Epithelial Notch signaling regulates lung alveolar morphogenesis and airway epithelial integrity

Po-Nien Tsao[a,b,1], Chisa Matsuoka[c], Shu-Chen Wei[d], Atsuyasu Sato[e], Susumu Sato[e], Koichi Hasegawa[e], Hung-kuan Chen[a], Thai-Yen Ling[b,f], Munemasa Mori[g], Wellington V. Cardoso[g], and Mitsuru Morimoto[c,1]

[a]Department of Pediatrics, National Taiwan University Hospital, Taipei 100, Taiwan; [b]Research Center for Developmental Biology and Regenerative Medicine, National Taiwan University, Taipei 100, Taiwan; [c]Laboratory for Lung Development, RIKEN Center of Developmental Biology, Kobe 650-0047, Japan; [d]Department of Internal Medicine, National Taiwan University Hospital, Taipei 100, Taiwan; [e]Department of Respiratory Medicine, Kyoto University Hospital, Kyoto 606-8507, Japan; [f]Graduate Institute of Pharmacology, College of Medicine, National Taiwan University, Taipei 10617, Taiwan; and [g]Columbia Center for Human Development, Department of Medicine, Columbia University, New York, NY 10027

Edited by Xin Sun, University of Wisconsin–Madison, Madison, WI, and accepted by Editorial Board Member Kathryn V. Anderson May 25, 2016 (received for review June 9, 2015)

Abnormal enlargement of the alveolar spaces is a hallmark of conditions such as chronic obstructive pulmonary disease and bronchopulmonary dysplasia. Notch signaling is crucial for differentiation and regeneration and repair of the airway epithelium. However, how Notch influences the alveolar compartment and integrates this process with airway development remains little understood. Here we report a prominent role of Notch signaling in the epithelial–mesenchymal interactions that lead to alveolar formation in the developing lung. We found that alveolar type II cells are major sites of Notch2 activation and show by Notch2-specific epithelial deletion (*Notch2*^{cNull}) a unique contribution of this receptor to alveologenesis. Epithelial Notch2 was required for type II cell induction of the PDGF-A ligand and subsequent paracrine activation of PDGF receptor-α signaling in alveolar myofibroblast progenitors. Moreover, Notch2 was crucial in maintaining the integrity of the epithelial and smooth muscle layers of the distal conducting airways. Our data suggest that epithelial Notch signaling regulates multiple aspects of postnatal development in the distal lung and may represent a potential target for intervention in pulmonary diseases.

Notch | alveolar myofibroblast | alveologenesis | bronchial smooth muscle | epithelial–mesenchymal interactions

In the mature lung, proper gas exchange is achieved by a vast network of alveolar structures comprised of epithelial type I and type II cells closely apposed to capillaries and a thin layer of mesenchymal lipofibroblasts and myofibroblasts. Formation of this compartment initiates prenatally with the establishment of a distal program of differentiation in lung epithelial progenitors and later with formation of primitive saccules. The mature murine alveoli, however, are generated postnatally through a dramatic remodeling of the primitive saccules known as alveologenesis (1). Abnormal alveologenesis has devastating effects and is present in human conditions such as bronchopulmonary dysplasia and prematurity. The mechanisms involved in alveolar formation include expansion of epithelial cells lining the primitive saccules and generation of secondary septa where interstitial alveolar myofibroblasts (AMYFs) deposit elastin (2). AMYFs are derived from a population of mesenchymal progenitors known to require platelet derived growth factor receptor-α (PDGFR-α) signaling to develop (3).

Notch signaling is essential for lung development. Notch genes encode single-transmembrane receptors that mediate communication between neighboring cells crucial for cell fate decisions during organ development (4). In mammals, four Notch receptors (*Notch1* to *Notch4*) and five ligands (*Jag1*, *Jag2*, *Dll1*, *Dll3*, and *Dll4*) mediate these signaling events, largely through *Rbpj* (or *CSL*) transcriptional effector. *O*-fucosyltransferase 1 (*Pofut1*), an additional component of this pathway, conjugates *O*-fucose to EGF repeats of Notch receptors, allowing efficient Notch–ligand interactions (5).

In the embryonic lung Notch regulates the balance of ciliated, secretory, and neuroendocrine cells in the airway epithelium (6, 7). Postnatally, epithelial Notch signaling prevents airway Club (Scgb1a1 positive/Clara) cells from differentiating into goblet cells and is critical for airway regeneration after injury (8–10). It is less clear, however, how Notch signaling influences the alveolar compartment. Analysis of *Rbpj* or *Pofut1* null mutants at late gestation shows that primitive saccules do not require Notch to develop. By contrast, Notch gain of function does interfere with differentiation of the developing distal lung compartment, from which alveoli will form postnatally (11). However, the Notch-overexpressing mutants die at birth, before the initiation of alveologenesis, thus limiting conclusions on the role of Notch in this process. Interestingly, analysis of Notch-deficient mice that survive postnatally, such as conditional *Jag1* or glycosyltransferase Lunatic fringe (*Lfng*) mutants, points to a role of Notch in alveologenesis (12). Nevertheless, these mutants shed little light on Notch-mediated events in alveolar development. Deletion of *Jag1* in lung epithelium had no effect on differentiation and maturation of alveolar epithelial cells (13). Deficiency in Lfng-mediated Notch signaling impaired myofibroblast differentiation, but it was unclear whether Notch was normally activated in these cells. Moreover, mice overexpressing Lfng in distal lung epithelium, including type II cells, show no lung abnormalities and survive to adulthood (14).

To better understand how Notch influences alveolar formation we investigated the impact of selective or pan-Notch receptor loss of function in the murine lung. Here we show that during neonatal life Notch2 is activated in type II cells to induce *PDGF-A* expression, triggering paracrine activation of PDGFR-α signaling in AMYF progenitors ultimately required for alveologenesis. We found a selectively dominant contribution of Notch2, compared with Notch1,

Significance

Formation of the gas-exchange region of the lung occurs largely postnatally through a process called alveologenesis. Alveolar abnormalities are a hallmark of neonatal and adult chronic lung diseases. Here we report that disruption of Notch signaling in mice, particularly by Notch2, results in abnormal enlargement of the alveolar spaces reminiscent of that seen in chronic lung diseases. We provide evidence that Notch is crucial to mediate cross-talk between different cell layers, including signals such as PDGF for formation of the alveoli and maintenance of the integrity of the conducting airways.

Author contributions: P.-N.T. and M. Morimoto designed research; P.-N.T., C.M., S.-C.W., A.S., S.S., K.H., H.-k.C., T.-Y.L., M. Mori, and M. Morimoto performed research; P.-N.T., A.S., S.S., T.-Y.L., W.V.C., and M. Morimoto analyzed data; and P.-N.T., W.V.C., and M. Morimoto wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. X.S. is a guest editor invited by the Editorial Board.

Freely available online through the PNAS open access option.

[1]To whom correspondence may be addressed. Email: tsaopn@ntu.edu.tw or mmorimoto@cdb.riken.jp.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1511236113/-/DCSupplemental.

in this process. Disruption of Notch signaling decreased *PDGF-A* expression, whereas overexpression of activated Notch2 rescued this negative effect of Notch inhibition. Notch signaling was also required for maintaining the integrity of the epithelial and bronchial smooth muscle (SM) layers of the distal airways. Thus, epithelial Notch signaling integrates postnatal morphogenesis of the distal bronchiole and alveoli via epithelial–mesenchymal interactions.

Results

Epithelial Pan-Notch Signaling Inactivation Disrupts Alveolar Development. To investigate Notch signaling in alveologenesis we examined mice of the *Shh-Cre*;*Pofut1* $^{flox/flox}$ line, which do not activate Notch in the lung epithelium but have some pups surviving up to 2–3 wk postnatally (7) (Fig. S1 *A* and *B*). Histological analysis revealed that whereas at embryonic day (E) 18.5 *Pofut1*^{cNull} lungs were indistinguishable from controls (Fig. 1 *A* and *B*), at postnatal day (P) 3 mutant lungs failed to initiate alveolization, secondary septa were less frequent, and distal airspaces were enlarged compared with controls (Fig. 1 *C* and *D*). By P21, when alveologenesis was mostly completed in controls, *Pofut1*^{cNull} lungs showed a major deficit in alveolar formation with an emphysema-like enlargement of distal airspaces [Fig. 1 *E–G*; mean alveolar cord length (mean ± SEM): control, 31 ± 0.6 μm and *Pofut1*^{cNull}, 61 ± 2.9 μm].

Immunohistochemistry for prosurfactant protein C (pro-SPC) and morphometric analysis at P3 showed no difference in the number of type II cells between control and mutants; however, by P21 this number was significantly decreased in *Pofut1*^{cNull} lungs (Fig. 1*H* and Fig. S2 *A* and *B*). T1-α (or podoplanin), which marks type I pneumocytes, was similarly expressed in control and *Pofut1*^{cNull} lungs and outlined the enlarged distal airspaces of mutant lungs (Fig. S2 *C–E*). Activated Caspase3 suggested no difference in cell apoptosis between control and *Pofut1*^{cNull} lungs (Fig. 1 *I* and *J*). However, we found decreased Ki-67 labeling in type II cells of *Pofut1*^{cNull} mice (5.6% ± 1.3) compared with control (27.4% ± 1.8) at P3 (Fig. 1 *K–M*). Decreased proliferation postnatally could at least in part have contributed to the distal abnormalities of Notch-mutant lungs. Thus, although dispensable for cell type I/type II cell fate decisions, Notch signaling could be necessary to control the number of type II cells in the postnatal lung (7).

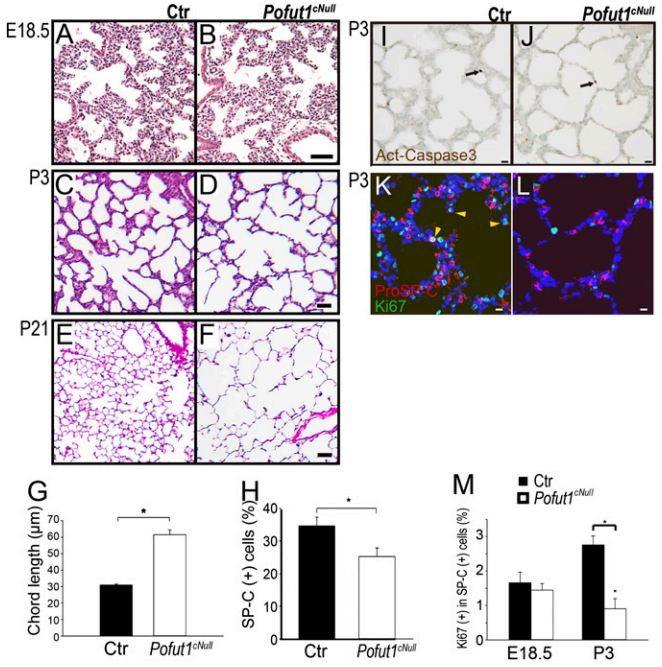

Fig. 1. Emphysema-like alveolar phenotype in *Pofut1*^{cNull} mice. H&E staining of controls (*A*, *C*, and *E*) and *Pofut1*^{cNull} (*B*, *D*, and *F*) at different ages. (*A* and *B*) No difference at E18.5. (*C* and *D*) Marked decrease in the secondary septa (arrowhead) in *Pofut1*^{cNull} mice compared with controls at P3. (*E* and *F*) At P21, *Pofut1*^{cNull} showed enlarged and simplified alveoli. (*G*) Significantly increased chord length in *Pofut1*^{cNull} mice at P21. (*H*) Pro-SPC–positive type II cells were significantly reduced at P21 in *Pofut1*^{cNull} lungs. The cell numbers were counted on 10 fields at 20× magnification of three mice for each genotype. Cleaved caspase-3 showed no difference between wild-type (*I*) and *Pofut1*^{cNull} lungs (*J*) at P3. Immunohistochemistry of Ki67 and SPC in control (*K*) and *Pofut1*^{cNull} lungs (*L*) revealed proliferation index significantly decreased in type II cells at P3 (*M*) ($n > 4$ in each group; *$P < 0.05$). (Scale bars, 50 μm in *A–F* and 10 μm in *I–L*.)

Notch2 Is Activated in Type II Cells During Alveologenesis. Given that in pan-Notch signaling inactivation only a limited number of *Pofut1*^{cNull} mice reached adulthood, we tested whether a Notch receptor-specific approach would allow better survival and provide additional insights into the role of Notch receptors in alveolar formation.

We limited our analysis to *Notch1* and *Notch2* because *Notch3* null mice show no alveolar abnormalities (15) and *Notch4* expression is restricted to the endothelium (16). First, we identified sites of Notch signaling activation during alveolar formation, by indirect immunofluorescence (IF) using antibodies that label selectively the Notch1 or 2 intracellular domains (N1-ICD and N2-ICD). Analysis of the distal lung at the onset of alveologenesis (P3) showed nuclear N1-ICD largely confined to endothelial cells with only weak epithelial signals (Fig. 2*A* and ref. 6). By contrast, N2-ICD strongly labeled type II cells (Fig. 2*B*).

We then investigated whether Notch2 is already active prenatally as primitive saccules form. N2-ICD signals were present at E18.5 in cells coexpressing pro-SPC (Fig. 2*C*), suggesting that Notch2 is activated already at the saccular stage concomitantly with the differentiation of type II cells. Thus, Notch2 is the predominant receptor activated in type II cells of primitive saccules, with a modest contribution of Notch1.

***Notch2*^{cNull} but Not *Notch1*^{cNull} Lungs Show Morphological and Functional Features of Emphysema-Like Phenotype.** To interrogate the function of Notch receptors individually in the developing lung, we inactivated *Notch1* or *Notch2* conditionally in the lung epithelium using the *Shh-Cre* mice, as we did for *Pofut1* mutants (henceforth referred to as *Notch1*^{cNull} and *Notch2*^{cNull} mice).

Notch1^{cNull} and *Notch2*^{cNull} pups were viable and reached adulthood (Fig. S1 *C* and *D*). Morphology and marker analyses of E18.5 and neonatal (P3) lungs was unremarkable in these mutants, including normal sacculation and differentiation of type I and type II cells, as confirmed by T1-α, Pro-SPC, Nkx2.1, and ABCa3 staining (Fig. 2 *D* and *E* and Fig. S3). Quantitative analysis showed no significant difference in the number of type II cells in *Notch2*^{cNull} compared with controls (Fig. 2 *F* and *G*), confirming that Notch signaling is not required for type I versus type II cell fate decision in the lung (7).

Adult lungs from *Notch2*^{cNull}, however, were markedly different from those of *Notch1*^{cNull}. Only *Notch2*^{cNull} exhibited the emphysema-like enlargement of distal airspaces seen in *Pofut1*^{cNull} (Fig. 2 *H–J* and *L*). As in *Pofut1*^{cNull} lungs (Fig. 1*M*), Ki-67 labeling in type II cells of *Notch2*^{cNull} lungs was significantly reduced (Fig. 2*M*). In addition, the number of type II cells was significantly decreased in adult *Notch2*^{cNull} lungs (Fig. 2*N*), consistent with the findings in *Pofut1*^{cNull} lung (Fig. 1*H*). We then isolated type II cells from control and *Notch2*^{cNull} mice at P14 (late stage of alveologenesis) using FACS; PCR analysis showed decreased expression of *SP-C*, *ABCa3*, and *LysM* genes in mutants (17) (Fig. 2*O*). We concluded that, although not required for sacculation, Notch2 activity is essential for proliferation and maturation of type II cells when definitive alveoli are forming.

To evaluate the impact of *Notch2* deficiency in the function of these lungs we assessed key physiologic parameters in adult mutants and age/sex-matched controls by FlexiVent analysis. We found a twofold increase in static lung compliance (Cst) in *Notch2*^{cNull} homozygous compared with *Notch2*^{cHet} and control (Cre-negative) littermates (Fig. S4*A*). We also detected a decrease in airway resistance (Rn) to high-pressure airflow (positive end

DEVELOPMENTAL BIOLOGY

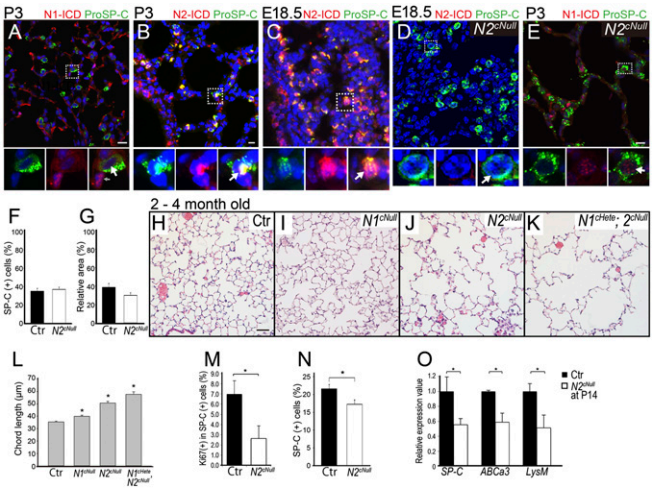

Fig. 2. Notch2 activation in type II cells and requirement for type II cell proliferation and maturation. (*A*) Coimmunostaining of N1-ICD and pro-SPC at P3 showed that type II cells (white arrow) were not the main Notch1-active population (gray arrow). (*B–D*) N2-ICD detected in pro-SPC–positive type II cells (white arrows) at P3 (*B*) and E18.5 (*C*). N2-ICD antibody validated by lack of staining in *Notch2*^{cNull} lungs (*D*). At P3, spotted N1-ICD signals in the nuclei of type II cells (white arrow) were increased in *Notch2*^{cNull} (*E*) in compare with wild type (*A*). Quantification of type II (*F*) and type I (*G*) cells showed no difference between *Notch2*^{cNull} and control lungs at P3. H&E staining of control (*H*), *Notch1*^{cNull} (*I*), *Notch2*^{cNull} (*J*), and *Notch1*^{cHete}; *2*^{cNull} (*K*) mutant mice at 2–4 mo old. Emphysema-like phenotype in *Notch2*^{cNull} and *Notch1*^{cHete};*2*^{cNull} mutant lungs but not in *Notch1*^{cNull}, and more severe in *Notch1*^{cHete};*2*^{cNull}. (*L*) Significantly increased chord length in Notch receptor mutants, especially in *Notch2*^{cNull} and *Notch1*^{cHete};*2*^{cNull} at 2–4 mo old. (*M*) Proliferation index determined by Ki67 in pro-SPC–positive type II cells in control or *Notch2*^{cNull} lungs at P3. (*N*) Type II cell number significantly decreased in adult *Notch2*^{cNull} lungs at 4 mo old. (*O*) Significant reductions of expression of the type II cell marker genes *SPC*, *ABCa*, and *LysM* in *Notch2*^{cNull} by quantitative RT-PCR of isolated the type II cells at P14. (Scale bars, 10 μm in *A–E* and 50 μm in *H–K*.)

expiratory pressure, PEEP) and a decrease in tissue elasticity and tissue damping (G) in homozygous *Notch2*^{cNull} (Fig. S4 *B–D*). These findings are consistent with the functional abnormalities associated with an emphysema-like enlargement of distal airspaces. Interestingly, N1-ICD and pro-SPC double IF in *Notch2*^{cNull} lungs showed N1-ICD nuclear signals in type II cells, stronger than in control type II cells (littermates without Cre gene) (Fig. 2 *A* and *E*). This suggested a potential compensatory up-regulation of N1-ICD in the absence of N2-ICD.

Given the potential functional redundancy between Notch1 and Notch2 (18) and the less severe phenotype of *Notch2*^{cNull} compared with *Pofut1*^{cNull}, we examined the effect of the ablating a *Notch1* allele in *Notch2*^{cNull} background. *Notch1*^{cHete};*2*^{cNull} showed a more marked enlargement of the distal airspaces suggestive of a more severe emphysema-like phenotype compared with single *Notch2*^{cNull} (Fig. 2 *K* and *L*). We concluded that although Notch2 plays a more prominent role in alveologenesis, activation of Notch1 also contributes to this process.

Disruption of Epithelial Notch Signaling Inhibits AMYF Proliferation and Differentiation Through Epithelium–Mesenchyme Interactions. Because AMYF plays a crucial role in secondary septa formation (3, 19), we investigated myofibroblast differentiation in the alveologenesis defect of Notch-deficient mice. In control lungs at P3 α-smooth muscle actin (αSMA)-positive AMYFs are normally seen at the tip of the emerging secondary septa. In *Pofut1*^{cNull} distal lungs at P3 these septa were nearly absent and the number of αSMA-positive AMYFs in primitive saccules was significantly decreased (Fig. 3 *A*, *B*, and *M*). By contrast, αSMA expression was unaffected in perivascular SM (Fig. 3 *B* and *K*, asterisk). Next, we analyzed the expression and distribution of elastin, which is deposited by AMYFs. In P3 controls, elastin was deposited at the tips of the septa and along the saccules, as expected (Fig. 3*C*). In contrast, elastic fiber formation was markedly decreased in the walls of P3 *Pofut1*^{cNull} saccules (Fig. 3*D*). Furthermore, tropoelastin mRNA expression was decreased to 65% of controls in the lungs of *Pofut1*^{cNull} mice (Fig. 3*E*). Quantitative analysis of the pool of PDGFR-α–positive AMYF progenitors showed a significant decrease in the number of proliferating PDGFR-α/Ki67 double-labeled cells in P3 *Pofut1*^{cNull} lungs (Fig. 3 *F–H*). However, at P3 the overall number of PDGFR-α–positive progenitors was still similar in control and *Pofut1*^{cNull} lungs. By contrast, by P7 the number of these progenitors significantly decreased in saccules walls of mutant lungs (Fig. 3*I*).

We investigated the effect of Notch deficiency in epithelial-derived paracrine signals that promote AMYF progenitor development. The epithelial-derived PDGF-A is essential for proliferation and differentiation of the PDGFR-α–positive AMYF progenitors (3, 20). PCR analysis of embryonic and postnatal lungs showed *PDGF-A* mRNA levels significantly decreased already at E15.5 and P3 *Pofut1*^{cNull} lungs (Fig. 3*J*). We proposed that disruption of Notch2 signaling leading to low epithelial PDGF-A expression could

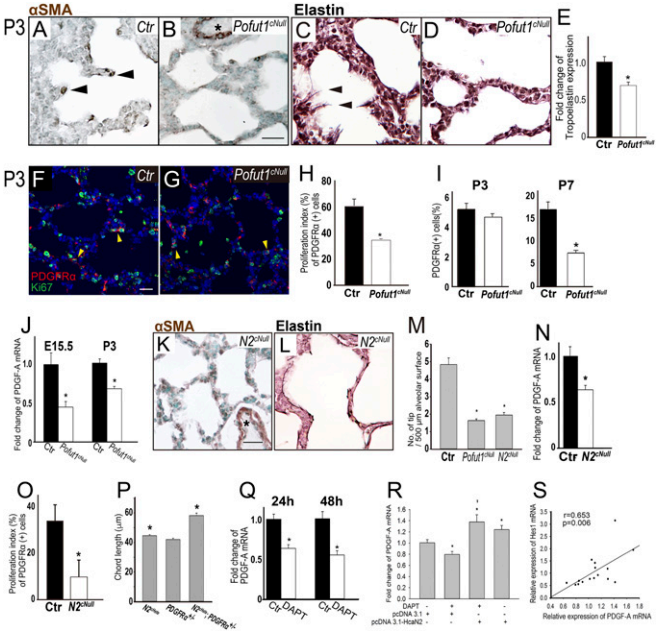

Fig. 3. Inactivation of epithelial Notch signaling inhibits proliferation of mesenchymal AMYF progenitors. (*A* and *B*) Decreased number of αSMA-positive AMYFs (arrowheads) in (*B*) *Pofut1*^{cNull} lungs compared with control (*A*) at P3. Asterisks indicate vascular SM. (*C* and *D*) Elastin staining revealed continuous elastin fibers with protrusions into the saccular space (arrowhead) in control (*C*). In contrast, only few protruding elastin fibers can be seen in *Pofut1*^{cNull} lungs (*D*). (*E*) *Tropoelastin* mRNA significantly decreased in *Pofut1*^{cNull} lungs. (*M*) Tip numbers of secondary septa significantly decreased in both *Pofut1*^{cNull} and *Notch2*^{cNull} lungs. (*F* and *G*) Proliferating AMYF progenitors are detected by PDGFR-α and Ki67 double staining of controls (*F*) and *Pofut1*^{cNull} (*G*) lungs. The proliferation index estimated from the double staining was significantly reduced in *Pofut1*^{cNull} and *Notch2*^{cNull} lungs (*H and O*). (*I*) The number of PDGFR-α–positive AMFY progenitors was significantly decreased in *Pofut1*^{cNull} lungs at P7, but not P3, during alveologenesis. (*J* and *N*) Real-time PCR showed significantly decreased *PDGF-A* mRNA expressions in *Pofut1*^{cNull} (*J*) and *Notch2*^{cNull} (*N*) lungs. AMYF differentiation (*K*) and elastin deposition (*L*) were also defective in *Notch2*^{cNull} lungs. (*P*) Quantitative analysis revealed significantly increased chord length in *Notch2*^{cHete};*PDGFR-α*$^{+/-}$ mice at 3–6 mo old. DAPT (10 μM) inhibited *PDGF-A* mRNA expression in MLE15 cells (*Q*), and constitutively activated Notch2 signaling by transfection with HcaN2 plasmids rescued this phenomenon (*R*). Strongly positive correlation between levels of *Hes1* and *PDGF-A* mRNA in MEL15 cells (*S*) ($n = 3–5$ in each group for quantification, $^*P < 0.05$). (Scale bars, 10 μm.)

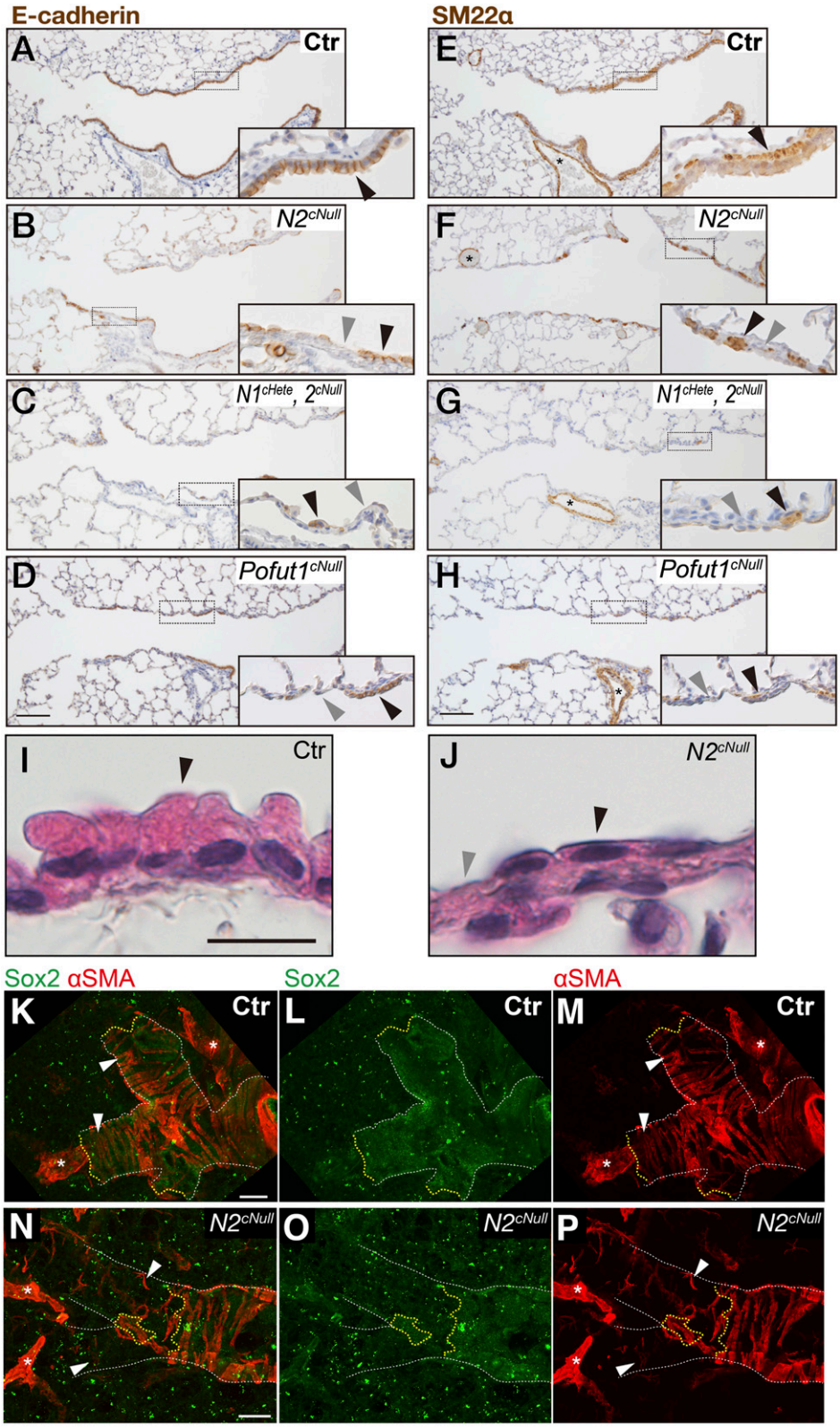

Fig. 4. Epithelial Notch signaling is required for SM development in distal airways. Immunohistochemistry for E-Cad (*A–D*) and SM22α (*E–H*) in *Pofut1*^{cNull} (pan-Notch signaling) compound *Notch1*^{cHete};*Notch2*^{cNull} or *Notch2*^{cNull} at P21 to 4 mo old. Distal airways showing discontinuous SM layer in areas of epithelial attenuation with squamous-like shape and loss of integrity, not seen in proximal regions. Phenotype is most severe in *Pofut1*^{cNull} and *Notch1*^{cHete};*Notch2*^{cNull}. (*I* and *J*) H&E staining revealed that the distal airway epithelium became squamous-like in appearance in adult *Notch2*^{cNull} lungs. Black and gray arrowheads indicate the epithelial cells and defect in epithelium (*A–D*), SM, and defect in the subepithelial layer (*E–H*), respectively. (*K–P*) Double IF staining for epithelial Sox2 (*K*, *L*, *N*, and *O*) and mesenchymal αSMA (*K*, *M*, *N*, and *P*) in thick sections of the distal bronchiole of *Notch2*^{cNull} showing correlation between epithelium and airway SM development. White and yellow lines outline shapes of the bronchioles and edges of epithelial sheet. White arrowheads indicate SM surrounding the distal-most bronchiole. In *Notch2*^{cNull} SM failed to develop band-like tissue structure where the epithelial sheet is defective (*N–P*). Asterisks indicate vessels. (Scale bars, 100 μm in *A–H* and *K–P* and 10 μm in *I* and *J*.)

ultimately result in the inability to form secondary septa postnatally. Indeed, as seen in *Pofut1*^{cNull} mice, lungs from *Notch2*^{cNull} mutants showed decreased proliferation and differentiation of PDGFR-α–expressing AMYF progenitors and decreased elastin deposition associated with low levels of epithelial PDGF-A expression (Fig. 3 *K–O*). Epistatic relation between Notch2 and PDGF signaling in alveologenesis was determined by generating *Notch2*^{cHete};*PDGFR-α*$^{+/-}$ mice. The double heterozygous adult mice showed longer mean alveolar cord length compared with single heterozygous [Fig. 3*P*; mean alveolar cord length (mean ± SEM): *Notch2*^{cHete}, 44.6 ± 0.7 μm; *PDGFR-α*$^{+/-}$, 42.1 ± 0.8 μm; *Notch2*^{cHete}, *PDGFR-α*$^{+/-}$, 58.2 ± 1.7 μm; Fig. S5].

Consistent with this, blocking Notch signaling pharmacologically with gamma-secretase inhibitor (N-[N-(3, 5-difluorophenacetyl)-l-alanyl]-S-phenylglycine *t*-butyl ester, DAPT) (21) in the alveolar type II cell line MLE 15 (22) decreased *PDGF-A* mRNA (Fig. 3*Q*). Notably, this effect could be reverted by expressing a constitutively active Notch2 (HcaN2) (Fig. 3*R*). In addition, expression of the *Hes1* gene, a known target of Notch signaling, positively correlated with *PDGF-A* expression (Fig. 3*S*). Thus, Notch signaling controls proliferation of epithelial type II cells and mesenchymal AMYF progenitors through regulating *PDGF-A* expression.

Disrupted Epithelial Integrity and Impaired SM Development in Airways of Notch Null Mutants. FlexiVent analyses of *Notch2*^{cNull} mice (Fig. S4) revealed functional changes compatible with abnormalities in the alveolar and the distal airway compartments. This prompted us to examine whether small airways were also defective in Notch null mutant lungs. Markers analyses of adult *Notch2*^{cNull} lungs revealed the expansion of the ciliated cell population at the costs of Club cells previously reported in other Notch-deficient mice (6, 7, 18). However, we also noticed E-cadherin (E-Cad) staining markedly attenuated in the distal airway epithelium *Notch2*^{cNull} lungs compared with controls (Fig. 4 *A* and *B*). In some of these regions the epithelium became squamous-like (Fig. 4 *I* and *J*) and appeared discontinuous.

Interestingly, expression of the SM marker SM22α showed poorly developed or absence of airway SM in areas associated with the discontinuous epithelium (Fig. 4 *E* and *F*). *Notch1*^{cHete};*2*^{cNull} and *Pofut1*^{cNull} lungs displayed similar but more severe disruption of integrity of airway epithelial cells and SM (Fig. 4 *C*, *D*, *G*, and *H*). This defect was not seen in proximal airways (Fig. S6). To further check the topological correlation between epithelial and mesenchymal defect, double staining for epithelial Sox2 and mesenchymal αSMA (an SM marker) was performed in 150-μm-thick sections of the distal bronchioles (Fig. 4 *K*–*P*). This confirmed the preserved airway SM in distal airways associated with the Sox2-positive epithelium in controls, not present where the epithelium was discontinuous in the *Notch2*^{cNull} airways (Fig. 4 *N*–*P*). The phenotype was not observed in E18.5 lungs (Fig. 5 *A*–*E* and Fig. S7), suggesting that these abnormalities occurred only postnatally.

Because Shh-Cre does not target mesenchymal cells (23), the aberrant SM phenotype of *Notch2*^{cNull} was likely to result primarily from an epithelial defect. In addition, we performed costaining of Scgb1a1b and αSMA for *Notch2*^{cNull} or control and detected no topological relation of Club cells and airway SM cells (Fig. S8). This suggests that SM development is independent of the presence of Club cells in the developing airways.

We compared the proliferation ratio of bronchiolar epithelial cells in control and *Notch2*^{cNull} lungs by coimmunostaining for phosphor Histone H3 (pHH3) or Ki67 and E-Cad (Fig. S9 *A*, *B*, *D*, and *E*). Morphometric analysis of *Notch2*^{cNull} bronchioles showed a reduction in the proliferating epithelial population (Fig. S9 *C* and *F*). This proliferation defect could be related to the reduction of Club cells, the major progenitor cells responsible for epithelial tissue maintenance in intrapulmonary airways (24).

We examined other signals present in the airway epithelium potentially associated with airway SM development. Epithelial-secreted *Wnt7b* has been reported as a crucial inducer of airway SM in the developing lung (25). In situ hybridization of control lungs confirmed epithelial Wnt7b expression throughout the airway epithelium of P3 to P75 (Fig. 5 *F*–*H*), suggesting continuous contribution of this paracrine factor to postnatal airway SM development. Quantitative RT-PCR of *Pofut1*^{cNull} lungs revealed reduction in *Wnt7b* expression particularly prominent at P21 compared with control (Fig. 5*J*). *Notch2*^{cNull} adult lungs also showed decreased *Wnt7b* expression (Fig. 5*I*) (25, 26). Thus, disruption of Wnt7b-mediated epithelial–mesenchymal interactions could well contribute to the SM phenotype of Notch-deficient mice. However, we cannot exclude the possibility that other epithelium-secreted factors may also contribute to the defective SM phenotype.

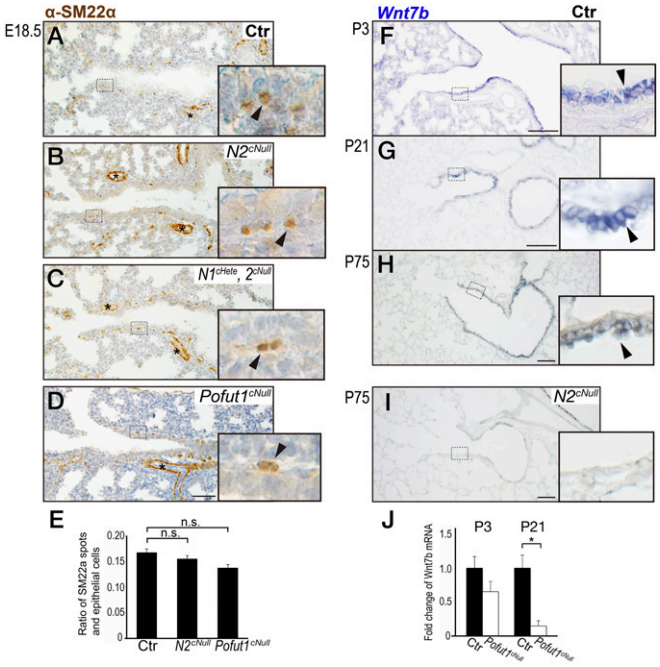

Fig. 5. Integrity of the airway epithelium is required for SM development. (*A*–*D*) SM22α immunostaining of E18.5 lung showing continuous epithelial layer and SM layer unaltered in Notch mutants and control. (*E*) Quantification of the subepithelial αSMA-positive spots in Notch mutants and controls. (*F* and *G*) *Wnt7b* in situ hybridization in airway epithelium at P3 and P21. (*H* and *I*) *Wnt7b* expression significantly decreased in *Notch2*^{cNull} lungs (*I*) compared with control (*H*). (*J*) RT-PCR showed a trend toward a decrease in expression in *Wnt7b* mRNA at P3 that becomes significant at P21 in *Pofut1*^{cNull} mice compared with controls (n = 3–9 in each group). Error bars represent SEM. (Scale bars, 50 μm in *A*–*D* and 100 μm in *F*–*I*.)

Discussion

Despite the reported association between altered Notch signaling and abnormal distal lung development in mice, the role of Notch in lung alveolar formation has not been clearly defined. Issues include lack of unambiguous evidence that Notch is normally activated in the developing alveolar epithelium and uncertainties about alveolar epithelial sites of ligand expression.

Here we provide direct evidence of Notch2 activation in type II cells and genetic data supporting a unique contribution of Notch2 to alveologenesis. We show that Notch2 signaling is required for type II cell proliferation and maturation. Disruption of Notch signaling in the distal lung epithelium resulted in decreased expression of *PDGF-A*, indispensible for AMYF development in the distal mesenchyme, leading to defective alveologenesis. Moreover, we found decreased cellularity and loss of epithelial integrity in distal bronchioles with defective development of the adjacent SM layer. We ascribe the defects in both the alveolar and airway compartments to impaired epithelial–mesenchymal interactions in Notch-deficient mice

Lineage studies suggest that bronchiolar Club cells serve as a progenitor cell source, maintaining the bronchiolar epithelium at the postnatal period, but are not required for the integrity of the alveolar compartment (24). Indeed, alveolar defects have not been reported in mouse mutants in which Club cells have been ablated, such as in Scgb1a1-Cre–driven *Sox2* deletion or in *Scgb1a1-rtTA/tetO-Cre/R26-lacZ:DT-A* transgenic mice (27, 28). Thus, the defect in alveologenesis is unlikely to be causally linked to the loss of Club cells. Interestingly, our Notch mutants lack the Club cells before birth (7), in contrast to the models described above. This may account for the different outcome, raising the possibility that integrity of Club cells prenatally, although not affecting saccular formation, may be required for normal alveologenesis.

Alternatively, lung epithelial activation of Notch signaling prenatally could be required for alveologenesis, as suggested by the Notch-deficient disruption of AMYF differentiation.

We propose that Notch2 activity in epithelial cells regulates epithelial–mesenchymal interactions crucial for development and homeostasis of the distal lung (alveoli and distal bronchiole) during postnatal life. Our study shows a clear association between the epithelial defect and aberrant SM in Notch2-deficient mutants. Notch2 exert its effects likely thorough epithelial-derived paracrine factors, such as Wnt7b and PDGFA, candidate mediators of SM and AMYF differentiation in the bronchoalveolar compartment. In addition, the more severe distal lung phenotype in $Notch1^{cHete}$;2^{cNull} compared with $Notch2^{cNull}$ mice suggests a cooperative effect of Notch1 and Notch2 in alveolar formation. Disrupted morphogenesis of distal airways has been associated with lung immaturity in conditions such as bronchopulmonary dysplasia in premature infants (29). Abnormal repair of alveolar and small airways after lung injury also lead to insufficient gas-exchange surface associated with chronic obstructive pulmonary disease in the adult (30).

Gene mutations resulting in loss of function of NOTCH2 or the ligand JAG1 have been causally linked to Alagille syndrome. This multisystem disorder includes abnormalities in liver, skeleton, eye, heart, and kidney (31). Interestingly, we have preliminary radiological evidence of diffuse emphysema-like changes in Allagile patients carrying *JAG1* mutation, although this limited cohort did not contain patients with NOTCH2 mutations. In humans, 94% of Alagille patients harbor *Jag1* mutations, but the disease can also result from loss of one *Notch2* allele (31). This is of interest, given that disruption of alveolar formation has been previously described in mice with conditional deletion of *Jag1* in lung epithelium (13), a phenotype also seen in our $Notch2^{cNull}$ mice.

The accumulated evidence of the role of Notch in the lung suggests the potential benefit of targeting this pathway in pulmonary disorders affecting the airway or alveolar epithelium.

Methods

$Pofut1^{F/F}$ (5), $Notch1^{F/F}$, and $Notch2^{F/F}$ were mated to mice carrying the ShhCre allele (23) to generate mutant embryos as in our previous reports (7, 18). PDGFR-$\alpha^{+/-}$ mice (32) were mated with *ShhCre*;$Notch2^{fl/+}$ for epistatic analysis. All mice were maintained on the C57BL/6 × CD1 mixed background, and *ShhCre* mice were on a C57BL/6 background. All animal experiments were approved by the Experimental Animal Use Committee of RIKEN Center of Developmental Biology or National Taiwan University Hospital. All other detailed methods, including information on antibodies (Table S1), the sequence of PCR primers for real-time PCR (Table S2), and the validation of the N2-ICD antibody (Fig. S10), are described in the *SI Methods*.

ACKNOWLEDGMENTS. We thank the second Core Laboratory of the Department of Medical Research of National Taiwan University Hospital and National Laboratory Animal Center for technical assistance. This work was supported by funding from Grant-in-Aid for Young Scientists (A) 26713029 and 23689044 and Challenging Exploratory Research Grant 16K15463 of the Japan Society for the Promotion of Science (to M. Mitsuru); National Health Research Institutes Grants EX102-9924SC, Ministry of Science and Technology Grant 101-2628-B-002-002-MY4 and 104-2627-M-002-012, National Taiwan University Grant 105R7751, and National Taiwan University Hospital Grant 105-P07 (to P.-N.T.); and NIH Grant 5R01HL105971 (to W.V.C.).

1. Massaro D, Massaro GD (2007) Developmental alveologenesis: Longer, differential regulation and perhaps more danger. *Am J Physiol Lung Cell Mol Physiol* 293(3):L568–L569.
2. Wendel DP, Taylor DG, Albertine KH, Keating MT, Li DY (2000) Impaired distal airway development in mice lacking elastin. *Am J Respir Cell Mol Biol* 23(3):320–326.
3. Boström H, et al. (1996) PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell* 85(6):863–873.
4. Kopan R, Ilagan MX (2009) The canonical Notch signaling pathway: Unfolding the activation mechanism. *Cell* 137(2):216–233.
5. Shi S, Stanley P (2003) Protein O-fucosyltransferase 1 is an essential component of Notch signaling pathways. *Proc Natl Acad Sci USA* 100(9):5234–5239.
6. Morimoto M, et al. (2010) Canonical Notch signaling in the developing lung is required for determination of arterial smooth muscle cells and selection of Clara versus ciliated cell fate. *J Cell Sci* 123(Pt 2):213–224.
7. Tsao PN, et al. (2009) Notch signaling controls the balance of ciliated and secretory cell fates in developing airways. *Development* 136(13):2297–2307.
8. Rock JR, et al. (2011) Notch-dependent differentiation of adult airway basal stem cells. *Cell Stem Cell* 8(6):639–648.
9. Tsao PN, et al. (2011) Notch signaling prevents mucous metaplasia in mouse conducting airways during postnatal development. *Development* 138(16):3533–3543.
10. Xing Y, Li A, Borok Z, Li C, Minoo P (2012) NOTCH1 is required for regeneration of Clara cells during repair of airway injury. *Stem Cells* 30(5):946–955.
11. Guseh JS, et al. (2009) Notch signaling promotes airway mucous metaplasia and inhibits alveolar development. *Development* 136(10):1751–1759.
12. Xu K, et al. (2010) Lunatic Fringe-mediated Notch signaling is required for lung alveogenesis. *Am J Physiol Lung Cell Mol Physiol* 298(1):L45–L56.
13. Zhang S, Loch AJ, Radtke F, Egan SE, Xu K (2013) Jagged1 is the major regulator of Notch-dependent cell fate in proximal airways. *Dev Dyn* 242(6):678–686.
14. van Tuyl M, et al. (2005) Overexpression of lunatic fringe does not affect epithelial cell differentiation in the developing mouse lung. *Am J Physiol Lung Cell Mol Physiol* 288(4):L672–L682.
15. Krebs LT, et al. (2003) Characterization of Notch3-deficient mice: Normal embryonic development and absence of genetic interactions with a Notch1 mutation. *Genesis* 37(3):139–143.
16. Uyttendaele H, et al. (1996) Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene. *Development* 122(7):2251–2259.
17. Desai TJ, Brownfield DG, Krasnow MA (2014) Alveolar progenitor and stem cells in lung development, renewal and cancer. *Nature* 507(7491):190–194.
18. Morimoto M, Nishinakamura R, Saga Y, Kopan R (2012) Different assemblies of Notch receptors coordinate the distribution of the major bronchial Clara, ciliated and neuroendocrine cells. *Development* 139(23):4365–4373.
19. Amy RW, Bowes D, Burri PH, Haines J, Thurlbeck WM (1977) Postnatal growth of the mouse lung. *J Anat* 124(Pt 1):131–151.
20. Lindahl P, et al. (1997) Alveogenesis failure in PDGF-A-deficient mice is coupled to lack of distal spreading of alveolar smooth muscle cell progenitors during lung development. *Development* 124(20):3943–3953.
21. Tsao PN, et al. (2008) Gamma-secretase activation of notch signaling regulates the balance of proximal and distal fates in progenitor cells of the developing lung. *J Biol Chem* 283(43):29532–29544.
22. Wikenheiser KA, et al. (1993) Production of immortalized distal respiratory epithelial cell lines from surfactant protein C/simian virus 40 large tumor antigen transgenic mice. *Proc Natl Acad Sci USA* 90(23):11029–11033.
23. Harris KS, Zhang Z, McManus MT, Harfe BD, Sun X (2006) Dicer function is essential for lung epithelium morphogenesis. *Proc Natl Acad Sci USA* 103(7):2208–2213.
24. Rawlins EL, et al. (2009) The role of Scgb1a1+ Clara cells in the long-term maintenance and repair of lung airway, but not alveolar, epithelium. *Cell Stem Cell* 4(6):525–534.
25. Shu W, Jiang YQ, Lu MM, Morrisey EE (2002) Wnt7b regulates mesenchymal proliferation and vascular development in the lung. *Development* 129(20):4831–4842.
26. Cohen ED, et al. (2009) Wnt signaling regulates smooth muscle precursor development in the mouse lung via a tenascin C/PDGFR pathway. *J Clin Invest* 119(9):2538–2549.
27. Tompkins DH, et al. (2009) Sox2 is required for maintenance and differentiation of bronchiolar Clara, ciliated, and goblet cells. *PLoS One* 4(12):e8248.
28. Stripp BR, et al. (1996) Clara cell secretory protein: A determinant of PCB bioaccumulation in mammals. *Am J Physiol* 271(4 Pt 1):L656–L664.
29. Gien J, Kinsella JP (2011) Pathogenesis and treatment of bronchopulmonary dysplasia. *Curr Opin Pediatr* 23(3):305–313.
30. Tuder RM, Petrache I (2012) Pathogenesis of chronic obstructive pulmonary disease. *J Clin Invest* 122(8):2749–2755.
31. Turnpenny PD, Ellard S (2012) Alagille syndrome: Pathogenesis, diagnosis and management. *Eur J Hum Genet* 20(3):251–257.
32. Ding G, Tanaka Y, Hayashi M, Nishikawa S, Kataoka H (2013) PDGF receptor alpha+ mesoderm contributes to endothelial and hematopoietic cells in mice. *Dev Dyn* 242(3):254–268.
33. Cheng HT, et al. (2007) Notch2, but not Notch1, is required for proximal fate acquisition in the mammalian nephron. *Development* 134(4):801–811.
34. Alanis DM, Chang DR, Akiyama H, Krasnow MA, Chen J (2014) Two nested developmental waves demarcate a compartment boundary in the mouse lung. *Nat Commun* 5:3923.
35. Hsia CC, Hyde DM, Ochs M, Weibel ER (2010) An official research policy statement of the American Thoracic Society/European Respiratory Society: Standards for quantitative assessment of lung structure. *Am J Respir Crit Care Med* 181(4):394–418.
36. Fust A, Bates JH, Ludwig MS (2004) Mechanical properties of mouse distal lung: In vivo versus in vitro comparison. *Respir Physiol Neurobiol* 143(1):77–86.
37. Ikegami M, et al. (2002) Deficiency of SP-B reveals protective role of SP-C during oxygen lung injury. *J Appl Physiol* 92(2):519–526.
38. Small D, et al. (2001) Soluble Jagged 1 represses the function of its transmembrane form to induce the formation of the Src-dependent chord-like phenotype. *J Biol Chem* 276(34):32022–32030.