in the Bmpr1a;b mutant foregut, although inactivation of Fgfr2 suppressed the formation of ectopic buds in Bmpr1a;b;Fgfr2 embryos. It therefore appears plausible that ectopic buds form in Bmpr1a;b mutants due to enhanced cellular responsiveness to the chemoattractive effects of FGF downstream of core BMP signaling pathway. Placement of an FGF10 bead adjacent to trachea induces lung bud formation (Ohtsuka et al., 2001), suggesting that early differences in FGF signaling in prospective trachea versus lung may contribute to the adoption of distinct morphogenetic programs following respiratory induction. Interestingly, this antagonistic relationship between FGF and BMP in bud induction is different from their relationship in specification, where both promote respiratory fate. This suggests that these pathways may employ distinct downstream effectors to execute their roles in respiratory specification and morphogenesis.

A putative mechanism for the role of BMP signaling in congenital foregut defects

Tracheal agenesis is a rare, but often fatal, birth defect, the etiology of which is currently unknown (Heimann et al., 2007; Saleeby et al., 2003). Intriguingly, the phenotypes of Bmpr1a;b and conditional Bmp4 mutant mice resemble that of individuals suffering from this defect. Mutations in Sox2 and chromosomal deletions spanning Nog have been identified in patients with esophageal atresia (Que et al., 2006), suggesting that precise regulation of BMP and its downstream genes is likely essential for proper foregut patterning in human.

Just as Bmpr1a;b mutant mice display ectopic buds, individuals with ectopic or mislocated bronchial branches have been described (Desir and Ghaye, 2009; Evans, 1990; Panigada et al., 2009). The frequency of these aberrations is unknown, as individuals with them may be asymptomatic for respiratory ailments. However, these bronchi defects have been detected in treatment for recurrent respiratory infections. It has been proposed that these defects may
cause retained secretions, possibly leading to increased susceptibility to infection. Our study therefore provides greater mechanistic insights into the potential causes of these congenital abnormalities.

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References


BMP signaling patterns the foregut endoderm

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