Cardiovascular, Pulmonary and Renal Pathology

Prenatal Lung Epithelial Cell-Specific Abrogation of Alk3-Bone Morphogenetic Protein Signaling Causes Neonatal Respiratory Distress by Disrupting Distal Airway Formation

Jianping Sun,* Hui Chen,* Cheng Chen,[†]
Jeffrey A. Whitsett,[‡] Yuji Mishina,[§]
Pablo Bringas, Jr.,[¶] Jeffrey C. Ma,*
David Warburton,* and Wei Shi *^{¶¶}

From the Developmental Biology Program,* Childrens Hospital Los Angeles, Los Angeles, California; The Center of Craniofacial Molecular Biology,[¶] University of Southern California School of Dentistry, Los Angeles, California; the Department of Pediatrics,[‡] Division of Pulmonary Biology, Cincinnati Children's Hospital Medical Center and the University of Cincinnati College of Medicine, Cincinnati, Ohio; the Molecular Developmental Biology Group,[§] Laboratory of Reproductive and Developmental Toxicology, National Institutes of Health, Research Triangle Park, North Carolina; and the Department of Developmental Biology,[†] China Medical University, Shenyang, Peoples Republic of China

Bone morphogenetic proteins (BMPs) play important roles in regulating lung development and function although the endogenous regulatory effects of BMP signaling are still controversial. We found that BMP type I receptor Alk3 is expressed predominantly in airway epithelial cells during development. The function of Alk3 in lung development was determined using an inducible knockout mouse model by crossing epithelial cell-specific Cre transgenic mice SPC-rtTA/TetO-Cre and floxed-*Alk3* mice. Abrogation of Alk3 in mouse lung epithelia from either early lung organogenesis or late gestation resulted in similar neonatal respiratory distress phenotypes accompanied by collapsed lungs. Early-induction of *Alk3* knockout in lung epithelial cells caused retardation of early lung branching morphogenesis, reduced cell proliferation, and differentiation. However, late gestation induction of the knockout caused changes in cell proliferation and survival, as shown by altered cell biology, reduced expression of peripheral epithelial markers (Clara cell-specific protein, surfactant protein C, and aquaporin-5), and lack of surfactant secretion. Furthermore, canonical Wnt signaling was

perturbed, possibly through reduced Wnt inhibitory factor-1 expression in *Alk3*-knockout lungs. Therefore, our data suggest that deficiency of appropriate BMP signaling in lung epithelial cells results in prenatal lung malformation, neonatal atelectasis, and respiratory failure. (Am J Pathol 2008, 172:571–582; DOI: 10.2353/ajpath.2008.070286)

Neonatal respiratory distress caused by immaturity of the fetal lung continues to be an important clinical problem. The molecular mechanism of lung development and its relationship to neonatal pulmonary diseases remains incompletely understood. Disruption of normal lung developmental processes can result in either neonatal respiratory failure if lung formation is severely affected, or susceptibility to lung diseases during later life if mild changes occur in the developing lung.¹

Lung development is initiated by the formation of a pair of primary epithelial buds that evaginate from the laryngo-tracheal groove of endoderm into the surrounding splanchnic mesenchyme.^{2,3} The respiratory tree then develops by branching morphogenesis, in which reiterated outgrowth, elongation, and subdivision of epithelial buds occurs,^{4,5} followed later on by alveolarization to form a large gas-exchange surface. Because the lung developmental process is quite well conserved, mouse lung development is an ideal model for studying the mechanism of lung organogenesis and congenital respiratory diseases in man. In the mouse, lung development begins at embryonic day (E) 9.5, and is divided into pseudoglandular stage (E9.5 to E16.5), canalicular stage (E16.6 to

Supported by the National Institutes of Health (grant HL68597 to W.S.; and grants HL60231, HL44060, HL44977, and HL75773 to D.W.) and the Webb Foundation (to D.W.).

Accepted for publication November 16, 2007.

J.C.M. is a summer volunteer student.

Address reprint requests to Dr. Wei Shi, Developmental Biology Program, Department of Surgery, Childrens Hospital Los Angeles, 4650 Sunset Blvd., MS 35, Los Angeles, CA 90027. E-mail: wshi@chla.usc.edu.

E17.4), saccular stage (E17.5 to postnatal day 5 or P5), and alveolar stage (P5 to P30).² Although the proximal-distal axis as seen by specific gene expression is already evident in the E10.5 mouse lung,^{6,7} functional distal lung epithelial cells are induced at late gestation stage (E17.5) with characteristic morphological changes from early columnar cells to late flat cuboidal and squamous cells, whereas the terminal differentiation of functional type I and type II alveolar epithelial cells (AECI and AECII) only occurs after birth in mice.⁸ Lung development is regulated by many growth factors, including bone morphogenetic proteins (BMPs).⁴

BMPs, with more than 20 family members, have been shown to regulate many fundamental biological processes including cell proliferation, differentiation, apoptosis, migration, and adhesion.⁹ Furthermore, they are involved in the development of almost all tissues and organs, as well as the specification of the basic embryonic body plan, such as dorso-ventral patterning, left-right asymmetric axis, and proximal-distal axis formation.¹⁰ As extracellular growth factors, BMPs bind to heteromeric complexes of BMP serine/threonine kinase type I and type II receptors.^{11,12} Upon ligand-induced aggregation of the receptors, constitutively activated BMP type II receptor kinase phosphorylates and activates the type I receptor, which subsequently recognizes and phosphorylates receptor-bound BMP-specific Smad proteins (Smad1, Smad5, and Smad8) on the carboxyl terminal SSXS motif. These Smads dissociate from the receptors, form complexes with a common partner Smad4, translocate into the nucleus, bind to BMP responsive element, and act as transcriptional co-modulators to induce or repress BMP target gene expression.¹³ The specificity of the biological response to BMP ligand is maintained by the utilization of specific type I receptors and Smad proteins. Three cognate BMP type I receptors (Alk2, Alk3, and Alk6) have been identified. Alk3, also called BMP receptor type IA (BMPRI-IA), plays an essential role during early embryonic development, particularly in mesoderm formation and gastrulation. The conventional Alk3 gene null mutation is early embryonic lethal in mice (E7.5 to 9.5) before lung organogenesis.¹⁴

BMP4 is an important BMP member that plays a key role in normal lung development.¹⁵ Addition of exogenous BMP4 to intact embryonic lung explant culture stimulates lung branching, as reported by us and other groups.^{16,17} However, in isolated E11.5 mouse lung endoderm cultured in Matrigel, addition of BMP4 inhibited epithelial growth induced by the morphogen FGF10.¹⁸ On the other hand, overexpression of BMP4 in the distal endoderm of fetal mouse lung, driven by a 3.7-kb human surfactant protein C (SP-C) promoter, causes abnormal lung morphogenesis with cystic terminal sacs.⁷ In contrast, SP-C promoter-driven overexpression of either the BMP antagonist Noggin or Gremlin to block BMP signaling, results in severely reduced distal epithelial cell phenotypes and increased proximal cell phenotypes in the lungs of transgenic mice.^{19,20} Although these studies suggest that BMP4 signaling is essential for normal lung morphogenesis, the data obtained from *in vitro* and transgenic animal studies are confusing. In particular, the

specific physiological functions in lung epithelia during different developmental stages have not been determined. Herein, we have used an inducible lung epithelial *Alk3* conditional knockout mouse model to dissect *Alk3*-mediated BMP signaling in promoting lung development and its role in preventing neonatal respiratory diseases.

Materials and Methods

Mouse Strains and Breeding

Alk3 heterozygous null mutant (*Alk3*^{+/-}) and floxed *Alk3* (*Alk3*^{lox/lox}) mice were generated in Dr. Yuji Mishina's laboratory.²¹ In *Alk3*^{lox/lox}, the exon 2 of *Alk3* gene was flanked with two loxP DNA elements. Deletion of exon 2 will cause frameshift and eliminate functional Alk3 protein expression. Inducible lung epithelial cell-specific Cre transgenic mice (*SPC-rtTA/TetO-Cre*) were generated in Dr. Jeffrey Whitsett's laboratory.²² Timed mating between *Alk3*^{lox/lox} and *Alk3*^{+/-}/*SPC-rtTA/TetO-Cre* mice generated lung epithelial-specific *Alk3* conditional knockout (CKO) mice (*Alk3*^{lox/-}/*SPC-rtTA/TetO-Cre*), heterozygous *Alk3* knockout (HT) mice (*Alk3*^{lox/-}, or *Alk3*^{lox/-}/*SPC-rtTA*, or *Alk3*^{lox/-}/*TetO-Cre*, or *Alk3*^{lox/+}/*SPC-rtTA/TetO-Cre*), and control mice (*Alk3*^{lox/+}, or *Alk3*^{lox/+}/*SPC-rtTA*, or *Alk3*^{lox/+}/*TetO-Cre*) when inducing agent doxycycline (Dox) was present. Because lung development in the control mice is the same as in wild-type mice (*Alk3*^{+/+}), they are all classified as wild-type (WT) group in this study. Administration of Dox started from different gestation stages (E7.5, or E17.5, or P1) to the end point of experiment by feeding the pregnant mice with Dox food (625 mg/kg; TestDiet, Richmond, IN) and drinking water (0.5 mg/ml; Sigma, St. Louis, MO). All mice were bred in C57BL/6 strain background, and genotyped by genomic DNA polymerase chain reaction (PCR). Mice used in this study were housed in pathogen-free conditions according to the protocol approved by Institutional Animal Care and Use Committee at Saban Research Institute of Children's Hospital Los Angeles.

Histology and Morphometric Analysis

Embryonic lung was fixed with 4% buffered paraformaldehyde at 4°C overnight, dehydrated, and embedded in paraffin. Five- μ m sections were stained with hematoxylin and eosin (H&E), as reported previously.²³ Quantification of air sac space in lung tissue was performed by measuring air space area in five different tissue sections of every 100- μ m distance using MetaMorph software (Molecular Devices, Sunnyvale, CA). Elastin was stained using Hart's resorcin-fuchsin solution, and counterstained with 0.5% tartrazine.

Immunohistochemistry and Immunofluorescence Staining

Antibodies used in these studies: Alk3 goat polyclonal antibody (sc-5676), Clara cell-specific protein (CCSP) goat polyclonal antibody (sc-9772), and SP-C goat poly-

clonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). β -Tubulin IV mouse monoclonal antibody was obtained from BioGenex (San Ramon, CA).

Alk3 immunofluorescence staining was performed on paraffin sections from 4% paraformaldehyde-fixed lung tissue. After deparaffinizing and rehydration, sections were blocked in 2.5% bovine serum for 1 hour at room temperature, followed by incubation with primary antibody for 1 hour, and detected using Alexa Fluor 488-labeled donkey anti-goat IgG (Invitrogen, Carlsbad, CA). Immunohistochemical staining of SP-C, CCSP, and β -tubulin IV were performed using a HistoStain kit from Zymed Laboratories (South San Francisco, CA) according to the manufacturer's instructions. Either 3-amino-9-ethylcarbazole or 3,3'-diaminobenzidine was used as chromogenic substrate.

Cell Proliferation and Apoptosis

Cell proliferation was analyzed by proliferating cell nuclear antigen (PCNA) staining using a Zymed PCNA staining kit, and by Ki-67 immunostaining (NeoMarkers, Fremont, CA). Cell apoptosis was evaluated by terminal dUTP nick-end labeling (TUNEL) staining using an ApopTag kit (Millipore, Billerica, MA), as published previously.²⁴

Cellular Structure under Transmission Electron Microscopy

One-mm-thick lung tissue was fixed in 2% glutaraldehyde/1% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 10 minutes at 37°C, followed by 4 hours at room temperature. Tissue blocks were then post-fixed overnight at 4°C in 1.5% osmium tetroxide in veronal acetate buffer (pH 7.4). After rinsing the specimen, the blocks were stained in 1.5% uranyl acetate (pH 5.2) for 1 hour at room temperature. Tissue was then dehydrated in graded acetone, infiltrated with propylene and oxide-Epon mixture, embedded in Epon. Ultra thin sections were then cut and observed under transmission electron microscopy (JEOL-1200EX; JEOL Ltd., Tokyo, Japan).

Western Blot

Detection of lung proteins has been previously described.²⁵ Briefly, fresh lung tissues were lysed on ice in RIPA buffer containing 1 mmol/L phenylmethyl sulfonyl fluoride, 0.2 U/ml aprotinin, and 1 mmol/L sodium orthovanadate. Protein concentration was measured by the Bradford method using reagents purchased from Bio-Rad Laboratories (Hercules, CA). Equal amounts (40 μ g) of total tissue lysate proteins were separated in NuPAGE 4 to 12% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels using a MOP buffering system (Invitrogen). After protein was transferred into polyvinylidene difluoride membrane, proteins of interest were detected by specific antibodies. Antibodies for Smad1, pSmad1, Smad2, and pSmad2 were purchased from Cell Signaling Technology (Danvers, MA). Active β -catenin

antibody was purchased from Chemicon (Temecula, CA). pLRP6 antibody was kindly provided by Dr. Xi He at Harvard University, Boston, MA.²⁶ Fibrillin-1, Patched, and Sprouty 2 antibodies were obtained from Santa Cruz Biotechnology.

Real-Time PCR Analysis and Primers

Total tissue RNAs were isolated from snap-frozen lung tissue using a RNeasy kit (Qiagen, Valencia, CA). The quality was checked by an Experion automated electrophoresis system using an Experion RNA HighSens analysis kit (Bio-Rad Laboratories). Synthesis of cDNA and quantitative reverse transcriptase (RT)-PCR analysis were performed using iScript cDNA synthesis kit and SYBR Green I dye on iCycler-iQ system (Bio-Rad), as reported previously.²⁴ The PCR primers for *SP-C*, *CCSP*, and *GAPDH* were previously published.²⁷ Other PCR primer sequences are: *FoxJ1* (5'-CCACCTGGCAGAAT-TCCAT-3'; 5'-CCTCCGCTTCTTGAAGGC-3'), *SP-B* (5'-CGCTTCTGGCTAGACAGGC-3'; 5'-GGAGCAGGCTG-CTGGAGA-3'), *AQP5* (5'-ATCTCTGAGGTCTGAGCT-GTGG-3'; 5'-CATGCCGCACACGGGGAT-3'), Wnt inhibitory factor-1 (*WIF-1*, 5'-CACTGCAATAAGAGGTATG-GAGC-3'; 5'-GGGTTACCAGATGTATTGGA-3'), respectively. *GAPDH* was used to normalize equal loading of template cDNA.

Data Presentation and Statistical Analysis

At least three pairs of *Alk3* gene CKO and WT littermate control mice from different dams were analyzed in each experimental subgroup. All experiments were repeated three times, and data represent consistent results. All quantitative data were expressed as mean $\pm$ SD. A Student's *t*-test was used for comparison of statistical difference and *P* values <0.05 were considered as significant.

Results

Conditional Abrogation of BMP Type I Receptor *Alk3* in Mouse Lung Epithelial Cells during Fetal Lung Development

Mouse embryos with the conventional *Alk3*-null mutation died before lung organogenesis.²¹ Thus, the conventional *Alk3* knockout mouse model is not applicable for studying *Alk3* function in lung biology, and a lung-specific conditional *Alk3* knockout mouse model using a *Cre-loxP* system is required for this *in vivo* study. By immunofluorescence staining, endogenous *Alk3* protein expression was found to be predominantly localized in fetal mouse lung airway epithelial cells, with high intensity in peripheral airways, at early gestation stage (E12.5; Figure 1A). This *Alk3* epithelial expression pattern persists in fetal lungs at different developmental stages (E14.5 and E18.5 in Figure 1, B and C). Thus, lung-specific *Alk3* conditional knockout mice were then generated by crossing floxed *Alk3* mice with *SPC-rtTA/TetO-Cre* transgenic

Figure 1. *Alk3* expression and conditional knockout in developing mouse lung. *Alk3* protein expression was detected by immunofluorescence staining (A–D) in normal mouse embryonic lung at different developing stages, including E12.5 (A), E14.5 (B), and E18.5 (C). *SPC-rtTA/TetO-Cre*-induced floxed *Alk3* conditional knockout was also confirmed by *Alk3* immunofluorescence protein staining (D) and by RT-PCR for floxed *Alk3* exon 2 (E). *GAPDH* RT-PCR was used as a control.

mice,^{22,28} in which Cre expression is induced in airway epithelial cells of lung and bronchus by a lung epithelia specifically expressed rtTA, driven by a 3.7-kb human surfactant protein C promoter (SPC), in combination with the inducing agent Dox.²² Moreover, Cre-mediated floxed *Alk3* gene deletion could be started at different developmental stages by controlling the time point of Dox administration. Abrogation of *Alk3* protein expression early in lung organogenesis was achieved by feeding the pregnant mother with Dox food and water from E7.5. As a result of floxed-*Alk3* exon 2 deletion, lack of functional *Alk3* protein in *Alk3* knockout lung was confirmed by immunofluorescence staining using an *Alk3* antibody (Figure 1D). Furthermore, the WT version of *Alk3* mRNA transcript in *Alk3* conditional knockout (CKO) lung tissue was barely detected by a more sensitive RT-PCR using exon 2-specific primers, indicating a high efficiency of gene knockout in lung (Figure 1E).

Abrogation of Alk3 Gene Expression in Developing Lung Epithelia Early in Embryonic Mouse Lung Organogenesis Results in Dysplastic Lung Formation and Neonatal Respiratory Distress

The *Alk3* CKO mice died within a couple of hours after birth with severe gasping and cyanosis. Both gross view and H&E-stained neonatal lung tissue sections indicated that the terminal air sacs in *Alk3* CKO lung failed to inflate

with air during postnatal air breathing, accompanied with eosin-stained amorphous materials in terminal sacs (Figure 2, A and B), suggesting atelectasis and failure of postnatal lung fluid reabsorption. Under high magnification, instead of normal squamous alveolar cells lining the peripheral air sac, only columnar cells were observed in the peripheral lung of *Alk3* CKO mice (Figure 2B, insets), accompanied with thick and edematous mesenchyme. Lack of lamellar body formation and secretion were further observed in *Alk3* knockout P1 lung by transmission electron microscopy (Figure 2C).

To understand the mechanisms of *Alk3*-mediated BMP signal regulation during mouse fetal lung development, the lung tissue structure of *Alk3* CKO mice induced from E7.5 were then examined at a series of gestation stages. Compared to WT littermates, most of the E12.5 embryonic lung did not have observable changes of epithelial growth and branching morphogenesis. However, a small portion of E12.5 *Alk3* knockout lungs (two of six) already displayed reduced size and epithelial branching, suggesting a low penetrance of this early embryonic lung branching morphogenesis phenotype. Significant retardation of lung growth was observed in all E14.5 *Alk3* CKO lungs, as shown by reduced numbers of airway epithelial tubules, accompanied with dilated lumen and less compacted mesenchyme (Figure 2D). Then, retardation of distal lung epithelial cell proliferation and differentiation was clearly indicated by retention of a columnar shape in *Alk3* CKO mice versus changing to a low cuboidal shape in WT control at the beginning of the canalicular stage (E16.5, Figure 2E). Furthermore, at the end of gestation (E18.5, Figure 2F), peripheral airway sac formation was significantly decreased, and mesenchymal septation between air sacs was relatively thick in *Alk3* CKO lung compared to WT littermate control. Morphometric measurement of peripheral air spaces was then performed to quantify the changes of lung structure. The peripheral air-space in E18.5 *Alk3* CKO lung was increased by 22% ($51 \pm 5\%$ in WT versus $62 \pm 5\%$ in *Alk3* CKO lung, $P < 0.001$).

Conditional Knockout of Alk3 Function in Airway Epithelial Cells Early in Embryonic Lung Organogenesis Resulted in Abnormal Distal Epithelial Cell Proliferation, Apoptosis, and Differentiation

To determine the mechanism of the above phenotypic changes in *Alk3* CKO mouse lung, cell proliferation, apoptosis, and differentiation were then measured. By PCNA immunostaining (Figure 3A), the numbers of PCNA-positive cells continuously decreased in *Alk3* CKO mouse lungs from the early to the end of gestation (E14.5 and E18.5). This reduced cell proliferation was also confirmed by Ki-67 immunostaining, a different marker for cell proliferation (Figure 3B). Moreover, cell apoptosis was also evaluated by TUNEL labeling. The number of apoptotic cells was not significantly changed at earlier stages of lung development (E14.5), but increased in *Alk3* CKO lung later on at E18.5, particularly in epithelial

Figure 2. Phenotypes of mouse lung epithelial-specific *Alk3* conditional knockout induced from E7.5. **A:** Gross view of neonatal lung at P1. Inflation with air was clearly seen in WT, but not in *Alk3* CKO lungs. **B:** Collapsed lung structure in P1 *Alk3* CKO mice was shown by H&E-stained tissue section. The cell shape of lining epithelial cells inside air sacs was changed from normal squamous/flat cells in WT to round/cuboidal cells in *Alk3* CKO (inset). **C:** Surfactant production and secretion was barely detected in P1 *Alk3* CKO lung by transmission electron microscopy, as shown by lack of lamellar bodies both inside cells and in air spaces (arrows). **D–F:** H&E-stained embryonic lung sections at different developmental stages. **D:** Less epithelial tubules with dilated lumen were observed in E14.5 lung of *Alk3* CKO mice. **E:** At E16.5, peripheral airway epithelial cells still have columnar shape in *Alk3* CKO lung versus cuboidal shape in WT control. **F:** At late gestation stage E18.5, less saccular formation with thickened mesenchyme was evident in *Alk3* CKO lung.

cells (Figure 3C). Thus, less saccular formation accompanied with enlarged air spaces in E18.5 *Alk3* CKO lung could be caused by both reduced cell proliferation and increased cell apoptosis. In addition, expression of selected molecular markers for differentiated lung epithelial cells, including *SP-B*, *SP-C*, aquaporin 5, *CCSP*, and *FoxJ1*, was evaluated at the mRNA level using quantitative real-time PCR (Figure 4A). Consistently, expression of distal conducting airway and lung epithelial cell differentiation markers *CCSP*, *SP-C*, and *AQP5* was significantly reduced in *Alk3* CKO lung tissues at various developmental stages (E14.5 to E18.5), whereas expression of proximal epithelial cell marker *FoxJ1* was not changed. The RNA data were further confirmed at protein level by *SP-C*, *CCSP*, and β -tubulin IV immunostaining (Figure 4). At E14.5, the number of *SP-C*-positive peripheral epithelial cells in *Alk3* CKO lung were less than WT control, accompanied by reduced branching morphogenesis. Also, the intensity of *SP-C* staining signal was significantly reduced in *Alk3* CKO lung at early embryonic stage E14.5, when excessive cell apoptosis was not detected (Figure 4B), suggesting retarded peripheral epithelial cell differentiation and lineage expansion in early *Alk3* CKO lung. Whereas, at late gestation (E18.5), both *CCSP* and *SP-C* were barely detected in *Alk3* CKO pe-

ripheral lung, whereas the pattern of proximal conducting airway epithelial cell marker β -tubulin IV remained the same compared to WT control (Figure 4, B–D). Therefore, disruption of normal peripheral lung epithelial cell differentiation at early stage and increased apoptosis of differentiated cell lineages at late stage observed in the *Alk3* CKO may directly contribute to abnormal lung structure and function.

In addition, major extracellular proteins were also examined. No significant changes in collagen and fibronectin staining were observed (data not shown). Although elastin fragmentation was not detected, thickened elastin fibers surrounding dilated small airways were frequently observed in the peripheral lung of *Alk3* CKO mice, in contrast to fine elastin fibers in primary septa structures of air sacs of normal control peripheral lung (Figure 4E).

Alk3-Mediated BMP Signaling at Late Gestation Stage Is Essential for Neonatal Respiratory Function by Supporting Differentiated Epithelial Cell Lineages in Peripheral Lung

By taking advantage of the inducible gene conditional knockout approach, we then selectively abrogated *Alk3*

Figure 3. Altered cell proliferation and apoptosis of *Alk3* CKO lung induced from E7.5. **A** and **B:** Cell proliferation was measured by PCNA and Ki-67 immunostaining (brown color) for lungs at E14.5 and E18.5. **C:** Cell apoptosis was also examined by TUNEL assay (brown color). Only a few TUNEL-positive cells (arrows) were detected in E14.5 lungs of either WT or *Alk3* CKO mice.

activity from different lung developmental stages by controlling Dox administration, as indicated in Table 1. Most mice with homozygous *Alk3* gene knockout in lung epithelial cells that was induced either from E7.5 (*Alk3* CKO) or E17.5 (E17.5-*Alk3* CKO) suffered severe neonatal respiratory distress, and died within a couple of hours after birth. Interestingly, mice with *Alk3* CKO induced from E18.5 also died in the first 2 days after birth with similar collapsed lungs, although most of them survived longer than *Alk3* CKO induced earlier (E7.5 or E17.5). However, mice with *Alk3* CKO genotype that was induced after birth (P1-*Alk3* CKO) did not suffer from respiratory distress,

and the morphology of lung at the end of postnatal alveolarization (P30) appeared to be normal (Figure 5C). We therefore further characterized the lung phenotypes of *Alk3* CKO mouse lung induced after completion of lung branching morphogenesis at saccular stage (E17.5-*Alk3* CKO). As indicated by *Alk3* immunofluorescence staining, almost all *Alk3* protein expression was efficiently abrogated in lung epithelial cells of P1 lung of E17.5-*Alk3* CKO mice when Dox was given from E17.5 (Figure 5A). The collapsed lung morphology was similar to that observed in *Alk3* CKO lung induced from the beginning of lung morphogenesis, except that saccular structure formation was not affected (Figure 5B). Moreover, many apoptotic cells were detected in peripheral lung epithelia of E17.5-*Alk3* CKO mice, compared to the rare apoptotic cells occasionally detected in WT control lung (Figure 5D). Furthermore, cell proliferation was also increased as shown by PCNA immunostaining in P1 lung of E17.5-*Alk3* CKO mice (Figure 5E). Differentiated epithelial cell markers were also evaluated by quantitative RT-PCR and immunohistochemistry analyses for the related cell markers. At the mRNA level, expression of distal conducting and respiratory airway epithelial cell markers, including CCSP, SP-C, and AQP5, were dramatically reduced, especially AQP5, whereas FoxJ1, a marker for proximal conducting airway ciliated epithelial cells, was not changed (Figure 5F). Interestingly, protein immunostaining showed that the cells lining small bronchioles had CCSP expression (Clara cells) in P1 lung of E17.5-*Alk3* CKO mice, which is barely detected in *Alk3* CKO lung induced from early embryonic lung organogenesis (E7.5). However, these Clara cells in E17.5-*Alk3* CKO lung showed a variety of shapes, dissociation from basement membrane, and shedding into the lumen of airways (Figure 5G). Furthermore, SP-C-positive alveolar epithelial cells in P1 lung of E17.5-*Alk3* CKO mice were barely detected, compared to the abundant SP-C-positive cells in WT control lung (Figure 5H). Therefore, *Alk3*-mediated BMP signaling seems to be essential to maintain differentiated functional cell lineages of fetal and neonatal peripheral lung.

Abrogation of Alk3-Mediated BMP Signaling in Peripheral Lung Epithelial Cells Resulted in Altered Canonical Wnt Signal Activity

To understand the molecular mechanisms of neonatal respiratory distress caused by defective BMP signaling, we have examined other signal pathways, including FGF, SHH, Wnt, and TGF- β , which are known to be critical for normal lung formation and function. By detecting fibrillin-1 protein level (a regulatory protein for TGF- β ligand activation) and phosphorylation of TGF- β downstream Smad2 protein, no significant change in TGF- β signal activity was observed between E18.5 *Alk3* CKO and WT control lungs (Figure 6A). Similarly, no changes in FGF and SHH signal activities, as determined by protein levels of FGF-targeted gene Sprouty 2 and SHH-targeted gene Patched, were detected in E18.5 *Alk3* CKO lung (Figure 6A). In addition, expression at the mRNA level of a BMP

Figure 4. Expression of selected molecular markers of differentiated lung cells in *Alk3* CKO lungs. **A:** Gene expression at mRNA level was quantified by real-time RT-PCR. * $P < 0.05$. **B:** Changes in peripheral differentiated epithelial cells were detected by SP-C immunostaining in either E14.5 (red color) or E18.5 (brown color) lungs. **C** and **D:** Immunostaining of CCSP and β -tubulin IV was used to detect Clara cells (red color) and proximal ciliated epithelial cells (brown color), respectively. **E:** Elastin fibers (red-black color) in lung tissues were also stained using Hart's resorcin-fuchsin solution.

antagonist Gremlin, which also plays an important role in regulating BMP signaling activity during lung development, was not changed, as quantified by real-time RT-

PCR (data not shown). However, the canonical Wnt signaling activity in *Alk3* CKO lung tissue was increased, as indicated by increased phosphorylation of Wnt co-recep-

Table 1. Neonatal Lethality of Lung Epithelial Cell-Specific *Alk3* Conditional Knockout Associated with Abnormal Lung Phenotypes

Genotype	Dox induction							
	E7.5 (at 24 hours)		E17.5 (at 24 hours)		E18.5 (at 48 hours)		P1 (at P28)	
	Survival (n)	Death (n)	Survival (n)	Death (n)	Survival (n)	Death (n)	Survival (n)	Death (n)
WT	4	0	11	0	5	1*	11	0
Alk3 HT	12	1*	5	0	5	1*	13	0
Alk3 CKO	0	7	0	3	0	5	3	0

*Mice died without lung abnormality. Detailed *Alk3* genotypes in each group are listed in Materials and Methods.

Figure 5. Pulmonary phenotypes and related cell and molecular changes in neonatal P1 lung of *Alk3* CKO mice induced from E17.5 (E17.5-*Alk3* CKO). **A:** *Alk3* protein expression in lung epithelia was abrogated in E17.5-*Alk3* CKO. **B:** Collapsed peripheral lung sac structure filled with amorphous material in E17.5-*Alk3* CKO lung was shown in H&E-stained tissue section. **C:** Normal lung structure of *Alk3* CKO mice induced after birth (P1-*Alk3* CKO) was shown by H&E staining for the lung obtained at the end of postnatal alveolarization (age of postnatal day 30). **D** and **E:** Increased cell apoptosis and cell proliferation were detected by TUNEL (**D**) and PCNA immunostaining (**E**) in P1 lungs of E17.5-*Alk3* CKO mice. **F–H:** Reduced gene expression of peripheral lung epithelial cell differentiation markers in P1 lungs of E17.5-*Alk3* CKO mice was detected both at the mRNA level by real-time PCR quantification (**F**) and at the protein level by protein immunostaining for CCSP (**G**) and SP-C (**H**). * $P < 0.05$. Changes in CCSP-positive stained cells in terminal bronchioles were also shown under high magnification (**G**, insets).

Figure 6. Changes in other signal pathways of *Alk3* CKO mouse lung. **A:** TGF- β , FGF, and SHH signaling activities in E18.5 *Alk3* CKO mouse lung were detected by measuring protein phosphorylation, or protein levels of pathway-specific targeted genes. β -Actin is a loading control. **B:** Increased canonical Wnt signal pathway activity was observed in E18.5 *Alk3* CKO lung, as indicated by increased pLRP6 and active β -catenin by Western blot. GAPDH is a loading control. **C:** Reduced *WIF-1* gene expression at the mRNA level was detected in lung epithelial cell-specific *Alk3* CKO lung tissues at indicated stages. * $P < 0.05$. **D:** Endogenous Smad1 protein expression and phosphorylation (activation) were detected for normal lung tissues at indicated stages.

tor LRP6 (active form) and activation of downstream β -catenin in E18.5 lung tissue of *Alk3* CKO mice induced from E7.5 (Figure 6B). Further analysis for Wnt signaling components found that Wnt inhibitory factor-1 (*WIF-1*) expression at the mRNA level was consistently reduced in perinatal lung tissues of *Alk3* CKO mice that were induced from either E7.5 or E17.5 (Figure 6C). Because *WIF-1* is an antagonist of Wnt ligands, reduced *WIF-1* level may be one of the mechanisms for elevated Wnt canonical signaling activity in *Alk3* CKO lung. However, whether *Alk3*-mediated BMP signaling has direct regulatory effect on *WIF-1* gene expression and whether changed Wnt signal activity directly mediates abnormal peripheral lung cell proliferation, differentiation, and apoptosis in *Alk3* CKO lung requires further study.

In addition, endogenous BMP signaling activities in postnatal lung tissues were compared to prenatal lung, to distinguish whether the lack of pulmonary phenotype in *Alk3* CKO lung induced postnatally is simply attributable to down-regulation of BMP signaling. BMP downstream Smad1 protein expression was increased postnatally in WT control lung (Figure 6D), and activation of Smad1 protein, as measured by Smad1 protein phosphorylation, was higher in P1 lung than prenatal E18.5 lung. However, lower, but still detectable Smad1 activation was also observed in P14 lung, compared with E18.5 lung. The detectable activation of BMP signaling in postnatal lung is consistent with observations from another group.²⁹ These data suggest that lung epithelial BMP signaling may not play a critical role during normal postnatal alveolarization stage.

Discussion

Prematurity of the lung can cause neonatal respiratory distress, attributable to deficiency of pulmonary surfactant production and failure of fetal lung fluid clearance by transepithelial sodium reabsorption. Abrogation of *Alk3*-mediated BMP signaling in lung epithelial cells resulted in severe deficiency of functionally differentiated alveolar epithelial cells in neonatal mouse lung, as shown by our studies above, causing atelectasis with retention of fluid in air spaces. In particular, E17.5-induced *Alk3* CKO lung still suffered severe respiratory failure immediately after birth, despite nearly normal prenatal lung structure. As reported previously, airway epithelial cells in early embryonic mouse lung co-express multiple marker genes, such as *SP-A*, *CCSP*, as well as calcitonin gene-related peptide, which become specific to different cell lineages later on.³⁰ Deletion of *Alk3* function in early embryonic lung epithelial cells before E16.5 appears to disrupt early lung epithelial common progenitor cell differentiation and proliferation, and cause retention of distal epithelial cells in an apparently less differentiated status that is indicated by columnar shape with drastically reduced *SP-C* and *CCSP* expression, yet without excessive cell apoptosis. All of these changes result in retarded lung branching morphogenesis at the tissue level. However, abrogation of *Alk3*-mediated BMP signal at late fetal gestation after E16.5, when restricted cell lineages (Clara cells in conducting airway and *SP-C*-expressing progenitor cells in respiratory airway) are already substantially formed, disrupts the survival of the restrictively committed epithelial progenitor cells, as shown by reduced lineage marker gene expression (*CCSP* and *SP-C*) and increased cell apoptosis, in addition to reduced cell proliferation. The different cell proliferation responses between early and late induction of *Alk3* CKO in lung may be attributable to varied BMP effects on growing cells with different differentiation status, and/or a compensatory response of non-affected cell lineages to excessive cell death in P1 lung. Furthermore, conditional knockout of *Alk3* in lung epithelial cells postnatally, when BMP-specific downstream Smad1 activation is still detected albeit at lower levels, does not affect lung maturation and function. Therefore, *Alk3*-mediated BMP signaling in peripheral lung epithelial cells right before birth appears to be essential to expand and maintain the differentiated epithelial cell lineages during transition from the prenatal fluid-filled environment to the postnatal atmospheric environment.

As mentioned above, the biological effects of BMP4 in regulating lung epithelial cells have appeared to be contradictory, based on different culture systems *in vitro* and overexpression of BMP4 versus its antagonists *in vivo*.^{7,16,18–20} Our current studies with abrogation of endogenous BMP type I receptor *Alk3* in lung epithelial cells *in vivo* provide a role for BMP4-mediated regulation of early lung epithelial growth consistent with previously reported data from our mouse whole embryonic lung explant culture system, in which *Alk3*-mediated endogenous BMP signaling promoted embryonic lung branching morphogenesis during early-mid gestation.^{16,31} However, excessive BMP signaling, achieved by either overexpressing BMP4 in lung

epithelial cells,⁷ or knocking out the endogenous BMP4 antagonist Gremlin,³² also resulted in abnormal fetal lung formation and neonatal lung dysfunction. Therefore, appropriate levels of BMP4 signaling in a finely-tuned spatial-temporal pattern are essential for both early lung branching morphogenesis and late saccular formation. In particular, the BMP regulatory effects on peripheral lung epithelial cell lineage differentiation, expansion, and survival are dependent on the developmental status of the cells at different stages.

The specificity of the biological response to BMP ligand is maintained by the utilization of specific type I receptors and Smad proteins. Alk2, Alk3, and Alk6 are three identified BMP type I receptors. Conventional *Alk2* or *Alk3* gene null mutations are early embryonic lethal in mice (E7.5 to 9.5) before lung organogenesis.^{14,33,34} However, mice with *Alk6*-null mutation, whose expression is restricted to proximal airway epithelia of embryonic lung,³⁵ only display defects in limb skeleton development, and not in other soft organs including lung.^{36,37} Moreover, conditional abrogation of Alk2 function in embryonic mouse lung epithelium using a *Cre-loxP* approach did not cause any pulmonary phenotype (Saverio Bellusci, personal communication). In contrast, our study shows that conditional knockout of *Alk3* in fetal mouse lung epithelium resulted in early retarded lung branching morphogenesis as well as late disrupted peripheral epithelial cell survival and growth, accompanied with severe neonatal respiratory distress. It seems that BMP receptor Alk3 is a unique and major endogenous signaling component that mediates BMP's regulatory effect in lung epithelial development.

Recently, Eblaghie and colleagues³⁵ reported conditional knockout of *Alk3* in lung epithelial cells using a different noninducible *SPC-Cre* approach that also resulted in abnormal lung development with excessive peripheral epithelial cell apoptosis. Interestingly, the phenotypic changes of the developing lung in their study are not completely the same as those observed in our studies above. Abnormal early lung branching morphogenesis was not observed until late gestation stage E16.5 in the study of Eblaghie and colleagues.³⁵ In contrast, reduced lung branching morphogenesis was found in 30% of E12.5 lungs, and in all of E14.5 lungs with *Alk3*-null mutant genotype in our study. Although growth of respiratory airway sacs was significantly decreased in our *Alk3* CKO lung at E18.5, the air sac enlargement was not so severe as the huge cystic sacs observed by Eblaghie and colleagues.³⁵ In addition, we also found that the mesenchymal septae between air sacs was relatively thick in *Alk3* CKO lung, and significant reduction of distal conducting airway epithelial cell differentiation marker CCSP at both mRNA and protein level occurred as early as E14.5 if abrogation of Alk3 was induced from early embryonic lung organogenesis. Moreover, CCSP gene expression at the mRNA level was also significantly decreased when *Alk3* CKO was induced from late fetal gestation stage (E17.5). In contrast, a change in CCSP was not observed in the study of Eblaghie and colleagues.³⁵ The discrepancy of *Alk3* conditional knockout phenotypes between Eblaghie and colleague's study³⁵ and our E7.5-induced

Alk3 knockout lung could be caused by variation of Cre-mediated DNA recombination. In our *SPC-rtTA/TetO-Cre* transgenic line, it has been shown that Cre-mediated DNA recombination is induced even before lung formation (E6.5-E8.5),²² whereas SPC-Cre-mediated DNA recombination in the study by Eblaghie and colleagues³⁵ was reported to be detected from E10.5 to E12.5. Also, Cre expression in our studies was induced under Dox administration, which may have impact on lung phenotypes. However, phenotypic comparison was always performed between *Alk3* CKO and WT control littermates that had the same dosage and time window of Dox exposure in our studies. Moreover, no changes in lung morphology were detected between control mice with and without Dox administration (data not shown). In addition, Cre expression level and other biological responses in different mouse strain backgrounds could be another factor that affected the efficacy of Cre-mediated loxP DNA recombination and the resulted pathological changes. For example, Eblaghie and colleagues³⁵ reported that ~46% of heterozygous embryonic lungs (*Alk3*^{+/fx}/*SPC-Cre*⁺) in ICR and C57BL/6 mixed strain background had abnormal lung phenotype, whereas this phenomenon was not seen when the Cre transgene was carried on the C57BL/6 background. In our studies, all mice were bred in C57BL/6 strain background for more than six generations.

Most importantly, by taking advantage of our inducible Cre-mediated gene knockout approach, we selectively abrogated Alk3 function in lung epithelial cells at different developmental stages to dissect the temporal-specific roles of Alk3-mediated signaling in lung formation and function, and hence have avoided obtaining the compounding phenotypes that result from accumulated abnormalities during all of lung development. In particular, blockade of Alk3 function in peripheral lung epithelial cells shortly before birth (E17.5 to E18.5) resulted in neonatal respiratory distress despite normal terminal air sac formation.

We therefore focused on the changes in P1 neonatal lung of *Alk3* CKO mice induced from E17.5. Increased peripheral lung epithelial cell apoptosis was evident, accompanied by dramatically reduced SP-C-positive cells. CCSP expression at the mRNA level was significantly reduced, and CCSP protein-positive cells appeared to be altered in cell shape, cell-cell connections, as well as dissociated from airway basement membrane, although CCSP-positive cells were still detected in terminal bronchioles.

The molecular mechanisms of Alk3-mediated BMP signaling in regulating lung epithelial cells were also explored in our studies. One of the downstream cross talk pathways may be Wnt canonical signaling. It is well known that Wnt signaling is required to maintain stem/progenitor cell self-renewal in many tissues and prevent these cells from differentiating,³⁸ whereas BMP is known to promote many progenitor cells to exit from cell cycle and differentiate into functional cells, as well as maintain cell survival.³⁹ Finely tuned Wnt signaling activity at the right place and right time is also critical to normal lung development and function. In mouse developing lung,

conditional knockout of Wnt downstream β -catenin or overexpression of Wnt antagonist DKK1 disrupts peripheral lung epithelial progenitor cell differentiation,^{40,41} whereas overexpression of constitutively active β -catenin-Lef1 fusion protein also results in distal lung epithelial cell dysplasia, including highly proliferative, cuboidal epithelial cells that lose fully differentiated lung cell characteristics.⁴²

Therefore, multiple, finely coordinated regulatory pathways are required for correct lung epithelial progenitor cell differentiation and lineage formation. Our studies suggest that WIF-1 may be one of the key cross talkers between two important signaling pathways. Blockade of Alk3 function in lung epithelial cells *in vivo* resulted in reduced levels of WIF-1 expression, accompanied by increased Wnt co-receptor LRP6 phosphorylation and downstream β -catenin activation. However, no changes in other major signal pathways that are involved in regulating lung development, including TGF- β , FGF, and SHH, were detected. These findings taken together suggest that peripheral lung epithelial cells with Alk3 deficiency could have higher Wnt activity that may disrupt progenitor cell growth during branching morphogenesis, and/or fail to maintain survival of differentiated cells during terminal saccular formation. As a consequence, the lung fails when the sudden changes of lung epithelial cell environment from relative intrauterine hypoxia to neonatal normoxia occurs.

In summary, Alk3-mediated BMP signaling in embryonic lung epithelial cells appears to be essential to promote early lung branching morphogenesis, as well as late saccular structure formation. Most importantly, Alk3-mediated BMP signaling in peripheral lung epithelial progenitor cells may promote survival and amplification of related cell lineages, which mediate surfactant production and facilitate fluid clearance from the air sacs of neonatal lung. All these functions are essential for normal neonatal respiratory transition at birth. Deficiency of appropriate BMP signaling in these key epithelial cells therefore results in neonatal atelectasis and respiratory failure.

Acknowledgments

We thank Drs. Xi He and Xin Zeng at Harvard University for providing pLRP6 antibody and Dr. Denise Tefft at Childrens Hospital Los Angeles for her advice on Sprouty 2 detection.

References

- Warburton D, Gaudie J, Bellusci S, Shi W: Lung development and susceptibility to chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2006, 3:668–672
- Ten Have-Opbroek AA: Lung development in the mouse embryo. *Exp Lung Res* 1991, 17:111–130
- Hilfer SR: Morphogenesis of the lung: control of embryonic and fetal branching. *Annu Rev Physiol* 1996, 58:93–113
- Hogan BL: Morphogenesis. *Cell* 1999, 96:225–233
- Hogan BL, Grindley J, Bellusci S, Dunn NR, Emoto H, Itoh N: Branching morphogenesis of the lung: new models for a classical problem. *Cold Spring Harb Symp Quant Biol* 1997, 62:249–256
- Wert SE, Glasser SW, Korfhagen TR, Whitsett JA: Transcriptional elements from the human SP-C gene direct expression in the primordial respiratory epithelium of transgenic mice. *Dev Biol* 1993, 156:426–443
- Bellusci S, Henderson R, Winnier G, Oikawa T, Hogan BL: Evidence from normal expression and targeted misexpression that bone morphogenetic protein (Bmp-4) plays a role in mouse embryonic lung morphogenesis. *Development* 1996, 122:1693–1702
- Warburton D, Schwarz M, Tefft D, Flores-Delgado G, Anderson KD, Cardoso WV: The molecular basis of lung morphogenesis. *Mech Dev* 2000, 92:55–81
- Sporn MB, Roberts AB: The transforming growth factor β s. *Peptide Growth Factors and Their Receptor: Handbook of Experimental Pharmacology*. Edited by MB Sporn and AB Roberts. Heidelberg, Springer-Verlag, 1991, pp 419–472
- Hogan BL: Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev* 1996, 10:1580–1594
- Shi Y, Massague J: Mechanisms of TGF- β signaling from cell membrane to the nucleus. *Cell* 2003, 113:685–700
- Massagué J: TGF- β signal transduction. *Annu Rev Biochem* 1998, 67:753–791
- Attisano L, Wrana JL: Smads as transcriptional co-modulators. *Curr Opin Cell Biol* 2000, 12:235–243
- Mishina Y, Suzuki A, Ueno N, Behringer RR: Bmpr encodes a type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis. *Genes Dev* 1995, 9:3027–3037
- Hogan BL: Bone morphogenetic proteins in development. *Curr Opin Genet Dev* 1996, 6:432–438
- Shi W, Zhao J, Anderson KD, Warburton D: Gremlin negatively modulates BMP-4 induction of embryonic mouse lung branching morphogenesis. *Am J Physiol* 2001, 280:L1030–L1039
- Bragg AD, Moses HL, Serra R: Signaling to the epithelium is not sufficient to mediate all of the effects of transforming growth factor beta and bone morphogenetic protein 4 on murine embryonic lung development. *Mech Dev* 2001, 109:13–26
- Weaver M, Dunn NR, Hogan BL: Bmp4 and Fgf10 play opposing roles during lung bud morphogenesis. *Development* 2000, 127:2695–2704
- Weaver M, Yingling JM, Dunn NR, Bellusci S, Hogan BL: Bmp signaling regulates proximal-distal differentiation of endoderm in mouse lung development. *Development* 1999, 126:4005–4015
- Lu MM, Yang H, Zhang L, Shu W, Blair DG, Morrissey EE: The bone morphogenic protein antagonist gremlin regulates proximal-distal patterning of the lung. *Dev Dyn* 2001, 222:667–680
- Mishina Y, Hanks MC, Miura S, Tallquist MD, Behringer RR: Generation of Bmpr/Alk3 conditional knockout mice. *Genesis* 2002, 32:69–72
- Perl AK, Wert SE, Nagy A, Lobe CG, Whitsett JA: Early restriction of peripheral and proximal cell lineages during formation of the lung. *Proc Natl Acad Sci USA* 2002, 99:10482–10487
- Chen H, Sun J, Buckley S, Chen C, Warburton D, Wang XF, Shi W: Abnormal mouse lung alveolarization caused by Smad3 deficiency is a developmental antecedent of centrilobular emphysema. *Am J Physiol* 2005, 288:L683–L691
- Shi W, Chen H, Sun J, Buckley S, Zhao J, Anderson KD, Williams RG, Warburton D: TACE is required for fetal murine cardiac development and modeling. *Dev Biol* 2003, 261:371–380
- Zhao J, Shi W, Chen H, Warburton D: Smad7 and Smad6 differentially modulate transforming growth factor beta-induced inhibition of embryonic lung morphogenesis. *J Biol Chem* 2000, 275:23992–23997
- He XC, Zhang J, Tong WG, Tawfik O, Ross J, Scoville DH, Tian Q, Zeng X, He X, Wiedemann LM, Mishina Y, Li L: BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. *Nat Genet* 2004, 36:1117–1121
- Chen C, Chen H, Sun J, Bringas Jr P, Chen Y, Warburton D, Shi W: Smad1 expression and function during mouse embryonic lung branching morphogenesis. *Am J Physiol* 2005, 288:L1033–L1039
- Wert SE, Dey CR, Blair PA, Kimura S, Whitsett JA: Increased expression of thyroid transcription factor-1 (TTF-1) in respiratory epithelial cells inhibits alveolarization and causes pulmonary inflammation. *Dev Biol* 2002, 242:75–87
- Alejandro-Alcázar MA, Kwapiszewska G, Reiss I, Amarie OV, Marsh LM, Sevilla-Perez J, Wygrecka M, Eul B, Kobrich S, Hesse M, Schermuly RT, Seeger W, Eickelberg O, Morty RE: Hyperoxia modulates

- TGF-beta/BMP signaling in a mouse model of bronchopulmonary dysplasia. *Am J Physiol* 2007, 292:L537–L549
30. Wuenschell CW, Sunday ME, Singh G, Minoo P, Slavkin HC, Warburton D: Embryonic mouse lung epithelial progenitor cells co-express immunohistochemical markers of diverse mature cell lineages. *J Histochem Cytochem* 1996, 44:113–123
 31. Shi W, Chen H, Sun J, Chen C, Zhao J, Wang YL, Anderson KD, Warburton D: Overexpression of Smurf1 negatively regulates mouse embryonic lung branching morphogenesis by specifically reducing Smad1 and Smad5 proteins. *Am J Physiol* 2004, 286:L293–L300
 32. Michos O, Panman L, Vintersten K, Beier K, Zeller R, Zuniga A: Gremlin-mediated BMP antagonism induces the epithelial-mesenchymal feedback signaling controlling metanephric kidney and limb organogenesis. *Development* 2004, 131:3401–3410
 33. Gu Z, Reynolds EM, Song J, Lei H, Feijen A, Yu L, He W, MacLaughlin DT, van den Eijnden-van Raaij, Donahoe PK, Li E: The type I serine/threonine kinase receptor ActRIA (ALK2) is required for gastrulation of the mouse embryo. *Development* 1999, 126:2551–2561
 34. Mishina Y, Crombie R, Bradley A, Behringer RR: Multiple roles for activin-like kinase-2 signaling during mouse embryogenesis. *Dev Biol* 1999, 213:314–326
 35. Eblaghie MC, Reedy M, Oliver T, Mishina Y, Hogan BL: Evidence that autocrine signaling through Bmpr1a regulates the proliferation, survival and morphogenetic behavior of distal lung epithelial cells. *Dev Biol* 2006, 291:67–82
 36. Yi SE, Daluiski A, Pederson R, Rosen V, Lyons KM: The type I BMP receptor BMPRII is required for chondrogenesis in the mouse limb. *Development* 2000, 127:621–630
 37. Baur ST, Mai JJ, Dymecki SM: Combinatorial signaling through BMP receptor IB and GDF5: shaping of the distal mouse limb and the genetics of distal limb diversity. *Development* 2000, 127:605–619
 38. Reya T, Clevers H: Wnt signalling in stem cells and cancer. *Nature* 2005, 434:843–850
 39. Sneddon JB, Zhen HH, Montgomery K, van de, Rijn M, Tward AD, West R, Gladstone H, Chang HY, Morganroth GS, Oro AE, Brown PO: Bone morphogenetic protein antagonist gremlin 1 is widely expressed by cancer-associated stromal cells and can promote tumor cell proliferation. *Proc Natl Acad Sci USA* 2006, 103:14842–14847
 40. Mucenski ML, Wert SE, Nation JM, Loudy DE, Huelsken J, Birchmeier W, Morrisey EE, Whitsett JA: {beta}-Catenin is required for specification of proximal/distal cell fate during lung morphogenesis. *J Biol Chem* 2003, 278:40231–40238
 41. Shu W, Guttentag S, Wang Z, Andl T, Ballard P, Lu MM, Piccolo S, Birchmeier W, Whitsett JA, Millar SE, Morrisey EE: Wnt/beta-catenin signaling acts upstream of N-myc, BMP4, and FGF signaling to regulate proximal-distal patterning in the lung. *Dev Biol* 2005, 283:226–239
 42. Okubo T, Hogan BL: Hyperactive Wnt signaling changes the developmental potential of embryonic lung endoderm. *J Biol* 2004, 3:11