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Claudin-18 Deficiency Results in Alveolar Barrier Dysfunction and Impaired Alveologenesis in Mice

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Abstract

Claudins are a family of transmembrane proteins that are required for tight junction formation. Claudin (CLDN)-18.1, the only known lung-specific tight junction protein, is the most abundant claudin in alveolar epithelial type (AT) 1 cells, and is regulated by lung maturational agonists and inflammatory mediators. To determine the function of CLDN18 in the alveolar epithelium, CLDN18 knockout (KO) mice were generated and studied by histological, biochemical, and physiological approaches, in addition to whole-genome microarray. Alveolar epithelial barrier function was assessed after knockdown of CLDN18 in isolated lung cells. CLDN18 levels were measured by quantitative PCR in lung samples from fetal and postnatal human infants. We found that CLDN18 deficiency impaired alveolar epithelial barrier function *in vivo* and *in vitro*, with evidence of increased paracellular permeability and architectural distortion at AT1–AT1 cell junctions. Although CLDN18 KO mice were born without evidence of a lung abnormality, histological and gene expression analysis at Postnatal Day 3 and Week 4 identified impaired alveolarization. CLDN18 KO mice also had evidence of postnatal lung injury, including acquired AT1 cell damage. Human fetal lungs at 23–24 weeks gestational age, the highest-risk period for developing bronchopulmonary dysplasia, a disease of impaired alveolarization, had significantly lower CLDN18 expression relative to postnatal lungs. Thus, CLDN18 deficiency results in epithelial barrier dysfunction, injury, and impaired alveolarization in mice. Low expression of CLDN18 in human fetal lungs supports further investigation into a role for this tight junction protein in bronchopulmonary dysplasia.

Keywords: claudins; tight junctions; bronchopulmonary dysplasia; lung injury

Clinical Relevance

Claudin (CLDN)-18 is required for normal alveolar epithelial barrier function *in vivo* and *in vitro*. Furthermore, CLDN18 knockout mice have impaired alveolarization. As data support developmental regulation of CLDN18 during the third trimester in human fetal lungs, further investigation is warranted to assess the role of this lung-specific tight junction protein in bronchopulmonary dysplasia.

Bronchopulmonary dysplasia (BPD), the most common chronic lung disease of infancy in the United States, is paradoxically rising in incidence despite medical advances (1). This disease was initially described in slightly premature infants that had been exposed to mechanical ventilation and high concentrations of supplemental oxygen (2). Although improved respiratory care and

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the introduction of therapies such as surfactant replacement have significantly reduced this "old" form of BPD, a new pattern of lung injury has emerged in infants born at earlier gestational ages (3). The main feature of "new" BPD is diffusely reduced alveolar development associated with a clinically significant loss of surface area for gas exchange (4). This new form of BPD is interpreted as developmental arrest secondary to injury in the immature lung (5). As the pathogenesis of new BPD remains unclear and treatment remains empirical, investigation of novel pathways is required to identify therapeutic targets.

Claudins constitute a protein family of at least 24 members in humans, and are critical constituents of the tight junction (6, 7) with a role in regulating paracellular transport (8, 9). The lung-specific splice variant of claudin (CLDN)-18, CLDN18.1, is the only known lung epithelium–specific tight junction protein (10). Our previous work identified CLDN18 as highly expressed in alveolar epithelial type (AT) 2 cells, and the most abundantly expressed claudin in AT1 cells (11). Clinically relevant proinflammatory innate immune mediators cause a specific loss of CLDN18 from the apical junction complex in association with barrier dysfunction (12). Conversely, pharmacologic agents that promote lung maturation cause a specific induction of CLDN18 in human fetal alveolar epithelial cells and augment barrier function (13). Although these data support a potential role for CLDN18 in regulating alveolar epithelial barrier function, the *in vivo* role of CLDN18 in lung biology requires further investigation.

We hypothesized that CLDN18 has a unique role in the establishment and maintenance of alveolar epithelial barrier function. To test this hypothesis, we generated a novel CLDN18 null mouse and examined the effects of CLDN18 deficiency on lung structure and function, including whole-genome mRNA microarray analysis. Alveolar epithelial barrier properties attributable to CLDN18 were also examined in cultured cells. Our results demonstrate a unique role for CLDN18 in determining alveolar epithelial barrier function, and indicate that CLDN18 deficiency results in lung injury and impaired alveolarization. In a complementary line of investigation, we found that human fetal lungs at 23–24 weeks gestational age, a high-risk period for developing BPD with extreme preterm birth (5), have significantly lower CLDN18 expression levels than postnatal human lungs. Taken together, these data indicate that CLDN18 deficiency represents a novel model of alveolar epithelial barrier dysfunction and impaired alveolarization in mice. Further study is warranted to assess the role of this developmentally regulated, lung-specific tight junction protein in clinical BPD.

Materials and Methods

See the online supplement for additional information.

Animal Model

Heterozygote *cldn18*$^{+/-}$ mice (C57Bl/6J background) used to breed CLDN18 knockout (KO) and wild-type (WT) mice, were generated by the trans–National Institutes of Health Knock-Out Mouse Project (KOMP) (www.komp.org). For some studies, additional WT C56Bl/6J mice used for breeding were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were maintained in pathogen-free conditions according to protocols approved by the Institutional Animal Care and Use Committee at the San Francisco Veterans Administration Medical Center. Mouse genotypes were identified by PCR using genomic DNA from the tails of postnatal mice. All experiments were performed with age- and sex-matched CLDN18 KO and WT mice.

Barrier Function Studies

Bidirectional alveolar epithelial permeability to albumin was determined *in vivo* in CLDN18 KO and WT mice as previously described (14) with minor modification. For *in vitro* studies, primary rat AT2 cells were isolated and grown in culture as previously described (11, 15). CLDN18 was transiently knocked down in primary cells by small interfering RNA (siRNA) using cationic lipid (16). After confirmation of knockdown by immunoblot, transepithelial electrical resistance of stable monolayers and paracellular permeability to a 0.5-kD tracer were measured as previously described (11).

Histopathology

Standard inflation-fixed lungs were paraffin embedded. The intra-alveolar distance on hematoxlin and eosin–stained sections was measured as the mean linear intercept (MLI) (17). MLI is inversely proportional to the surface area of the lung (18, 19).

Microarrays

mRNA was extracted from WT (n = 5) and CLDN18 KO (n = 4) mouse lungs at 6 weeks of age. Labeled cRNA was hybridized to Agilent Whole Mouse Genome 4 × 44K arrays (Agilent Technologies, Santa Clara, CA). Array data were analyzed using DAVID (the Database for Annotation, Visualization, and Integrated Discovery) v6.7 (Laboratory of Immunopathogenesis and Bioinformatics, Frederick, MD).

Human Lung Samples

Lung tissue samples from human fetuses (n = 6, gestational ages 23–24 wk) and postnatal infants who were born at full term (n = 7, ages 0–3 mo) were obtained *post mortem* at the Children's Hospital of Philadelphia. Gene expression levels in whole-lung homogenates were determined using quantitative real-time PCR. The protocol for pathological specimens was approved by the Institutional Review Board of Children's Hospital of Philadelphia.

Statistical Analyses

Differences between groups were compared using unpaired t tests or ANOVA with *post hoc* Bonferroni's correction for multiple comparisons. A P value of 0.05 was considered significant. Data are reported as means (± SD) or means (± SE), as indicated.

Results

Confirmation of CLDN18 KO in the Lung

Protein expression of CLDN18 was confirmed in the lungs of WT mice by immunoblot; in contrast, in the lungs of CLDN18 KO mice, expression of CLDN18 was absent (Figure E1A in the online supplement). At birth, there was no difference in the expression level of CLDN4 and -3, the other dominant claudins in the alveolar epithelium (11), as measured by immunoblot of whole-lung homogenates (Figure E1B). By Postnatal Day 7, CLDN18 KO mice had increased expression of CLDN4 and -3 relative to WT mice (Figure E1C). CLDN18 KO mice appear grossly normal at Postnatal Day 1 and exhibit no signs of respiratory distress.

Alveolar Epithelial Barrier Structure and Function in CLDN18 KO Lungs

CLDN18 KO mice demonstrated a fixed alveolar permeability defect at 4 wk, 12 wk,

and 16 weeks of age as measured by the accumulation of FITC–albumin in lavage fluid 4 h after intraperitoneal instillation of labeled tracer (Figure 1A). Intratracheal instillation of ^{125}I-albumin followed by sampling of plasma from the inferior vena cava produced a similar result, further supporting a bidirectional alveolar epithelial permeability defect (Figure 1B). Ultrastructural analysis of distal airspaces in CLDN18 KO and WT mice using transmission electron microscopy revealed membrane ruffling and splaying at AT1–AT1 cell junctions in KO mice (Figure 1C). The observed ultrastructural differences may represent altered tight junction structure or function in the absence of CLDN18.

Barrier Function in Primary Alveolar Epithelial Cells with CLDN18 Knockdown

To investigate whether CLDN18 plays a specific role in regulating alveolar epithelial barrier function, CLDN18 expression was experimentally decreased in primary alveolar epithelial cells. Primary rat alveolar epithelial cells were transfected with CLDN18-targeting or control nonsilencing siRNA using cationic lipid. Knockdown of CLDN18 was confirmed by immunoblot and densitometric analysis (Figure 2A). Transepithelial electrical resistance was significantly decreased (Figure 2B) and paracellular permeability to a 0.5-kD fluorescent tracer was significantly increased with CLDN18 knockdown (Figure 2C). These data support a specific role for CLDN18 in regulating paracellular permeability consistent with the alveolar epithelial barrier dysfunction identified in CLDN18 KO mice *in vivo*.

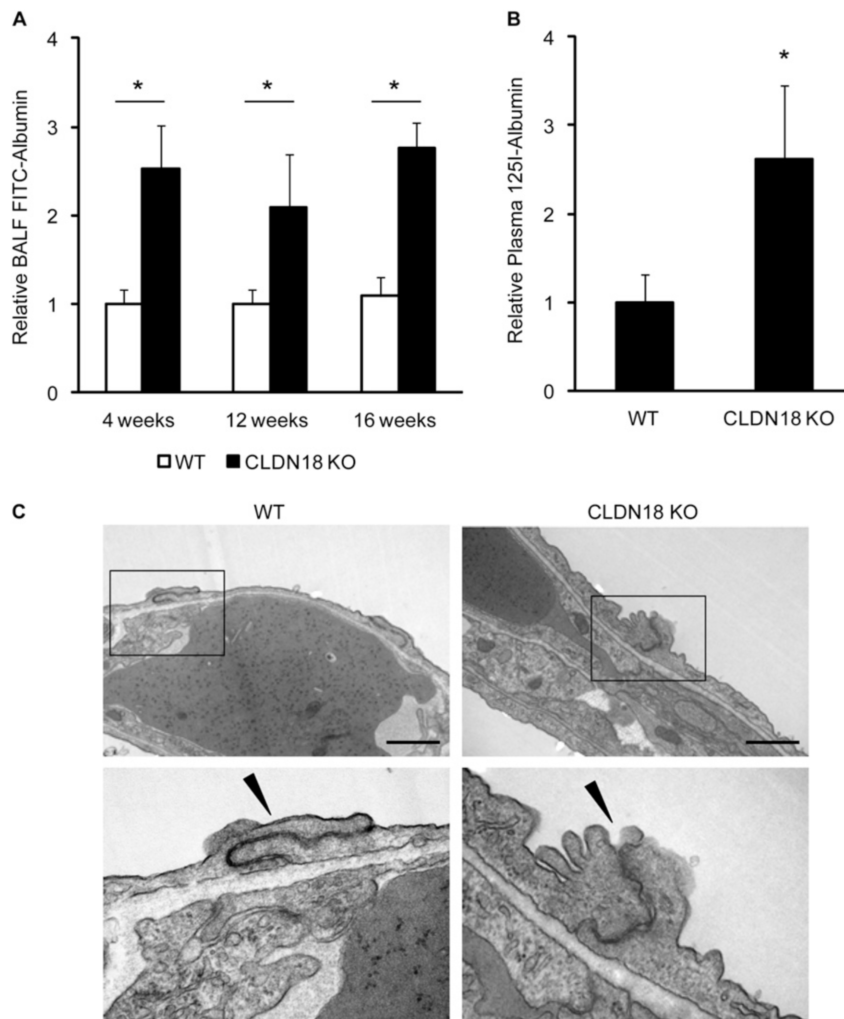

Figure 1. Alveolar epithelial barrier dysfunction in claudin (CLDN)-18 knockout (KO) mice. (*A* and *B*) Bidirectional alveolar permeability in wild-type (WT) and CLDN18 KO mice as measured by (*A*) the accumulation of FITC–albumin in lavage fluid 4 hours after intraperitoneal instillation of tracer at 4, 12, and 16 weeks of age or (*B*) the accumulation of ^{125}I-labeled albumin in plasma 4 hours after intratracheal instillation of tracer at 8 weeks of age. Relative permeability is expressed as the ratio of tracer concentration or plasma radioactivity in CLDN18 KO mice versus WT mice. CLDN18 KO mice have a fixed permeability defect (n = 4–5 per group, $^*P < 0.05$). (*C*) Representative transmission electron microscopy images of junctions between alveolar epithelial type (AT) 1 cells in WT and CLDN18 KO lungs at 8 weeks of age revealed membrane ruffling and splaying at AT1–AT1 cell junctions in CLDN18 KO mice, suggesting altered junction morphology in the absence of CLDN18. *Arrowheads* indicate AT1–AT1 junctions. *Boxes* indicate magnified image in *lower panels*. *Scale bar*, 1 μm.

Impaired Postnatal Alveolarization in CLDN18 KO Mice

Previous studies identified that CLDN18 is specifically induced in human fetal lung cells by maturational agonists, and that this induction is associated with augmented barrier function (13). Mice, unlike humans, are born in the late saccular phase of lung development, with alveolarization occurring after term birth (20). We hypothesized that CLDN18 is required for normal alveolarization in the postnatal mouse lung. Therefore, we studied WT and CLDN18 KO mice immediately before (Postnatal Day 3) and after (Postnatal Week 4) alveolarization. Hematoxylin and eosin staining of inflation-fixed distal lung sections demonstrated similar histology between genotypes at Postnatal Day 3, but markedly impaired alveolarization in CLDN18 KO mice by 4 weeks of age (Figure 3A). Quantification of MLI, which is inversely proportional to lung surface area (18, 19), confirmed similar lung structure between genotypes at Postnatal Day 3, but markedly decreased surface area in CLDN18 KO mice at 4 weeks as compared with WT controls (Figure 3B).

Evidence of Impaired Alveolarization by DNA Microarray Analyses in CLDN18 KO Lungs

To comprehensively investigate mRNA expression changes in the context of evidence of impaired alveolarization, we performed cDNA microarray analyses using whole mouse lungs at age 6 weeks (Figure 4A). Functional analysis clustering of the genes differentially expressed between WT lungs and CLDN18 KO lungs was performed using the DAVID knowledge database. As expected, an

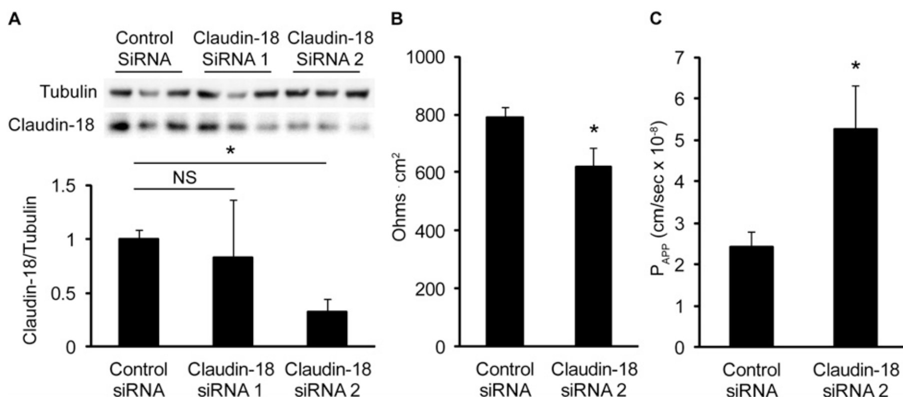

Figure 2. Alveolar epithelial barrier dysfunction with CLDN18 knockdown *in vitro*. (*A*) The expression of CLDN18 was significantly (> 50%) down-regulated in primary rat alveolar epithelial cells with knockdown using sequence CLDN18 small interfering RNA (siRNA) 2, not sequence CLDN18 siRNA1 compared with cells treated with control nonsilencing siRNA. The immunoblot was also labeled for β-tubulin to normalize for total sample protein content with densitometric analysis. CLDN18 siRNA2 was used for all subsequent experiments in primary rat alveolar epithelial cells. $^{*}P < 0.05$. (*B* and *C*) Transepithelial electrical resistance (TER) was significantly decreased (*B*), and apparent permeability (P_{APP}) to a 0.5-kD labeled tracer was significantly increased (*C*) in CLDN18 knockdown primary alveolar epithelial cells (n = 6 biologic replicates). $^{*}P < 0.05$. NS, not significant.

annotation cluster consisting of gene ontology term "lung development" was enriched in the set of differentially expressed genes (Figure 4B). This annotation cluster for lung development consisted of 22 total genes (Table E1). Differential expression was confirmed for several genes from this annotation cluster with quantitative real-time PCR (Figure E2). Moreover, differential expression of several other genes involved in lung development, such as elastin (21) and vascular endothelial growth factor-α (22), were identified by microarray analysis and confirmed by quantitative real-time PCR (Figure E2). Furthermore, as histology did not differ between WT and CLDN18 KO mice in the early postnatal period, we hypothesized that the lung development genes identified by microarray would not be differentially expressed at birth, before alveolarization. Therefore, we investigated several of these lung development genes at Postnatal Day 3 and Week 4. This time course evaluation revealed similar mRNA expression levels at Postnatal Day 3, but differential expression at 4 weeks (Figure 4C), supporting the hypothesis that CLDN18 deficiency leads to impaired postnatal lung development.

Early Alveolar Epithelial Injury in CLDN18 KO mice

Even minimal injury in the developing lung can impair the normal processes of alveolarization (5). Thus, we hypothesized that a consequence of CLDN18 deficiency could be alveolar epithelial cell injury (specifically AT1 cell injury) in the postnatal mouse lung after conversion to air breathing. We assessed bronchoalveolar lavage fluid levels of the AT1 cell–specific protein, podoplanin, a validated marker of AT1 cell injury (23) in neonatal mice (Postnatal Day 3) and at Postnatal Week 4. CLDN18 KO mice showed a small, but significant, increase in bronchoalveolar lavage fluid podoplanin at Postnatal Day 3, and a larger increase at 4 weeks, as compared with WT mice (Figure 5A). This increase in podoplanin at Postnatal Day 3 preceded histological (Figure 3A) and gene expression (Figure 4), evidence of impaired alveolarization.

To determine the relative abundance of AT2 cells, we used immunostaining for pro–surfactant protein C (pro-SPC) and quantification of pro-SPC–stained cells per field. These studies demonstrated significantly more AT2 cells in CLDN18 KO lungs by 4 weeks of age (Figure 5B), which was further supported by increased pro-SPC by Western blot in lung homogenate from CLDN18 KO mice at 4 weeks of age. However, there was no difference in pro-SPC expression by immunoblot of whole-lung lysates on Postnatal Day 1 in CLDN18 KO lungs (Figure 5C). Collectively, these data support an injured and reactive epithelium in the setting of CLDN18 deficiency, and that these changes manifest after birth.

CLDN18 Levels in Fetal Human Lungs

Extremely preterm human infants born 24–28 weeks gestational age are at the highest risk of developing BPD (5). In mice, CLDN18 expression increases before term birth and then remains stable from Postnatal Day 0 through 4 weeks of age (Figure E3). Given impaired alveolarization and lung injury in CLDN18 null mice, we investigated whether CLDN18 expression is low in preterm human lungs born at the highest risk period for developing BPD, a disease of impaired alveolarization. We analyzed transcript levels of CLDN18 and CLDN4 in human fetal lungs (n = 6, gestational age 23–24 wk) and normal lungs from the early postnatal period after birth at term (n = 7, age 0–3 mo). CLDN18 expression levels are significantly decreased in fetal lungs relative to the postnatal period, indicating developmental regulation during the third trimester. Conversely, there is no difference in CLDN4 expression between fetal and postnatal human lungs (Figure 6). These data are consistent with the hypothesis that there is a specific, relative CLDN18 deficiency in extremely preterm infants born in the risk period for developing BPD.

Discussion

Here, we provide novel evidence that CLDN18 deficiency results in alveolar epithelial barrier dysfunction and impaired alveolarization in mice. This study represents the first report of a claudin KO mouse with a clinically relevant pulmonary phenotype (impaired alveolarization), and implicates a role for CLDN18 in the critical process of barrier function regulation. Moreover, analysis of human samples indicates that extremely preterm human lungs have decreased CLDN18 expression relative to postnatal lungs. Taken together, these data support further investigation into a role for CLDN18 in BPD.

The lung maintains a highly specialized barrier between the atmosphere and fluid-filled tissues to enable gas exchange. Failure of this barrier can have catastrophic clinical consequences, as is best exemplified by the alveolar flooding characteristic of acute lung injury (24). The extensive surface area of the alveolar epithelium mandates

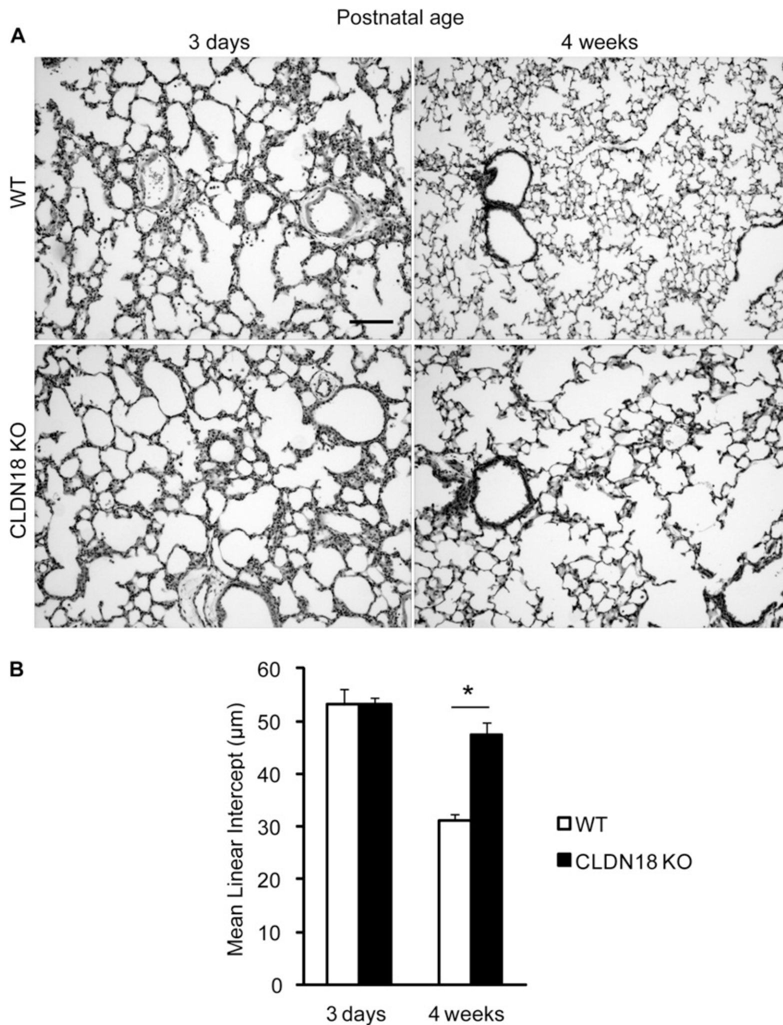

Figure 3. CLDN18 KO mice have impaired alveolarization. (*A*) Representative lung sections from WT and CLDN18 KO mice at Postnatal Day 3 (*left panels*) and Week 4 (*right panels*) stained with hematoxylin and eosin. Although mice have similar lung structure at Postnatal Day 3 (before alveolarization), CLDN18 KO mice have evidence of impaired alveolarization at 4 weeks. *Scale bar*, 100 μm. (*B*) Mean linear intercept (MLI; μm) was measured in WT and CLDN18 KO mouse lungs at Postnatal Day 3 and Week 4. MLI is inversely proportional to lung surface area, and quantifies impaired alveolarization in CLDN18 KO mice at 4 weeks of age (n = 3–4 per group). $^*P < 0.001$.

fine regulation of paracellular permeability, the movement of fluids and solutes between cells. Tight junctions regulate paracellular permeability, and claudins are the primary structural constituents of tight junctions serving this function (25, 26). As claudins demonstrate tissue-specific expression patterns, the claudin profile of a given epithelium is a critical determinant of the integrity of its barrier (8). Moreover, human disease has been linked to mutations in individual claudins, including CLDN1 (ichthyosis and sclerosing cholangitis) (27), CLDN14 (nonsyndromic deafness) (28), and CLDN16 and -19 (familial hypomagnesemia with hypercalciuria and nephrocalcinosis) (29). Data are now emerging for roles of individual claudins in the pathogenesis of lung disease. For instance, *in vitro* and *in vivo* studies and studies in *ex vivo* perfused human donor lungs support a functional role for CLDN4 in regulating alveolar fluid clearance (16, 30).

Previously published data support a unique role for CLDN18 in the alveolar epithelium. The lung-specific splice variant of CLDN18 is the only known tight junction protein solely expressed in the lung (10). Moreover, pulmonary expression of CLDN18 appears to be restricted to the epithelium, without evidence of endothelial expression (31). CLDN18 is the most abundantly expressed claudin in freshly isolated AT1 cells (11), the cell type that covers over 95% of the surface area of the lung (32). Exposure of primary human alveolar epithelial cells to clinically relevant proinflammatory innate immune mediators results in a specific loss of CLDN18 from the apical junction complex associated with impaired barrier function. Treatment with mesenchymal stem cells prevents this loss of CLDN18 and preserves barrier function (12). In light of these previous findings, the aim of this study was to directly test the function of CLDN18 in the alveolar epithelium. Our data indicate a primary role for CLDN18 in regulating alveolar epithelial barrier function. Furthermore, while this article was in revision, Li and colleagues (33) published complementary data indicating a macromolecular permeability defect in a different CLDN18 KO mouse *in vivo* and *in vitro* in alveolar epithelial cell monolayers from KO mice. This is independent support for a unique role for CLDN18 in regulating alveolar epithelial barrier function. In that study, KO mouse alveolar epithelial cell monolayers had similarly increased permeability to both small (376 Da) and large (155,000 Da) molecular weight tracers. Our study further adds that partial loss of CLDN18 through specific siRNA knockdown is sufficient to induce barrier dysfunction in primary alveolar epithelial cells *in vitro* (Figure 2). This raises the possibility that partial, not just total, loss of CLDN18 *in vivo* could result in significant alterations in barrier function. Moreover, that barrier function persists in CLDN18 KO mice despite postnatal acquisition of changes in other claudin expression levels (Figure E1) supports a unique, nonredundant role for CLDN18 in the alveolar epithelium.

Recent advances in perinatal care have enabled the delivery of prematurely born infants as early as 23–24 weeks gestation. However, despite improved survival, significant morbidity persists (34). BPD, a disease characterized by developmental arrest secondary to injury in the immature lung, remains the most common severe complication of preterm birth (4). Although recent studies have offered insights into the pathogenesis of this disease, there remain no highly effective preventive treatments. As such, investigation of novel pathways is required to open new potential therapeutic avenues.

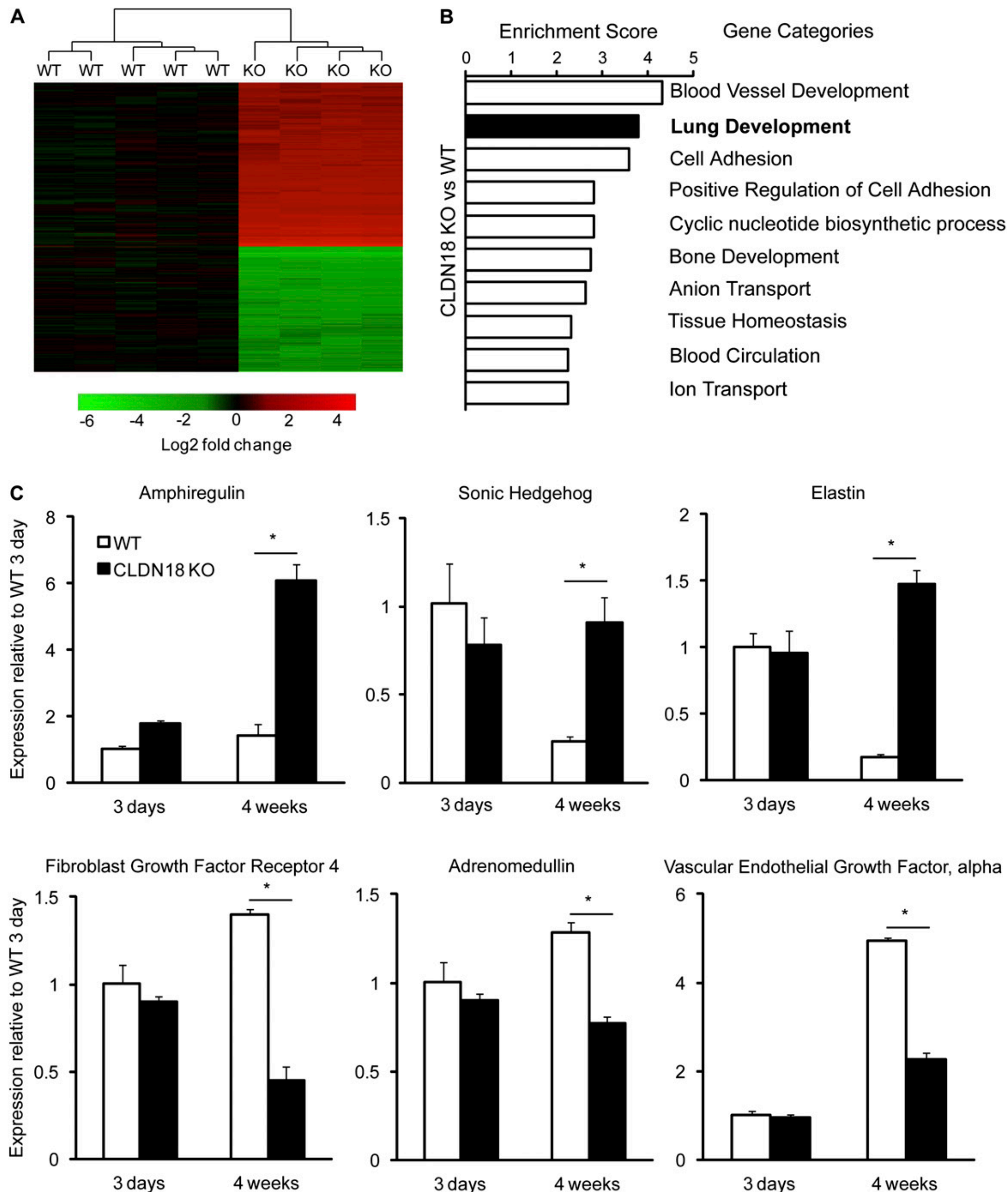

Figure 4. Microarray analysis indicates impaired postnatal alveolarization in CLDN18 KO mice. (*A*) Heat map demonstration of 893 differentially expressed probes identified by microarray using a fold change cutoff of 2 and adjusted *P* less than 0.05 (n = 4–5 per group). (*B*) The list of genes differentially expressed between WT and CLDN18 KO lungs at 6 weeks of age was analyzed using DAVID (the Database for Annotation, Visualization, and Integrated Discovery) v6.7 and enriched functional gene categories were identified. Results shown are the top 10 gene categories differentially expressed between WT and CLDN18 KO lungs by functional analysis clustering of gene ontology terms. Enrichment score, the geometric mean (in –log scale) of member's *P* values in a corresponding annotation cluster, is used to rank biological significance with the top-ranked annotation groups having consistently lower *P* values for their annotation members. The functional annotation cluster for lung development had an enrichment score of 3.79. (*C*) CLDN18 KO mice acquire differential expression of lung development genes in the postnatal period. mRNA expression levels of select lung development genes identified by microarray analysis and functional analysis clustering were measured in WT and CLDN18 KO lungs at Postnatal Day 3 and Week 4. Amphiregulin, sonic hedgehog, elastin, fibroblast growth factor receptor 4, adrenomedullin, and vascular endothelial growth factor-α were differentially expressed at 4 weeks of age only, not at Postnatal Day 3 before the onset of alveolarization (n = at least 3 per group). *$P < 0.05$.

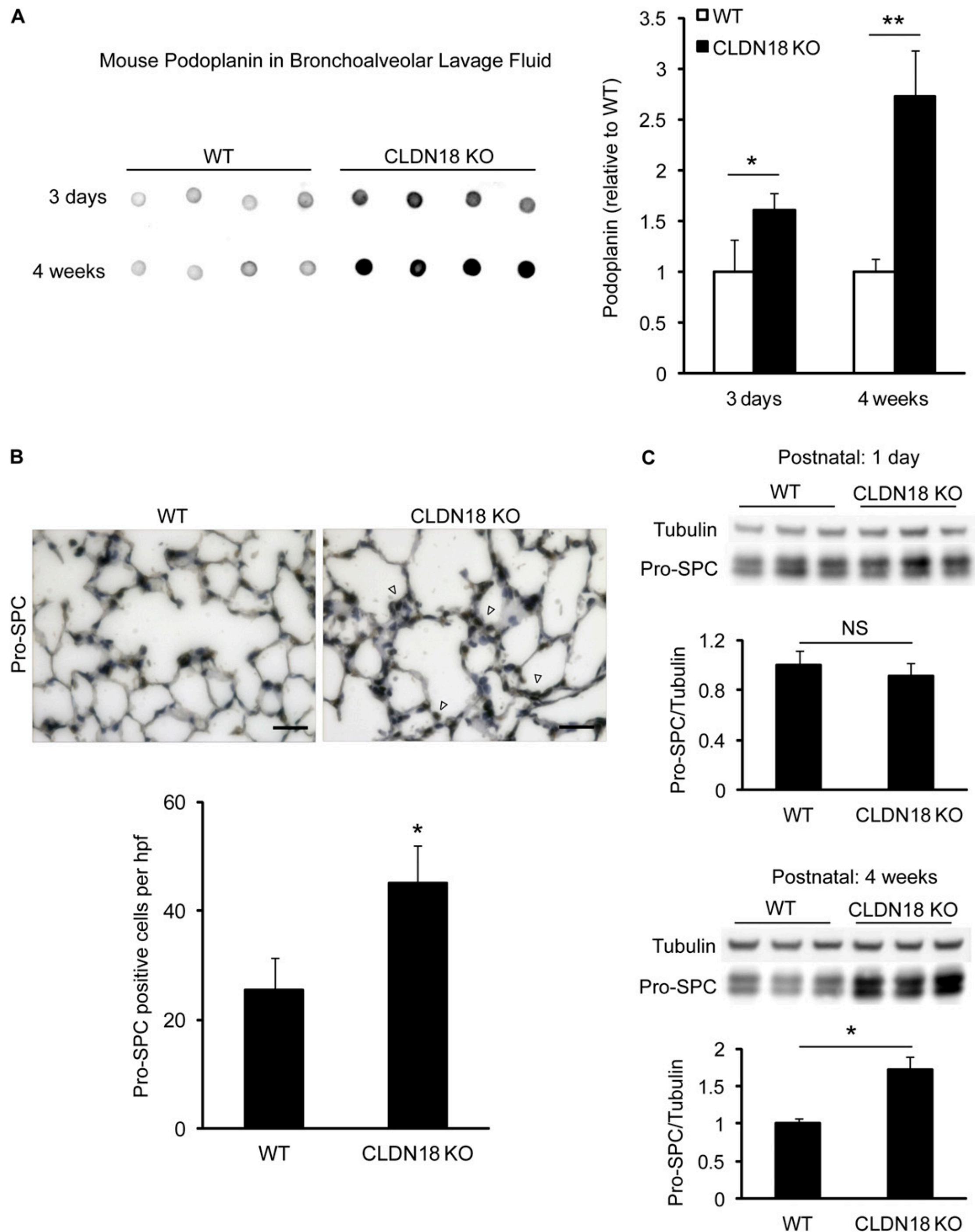

Figure 5. Alveolar epithelial cell injury in CLDN18 KO mice. (*A*) Accumulation of the AT1 cell protein, podoplanin, a validated marker of AT1 cell injury, was measured by dot blot analysis of bronchoalveolar lavage fluid with equal protein load from WT and CLDN18 KO mice at Postnatal Day 3 and Week 4 ($n = 4$ per group). $^*P < 0.05$ (3 d) and $^{**}P < 0.001$ (4 wk). (*B*) Representative lung sections from WT and CLDN18 KO mice at 4 weeks of age stained for pro–surfactant protein C (pro-SPC) to mark AT2 cells. AT2 cell number was increased in CLDN18 KO mice, as measured by counting of pro-SPC–positive cells in standard inflation-fixed lungs. In addition, clusters of contiguous pro-SPC–positive cells lining alveoli (*open arrowheads*) were frequently encountered in CLDN18 KO mice ($n = 4$ per group). $^*P < 0.001$. *Scale bar*, 30 μm. (*C*) The expression of pro-SPC is similar between WT and CLDN18 KO mice at Postnatal Day 1, but increases in CLDN18 KO mice at 4 weeks of age, consistent with acquired increases in AT2 cell number in the postnatal period ($n = 3$ per group). $^*P < 0.05$. NS, not significant.

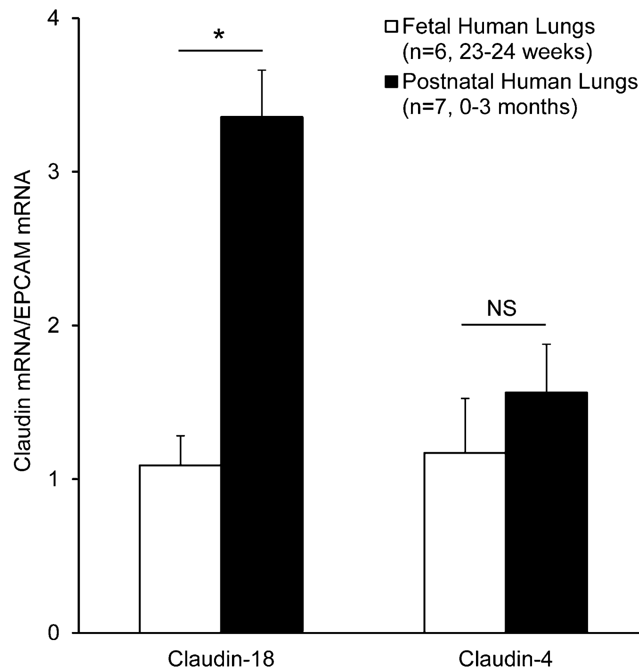

Figure 6. CLDN18 expression is reduced in fetal human lungs. The transcript levels of CLDN18 and CLDN4 were measured by quantitative real-time PCR in *post mortem* whole-lung samples from fetuses during the risk period for bronchopulmonary dysplasia (n = 6, gestational age 23–24 wk) and in lung samples from the early postnatal period after term birth (n = 7, age 0–3 mo). Relative to postnatal samples, CLDN18 has significantly lower expression level in human fetal lungs. CLDN4, another highly expressed claudin in the alveolar epithelium, has stable expression at both developmental ages. Expression levels are normalized to the epithelial cell–specific marker, epithelial cell adhesion molecule (EPCAM), to account for epithelial cell content. The same statistically significant result was obtained when claudin gene expression levels were normalized to 18S RNA (data not shown). *$P < 0.001$. NS, not significant. Data presented are means ± SEM.

Existing models of BPD use exogenous epithelial injury to trigger alveolar hypoplasia (35–37). Genetically modified mice that lack specific growth and differentiation factors also exhibit impaired septation and angiogenesis, among other defects (38, 39). These models have proven invaluable in deciphering mechanisms of development and repair, but our understanding of the causes of BPD remains incomplete. Although epithelial injury is a cardinal feature of respiratory failure in the preterm infant, whether alveolar barrier dysfunction alone contributes to the pathogenesis of this disorder is uncertain. Emerging evidence supports a primary role for alveolar epithelial barrier dysfunction in the pathogenesis of multiple pulmonary diseases, including acute lung injury and idiopathic pulmonary fibrosis (24, 40, 41). The present study supports a model of alveolar epithelial dysfunction as sufficient to injure and impair postnatal alveolarization in mice. That the injury is acquired in the postnatal period is supported by both histology (Figure 3) and postnatal differential expression of key lung development gene identified by microarray (Figure 4). Moreover, as several of these lung development genes show differential expression patterns consistent with established experimental models of impaired alveologenesis, such as elastin up-regulation in fibroblast growth factor receptor 3/ fibroblast growth factor receptor 4–deficient mice (42), there is further support of CLDN18 deficiency as a novel model of BPD. Future studies will examine potential mechanistic links between barrier dysfunction and alveolar epithelial cell injury in the context of impaired alveologenesis. Alternatively, increased AT2 cell numbers in KO mouse lungs may represent impaired differentiation into AT1 cells in the setting of CLDN18 deficiency. The extent to which CLDN18 affects alveolar epithelial cell phenotype requires further investigation.

Inherent differences in mouse and human lung development represent a limitation to generalizing data from any mouse model to human disease. Term human infants are born in the alveolar phase of lung development, whereas mice are born in the saccular phase with alveolarization occurring after term birth (20). Despite this, the mouse lung adapts to air breathing, and lung development continues normally if CLDN18 is present. CLDN18 expression in WT mouse lungs increases during late gestation, and is maximal at the time of term birth. Contrary to WT mice, CLDN18 deficiency at term birth in KO mice disrupts the normal lung development program in the early postnatal period.

Evaluation of expression levels of CLDN18 during human lung development is limited by the availability of tissue samples. Nonetheless, in this study, we present data that CLDN18 levels are significantly lower in fetal lungs at 23–24 weeks gestational age (the high-risk period for developing BPD with extreme preterm birth) relative to expression in postnatal lungs after term birth. This dynamic change appears to be specific to CLDN18, as another highly expressed claudin in the alveolar epithelium, CLDN4, is expressed at similar levels in preterm and postnatal human lung samples. Moreover, this lower expression of CLDN18 in fetal lungs is unlikely due to a change in epithelial cell content, as the result persists when normalized to epithelial cell adhesion molecule, an epithelial cell–specific marker. Of note, previous studies of human fetal lung alveolar epithelial cells demonstrated that CLDN18 is among the genes induced by cAMP and corticosteroids (13), a treatment that causes precocious maturation of lung structure and function.

In conclusion, this novel CLDN18 KO mouse exhibits a clinically relevant pulmonary phenotype characterized by alveolar epithelial cell injury and impaired alveolarization. Combined with *in vivo* and *in vitro* data demonstrating a role for CLDN18 in the alveolar epithelial permeability barrier, this model supports a link between primary epithelial barrier defects and the development of chronic lung disease. Evidence of low expression of CLDN18 in human fetal lungs supports further investigation into a role for this tight junction protein in BPD. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

References

1. Kinsella JP, Greenough A, Abman SH. Bronchopulmonary dysplasia. *Lancet* 2006;367:1421–1431.
2. Northway WH Jr, Rosan RC, Porter DY. Pulmonary disease following respirator therapy of hyaline-membrane disease: bronchopulmonary dysplasia. *N Engl J Med* 1967;276:357–368.
3. Walsh MC, Szefler S, Davis J, Allen M, Van Marter L, Abman S, Blackmon L, Jobe A. Summary proceedings from the bronchopulmonary dysplasia group. *Pediatrics* 2006;117:S52–S56.
4. Coalson JJ. Pathology of new bronchopulmonary dysplasia. *Semin Neonatol* 2003;8:73–81.
5. Baraldi E, Filippone M. Chronic lung disease after premature birth. *N Engl J Med* 2007;357:1946–1955.
6. Furuse M, Sasaki H, Fujimoto K, Tsukita S. A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. *J Cell Biol* 1998;143:391–401.
7. Morita K, Furuse M, Fujimoto K, Tsukita S. Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. *Proc Natl Acad Sci USA* 1999;96:511–516.
8. Angelow S, Ahlstrom R, Yu AS. Biology of claudins. *Am J Physiol Renal Physiol* 2008;295:F867–F876.
9. Frank JA. Claudins and alveolar epithelial barrier function in the lung. *Ann N Y Acad Sci* 2012;1257:175–183.
10. Niimi T, Nagashima K, Ward JM, Minoo P, Zimonjic DB, Popescu NC, Kimura S. Claudin-18, a novel downstream target gene for the T/EBP/NKX2.1 homeodomain transcription factor, encodes lung- and stomach-specific isoforms through alternative splicing. *Mol Cell Biol* 2001;21:7380–7390.
11. LaFemina MJ, Rokkam D, Chandrasena A, Pan J, Bajaj A, Johnson M, Frank JA. Keratinocyte growth factor enhances barrier function without altering claudin expression in primary alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2010;299:L724–L734.
12. Fang X, Neyrinck AP, Matthay MA, Lee JW. Allogeneic human mesenchymal stem cells restore epithelial protein permeability in cultured human alveolar type II cells by secretion of angiopoietin-1. *J Biol Chem* 2010;285:26211–26222.
13. Daugherty BL, Mateescu M, Patel AS, Wade K, Kimura S, Gonzales LW, Guttentag S, Ballard PL, Koval M. Developmental regulation of claudin localization by fetal alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L1266–L1273.
14. Frank J, Roux J, Kawakatsu H, Su G, Dagenais A, Berthiaume Y, Howard M, Canessa CM, Fang X, Sheppard D, *et al*. Transforming growth factor-beta1 decreases expression of the epithelial sodium channel alphaENaC and alveolar epithelial vectorial sodium and fluid transport via an ERK1/2-dependent mechanism. *J Biol Chem* 2003;278:43939–43950.
15. Dobbs LG. Isolation and culture of alveolar type II cells. *Am J Physiol* 1990;258:L134–L147.
16. Wray C, Mao Y, Pan J, Chandrasena A, Piasta F, Frank JA. Claudin-4 augments alveolar epithelial barrier function and is induced in acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2009;297: L219–L227.
17. Balasubramaniam V, Tang JR, Maxey A, Plopper CG, Abman SH. Mild hypoxia impairs alveolarization in the endothelial nitric oxide synthase–deficient mouse. *Am J Physiol Lung Cell Mol Physiol* 2003; 284:L964–L971.
18. Thurlbeck WM. The internal surface area of nonemphysematous lungs. *Am Rev Respir Dis* 1967;95:765–773.
19. Thurlbeck WM. Measurement of pulmonary emphysema. *Am Rev Respir Dis* 1967;95:752–764.
20. Mund SI, Stampanoni M, Schittny JC. Developmental alveolarization of the mouse lung. *Dev Dyn* 2008;237:2108–2116.
21. Bland RD, Xu L, Ertsey R, Rabinovitch M, Albertine KH, Wynn KA, Kumar VH, Ryan RM, Swartz DD, Csiszar K, *et al*. Dysregulation of pulmonary elastin synthesis and assembly in preterm lambs with chronic lung disease. *Am J Physiol Lung Cell Mol Physiol* 2007;292: L1370–L1384.
22. Thébaud B, Abman SH. Bronchopulmonary dysplasia: where have all the vessels gone? Roles of angiogenic growth factors in chronic lung disease. *Am J Respir Crit Care Med* 2007;175:978–985.
23. McElroy MC, Pittet JF, Hashimoto S, Allen L, Wiener-Kronish JP, Dobbs LG. A type I cell–specific protein is a biochemical marker of epithelial injury in a rat model of pneumonia. *Am J Physiol* 1995;268:L181–L186.
24. Ware LB, Matthay MA. The acute respiratory distress syndrome. *N Engl J Med* 2000;342:1334–1349.
25. Schneeberger EE, Lynch RD. The tight junction: a multifunctional complex. *Am J Physiol Cell Physiol* 2004;286:C1213–C1228.
26. Van Itallie CM, Anderson JM. Claudins and epithelial paracellular transport. *Annu Rev Physiol* 2006;68:403–429.
27. Hadj-Rabia S, Baala L, Vabres P, Hamel-Teillac D, Jacquemin E, Fabre M, Lyonnet S, De Prost Y, Munnich A, Hadchouel M, *et al*. Claudin-1 gene mutations in neonatal sclerosing cholangitis associated with ichthyosis: a tight junction disease. *Gastroenterology* 2004;127: 1386–1390.
28. Wilcox ER, Burton QL, Naz S, Riazuddin S, Smith TN, Ploplis B, Belyantseva I, Ben-Yosef T, Liburd NA, Morell RJ, *et al*. Mutations in the gene encoding tight junction claudin-14 cause autosomal recessive deafness DFNB29. *Cell* 2001;104:165–172.
29. Hou J, Renigunta A, Gomes AS, Hou M, Paul DL, Waldegger S, Goodenough DA. Claudin-16 and claudin-19 interaction is required for their assembly into tight junctions and for renal reabsorption of magnesium. *Proc Natl Acad Sci USA* 2009;106:15350–15355.
30. Rokkam D, Lafemina MJ, Lee JW, Matthay MA, Frank JA. Claudin-4 levels are associated with intact alveolar fluid clearance in human lungs. *Am J Pathol* 2011;179:1081–1087.
31. Ohta H, Chiba S, Ebina M, Furuse M, Nukiwa T. Altered expression of tight junction molecules in alveolar septa in lung injury and fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2012;302:L193–L205.
32. Dobbs LG, Johnson MD, Vanderbilt J, Allen L, Gonzalez R. The great big alveolar TI cell: evolving concepts and paradigms. *Cell Physiol Biochem* 2010;25:55–62.
33. Li G, Flodby P, Luo J, Kage H, Sipos A, Gao D, Ji Y, Beard LL, Marconett CN, Demaio L, *et al*. Knockout mice reveal key roles for claudin 18 in alveolar barrier properties and fluid homeostasis. *Am J Respir Cell Mol Biol* (In press)
34. Jobe AH. The new bronchopulmonary dysplasia. *Curr Opin Pediatr* 2011;23:167–172.
35. Buczynski BW, Maduekwe ET, O'Reilly MA. The role of hyperoxia in the pathogenesis of experimental BPD. *Semin Perinatol* 2013;37:69–78.
36. Velten M, Heyob KM, Rogers LK, Welty SE. Deficits in lung alveolarization and function after systemic maternal inflammation and neonatal hyperoxia exposure. *J Appl Physiol (1985)* 2010;108: 1347–1356.
37. Bland RD, Mokres LM, Ertsey R, Jacobson BE, Jiang S, Rabinovitch M, Xu L, Shinwell ES, Zhang F, Beasley MA. Mechanical ventilation with 40% oxygen reduces pulmonary expression of genes that regulate lung development and impairs alveolar septation in newborn mice. *Am J Physiol Lung Cell Mol Physiol* 2007;293:L1099–L1110.
38. Weinstein M, Xu X, Ohyama K, Deng CX. FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung. *Development* 1998;125:3615–3623.
39. Wan H, Kaestner KH, Ang SL, Ikegami M, Finkelman FD, Stahlman MT, Fulkerson PC, Rothenberg ME, Whitsett JA. Foxa2 regulates alveolarization and goblet cell hyperplasia. *Development* 2004;131: 953–964.
40. Young LR, Gulleman PM, Bridges JP, Weaver TE, Deutsch GH, Blackwell TS, McCormack FX. The alveolar epithelium determines susceptibility to lung fibrosis in Hermansky-Pudlak syndrome. *Am J Respir Crit Care Med* 2012;186:1014–1024.
41. King TE Jr, Pardo A, Selman M. Idiopathic pulmonary fibrosis. *Lancet* 2011;378:1949–1961.
42. Srisuma S, Bhattacharya S, Simon DM, Solleti SK, Tyagi S, Starcher B, Mariani TJ. Fibroblast growth factor receptors control epithelial–mesenchymal interactions necessary for alveolar elastogenesis. *Am J Respir Crit Care Med* 2010;181:838–850.