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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 BMPR1A;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 esophagus. By E11.5, the separation of the digestive and respiratory tracts is complete.

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) (Litingtung 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 (Wrana 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; Bellusci 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*$^{tm1(EGFP/cre)Cjt}$ (*Shh*^{cre}) (Harfe et al., 2004), *Bmpr1a*$^{tm2.1Bhr}$ (*Bmpr1a*^{fl}) (Mishina et al., 2002), *Bmpr1a*$^{tm2.2Bhr}$ (*Bmpr1a*$^{-}$) (Mishina et al., 2002), *Bmpr1b*^{tm1Kml} (*Bmpr1b*$^{-}$) (Yi et al., 2000), *Fgfr2*^{tm1Dor} (*Fgfr2*^{fl}) allele (Yu et al., 2003), *Ctnnb1*^{tm1Mmt} (β-*cat*^{act}) (Harada et al., 1999), *Ctnnb1*^{tm2Kem} (β-*cat*^{fl}) (Brault et al., 2001) and *Sox2*$^{tm4.1Skn}$ (*Sox2*^{fl}) (Favaro et al., 2009). Littermates were used as controls.

Embryos were fixed in 4% PFA at 4°C for 2 hours to overnight, then either 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). Paraffin 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 immunohistochemistry, antigen detection was performed with DAB kit (Vector Laboratories).

Primary antibodies used were mouse anti-NKX2-1 (also termed anti-TTF1, 8G7G3/1, Neomarkers, 1:100 dilution), rabbit anti-SOX2 (Novus Biologicals, 1:4000 dilution), mouse anti-P63 (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^{+} 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 $P \leq 0.05$. 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: *Nkx2-1* (5′-CGCCTTACCAGGACACCAT-3′ and 5′-CCCATGCCACTCATATTCAT-3′), *Axin2* (5′-AGGAGCAGCTCAGCAAAAAAG-3′ and 5′-CTTCGTACATGGGGAGCACT-3′) and β-actin (5′-CGGCCAGGTCATCACTATTGGCAAC-3′ and 5′-GCCACAGGATTCCATACCCAAGAAG-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-luc*, the –500 to +16 bp region of the *Sox2* promoter was amplified using the following primers: (5′-TGATCGCTAGCACAGAGCGCAGTGCCGCGGAT-3′ and 5′-TGATCAAGCTTTTGAACAAGTTAATAGACAACCAT-3′), and the fragment was cloned into pGl2 Basic (Promega). To make the reporter *Sox2*^{mut}*-luc*, in which substitution mutations were placed in the 5′- and 3′-most putative SMAD4-binding motifs, the following primers were used: 5′-TGATCGCTAGCAAATAGCGCAGTGCCGCGGAT-3′ and 5′-TGATCAAGCTTTTGAACAAGTTAATCTTCAACCAT-3′. The plasmid *pBmpr1a*^{ca} 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*^{ca} (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*^{ca}-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*^{cre}, and combining these alleles with a null allele of *Bmpr1b* to generate *Shh*$^{cre/+}$*; Bmpr1a*$^{fl/-}$*; Bmpr1b*$^{-/-}$ (hereafter termed *Bmpr1a;b*) embryos. Consistent with previous findings (Harris et al., 2006; Harris-Johnson et al., 2009), we show that recombinase activity in *Shh*^{cre} 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*^{cre}*;Bmpr1a*$^{fl/-}$ (*Bmpr1a*) 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.

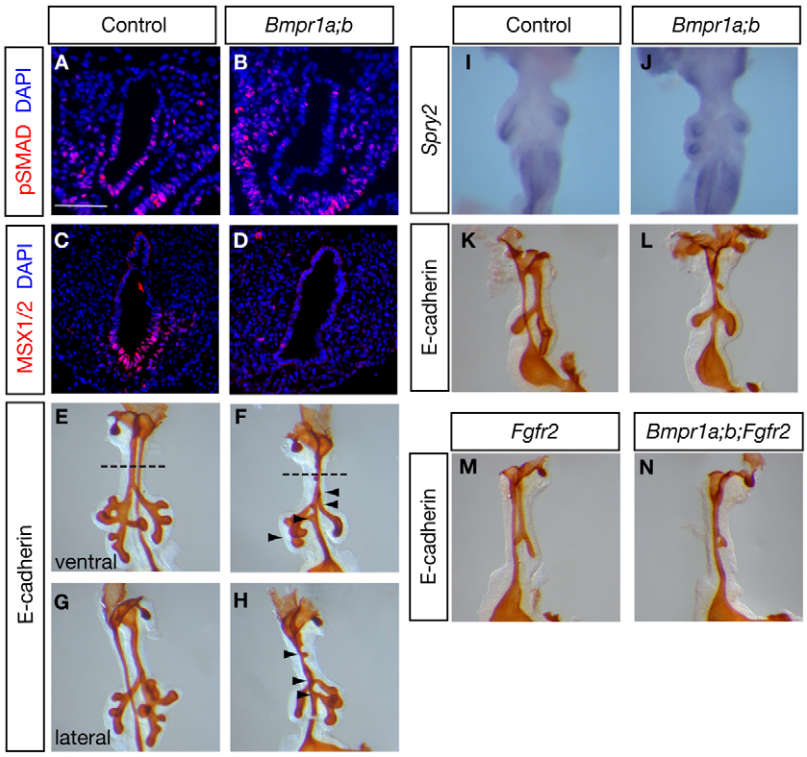

Fig. 1. Inactivation of *Bmpr1a;b* in foregut endoderm leads to defects in early respiratory development. (**A-D**) Antibody-stained frozen transverse sections of E9.5 embryos, taken at the level of the foregut. Colors are as labeled in all figures. Dorsal is towards the top. (**E-H**) Whole-mount immunohistochemistry for E-cadherin of foregut at E11.5. (E,F) Ventral views; (G,H) lateral views of the same respective samples. Arrowheads in F and H indicate buds. Broken lines in E and F indicate approximate plane of section for Fig. 2A-F. (**I**,**J**) *Spry2* expression in E11.5 foreguts assayed by whole-mount in situ hybridization. (**K-N**) Whole-mount immunohistochemical staining for E-cadherin of foregut endoderm at E11.5, lateral views. Scale bar: 40 μm.

Bmpr1a;b mutants died at birth with respiratory distress, while *Shh*^{cre}*;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 contrast to this, in *Bmpr1a;b* mutants, 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 two primary lung buds form, developing into the pair of bronchi bifurcating from the distal trachea. In *Bmpr1a;b* embryos, however, we observed a variable number of ectopic buds off the common foregut tube, a subset of which appear to develop into extra bronchi that lead into branching lung epithelium (Fig. 1F,H). Taken together, these data suggest that signaling through BMPR1A;B is required for normal initiation of the respiratory lineage.

Inactivation of *Fgfr2* suppresses ectopic bud formation in a *Bmpr1a;b* mutant background

To investigate the mechanism underlying the formation of ectopic buds in *Bmpr1a;b* mutants, we focused on FGF10 signaling through its receptor FGFR2 as it is the primary signaling pathway known to drive lung bud outgrowth (Arman et al., 1999; Bellusci et al., 1997; Sekine et al., 1999). Furthermore, data from organ culture experiments show that BMP4 protein can inhibit the chemoattractive property of FGF10 protein on isolated embryonic lung epithelium (Weaver et al., 2000). These results raised the possibility that the formation of ectopic buds in the *Bmpr1a;b* mutant was due to an increased ability of FGF signaling to stimulate the growth and migration of lung epithelium.

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,J; 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 BMPR1A;B to control bud formation. To test this possibility, we conditionally inactivated the receptor *Fgfr2* in the *Bmpr1a;b* mutant background (generating *Shh*^{cre}*;Bmpr1a*$^{fl/-}$*;Bmpr1b*$^{-/-}$*;Fgfr2*$^{fl/fl}$ 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

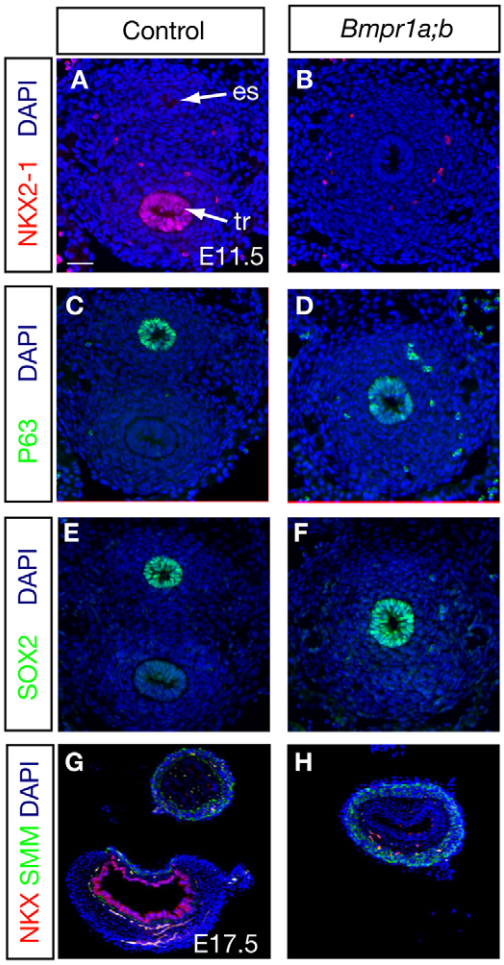

Fig. 2. Inactivation of *Bmpr1a;b* in foregut endoderm causes tracheal agenesis. (**A-H**) Antibody-stained adjacent paraffin transverse sections of E11.5 (A-F) and E17.5 (G,H) embryos, taken anterior to primary bronchi. Dorsal is towards the top. es, esophagus; tr, trachea. Scale bar: 40 μm.

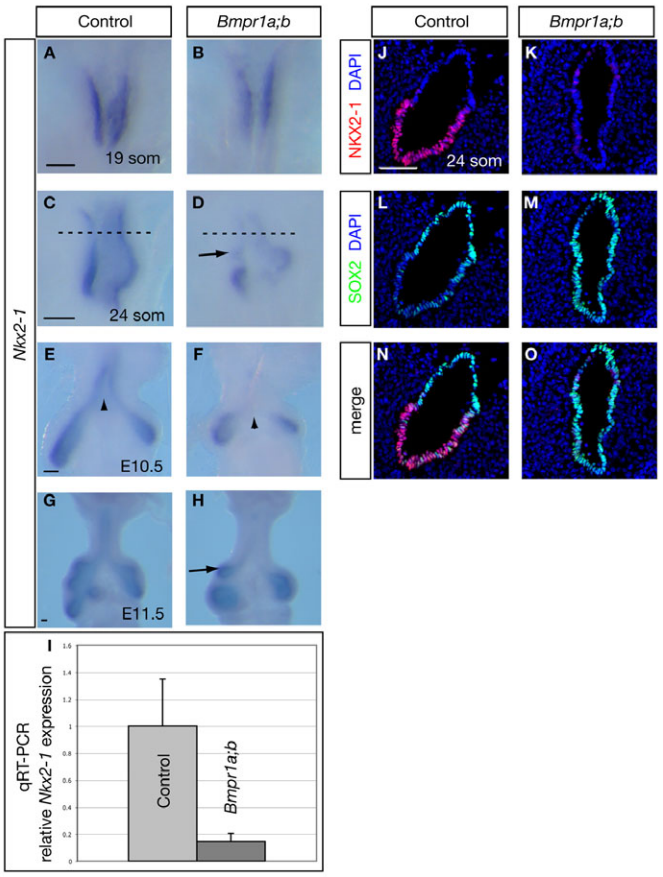

Fig. 3. Loss of trachea in *Bmpr1a;b* embryos results from foregut patterning defects. (**A-H**) Ventral view of foregut stained for *Nkx2-1* expression by whole-mount in situ hybridization at indicated stages. Broken lines in C and D indicate approximate plane of section for J-O. Arrows in D and H indicate 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.

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 several time points from E9.25 to E11.5 (Fig. 3A-H). At 19 somites (~E9.25), shortly after *Nkx2-1* is first detected by whole-mount in situ hybridization and *Shh*^{cre} is first active (Harris et al., 2006; Harris-Johnson et al., 2009), expression of *Nkx2-1* was similar between control and *Bmpr1a;b* embryos (Fig. 3A,B). By 24 somites (roughly E9.5), however, we observed an apparent downregulation of *Nkx2-1* throughout the anterior region of the respiratory field in *Bmpr1a;b* embryos relative to control (Fig. 3C,D). *Nkx2-1* remained expressed in the primary and ectopic buds in *Bmpr1a;b* embryos (arrow in Fig. 3D). At E10.5 and E11.5, robust *Nkx2-1* expression was detected throughout the entire respiratory lineage of control embryos (Fig. 3E,G). By contrast, *Nkx2-1* expression was greatly reduced from the presumptive tracheal region and main bronchi of *Bmpr1a;b* embryos at these stages, although it was present in the lung bud tips (Fig. 3F-I, *Bmpr1a;b* 0.145±0.06 versus control 1±0.35, n=3 each, P=0.04).

The progressive loss of *Nkx2-1* expression from the presumptive tracheal region of *Bmpr1a;b* embryos could result from changes in cell proliferation, cell survival, cell fate or any combination of these defects. To distinguish among these possibilities, we first examined cell death by staining for cleaved caspase 3 and by TUNEL

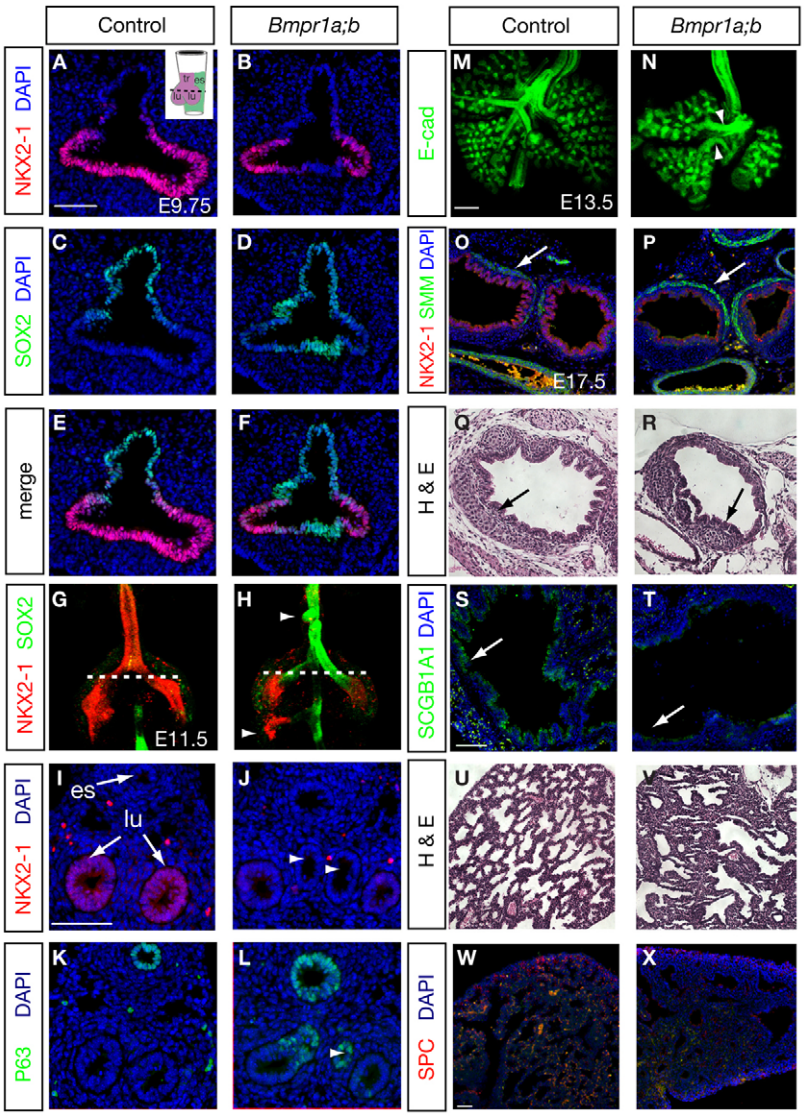

Fig. 4. Patterning and differentiation of the lung is disrupted in *Bmpr1a;b* embryos. (**A-F**) Antibody-stained transverse sections through the site of lung bud evagination in E9.75 (28 somite) embryos. Dorsal is towards the top. Inset in A illustrates approximate plane of section for A-F. (**G**,**H**) Stacked confocal images of E11.5 whole-mount foregut specimens. In control, the presence of NKX2-1 in the trachea obscures part of the SOX2 expression domain in the esophagus. Arrowheads in H indicate ectopic buds. Broken white lines indicate approximate plane of section for I-L. (**I-L**) Antibody-stained near-adjacent transverse sections through the esophagus and primary bronchi of E11.5 embryos. Dorsal is towards the top. Arrowheads in J and L indicate ectopic buds. (**M**,**N**) Stacked confocal images of E13.5 whole-mount foregut specimens. White arrowheads indicate ectopic bronchi in the mutant. (**O-X**) Antibody- or Hematoxylin and Eosin-stained transverse sections through the bronchi and lung of E17.5 embryos. Colors are as labeled. Bronchi of *Bmpr1a;b* embryos express NKX2-1 and show normal localization of smooth muscle (arrows in O,P) and cartilage (arrows in Q,R), similar to the control. Arrows in S,T indicate bronchiolar epithelium. (U,V) The number of distal airways is reduced in *Bmpr1a;b* embryos relative to control. Yellow signal in W,X is autofluorescence from red blood cells. es, esophagus; lu, lung. Scale bar: 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

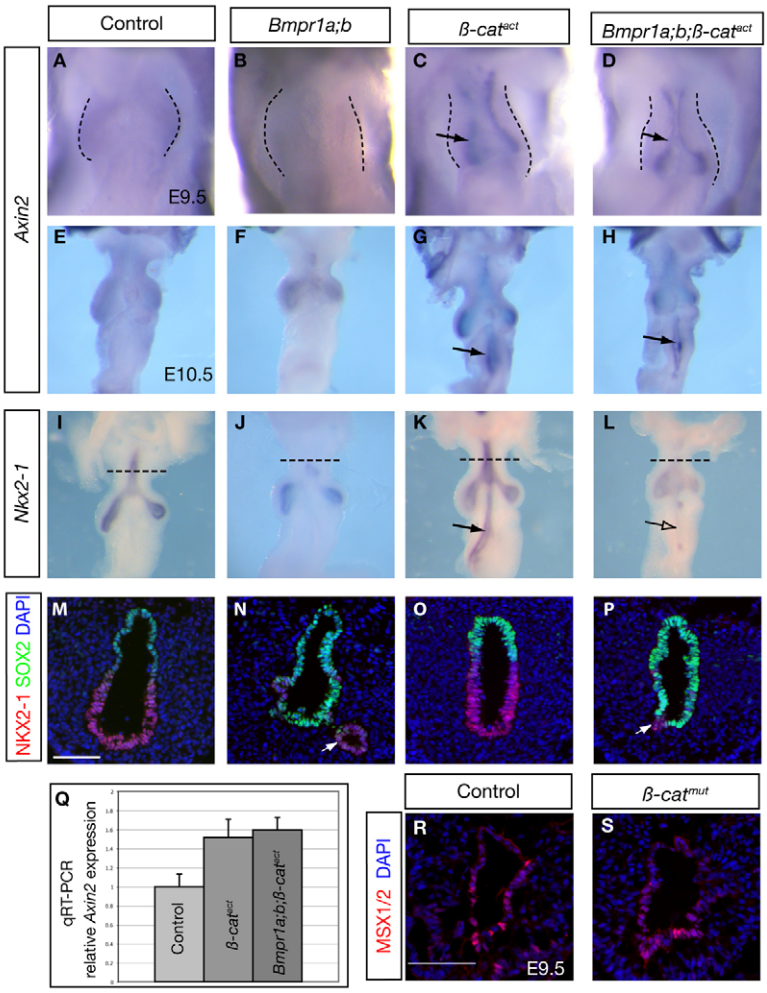

Fig. 5. Constitutive activation of β-catenin does not induce NKX2-1 expression in *Bmpr1a;b;* β-*cat*^{act} embryos. (**A-L**) Ventral view of foregut region stained by whole-mount in situ hybridization at E9.5 and E10.5. Arrows in C,D,G,H indicate *Axin2* expression. *Nkx2-1* is ectopically expressed in the ventral stomach in β-*cat*^{act} embryos (arrow in K) but not in *Bmpr1a;b;*β-*cat*^{act} embryos (arrow in L). Broken transverse lines indicate approximate level of section shown in M-P. (**M-P**) Antibody-stained transverse sections of E10.5 embryos. Arrows in N,P indicate ectopic buds. (**Q**) qRT-PCR results of *Axin2* expression at E10.5. Results are mean + s.e.m. (**R**,**S**) Antibody-stained transverse sections of E9.5 embryos. Scale bars: 40 μm.

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 *Shh*^{cre}*;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-*cat*^{act}, also termed *Ctnnb1*^{tm1Mmt}) (Harada et al., 1999) into *Bmpr1a;b* background, generating *Shh*$^{cre/+}$*; Bmpr1a*$^{fl/-}$*; Bmpr1b*$^{-/-}$*;* β-*cat*$^{act/+}$ (*Bmpr1a;b;*β-*cat*^{act}) embryos. In *Bmpr1a;b;*β-*cat*^{act} 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-*cat*^{act} and *Bmpr1a;b;*β-*cat*^{act} embryos compared with control (Fig. 5A-H,Q, *Bmpr1a;b;*β-*cat*^{act} 1.59±0.13, β-*cat*^{act} 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-*cat*^{act}, and *Bmpr1a;b;*β-*cat*^{act} 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-*cat*^{act} 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-

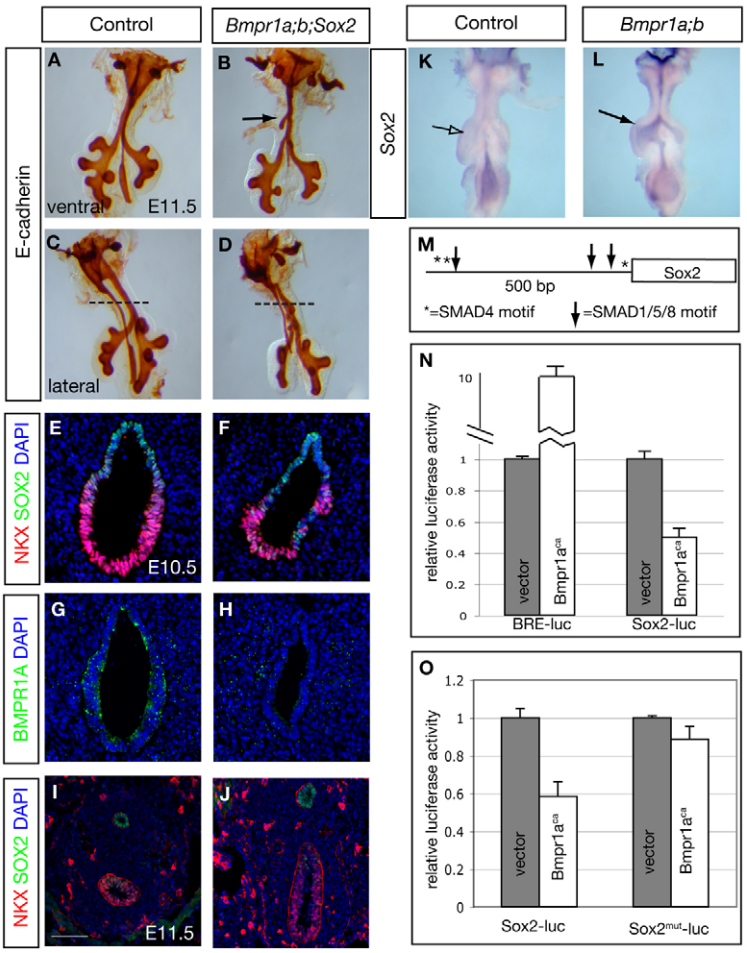

Fig. 6. Inactivation of *Sox2* rescues trachea formation and NKX2-1 expression in *Bmpr1a;b; Sox2* embryos, and BMP signaling inhibits *Sox2* promoter activity in vitro. (**A-D**) Whole-mount immunohistochemical staining at E11.5. Arrow in B indicates ectopic bud. (A,B) Ventral views, (C,D) lateral views of the same samples. Broken lines indicate approximate plane of section shown in I,J. (**E-J**) Antibody-stained transverse frozen sections of E10.5 (E-H) and E11.5 (I,J) 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*^{ca} 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.

cat^{act} 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*^{act} but is largely reduced in *Bmpr1a;b* or *Bmpr1a;b;*β-*cat*^{act} embryos (Fig. 5M-P). Interestingly, in both *Bmpr1a;b* and *Bmpr1a;b;*β-*cat*^{act} 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 *Shh*^{cre};β-*cat*$^{fl/fl}$ mutants (hereafter termed β-*cat*^{mut}) where WNT signaling is inactivated (Fig. 5R,S) (Goss et al., 2009; Harris-Johnson et al., 2009). Expression of MSX1/2 was similar between control and β-*cat*^{mut} embryos, suggesting that BMP activity is not dependent on WNT signaling in the ventral foregut endoderm.

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 *Shh*^{cre};*Bmpr1a*$^{fl/-}$;*Bmpr1b*$^{-/-}$;*Sox2*$^{fl/fl}$ embryos (*Bmpr1a;b;Sox2*) 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* mutants, 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. 6F,J 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 BMPR1A between control and *Bmpr1a;b;Sox2* mutants (Fig. 6G,H). Similar to *Bmpr1a* mutants (see Fig. S1C in the supplementary material), we found that BMPR1A 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 BMPR1A;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 BMPR1A;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 GCCGnCG) (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 a constitutively active form of *Bmpr1a* (*Bmpr1a*^{ca}). As a positive control for BMPR1A^{ca} activity, we found that a reporter containing a BMP-response element (*BRE-luc*) was robustly activated by expression of *Bmpr1a*^{ca} (*Bmpr1a*^{ca} 10.11±0.31 versus control 1±0.02, n=3 each, $P=3\times10^{-4}$) (Fig. 6N). By contrast, activity of the *Sox2* promoter was significantly repressed by *Bmpr1a*^{ca} (*Bmpr1a*^{ca} 0.50±0.06 versus control 1±0.05, n=3 each, $P=4\times10^{-4}$) (Fig. 6N). This repression was attenuated when substitution mutations were introduced in the first and last putative SMAD4-binding sites (*Sox2*^{mut}*-luc* 0.88±0.07 versus *Sox2-luc* 0.58±0.08, n=6 each, $P=3\times10^{-5}$) (Fig. 6O). From these data, we conclude that signaling though BMPR1A represses the *Sox2* 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 BMPR1A;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 BMPR1A;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 BMPR1A 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 BMPR1A 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-*cat*^{mut} 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 lens 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 *Bmp4*^{lacZ} reporter allele (Weaver et al., 2000). This suggests that BMP antagonizes a subset of downstream responses to FGF10, rather than antagonizing core FGF 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 FGF 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.

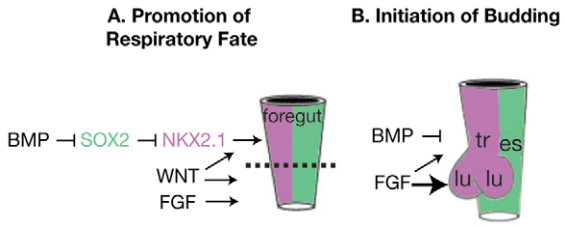

Fig. 7. A model for the roles of BMP signaling in early foregut development. (**A**) Signaling through BMPR1A;B in ventral foregut endoderm promotes *Nkx2-1* expression and trachea formation by repressing *Sox2*. Broken line indicates boundary between BMPR-dependent (prospective trachea) and -independent (prospective lung) respiratory progenitors. (**B**) Signaling through BMPR1A;B in foregut endoderm represses budding morphogenesis in the anterior foregut. Modified, with permission, from Que et al. (Que et al., 2006). es, esophagus; lu, lung; tr, trachea.

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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Competing interests statement
The authors declare no competing financial interests.

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