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Smad1 and its target gene *Wif1* coordinate BMP and Wnt signaling activities to regulate fetal lung development

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SUMMARY

Bone morphogenetic protein 4 (Bmp4) is essential for lung development. To define the intracellular signaling mechanisms by which Bmp4 regulates lung development, BMP-specific Smad1 or Smad5 was selectively knocked out in fetal mouse lung epithelial cells. Abrogation of lung epithelial-specific Smad1, but not Smad5, resulted in retardation of lung branching morphogenesis and reduced sacculation, accompanied by altered distal lung epithelial cell proliferation and differentiation and, consequently, severe neonatal respiratory failure. By combining cDNA microarray with ChIP-chip analyses, Wnt inhibitory factor 1 (*Wif1*) was identified as a novel target gene of Smad1 in the developing mouse lung epithelial cells. Loss of Smad1 transcriptional activation of *Wif1* was associated with reduced Wif1 expression and increased Wnt/β-catenin signaling activity in lung epithelia, resulting in specific fetal lung abnormalities. This suggests a novel regulatory loop of Bmp4-Smad1-Wif1-Wnt/β-catenin in coordinating BMP and Wnt pathways to control fetal lung development.

KEY WORDS: Lung morphogenesis, Bone morphogenetic protein, Smad1, Wif1, Mouse

INTRODUCTION

Disruption of normal lung development can result in a variety of neonatal respiratory problems (Warburton et al., 2006b), although the molecular mechanism of lung development and its relationship to neonatal pulmonary diseases remains incompletely understood. Lung development is composed of early branching morphogenesis and late alveogenesis processes. Through branching morphogenesis, the lung epithelial buds undergo reiterated outgrowth, elongation and subdivision to form complicated airway structures, accompanied by vasculogenesis (Hogan, 1999; Metzger et al., 2008). Then, terminal air sacs and alveoli are formed to generate a large gas-exchange surface. At the same time, distal lung epithelial cells differentiate into type I and type II alveolar epithelial cells (AECI and AECII). Cuboidal AECII undergo marked ultrastructural and biochemical changes, including depletion of glycogen, increased synthesis of surfactant proteins and lipids, increased numbers of lamellar bodies and secretion of surfactant proteins into the air space, which reduces surface tension at the air-liquid interface, while flat AECI cover the majority of the airspace surface and provide the capacity for fluid transportation across the respiratory membrane (Cardoso and Whitsett, 2008; Whitsett and Weaver, 2002; Williams, 2003). These complicated lung developmental processes are regulated by coordinated signaling networks, including bone morphogenetic proteins (BMPs) and Wnts (Cardoso, 2001; Warburton et al., 2006a).

Among the many BMP ligands, Bmp4 has been identified as a major ligand involved in lung developmental regulation (Eblaghie et al., 2006). However, the specific intracellular signaling pathway that mediates BMP regulatory effects during lung development has not been defined. Studies in vitro have demonstrated that BMP ligands bind to heteromeric BMP receptor complexes and activate the receptor serine/threonine kinases, which subsequently phosphorylate cytoplasmic Smad1, Smad5 and Smad8 (also known as Smad9 – Mouse Genome Informatics). These phosphorylated Smads then dissociate from the receptors, form complexes with Smad4, translocate into the nucleus and bind to BMP-responsive elements in their target genes to modulate gene expression (Shi and Massague, 2003). In addition, Smad-independent pathways have also been reported to transduce BMP signals (Derynck and Zhang, 2003). Previous studies by us and other groups have found that Alk3 [also known as BMP type IA receptor (Bmpr1a)], rather than Alk2 (Acvr1) and Alk6 (Bmpr1b), plays an essential role in mediating BMP regulatory signals during prenatal lung development in mice (Eblaghie et al., 2006; Sun et al., 2008). Abrogation of Alk3 in developing mouse lung epithelial cells causes immediate neonatal respiratory distress and lethality due to abnormal fetal lung formation and postnatal lung atelectasis.

In order to further define the intracellular BMP signaling mechanisms that regulate lung development, the role of BMP-specific downstream Smads must be addressed. As reported by other groups, blockade of Smad8 function by a conventional knockout approach in mice has no significant impact on fetal lung formation, although pulmonary arterial medial thickening and pulmonary adenoma are observed in the adult *Smad8* knockout lung (Arnold et al., 2006; Huang et al., 2009). Therefore, we focused on Smad1 and Smad5 in lung development in vivo. We employed a *loxP*/Cre genetic approach to generate lung epithelial cell-specific conditional gene knockout mice, as conventional deletion of the *Smad1* or *Smad5* gene results in early embryonic

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lethality before lung organogenesis (Chang et al., 1999; Lechleider et al., 2001; Tremblay et al., 2001; Yang et al., 1999). Furthermore, we also explored specific Smad1-modulated target genes in developing fetal mouse lung at the transcriptional level. One of these is Wnt inhibitory factor 1 (*Wif1*), which encodes a factor that acts as an antagonist for Wnt ligand and negatively regulates Wnt signaling activity (Hsieh et al., 1999). Reduction of *Wif1* gene expression and the subsequent increase in β-catenin activation result in specific distal lung abnormalities in *Smad1* conditional knockout fetal lung. Based on the data presented here, we define for the first time the intracellular BMP signaling pathway that is involved in regulating fetal lung development, and then propose a novel mechanism for signal crosstalk between the BMP and Wnt pathways in controlling lung organogenesis and homeostasis.

MATERIALS AND METHODS

Mouse strains and breeding

Floxed *Smad1* mice were generated in the laboratory of Dr Anita B. Roberts (Huang et al., 2002), in which the *Smad1* gene locus was targeted by *loxP*-PGKneo-*loxP*-exon2-*loxP*. We then removed the PGKneo cassette by crossing the floxed *Smad1* heterozygous mice with EIIa-Cre mice (Xu et al., 2001) to generate the $Smad1^{fx}$ allele (*loxP*-exon2-*loxP*) in order to avoid non-specific genetic interference caused by PGKneo cassette insertion. The *Smad1* heterozygous mouse line ($Smad1^{+/-}$) was also derived from the above crossbreeding. Floxed *Smad5* mice were as published previously (Umans et al., 2003). $Smad5^{+/-}$ mice were derived by crossing $Smad5^{+/fx}$ with X-linked CMV-Cre mice (Su et al., 2002). Inducible lung epithelial-specific Cre transgenic mice (*SPC-rtTA/TetO-Cre*) were provided by Dr Jeffrey Whitsett (Perl et al., 2002; Perl et al., 2009), in which line 2 *SPC-rtTA* mice were used. Lung epithelial-specific conditional knockout of *Smad1* (*Smad1* Ep-CKO) or *Smad5* (*Smad5* Ep-CKO) was induced by doxycycline (Dox) administration from E6.5 to the experimental end point by feeding the pregnant mice with Dox food (625 mg/kg; TestDiet, Richmond, IN, USA) and drinking water (0.5 mg/ml; Sigma, St Louis, MO, USA). Lung development was not affected in *SPC-rtTA/TetO-Cre* transgenic mice after Dox induction. In addition, floxed β-catenin mice ($Ctnnb1^{fx/fx}$) and BAT-lacZ Wnt signaling reporter mice (Brault et al., 2001; Maretto et al., 2003) were obtained from the Jackson Laboratory. All mice were bred in the C57/BL6 background and genotyped by genomic DNA PCR. Mice used in this study were housed in pathogen-free conditions according to the protocol approved by the Institutional Animal Care and Use Committee at the Saban Research Institute of the Childrens Hospital Los Angeles.

Morphological analysis

Lung tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Hematoxylin and Eosin (H&E)-stained sections were used for histological examination. Periodic acid-Schiff (PAS) staining was performed using a commercial kit (Sigma). Immunohistochemistry was carried out using a HistoStain kit (Invitrogen). Either 3-amino-9-ethylcarbazole or 3,3′-diaminobenzidine was used as the chromogenic substrate. The antibodies used were: rabbit anti-prosurfactant protein C (Pro-SP-C; Fitzgerald Industries International), rabbit anti-Clara cell-specific protein (CCSP) and FoxJ1 (Seven Hills Bioreagents), mouse monoclonal anti-Smad1 (Millipore) and mouse monoclonal anti-β-tubulin IV (BioGenex).

Transmission electron microscopy (TEM) was performed on E18.5 lung tissues as previously described (Sun et al., 2008). Ultrathin sections stained with uranyl acetate and lead citrate were viewed and photographed under a JEOL 1200CX electron microscope. In addition, X-gal staining of lung cryosections was used to detect *lacZ* expression following the protocol published previously (Xu et al., 2008).

Cell proliferation and apoptosis

Cell proliferation was analyzed by proliferating cell nuclear antigen (Pcna) staining using a PCNA staining kit (Invitrogen). Apoptosis was evaluated using an ApopTag kit (Chemicon).

Western blot and densitometric analysis

Protein detection in lung tissue by western blot has been described previously (Sun et al., 2006). Proteins were detected by specific antibodies against Smad1 (Millipore), Wif1 (goat polyclonal, Santa Cruz), active β-catenin (8E7, mouse monoclonal, Millipore), phosphorylated Lrp6 [Ab1490, kindly provided by Dr Xi He at Harvard Medical School (He et al., 2004)], β-actin (Santa Cruz) and Gapdh (6C5, mouse monoclonal, Fitzgerald). The intensities of the protein bands were quantified using Image J software and normalized to the protein loading control (Gapdh).

RNA isolation and real-time PCR analysis

Total RNA was isolated from lung tissues using the RNeasy kit (Qiagen) following the manufacturer's protocol. Real-time PCR reactions were performed as described previously (Chen et al., 2008a). Primer sequences are described in our previous publication (Sun et al., 2008).

cDNA microarray analysis

Total RNA (20 μg) isolated from E18.5 wild-type and *Smad1* Ep-CKO lungs was used to prepare biotinylated cRNA according to the protocol described in the Affymetrix expression analysis technical manual. Samples from three pairs of *Smad1* Ep-CKO mice and wild-type controls were used to hybridize with GeneChip Mouse Genome 430 2.0 arrays (Affymetrix). Images were acquired using a GeneChip Scanner 3000. Data were analyzed using an RMA method to perform background adjustment and quantitative normalization (Bolstad et al., 2003). The expression ratio between knockout and wild-type samples was computed. Probes that did not have detectable signals in both samples were discarded. Three pairs of experiments were then combined, and genes that were all upregulated or downregulated across all three experiments with an expression ratio greater than 2 were selected. Microarray data have been deposited at Gene Expression Omnibus with accession number GSE26502.

For gene ontology analysis, BioConductor (Gentleman et al., 2004) was used to classify differentially expressed genes into the following categories according to their gene ontology annotations: respiratory gaseous exchange, ion transport, fluid transport, lipid metabolic process, lipid transport, cell proliferation, and cell differentiation.

Chromatin immunoprecipitation combined with microarray technology (ChIP-chip)

Three E18.5 wild-type lung tissues were pooled, crosslinked with 1% formaldehyde and then quenched using 2.5 M glycine. After washing in PBS, the fixed lung tissues were homogenized in 3 ml cold whole-cell lysis buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate, 0.5% N-lauroyl sarcosine) with protease and phosphatase inhibitors. Lysates were sonicated using a Branson 250 Sonifier (30 seconds on/2 minutes off pulses, 100 times on high setting). After removing the debris, chromatin was immunoprecipitated with 25 μg rabbit monoclonal antibody against mouse phosphorylated Smad1 (Cell Signaling Technology) and mouse monoclonal antibody against RNA polymerase II (clone 8WG16, Covance) that had been pre-bound to Dynal protein G magnetic beads. These antibodies have been qualified in a previously published ChIP-seq study (Chen et al., 2008b). Following a washing series of increasing stringency, the antibody-bound chromatin was eluted and treated with RNase and proteinase K. The released DNA was then purified using phenol:chloroform:isoamyl alcohol (25:24:1, v/v). After T4 DNA polymerase fill-in and blunt-end ligation with a primer pair, the sample DNA was amplified by a ligation-mediated 24-cycle PCR. The PCR products were column purified using the QIAquick PCR purification kit (Qiagen), fluorescently labeled and hybridized to MM8 RefSeq promoter arrays (Roche Nimblegen), which cover ~2 kb upstream and 0.5 kb downstream of the 5′ transcriptional start site of 19,489 annotated mouse genes. The arrays were scanned and data were extracted and analyzed at Roche Nimblegen.

Chromatin immunoprecipitation (ChIP)

Smad1 ChIP using E18.5 wild-type lung tissue was performed as previously described (Xu et al., 2008) using antibodies against Smad1 (A-4, Santa Cruz), phosphorylated Smad1 (Cell Signaling Technology) and

RNA polymerase II (Covance). Immunoprecipitated samples were analyzed by PCR with primers spanning the potential Smad1 binding sites (–362 to –232, sense 5′-CCCCGTTTTCATTCACCTG-3′ and antisense 5′-CAAAAGGCGAGCACTGAGAG-3′; –152 to –55, sense 5′-TTTGCC-AGCATCGTGTCTC-3′ and antisense 5′-CTGGAGAGGAGGGAGC-AAG-3′) in the mouse *Wif1* gene promoter.

Transient transfection of *Wif1* promoter reporter DNA constructs

A region of the *Wif1* gene (–940 to +20) was amplified from mouse genomic DNA and subcloned into the pGL2-Basic vector (Promega) (Wif1-luc) and verified by DNA sequencing. The potential Smad1 binding element in Wif1-luc was mutated using the GeneTailor Site-Directed Mutagenesis System (Invitrogen) according to the manufacturer's protocol. Human *SMURF1* cDNA was subcloned into pcDNA3 and verified as previously described (Shi et al., 2004). Human *SMAD1* cDNA was subcloned into pcDNA3. The constitutively active Alk3 plasmid was as previously reported (Katagiri et al., 2002). A549 cells were transfected using FuGENE HD Transfection Reagent (Roche) following the manufacturer's instructions. Luciferase activity in cell lysates was detected using the Dual-luciferase Reporter Assay System (Promega) and normalized to Renilla luciferase activity of co-transfected pRL-CMV. Empty pCR-CMV vector was used to adjust the total amount of DNA for transfection.

Data presentation and statistical analysis

Thirty-two pairs of *Smad1* Ep-CKO and wild-type littermate control mice and five pairs of *Smad5* Ep-CKO and wild-type littermate control mice were generated for these studies. All experiments were repeated at least three times and data represent consistent results. The quantitative data are expressed as mean ± s.d. Statistical difference between two independent groups was assessed by an independent samples *t*-test; $P \leq 0.05$ was considered statistically significant.

RESULTS

Epithelial cell-specific deletion of intracellular BMP signaling molecules Smad1 or Smad5 during fetal mouse lung development

Previous studies by us and other groups have established that Alk3 is a major type I receptor that mediates BMP regulatory signaling in fetal lung development in vivo (Eblaghie et al., 2006; Sun et al., 2008). We now further dissect the BMP downstream signaling mechanisms during mouse lung development. As mentioned above, *Smad1* and *Smad5* are candidate genes that potentially play unique roles in regulating lung development. Conventional knockout of the *Smad1* or *Smad5* gene in mice results in early embryonic lethality before lung formation (Chang et al., 1999; Lechleider et al., 2001; Yang et al., 1999), which makes these mouse models unsuitable for studies of lung development. Therefore, to determine the role of Smad1- or Smad5-mediated BMP signaling in developing lung epithelial cells, we first generated fetal lung epithelial-specific *Smad1* or *Smad5* conditional knockout mice using an inducible *loxP/SPC-rtTA/TetO-Cre* approach, as published previously (Perl et al., 2002; Sun et al., 2008).

Floxed *Smad1* mice ($Smad1^{fx/fx}$) were crossed with *Smad1* heterozygous mice carrying *SPC-rtTA/TetO-Cre* transgenes ($Smad1^{+/-}$*/SPC-rtTA/TetO-Cre*). Dox was given to the pregnant mother at E6.5 and administration maintained to the experimental end point to induce lung epithelial cell-specific deletion of the *Smad1* gene (*Smad1* Ep-CKO), with a tail DNA genotype of $Smad1^{fx/-}$*/SPC-rtTA/TetO-Cre* (Fig. 1A). Since lung development in $Smad1^{fx/+}$, $Smad1^{fx/+}$*/SPC-rtTA*, or $Smad1^{fx/+}$*/TetO-Cre* mice was not affected, compared with the mice of wild-type genotype ($Smad1^{+/+}$), these normal control mice will be referred to simply as 'wild type (WT)' in this paper. Conditional *Smad1* heterozygous (HT) knockout refers to the genotype in lung epithelial cells only

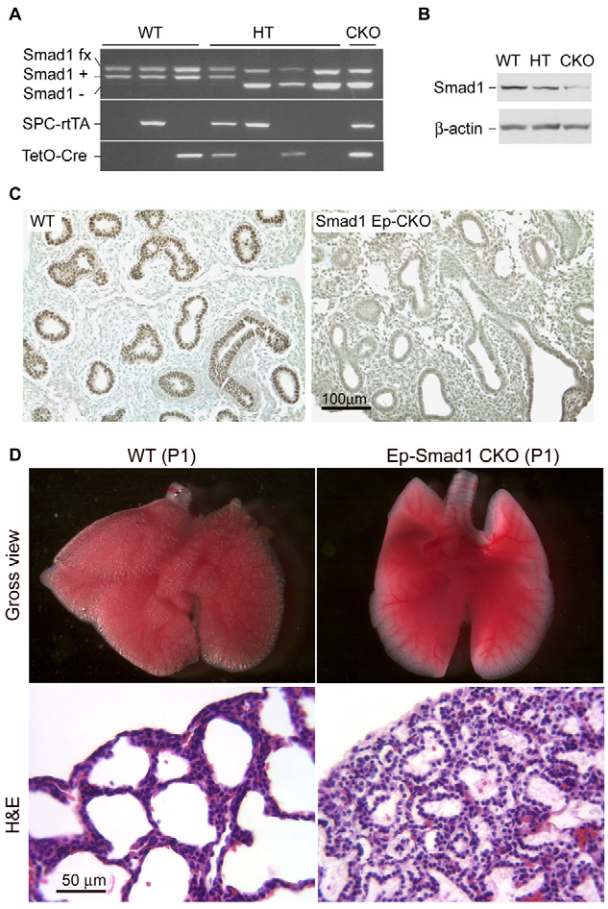

Fig. 1. Smad1 abrogation in lung epithelial cells causes neonatal pulmonary atelectasis. (**A**) PCR genotyping of offspring from intercrosses between $Smad1^{fx/fx}$ and $Smad1^{+/-}$*/SPC-rtTA/TetO-Cre* mice. (**B**,**C**) Smad1 protein levels in total lung tissue lysates (B) and in lung epithelial cells (C) were compared between *Smad1* knockout and littermate controls at E14.5. β-actin was used as a loading control. (**D**) Neonatal (P1) lungs of *Smad1* lung epithelial-specific conditional knockout (Ep-CKO) and control mice were compared in gross view and in Hematoxylin and Eosin (H&E)-stained tissue sections. WT, wild type (see main text); HT, *Smad1* heterozygous; CKO, *Smad1* conditional knockout.

($Smad1^{fx/+}$*/SPC-rtTA/TetO-Cre* with Dox induction), and conventional *Smad1* HT knockout ($Smad1^{fx/-}$*/SPC-rtTA*, $Smad1^{fx/-}$*/TetO-Cre* or $Smad1^{fx/-}$) refers to the genotype in the whole body. *Smad1* Ep-CKO in lung tissue was verified at the protein level by both western blot and immunohistochemistry. As illustrated in Fig. 1B, Smad1 protein in E18.5 lung tissue lysate was dramatically reduced in the conditional knockout lungs, compared with those of wild-type littermate controls, whereas an intermediate level of Smad1 was detected in *Smad1* heterozygous lungs. Consistently, Smad1 protein expression in airway epithelial cells at E14.5 was barely detected in *Smad1* Ep-CKO lungs as compared with the wild-type control (Fig. 1C).

Similarly, lung epithelial cell-specific *Smad5* conditional knockout mice (*Smad5* Ep-CKO) were obtained using the same approach as described for *Smad1*. Dox induction was initiated from E6.5, and *Smad5* Ep-CKO was also verified at the mRNA level by lung tissue RT-PCR (see Fig. S1 in the supplementary material) in addition to genomic DNA genotyping.

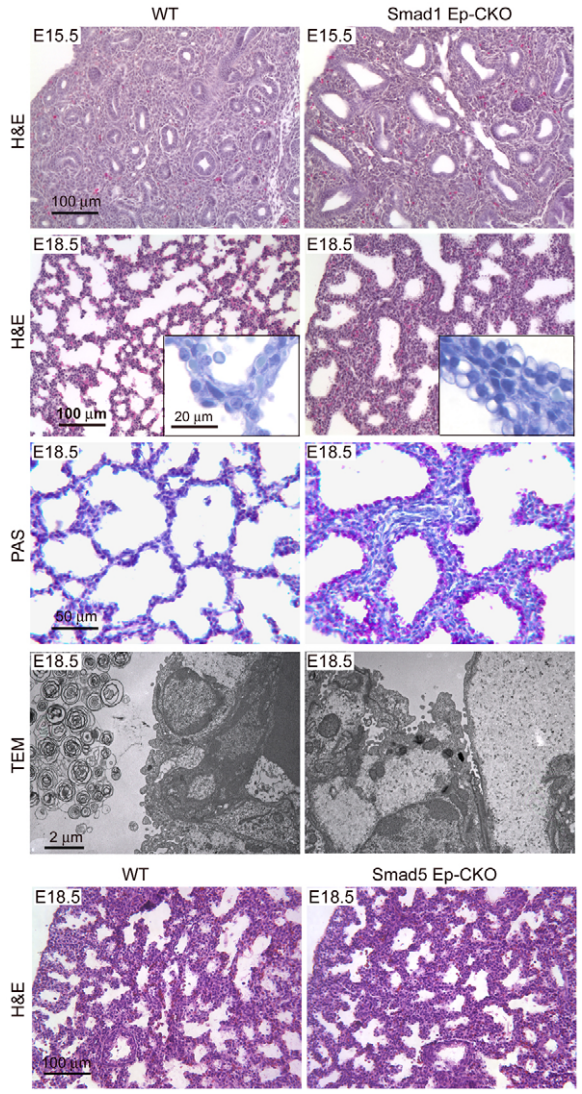

Fig. 2. Lung epithelial-specific deletion of *Smad1*, but not *Smad5*, results in abnormal fetal lung formation. Both embryonic lung branching morphogenesis and saccule formation were disrupted in *Smad1* Ep-CKO mice, as shown in H&E-stained lung sections obtained at E15.5 and E18.5. Changes in distal lung epithelial cell morphology (cuboidal in shape with large cytoplasmic vacuole) were also observed under high magnification in Ethylene Blue-stained thin (0.5 μm) tissue sections, as shown in the inset. The excessive accumulation of glycogen in the cytoplasm of *Smad1* Ep-CKO lung epithelial cells was determined by periodic acid-Schiff (PAS) staining. Alteration of the ultrafine structure in distal lung epithelial cells of E18.5 *Smad1* Ep-CKO mice was examined under transmission electron microscopy (TEM). By contrast, alteration of lung development was not detected in *Smad5* Ep-CKO mice at E18.5.

Lung epithelial cell-specific abrogation of Smad1, but not Smad5, results in abnormal fetal lung morphogenesis and neonatal respiratory distress

Newborn *Smad1* Ep-CKO mice developed severe respiratory distress with gasping and cyanosis and died within a couple of hours after birth. Unlike wild-type control lungs, the lungs isolated from newborn *Smad1* Ep-CKO mice before death [postnatal day (P) 1] were not inflated with air (Fig. 1D). H&E-stained lung tissue sections further showed amorphous material-filled air sacs, accompanied by thick and edematous air sac walls, suggesting atelectasis and a failure of postnatal lung fluid clearance (Fig. 1D). The specificity of the *Smad1* Ep-CKO phenotypes was verified by comparison with *Smad1*$^{fx/-}$*/SPC-rtTA/TetO-Cre* pups in the absence of Dox induction during gestation, which had a normal lung structure compared with their littermate controls. In addition, *Smad1* Ep-CKO mice with Dox induction from E6.5 to mid-gestation also suffered neonatal respiratory failure, similar to animals with continuous Dox administration. Thus, the abnormal lung phenotypes were not caused by overexpressed rtTA-induced toxicity, as reported elsewhere (Perl et al., 2009).

In order to characterize the abnormal lung formation observed in *Smad1* Ep-CKO mice, the prenatal lung morphology at different developmental stages was examined in detail. No apparent differences in early branching morphogenesis could be observed by directly counting the branches in wild-type versus *Smad1* Ep-CKO lungs at E12.5. However, later in gestation, ~50% of E14.5 (n=8) and 100% of E15.5 (n=9) *Smad1* Ep-CKO lungs displayed a decreased number of peripheral airways, accompanied by dilation of the lumen, as quantified in tissue sections (Fig. 2). These morphological abnormalities became more obvious at the end of gestation. The distal lungs of *Smad1* Ep-CKO mice at E18.5 exhibited profound pathological changes, characterized by a significantly decreased number of terminal sacs, enlarged saccular lumen and thickened mesenchymal tissues. The overall airspace area relative to the entire tissue area was significantly larger in *Smad1* Ep-CKO lungs (34.7±5.9%) than in wild-type controls (25.1±3.7%; P<0.05). Unlike normal control lungs, in which squamous peripheral epithelial cells covered the peripheral sac surface at E18.5, the epithelial cells lining the distal air sacs of *Smad1* Ep-CKO lungs remained cuboidal in shape with enlarged cytoplasm, as shown in Ethylene Blue-stained ultrathin (0.5 μm) lung tissue sections (Fig. 2). Furthermore, the cytoplasm was stained by PAS (Fig. 2), indicating an excessive accumulation of glycogen in these cells. Glycogen normally accumulates in immature lung epithelial cells and is converted into phospholipids and mobilized to lamellar bodies during lung epithelial cell differentiation and maturation. Therefore, glycogen accumulation reflects immaturity of peripheral lung epithelial cells. More detailed changes in peripheral lung epithelial cells in *Smad1* Ep-CKO lungs at E18.5 were detected at the ultrafine level by TEM. As shown in Fig. 2, control lungs at this stage had cuboidal type II-like epithelial cells that contained highly organized rosette glycogen, apical microvilli and numerous lamellar bodies, which are the intracellular storage forms of surfactant. Secreted lamellar bodies were also observed in the lumen of peripheral airspaces. By contrast, peripheral lung epithelial cells in *Smad1* Ep-CKO mice had enriched cytoplasmic glycogen, smaller apical microvilli and markedly decreased lamellar bodies. Secretion of surfactants into the airspace was barely detectable. Taken together, these observations in the distal lungs of *Smad1* Ep-CKO mice are consistent with pulmonary immaturity that contributes to extensive atelectasis at birth, suggesting that Smad1-mediated BMP signaling is essential for promoting peripheral lung epithelial cell differentiation and maturation during fetal development.

In contrast to *Smad1* Ep-CKO mice, newborn *Smad5* Ep-CKO mice became oxygenated and survived normally. There were no structural differences between E18.5 wild-type controls and *Smad5* Ep-CKO lungs (Fig. 2).

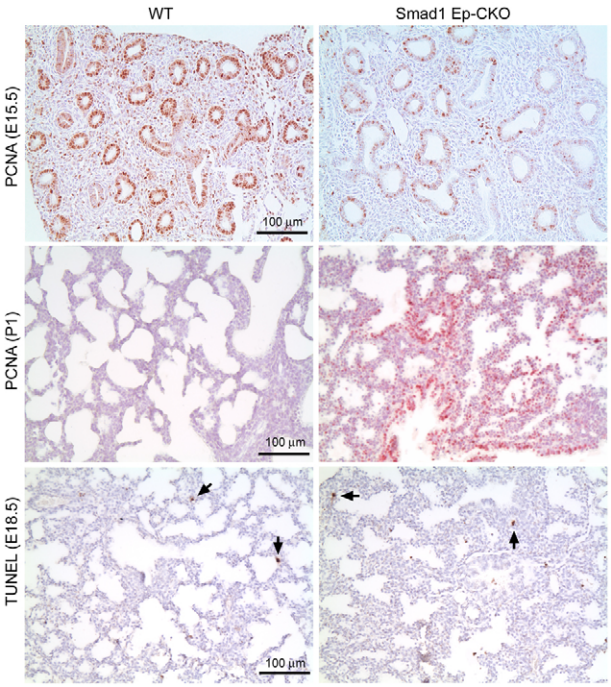

Fig. 3. Deletion of *Smad1* in developing lung epithelial cells results in altered cell proliferation but not apoptosis. Cell proliferation, as detected by Pcna immunostaining (red), appeared reduced in *Smad1* Ep-CKO mouse lungs at early to mid-gestation (E15.5). However, increased cell proliferation was detected in perinatal stages (P1). Apoptosis in lung tissues, as shown by TUNEL assay (brown, arrows), was unchanged in *Smad1* Ep-CKO compared with control littermates.

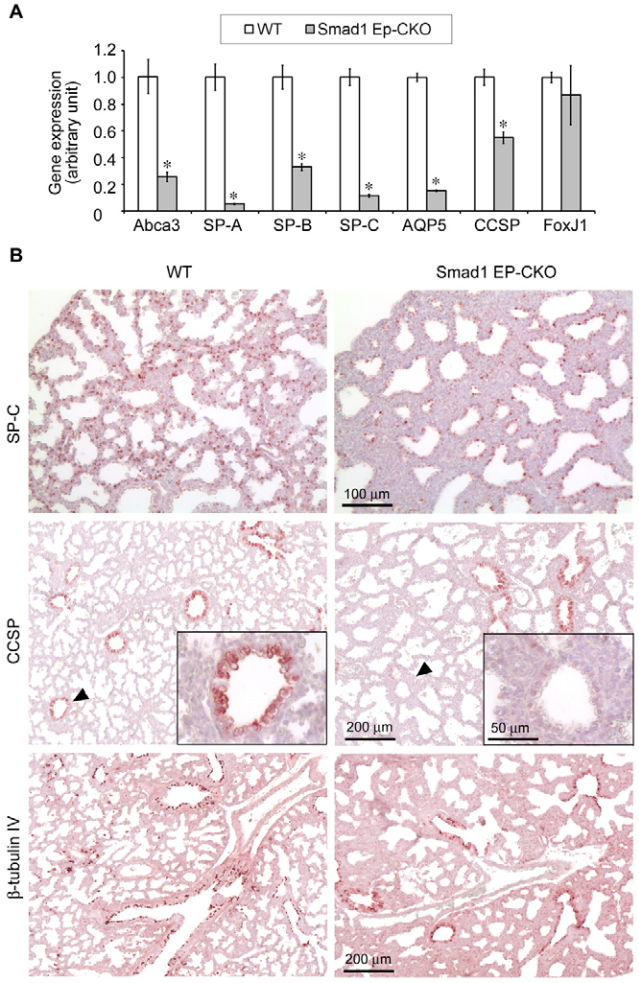

Fig. 4. Blockade of Smad1-mediated BMP signaling results in altered distal lung epithelial cell differentiation. (**A**) Real-time RT-PCR was performed to quantify mRNA expression of marker genes for a variety of airway/lung epithelial cells at E18.5. β-actin was used as an internal control for normalization. *, $P<0.05$ as compared with control. Data are mean ± s.d. (**B**) Changes in lung epithelial cell marker expression at the protein level were verified by immunohistochemistry (red). SP-C, CCSP and β-tubulin IV are markers for differentiated distal, mid-distal and proximal airway epithelial cells, respectively. CCSP staining in terminal airways (arrows) was also visualized under high magnification (insets).

Smad1-mediated BMP signaling in lung epithelial cells is essential for regulating cell proliferation and differentiation

Abnormal development of lung structure may be related to altered cell proliferation, differentiation or apoptosis. Cell proliferation was compared between *Smad1* Ep-CKO and wild-type lungs by detecting the cell proliferation marker proliferating cell nuclear antigen (Pcna). A significant reduction of Pcna-positive cells, particularly of lung epithelial cells, was detected in *Smad1* Ep-CKO lungs (Fig. 3) at E15.5, when less branching morphogenesis was observed, suggesting that reduced cell proliferation might be one of the causes for retarded airway branching in early gestation. However, at late gestation and neonatal stages, the percentage of Pcna-positive cells in the *Smad1* Ep-CKO distal lungs was significantly increased compared with control lungs (Fig. 3), suggesting that normal BMP-Smad1 signaling might negatively regulate distal lung cell expansion during lung maturation when cell differentiation takes priority over growth. Therefore, BMP-Smad1 signaling plays distinct roles in regulating distal lung cell proliferation at different developmental stages. No significant change in apoptosis was observed between wild-type and *Smad1* Ep-CKO lungs at E18.5 (Fig. 3) as shown by TUNEL assay, suggesting that the thickened mesenchyme is unlikely to be due to reduced apoptosis.

Lung cell differentiation is accompanied by molecular marker expression that reflects the degree of maturation of the lung epithelia. In normal perinatal lung, proximal airway epithelial cells differentiate into ciliated columnar cells with expression of several unique molecular markers including FoxJ1 and β-tubulin IV. The transitional region of airways from proximal to distal is lined with differentiated Clara cells, which can be easily recognized by Clara cell-specific protein (CCSP; also known as Scgb1a1) expression, whereas distal lung epithelial cells express Abca3, surfactant proteins [including surfactant protein (SP)-A, -B, -C and -D; also known as Sftpa-d], and other ion/water channel proteins [including Aqp5 and T1α (also known as Pdpn)], and have the potential to become AECI and AECII after birth. As the histological studies above indicated pulmonary immaturity in *Smad1* Ep-CKO fetuses, gene expression at the mRNA level was compared between E18.5 *Smad1* Ep-CKO and wild-type control lungs using a real-time PCR approach. As shown in Fig. 4A, expression of the genes marking mid-distal airway epithelial cells, including those encoding Abca3, SP-A, SP-B, SP-C, SP-D, Aqp5, and CCSP, was drastically reduced in the *Smad1* Ep-CKO lungs, whereas the proximal epithelial cell marker *Foxj1* was not affected by *Smad1* deletion.

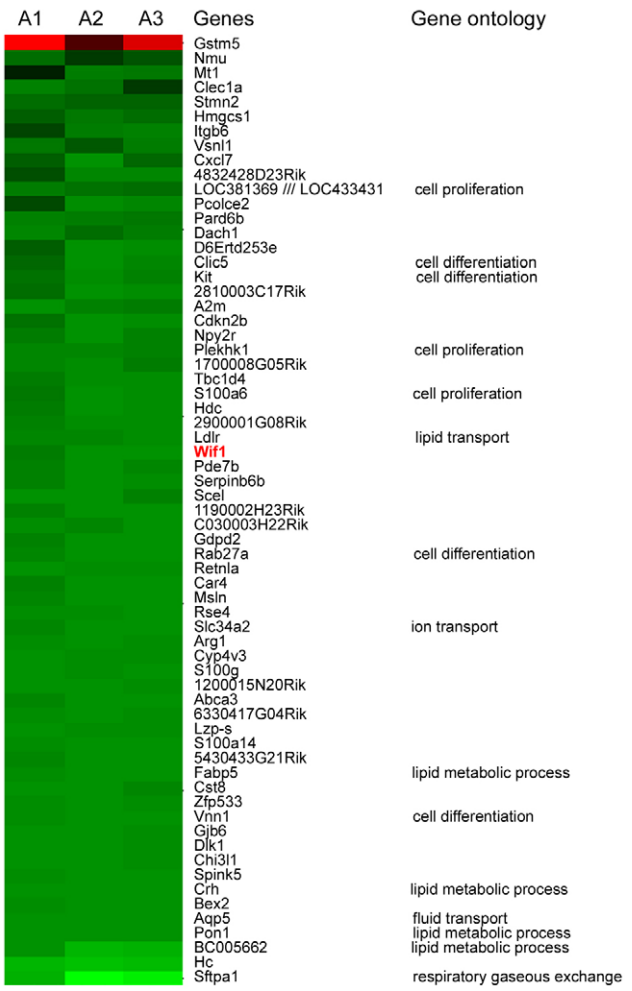

Fig. 5. Altered gene expression profile of mouse *Smad1* Ep-CKO lungs compared with wild-type controls as determined by cDNA microarray analysis. Genes that exhibited greater than 2-fold changes in all three paired microarray analyses are listed. Red, increased expression; green, decreased expression. *Wif1* is highlighted. In addition, ontological analysis shows that these include genes involved in respiratory gaseous exchange, ion or fluid transport, lipid metabolic process, lipid transport, cell proliferation, and cell differentiation, as indicated.

These data were further verified at the protein level by immunohistochemistry. As shown in Fig. 4B, there was no perceivable change in β-tubulin IV-positive epithelial cells in the proximal large airways. However, the number of distal SP-C-positive epithelial cells was significantly decreased. Interestingly, CCSP-positive epithelial cells in small terminal bronchioles were barely detected in the E18.5 *Smad1* Ep-CKO lungs, whereas these cells were still detected in relatively large airways. The absence of Clara cells in the small airways of *Smad1* Ep-CKO mice was not due to their replacement by proximal ciliated epithelial cells (see Fig. S2 in the supplementary material).

Altered gene expression profile in *Smad1* Ep-CKO lungs

Smad1 is a BMP-specific intracellular signaling component, the phosphorylated form of which is able to translocate into the nucleus and directly modulate target gene expression. Although blockade of Smad1-mediated function can directly affect target gene expression, the abnormally developed lung of *Smad1* Ep-CKO mice may also have subsequent changes in the gene expression profile. In order to compare gene expression profiles, total lung RNAs were isolated from wild-type control and *Smad1* Ep-CKO littermate fetuses at E18.5. cRNA probes were then prepared and hybridized with cDNA arrays on Affymetrix gene chips (Mouse Genome 430A 2.0 arrays). The data were analyzed using an RMA method (Irizarry et al., 2003). More than 65 genes were found to have greater than 2-fold changes in all three pairs of arrays (Fig. 5). The majority of these genes were downregulated. Some were involved in cell proliferation and differentiation, respiratory gaseous exchange, ion/water transport, lipid metabolism and transport (Fig. 5), as analyzed by gene ontology.

Screening Smad1 target genes in developing mouse lung

As changes in gene expression can be caused by blockade of Smad1 function directly or indirectly, potential BMP-Smad1 downstream target genes were further characterized using chromatin immunoprecipitation with subsequent mouse gene promoter array analysis (ChIP-chip). Thus, Smad1-bound DNA elements were pulled down from E18.5 lung tissue, and the amplified DNA probes were then hybridized with MM8 RefSeq promoter arrays (Roche Nimblegen), which cover ~2 kb upstream and 0.5 kb downstream of the 5′ transcriptional start site of 19,489 annotated mouse genes. In total, 2132 genomic DNA elements of 1103 genes were identified with detectable signal intensity (peak score of 2.3 and above, chosen based on the score from a verified Smad1 downstream target gene). The distribution of these genes is summarized in Fig. S3 in the supplementary material.

BMP-Smad1 signaling regulates Wif1, a protein that mediates crosstalk between BMP and Wnt pathways in the developing lung

Wif1 was one of the potential target genes identified by ChIP-chip. Altered *Wif1* gene expression in the *Smad1* Ep-CKO lungs was also detected by cDNA microarray (2.5- to 3.2-fold reduction; $P<0.05$). In order to validate these data, we further compared *Wif1* expression at both the mRNA and protein levels in lung tissues of *Smad1* Ep-CKO and control littermates. The mRNA levels of *Wif1* were reduced ~4-fold in the *Smad1* Ep-CKO lungs at E18.5 as measured by real-time RT-PCR (Fig. 6A). Consistently, Wif1 protein levels in the E18.5 *Smad1* Ep-CKO lungs were also significantly decreased by ~6-fold as compared with those of wild-type littermate controls (Fig. 6B and see Fig. S4 in the supplementary material).

Wif1 is a member of the secreted antagonists of the Wnt pathway, which bind directly to Wnt ligands to sequestrate their activities. Since the Wif1 protein level in *Smad1* Ep-CKO lungs was significantly reduced, altered Wnt signaling activity in the *Smad1* Ep-CKO lung was also expected. Canonical Wnt signaling activity in whole lung was evaluated by measuring the level of active β-catenin by western blot. As expected, β-catenin activity was increased 2-fold in *Smad1* Ep-CKO lungs at E18.5 (Fig. 6B and see Fig. S4 in the supplementary material). Increased Wnt signaling activity, particularly in distal lung epithelial cells at E18.5, was also confirmed in vivo by generating *Smad1* Ep-CKO in BAT-lacZ reporter mice, in which cells with active Wnt signaling can be detected by positive X-gal staining (Fig. 6C). Furthermore, expression of the related β-catenin target genes, cyclin D1 and *c-Myc*, was quantified at the mRNA level by real-

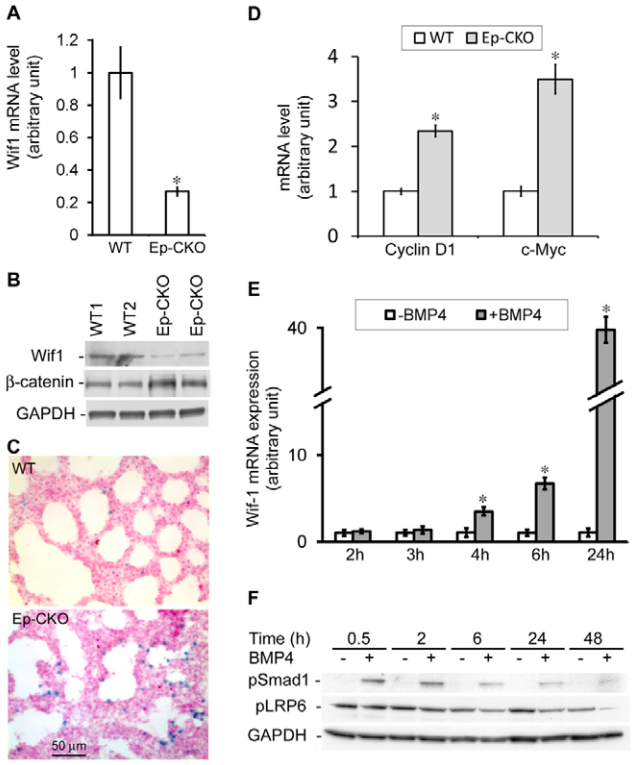

Fig. 6. Bmp4-Smad1 regulates Wnt signal activation through changing Wif1 expression. (**A-C**) Decreased *Wif1* expression at both the mRNA and protein levels in E18.5 *Smad1* Ep-CKO lungs as detected by real-time RT-PCR (A) and western blot (B). Increased Wnt signal activation in *Smad1* Ep-CKO lung was detected by western blot as increases in active β-catenin in total lung tissue lysate (B) and by X-gal staining in BAT-lacZ Wnt signaling reporter mouse lung (C). These increased *lacZ*-positive cells were mainly distal lung epithelial cells, based on their localization. (**D**) Upregulated expression of the Wnt target genes cyclin D1 and *c-Myc* was also detected in *Smad1* Ep-CKO lungs. (**E**,**F**) In cultured mouse lung epithelial cells (MLE12), addition of Bmp4 (100 ng/ml) immediately activated Smad1 (F) and subsequently upregulated *Wif1* gene expression (E). Consistently, Wnt pathway inhibition was detected by reduced phosphorylation levels of the Wnt co-receptor Lrp6 (F). Gapdh was a loading control. *, $P<0.05$. Data are mean ± s.d.

time PCR. Consistent with increased β-catenin activation, both cyclin D1 and *c-Myc* expression increased significantly (2-fold) in E18.5 *Smad1* Ep-CKO lungs compared with wild-type controls.

BMP-Smad1-Wif1-Wnt activity was also directly evaluated in cultured mouse lung epithelial cells (MLE12, Fig. 6E,F). Addition of Bmp4 ligand (100 ng/ml) to the cultured cells activated Smad1 immediately, as measured by Smad1 phosphorylation levels, which reached a peak at 2 hours and was sustained up to 48 hours. Moreover, *Wif1* gene expression at the mRNA level was induced after 4 hours of Bmp4 treatment and reached a ~40-fold increase after 24 hours. Wnt signaling activity was inhibited inversely with Wif1 induction 6 hours after Bmp4 stimulation, as shown by reduced phosphorylation levels of the Wnt co-receptor Lrp6. Taken together, these data suggest that Wif1, which may be one of the key molecules regulated by the BMP-Smad1 pathway, mediates a BMP-Smad1 negative regulatory effect on the canonical Wnt pathway, thus coordinating these signaling pathways in the regulation of lung epithelial cell proliferation and differentiation during late gestation.

Smad1 directly binds and activates the *Wif1* promoter

Previous studies have confirmed that Smad1 activates mouse inhibitor of DNA binding 1 (*Id1*) gene transcription by directly binding to the imperfect palindrome DNA sequences (CCGCCCGCGCGG) in its promoter with high affinity (Katagiri et al., 2002). By analyzing *Wif1* promoter sequences (0.5 kb upstream of the transcriptional start site that was included in the promoter array of the ChIP-chip study above), two DNA sequences highly homologous to the Smad1 binding site in the *Id1* promoter were found in the mouse *Wif1* promoter (Fig. 7A). One of these *Wif1* promoter sequences is conserved between mouse and human, suggesting a potential Smad1 binding site on the *Wif1* promoter that might account for the positive result obtained from the ChIP-chip assay (Fig. 7B).

To further map these Smad1 binding sites, conventional Smad1 ChIP analyses using E18.5 wild-type mouse lung tissues were performed. ChIP was repeated with two different antibodies that recognized Smad1 and phosphorylated Smad1 (pSmad1), respectively (Fig. 7C). A region spanning –362 to –232 (P1), but not the region –152 to –55 (P2), of the mouse *Wif1* promoter could be reproducibly amplified from ChIP products. ChIP assay using an antibody to RNA polymerase II was used as a positive control for transcriptional complex-DNA binding, and a positive P1 amplification was detected in the RNA polymerase II ChIP assay. Thus, P1 may be a Smad1 binding site for BMP-Smad1-mediated transcriptional regulation of *Wif1*.

In order to confirm that Smad1 activates the *Wif1* promoter through direct binding to the P1 site, a wild-type (Wif1-luc) and two mutated (Wif1-MutA-luc and Wif1-MutB-luc) *Wif1* promoter/luciferase reporter constructs were transiently transfected into cultured A549 lung epithelial cells (Fig. 7D). Compared with the wild-type *Wif1* promoter, mutations in *Wif1* P1 sites significantly reduced *Wif1* promoter activities by 3- to 4-fold. Overexpression of a constitutively active form of BMP receptor IA (c.a. Alk3) and/or Smad1 drastically enhanced *Wif1* promoter activation (Fig. 7E), which could be partially inhibited by co-expression of Smurf1, an E3 ubiquitin ligase that promotes Smad1 and Smad5 ubiquitylation and degradation (Shi et al., 2004; Zhu et al., 1999). These data suggest that Smad1 can activate *Wif1* gene transcription in lung epithelial cells by directly binding to the P1 site in the *Wif1* promoter. Thus, *Wif1* is a Smad1 downstream target gene.

Increased Wnt activity caused by deficient Smad1-Wif1 regulation in fetal lung epithelial cells results in specific developmental lung abnormalities

In order to understand the role of BMP and Wnt pathway crosstalk in regulating fetal lung development, increased β-catenin activation in *Smad1* Ep-CKO lung epithelial cells was restored to normal levels via heterozygous deletion of β-catenin (*Ctnnb1*$^{del/+}$). By crossing *Smad1*$^{fx/fx}$;*Ctnnb1*$^{fx/+}$ mice with *Smad1*$^{+/-}$/*SPC-rtTA/TetO-Cre* mice, *Smad1* Ep-CKO mice with or without the β-catenin heterozygous knockout in fetal lung epithelial cells were obtained after Dox induction from E6.5 (Fig. 8A). The increased level of active β-catenin in *Smad1* Ep-CKO lung tissue lysates (2.3±0.3-fold over wild type), as measured by western blot and densitometric analysis (Fig. 8B,C), was restored to the normal level (1.1±0.1-fold over wild type) in the lungs of *Smad1* Ep-CKO/*Ctnnb1*$^{del/+}$ mice. Consistently, expression of the Wnt-regulated cyclin D1 and c-Myc in *Smad1* Ep-CKO/*Ctnnb1*$^{del/+}$ lung was comparable to that in the wild type (data not shown). In

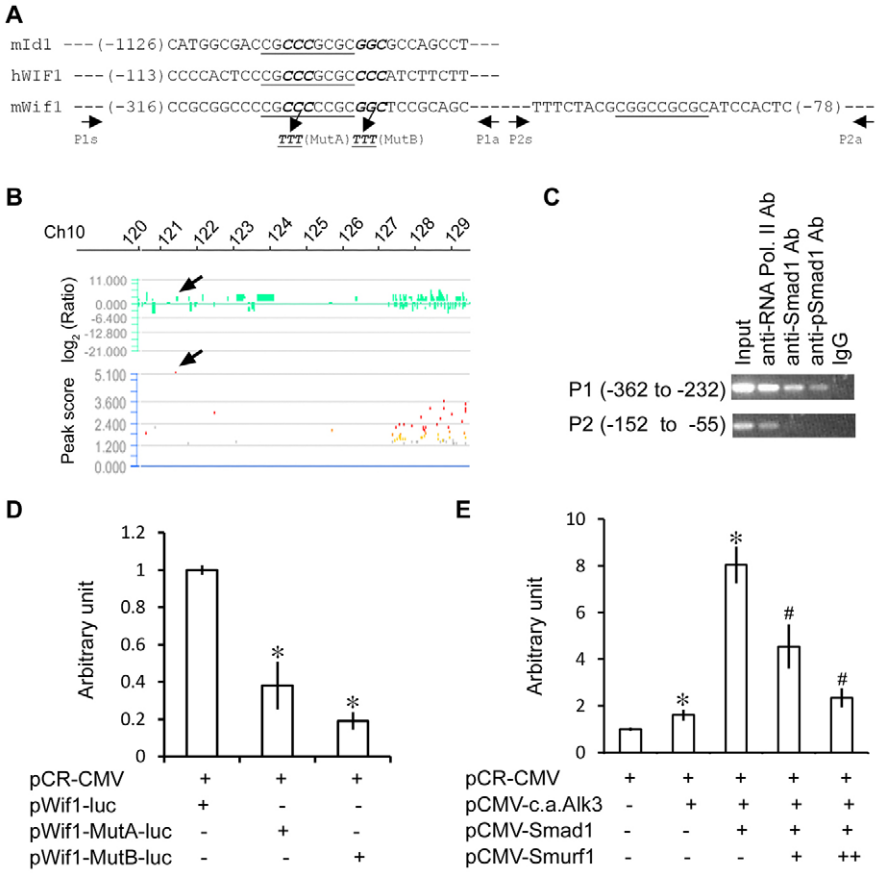

Fig. 7. Smad1 binds to the *Wif1* gene promoter and regulates Wif1 expression. (**A**) Comparison of a well-characterized Smad1-binding DNA sequence (underlined) in the *Id1* promoter with potential Smad1-binding sequences identified in the promoter region of the human (h) and mouse (m) *Wif1* genes. Mutated DNA sequences used in the promoter assay below are indicated. The positions of PCR primer pairs used for chromatin immunoprecipitation (P1 and P2) are marked. (**B**) Smad1 ChIP-chip demonstrated significant occupancy of the *Wif1* gene promoter on mouse chromosome 10 as shown by hybridization signal intensities (on different scales). Arrows indicate the positive signals for the *Wif1* genomic locus. (**C**) Independent ChIP assays using E18.5 wild-type lungs with antibodies to Smad1, phosphorylated Smad1, RNA polymerase II (positive control) and mouse IgG (negative control). For the input lane, 1% of total chromatin was used as the PCR template. (**D**) *Wif1* promoter/reporter activity was significantly inhibited by mutating the potential Smad1-binding sequences of the *Wif1* promoter as demonstrated in an in vitro promoter assay (*, $P<0.05$). (**E**) Overexpression of BMP signaling components, including constitutively active Alk3 (c.a. Alk3) and/or Smad1 activated the *Wif1* promoter/reporter (*, $P<0.05$). The specificity of this activation was verified by co-expressing Smurf1, which facilitates degradation of Smad1 protein through ubiquitylation to inhibit Smad1-mediated *Wif1* promoter activation (#, $P<0.05$, as compared with that of Alk3 and Smad1 co-expression without Smurf1). Overexpressing Smurf1 exerted a dose-related inhibitory effect on the *Wif1* promoter. Data are mean ± s.d.

correlation with the restoration of the active β-catenin level, heterozygous deletion of β-catenin in the *Smad1* Ep-CKO lung was also able to rescue certain *Smad1* Ep-CKO-specific lung abnormalities. Fetal lung morphology and distal lung epithelial cell proliferation were restored to wild-type (Fig. 8D,E versus Figs 2 and 3). However, abnormal distal lung epithelial cell differentiation in the *Smad1* Ep-CKO/*Ctnnb1*$^{del/+}$ lung was not completely rescued. In particular, Abca3 expression was still significantly reduced, although expression of some mid-distal fetal lung epithelial cell markers, such as SP-A, SP-C, Aqp5 and CCSP, was restored (Fig. 8F versus Fig. 4A). Overall, these data indicate that BMP-regulated Wnt signaling in distal lung epithelial cells is crucial for fetal lung development.

DISCUSSION

Abnormal fetal lung development and consequent pulmonary immaturity at birth result in insufficient respiratory gas exchange, causing neonatal respiratory distress, particularly in preterm infants (Whitsett et al., 2004). The molecular mechanisms controlling lung development and maturation are still not fully understood. Studies by our group and others have demonstrated that Bmp4 and its receptor Alk3 play important roles in regulating normal lung formation (Bellusci et al., 1996; Eblaghie et al., 2006; Shi et al., 2001; Sun et al., 2008). However, BMP intracellular signaling mechanisms involved in lung developmental regulation have never been systemically studied. Here, using a gene conditional deletion strategy, we showed that *Smad1* knockout in lung epithelial cells altered cell proliferation and differentiation, resulting in retarded branching morphogenesis, impaired terminal sacculation, neonatal lung inflation and fluid clearance, and neonatal respiratory failure. These observations suggested that intracellular Smad1, but not Smad5, mediates BMP regulatory effects in lung development and maturation, and that Smad1-mediated BMP signaling in lung epithelial cells is essential for embryonic lung branching morphogenesis and lung structural, biochemical and functional maturation prior to birth.

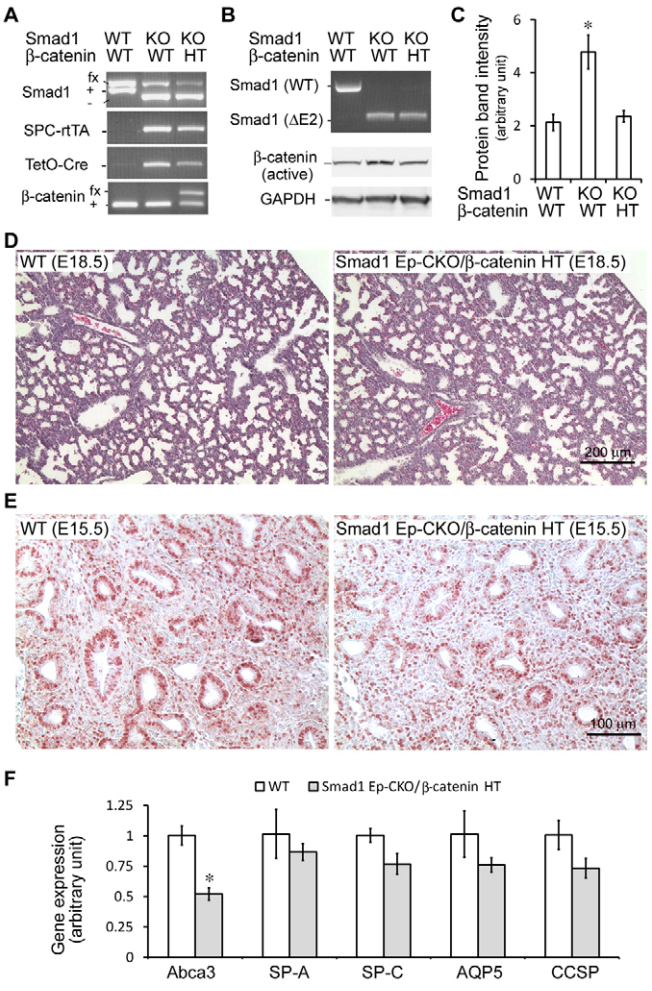

Fig. 8. *Smad1* Ep-CKO fetal lung development was partially rescued by restoring active β-catenin levels in lung epithelial cells. (**A-C**) *Smad1*/β-catenin genotypes were confirmed by genomic DNA PCR (A) and RT-PCR (B) of mouse lung tissues. Restoration of the active β-catenin level was confirmed by western blot and densitometric analysis (B,C). Gapdh was used as a loading control for normalization. *, $P<0.05$, compared with the wild type. (**D**,**E**) Fetal lung morphology and cell proliferation in *Smad1* Ep-CKO/β-catenin heterozygous (HT) lungs appeared normal, as shown by H&E staining (D) and Pcna immunostaining (red in E). (**F**) Expression of genes associated with peripheral lung epithelial cell differentiation and maturation was measured at the mRNA level by real-time PCR. *, $P<0.05$. Data are mean ± s.d.

As mentioned above, Alk3 is a key component in BMP-regulated mouse lung development (Eblaghie et al., 2006; Sun et al., 2008) and is an upstream activator of Smads. Abrogation of Alk3 in the prenatal lung epithelia (*Alk3* Ep-CKO), using the same SPC-rtTA/TetO-Cre-driven gene deletion, causes neonatal respiratory distress and lethality (Sun et al., 2008), possibly owing to retardation of branching morphogenesis and altered alveolar epithelial cell differentiation. The abnormal phenotypes in *Alk3* Ep-CKO lungs are similar to those observed in *Smad1* Ep-CKO lungs in current study, particularly at late gestation. However, retarded lung branching morphogenesis was detected earlier in *Alk3* Ep-CKO mice [phenotypic penetrance of 50% at E12.5 and 100% at E14.5 (Sun et al., 2008)] than in *Smad1* Ep-CKO mice (phenotypic penetrance of 50% at E14.5 and 100% at E15.5). In addition, extensive apoptosis, which was observed in *Alk3* Ep-CKO lung at late gestation (Eblaghie et al., 2006; Sun et al., 2008), was not detected in *Smad1* Ep-CKO lung at the same stage. Expression of genes associated with apoptosis, such as Bax, Birc5 and Birc4 (also known as Xiap), which was reported to be reduced in *Alk3* Ep-CKO lungs (Eblaghie et al., 2006), was not significantly changed in the *Smad1* Ep-CKO lung (data not shown). Whether the phenotypic differences between *Alk3* and *Smad1* Ep-CKO lungs are due to functional compensation by other BMP-specific Smads or to the involvement of Smad-independent pathways remains to be determined.

Studies in vitro suggest that activated Smad1 can form complexes with other transcriptional factors to directly modulate gene expression, which might be one of the molecular mechanisms underlying lung phenotypic changes. However, altered gene expression in the *Smad1* Ep-CKO lung could also be a consequence of malformed lung structure in addition to direct loss of Smad1-mediated gene regulation. Thus, the overall gene expression profile was first compared between *Smad1* Ep-CKO and control mouse lungs using a cDNA microarray approach. We then employed a strategy to find Smad1-interacting DNA sequences in gene promoters globally, using a ChIP-chip approach. Several novel target genes were identified by combining both approaches (data not shown). One of these was *Wif1*, an antagonist of Wnt ligand activity (Hsieh et al., 1999). Smad1 acts as a transcription factor in regulating *Wif1* gene expression, which was further verified in our study by determining the Smad1 binding site on the *Wif1* promoter and its activation of *Wif1* gene transcription. Other BMP-Smad1 target genes that are involved in lung development and maturation remain to be confirmed in future studies.

Blockade of Smad1 function in fetal lung epithelial cells results in downregulation of *Wif1* gene expression. As a result, an abnormally increased Wnt/β-catenin signaling activity occurs in the developing lung, as shown by increased β-catenin activation and upregulation of the Wnt target genes cyclin D1 and *c-Myc*, as well as increased *lacZ*-positive cells in BAT-lacZ reporter mouse lung. Wnt/β-catenin signaling is known to play a crucial role in lung branching morphogenesis and cell differentiation by establishing the proximal-distal cell fate in the respiratory epithelium. Although loss-of-function mutation of the canonical Wnt pathway by deleting or inhibiting β-catenin in respiratory epithelial cells in vivo impairs branching morphogenesis and peripheral airway cell differentiation (Mucenski et al., 2003; Shu et al., 2005), constitutive activation of Wnt/β-catenin signaling by overexpressing a β-catenin-Lef1 fusion protein in transgenic mice also leads to an embryonic-lethal phenotype with severe lung defects (Okubo and Hogan, 2004). This suggests that an appropriate level of Wnt signaling activity in respiratory epithelium is crucial for normal lung development, although the related molecular mechanisms for fine-tuning this signaling pathway activity in developing lung are currently unknown. Interestingly, there are some similarities between the abnormal phenotypes of β-catenin-Lef1 transgenic mouse lungs and *Smad1* Ep-CKO lungs at late gestation, which include reduced terminal air sac formation, altered epithelial cell morphology in distal airways, undifferentiated peripheral lung epithelial cells with accumulated glycogen and reduced lamellar bodies and surfactant proteins. However, other phenotypes in β-catenin-Lef1 transgenic mouse lung, such as the cell lineage switch to intestinal cells, were not detected in *Smad1* Ep-CKO lung (data not shown). This might be due to different levels of Wnt signal

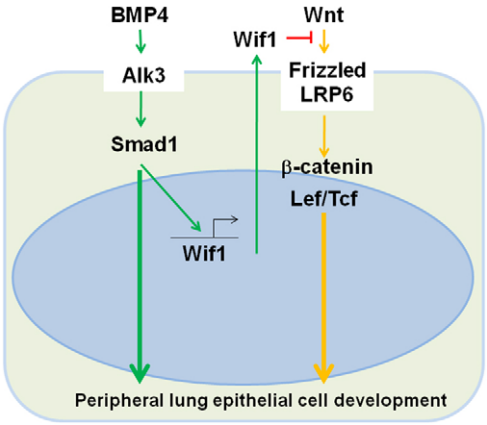

Fig. 9. Model of BMP-Wnt signaling crosstalk in the regulation of peripheral lung epithelial cell growth during fetal lung development. Green area, cytoplasm; blue oval, nucleus; green and yellow arrows, activation; red bar, inhibition. Other target genes of the BMP and Wnt pathways are excluded for simplicity.

activation and/or differences in altered downstream gene profiles between these two mouse models. Further, restoration of β-catenin signaling to wild-type levels by deleting one allele of β-catenin in vivo rescues the abnormal lung branching morphogenesis and epithelial cell proliferation, and also partially rescues the distal lung epithelial cell differentiation seen in the *Smad1* Ep-CKO lung. Therefore, Wif1 downregulation and subsequent Wnt signal activation caused by *Smad1*-null mutation are responsible for the specific fetal lung abnormalities in *Smad1* Ep-CKO mice.

Wnt ligand activity can be negatively regulated by several extracellular antagonists, including Wif1, which can directly bind to Wnt to prevent Wnt interaction with the receptor frizzled (Hsieh et al., 1999). Our current study suggests that increased Wnt/β-catenin signaling activity in *Smad1* Ep-CKO lungs is likely to be due to loss of Smad1 transcriptional activation of the *Wif1* promoter. Interestingly, reduced Bmp4 expression has been reported in lung with inhibited β-catenin activation (Shu et al., 2005), and it was proposed that the Wnt/β-catenin pathway functions upstream of Bmp4 signaling in developing pulmonary epithelial cells. We now show that the BMP-Smad1 signaling pathway also acts as a negative regulator of Wnt signaling activity by modulating expression of the Wnt antagonist Wif1. These findings suggest that finely tuned and coordinated Wnt and BMP signaling pathways might be essential for regulating lung epithelial cell development and maturation (Fig. 9).

In conclusion, the present study establishes a novel role for epithelial-specific BMP-Smad1 signaling in branching morphogenesis and lung maturation. Smad1 directly regulates *Wif1* gene expression, which coordinates the activation of the BMP and Wnt pathways. Changes in the activation of these pathways result in altered distal lung epithelial cell proliferation and differentiation and in disruption in the production of surfactant protein and Abca3, which is a common cause of neonatal respiratory distress, particularly in premature infants (Ramet et al., 2000; Shulenin et al., 2004; Whitsett et al., 2004). Thus, the data obtained from the current study greatly advance our understanding of the molecular basis of lung development and neonatal respiratory diseases, and suggest potential targets for therapeutic intervention.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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