Identification of P311 as a Potential Gene Regulating Alveolar Generation

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Smoking-related destructive lung diseases such as chronic obstructive pulmonary disease (COPD) and emphysema are a major cause of morbidity and mortality worldwide. The immediate cause of emphysema is the obliteration of alveoli that are key functional units of the lungs where gas exchange takes place. Alveolar generation/regeneration under normal and pathologic conditions is a poorly understood process, but may hold the key to treatment of human emphysema. We used suppression subtractive hybridization to identify genes that may control alveolar generation during periods of pre- and postnatal active alveolar development. P311, a putative neuronal protein originally identified for its high expression in late-stage embryonic brain, was highly differentially expressed during periods of active distal lung morphogenesis. Quantitative real-time RT-PCR showed that the expression of P311 is developmentally regulated, with peak levels occurring during saccular and alveolar formation. Intriguingly, P311 gene expression was significantly decreased in lungs of individuals with emphysema compared with control subjects. Consistent with a role for this gene in alveolar formation, inhibition of alveolization by dexamethasone treatment *in vivo* resulted in decreased expression of P311. Together our data suggest that P311 expression is tightly regulated during the critical periods of alveolar formation, and that under pathologic conditions, its relative absence may contribute to failure of alveolar regeneration and lead to the development of human emphysema.

Keywords: alveolization; COPD; emphysema; lung development; P311

Chronic obstructive pulmonary disease (COPD) is characterized by irreversible reduction of maximum expiratory airflow and is currently the fourth leading cause of death in the United States. Worldwide, COPD is the 12th most prevalent disease and is estimated to rise to the 5th in the next two decades (1–5). Exposure to first- or second-hand tobacco smoke accounts for the majority of clinically significant COPD and leads to $\sim$ 440,000 deaths annually in the United States, and both morbidity and mortality due to COPD is on the rise, despite increased use of health care resources (6, 7).

There is a great need for a better understanding of the pathophysiology of smoking-related obstructive and destructive lung diseases under stable and progressive phases of illness. Of particular interest are better insights into the nature and causes of lung processes that are responsible for loss of alveoli and subsequent worsening of respiratory failure in current and former smokers. Alveoli are the key functional units of the lungs where gas exchange takes place, yet the regulation of their formation is currently not well understood. Alveolar generation occurs during the development of the distal lung that takes place during the canalicular, saccular, and alveolar stages of lung development (8). During the canalicular stage, the terminal epithelial buds elongate and branch to form small clusters of tubules and buds. These develop further during the saccular stage through the process of primary septation to form saccules, which are dilated smooth-walled structures containing two layers of capillaries lined on either side by epithelium. During the last month of gestation in humans and after birth (around Postnatal Day [P]5) in mice, the saccules are further developed into alveoli by a process of secondary septation and remodeling of the saccular wall. Secondary septa form from the saccular walls and divide the saccules into pre-alveolar shallow sacs. The walls are subsequently remodeled into thin layer containing a single capillary network. Several years after birth in humans or several weeks after birth in mice, alveolar formation by secondary septation ceases. However, alveoli may continue to form in small numbers by a different mechanism (9). The formation of alveoli accounts for the tremendous increase in the respiratory gas exchange surface of the lungs.

Studies of mice with targeted gene inactivation have identified several genes that impact either saccule formation or secondary septation. Factors that have been found to be important for saccule formation include corticotropin-releasing hormone (10, 11), transforming growth factor-β3 (12), latent TGF-binding protein (LTBP)-4 (13), epidermal growth factor (EGF) receptor (14), the transcription factor Hoxa-5 (15), and the extracellular matrix molecule fibulin-5 (16). Genes that have been found to be necessary for alveolar formation include platelet-derived growth factor-A (17), fibroblast growth factor receptor (FGFR) family members (18), and fibrillin-1 (19). The retinoic acid signaling pathways have also been implicated in alveolar formation (20–22).

Even though many genes have been implicated in alveolar formation, the mechanism and regulation of alveolar generation remain unclear. Since the formation of alveoli occurs within a defined period, we postulated that genes regulating alveolar generation must be differentially expressed during periods of active alveolar development. In this study we used suppression subtractive hybridization (SSH) to isolate genes differentially expressed during saccular and alveolar formation, and identified P311 as a differentially expressed gene that may play a role both in alveolar generation and in the pathogenesis of human emphysema.

MATERIALS AND METHODS

SSH and Differential Screenings

Polyadenylated RNA and total RNA from mouse lungs were isolated using Poly(A)Pure mRNA Purification Kit (Ambion, Austin, TX) and TRIzol Reagent (Invitrogen, Carlsbad, CA), respectively. SSH was performed using a PCR-Select cDNA Subtractive Kit (Clontech,

(*Received in original form December 21, 2005 and in final form February 7, 2006*)

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This work was supported by grants from the March of Dimes research foundation (5-FY01-422 to T.H.V.), from the National Institutes of Health (HL69925, HL73823, and HL75680 to T.H.V.), and from the Alpha-1 foundation (to F.K.). L.Z. was supported by a Fellowship from the American Heart Association Western Affiliates. J.K.L. was supported by an NIH Ruth L. Kirschstein NRSA Individual Fellowship.

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Am J Respir Cell Mol Biol Vol 35. pp 48–54, 2006
Originally Published in Press as DOI: 10.1165/rcmb.2005-0475OC on February 16, 2006
Internet address: www.atsjournals.org

Mountain View, CA), according to the manufacturer's instructions. Polyadenylated RNA from E18.5 (the day of the vaginal plug is considered E0.5), P5, and P30 were used to generate two subtracted libraries: E18.5–P30 and P5–P30, which should contain cDNAs enriched in E18.5 or P5 compared with P30, respectively. Approximately 600 clones were picked randomly from each library for further differential screening. The clones were dotted onto membrane and probed either with forward and reverse subtracted cDNA, or with tester (E18.5 or P5) and driver (P30) cDNA, labeled with [α-^{32}P]dCTP. Clones that showed more than 5-fold difference in signal intensity between the probes were again dotted onto membranes and cross-hybridized with each other to identify replications.

Northern Blot Analysis

Twenty micrograms of total RNA was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde and then transferred to nylon membrane (Hybond N$^+$; Amersham Biosciences, Pistacaway, NJ) in 20× SSC using the Rapid Downward Transfer System (Schleicher and Schuell Biosciences, Keene, NH) and cross-linked by ultraviolet light. Specific probes were generated by labeling the differentially expressed cDNA fragments with [α-^{32}P] dCTP using Ready-To-Go DNA Labeling Beads (-dCTP) (Amersham Biosciences). Hybridization was performed in QuikHyb solution (Stratagene, La Jolla, CA) overnight at 68°C with 10^6 cpm/ml of labeled probe. The blot was washed to a final stringency of 0.2× SSC and 0.1% SDS at 60°C, and exposed to X-ray film with an intensifying screen at −70°C.

Immunohistochemistry

Paraffin-embedded human autopsy lung sections (from the Department of Anatomic Pathology, UCSF) were deparaffinized to PBS, treated with 0.1% Trypsin for 10 min at 37°C, washed with 0.1 M PBS (11.5 g Na2HPO4, 2.62 g NaH2PO4, 9.0 g NaCl, pH7.4/1L) + 0.2% Tween 20, and quenched with 3% H_2O_2 in methanol for 20 min at ambient temperature. After washing with 0.1 M PBS + 0.2% Tween 20, the sections were treated with streptavidin/biotin blocking reagent (Vector Laboratory, Burlingame, CA), then incubated with blocking solution (0.1 M PBS + 0.2% Tween 20 with 5% normal goat serum) for 2 h at ambient temperature. The sections were then incubated with rabbit anti-P311 antisera (generated against the C-terminal peptide CGSSELRSPRISYLHFF of P311, a kind gift from Dr, Gregory A. Taylor, Department of Medicine and Immunology, Duke University) diluted 1:1,000 in blocking solution overnight at 4°C. After primary antibody incubation, the sections were washed with 0.1 M PBS + 0.2% Tween 20, incubated with HRP-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Westgrove, PA) diluted 1:500 in blocking solution for 1 h at room temperature, washed in 0.1 M PBS + 0.2% Tween 20, then incubated with Biotinylated Tyramide diluted 1:50 in the amplification buffer (Perkin Elmer Life and Analytical Sciences, Boston, MA). The sections were then washed in 0.1 M PBS + 0.2% Tween 20, and incubated with Vector Elite ABC reagent (Vector Laboratory) for 1 h at room temperature, washed in 0.1 M PBS + 0.2% Tween 20, and developed with DAB substrate.

Quantitative Real-Time RT-PCR

Two micrograms of total RNA was treated with Dnase I (Invitrogen) and reverse transcribed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). PCR was performed in the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers and probes were designed using the Primer Express program from the manufacturer. The sequences for the primers/probes used are as follows. P311: probe, 5′-AGACTGGCGCTGCCTCGCTGAC-3′; forward primer, 5′-GAAGTGAACCGAAAGAAGATGAG-3′; reverse primer, 5′-GAATTCACGGCTGCCTGG-3′; GAPDH: probe, 5′-CCGCCTGG AGAAACCTGCCAAGTATG-3′; forward primer, 5′-TGTGTCCG TCGTGGATCTGA-3′; reverse primer, 5′-CCTGCTTCACCACCTTC TTGAT-3′.

The 5′ and 3′ modification of all the probes are FAM and TAMRA, respectively. Thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 repetitive cycles of 95°C for 15 s and 60°C for 1 min. After PCR, quantification of gene expression was made by determining the number of cycles to threshold (C_T) of fluorescence detection within the geometric region of the semilog plot of fluorescence detection. Relative gene expression was determined using the comparative C_T method as described previously (23). Briefly, the ΔC_T value was obtained by subtracting the GAPDH C_T value from the C_T value of the gene tested in the same sample. The $\Delta\Delta C_T$ was obtained by subtracting the highest mean ΔC_T as an arbitrary comparison sample. Fold changes in the relative gene expression were determined by $2^{-\Delta\Delta CT}$. Statistical analysis was performed using InStat 2.00 program (GraphPad, San Diego, CA).

Animal and Tissue Preparation

For dexamethasone (Dex) treatment, newborn CD1 mice were used. Dex (0.1–5.0 mg/kg body weight) was injected intraperitoneally daily from P1 or P5. Control animals were injected with similar volumes of saline. For each experimental condition five to six animals were used. The animals were killed 24 h after the last administration of Dex or saline. Lungs were fixed at 20 cm H_2O constant pressure before being processed for paraffin embedding.

Human Lung Sample Collection and Analyses

Sixteen nonatopic current and/or ex-smoker subjects undergoing medically necessary lung resection were entered into the study: 11 subjects with no COPD and no evidence of emphysema (control group); and 5 subjects (COPD/emphysema group) with moderate to severe COPD and evidence of emphysema as determined by pulmonary function tests, high-resolution computed tomography (CT), and/or conventional CT scan (*see* Table 1). Ex-smoking subjects had quit smoking for an average of 25 ± 18 and 15 ± 11 yr in control and COPD/emphysema groups, respectively. COPD was diagnosed according to the criteria recommended by the NIH/WHO workshop summary (3). Subjects in the control and COPD/emphysema groups had similar (36 ± 18 and 59 ± 19, respectively) "pack-year" smoking histories, where smoking one pack of cigarettes per day each year is defined as one "pack-year." All subjects were recruited from the surgical clinic at the Michael E. DeBakey Veterans Affairs Medical Center, and the Methodist Hospital, undergoing lung resection for diagnostic or therapeutic purposes. Study protocols were approved by the institutional review board for human studies at Baylor College of Medicine, and informed consent was obtained from all subjects. The subjects had no history of allergy or asthma and had not received oral/systemic corticosteroids during the last 6 mo. At the time of study, all subjects were free of acute symptoms suggestive of upper or lower respiratory tract infection in the 6 wk preceding the study.

Total lung RNA was extracted from lung tissue samples and stored at −80°C. Two-step real-time RT-PCR was used to determine the relative expression of P311 mRNA using the ABI Perkin Elmer Prism 5700 Sequence Detection System (Applied Biosystems) as previously described (24). Primers and probe for the 18S rRNA used as internal control were purchased from Applied Biosystems. The sequences of the primers and probes for human P311 are as follows. P311: probe, 5′-TCCACAACTCAAACTCCCACCGCG-3′; forward primer, 5′-CCTGGTTCGCTACAACAATGTC-3′; reverse primer, 5′-CAG GAGTGGACCGGTTGTGT-3′.

Data were expressed as the fold difference between P311 mRNA compared with 18S rRNA in each sample. Two-tailed nonparametric

TABLE 1. CLINICAL CHARACTERISTICS OF THE PATIENT POPULATION

Characteristic	Control	Emphysema
N	11	5
Age (± STDV)	61 ± 15	67 ± 9
Male	10	4
Lung function		
%FEV1 (± STDV)	86 ± 20	49 ± 25
Smoking history		
Ex-smokers	10	4
Current smoker	1	1
PPY (± STDV)	36 ± 18	59 ± 19
Quitting time (± STDV)	25 ± 18	15 ± 11

t test (Mann-Whitney test) was used to analyze the differences between P311 expression in the normal versus COPD/emphysema lung samples.

RESULTS

Identification of P311 as a Differentially Expressed Gene during Saccular and Alveolar Development

To identify genes differentially expressed during saccular and/or alveolar formation, two subtractive cDNA libraries were constructed by suppression subtractive hybridization (SSH). The E18.5-P30 cDNA library was obtained using cDNAs from E18.5 lungs as tester and cDNAs from P30 lungs as driver cDNAs. It is enriched for cDNAs expressed at E18.5, which is at the saccular stage of lung development, compared with P30. The P5-P30 cDNA library was obtained using cDNAs from P5 lungs as tester and cDNAs from P30 lungs as driver cDNAs. It is enriched for cDNAs expressed at P5, which is at the alveolar stage of lung development, compared with P30. We selected P30 lungs as the stage to compare, because P30 lungs are structurally similar to lungs in the saccular and alveolar stages except that active saccular and alveolar formation have ceased.

We picked 600 clones from each subtraction library for further differential screening by hybridization with cDNA probes made from P30 mRNA and from either E18.5 or P5 mRNA. Clones that hybridized with $>$ 5-fold intensity to either of the E18.5 or P5 probes compared with P30 probes were considered differentially expressed. From this screen, 175 clones from the E18.5-P30 and 71 clones from the P5-P30 library met the differential expression criteria. We then eliminated duplicate clones by hybridizing them to each other. From this we identified 75 unique clones from the E18.5-P30 library and 43 clones from the P5-P30 library. These were then completely sequenced. After obtaining the sequences of the cDNA clones, we searched for homology with sequences deposited in Genbank databases using the online BLAST search. Among the cDNA clones from the E18.5-P30 library, 53% is homologous to known genes, 24% is homologous to ESTs or predicted genes from genomic sequences, and the rest shows no significant homology to any database entry. Among the cDNA clones from the P5-P30 library, 79% is homologous to known genes, 7% to ESTs or predicted genes, and the rest has no significant homology to deposited sequences. We then performed Northern blotting using these clones as probes on blots with total RNA from E18.5, P5, and P30 lungs to confirmed differential expression. Only a few of the clones showed differential expression on Northern blots (9 known genes and 7 unknown genes differentially expressed at P5, and 3 genes differentially expressed at E18.5). The list of the differentially expressed known genes is shown in Table 2.

We selected one cDNA for further studies on the basis of it being highly expressed in both E18.5 and P5 lungs, and at a much lower level in P30 lungs, as confirmed by Northern Blotting (Figure 1A). Sequencing results showed that this cDNA fragment corresponded to $\sim$ 800 nucleotides in the 3′ untranslated region of Mus musculus P311 mRNA (GenBank Accession No. X70398). P311 is a neuronal protein that was initially isolated from a late stage mouse embryonic brain cDNA library (25). It encodes for a small 8-kD protein with unknown function. The protein does not belong to any known family of proteins and has no structural motifs that provide clues to its function.

Expression of P311 mRNA during Lung Development

To have a better understanding of the function of P311 in lung development, we assayed for the level of P311 expression at different stages of lung development using quantitative real-time PCR. We found that P311 expression was highly developmentally regulated (Figure 1B). It was expressed at low levels at the

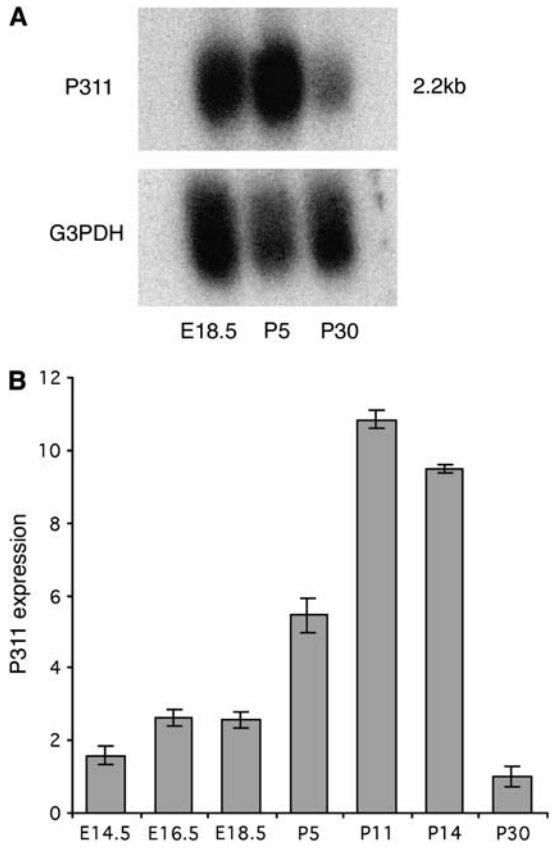

Figure 1. P311 gene expression during mouse embryonic lung development. (*A*) Northern blot of total RNA hybridized with α^{32}P-labeled P311 probe showing higher expression of P311 in E18.5 and P5 lungs compared with P30 lungs. (*B*) Quantification of P311 mRNA expression at different stages of lung development by real-time RT-PCR. Results are shown as relative expression of each stage compared with P30, which is set arbitrarily at 1. The expression of P311 in pseudoglandular stage (E14.5) is slightly higher than that in P30 lungs ($P = 0.10$). It increases further in the canalicular (E16.5, $P = 0.01$) and saccular (E18.5, $P = 0.01$) stages. Peak expression of P311 occurs during alveolar formation (P5, P11, and P14, $P < 0.01$).

TABLE 2. GENES DIFFERENTIALLY EXPRESSED IN E185 OR P5 LUNGS COMPARED TO P30 LUNGS

Genes differentially expressed at E18.5 compared to P30:
1. Myristoylated alanine rich protein kinase C substrate (MARCKS)
2. Osteoglycin
3. P311

Genes differentially expressed at P5 compared to P30:
1. Chaperonin subunit 4
2. GABA receptor–associated protein
3. Lectin, galactose binding, soluble 1 (Galectin)
4. Glia Maturation Factor β
5. Nucleophosmin 1
6. P311
7. Mus Musculus similar to peptidyl-prolyl cis-trans isomerase A
8. Mus Musculus similar to hypothetical protein KIAA0419
9. Mus Musculus similar to hypothetical protein MGC12981

Definition of abbreviations: E18.5, embryonic day 18.5; P5, postnatal day 5; P30, postnatal day 30.

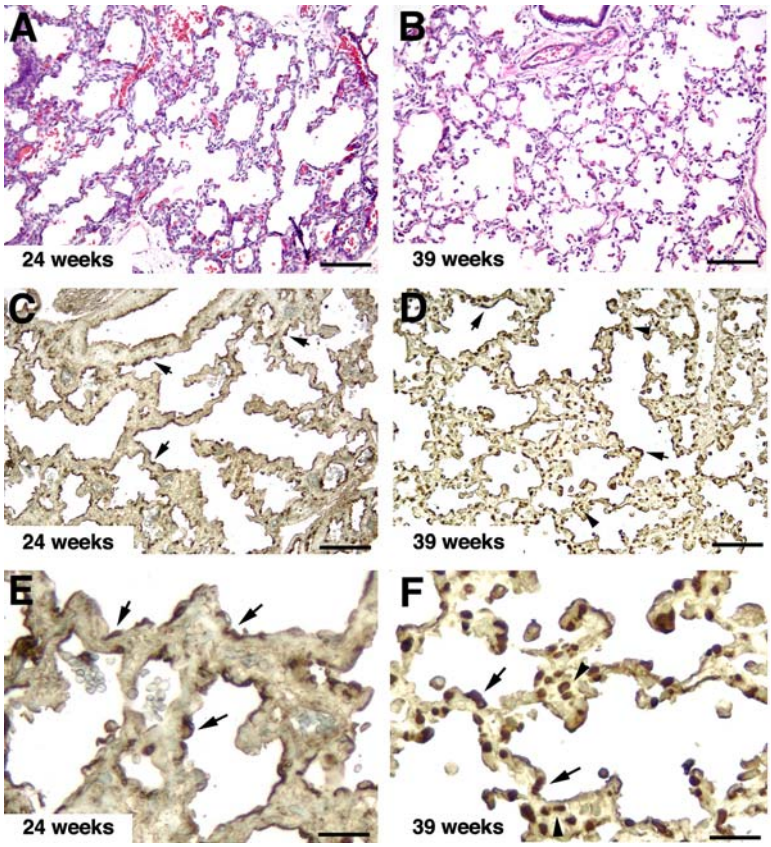

Figure 2. Cellular expression of P311 protein in human fetal lungs. (*A* and *B*) Hematoxylin and eosin (H&E)-stained sections of human lungs at 24 (*A*) and 39 (*B*) wk gestation. The 24-wk gestation lung shows the saccular stage of lung development with large terminal air sacs with thick walls. The 39-wk gestation lung shows the alveolar stage of lung development with smaller terminal air sacs with thin walls. (*C–F*) immunostaining of 24 (*C* and *E*)- and 39 (*D* and *F*)-wk gestation lung sections with an antibody to P311. In the saccular stage, P311-expressing cells were found lining the saccular surfaces (*C* and *E*, *arrows*). In the alveolar stage, P311-expressing cells were found both lining the alveolar surface (*D* and *F*, *arrows*) and in the mesenchyme (*D* and *F*, *arrowheads*). *Bar*: *A–D*, 100 μm; *E* and *F*, 32 μm.

pseudoglandular stage of embryonic development (E14.5). The expression starts to increase at the beginning of distal lung development (E16.5), and remains at about the same level during saccular development (E18.5). However, at the beginning of and during alveolar development, the expression of P311 increased significantly (P5-P14), before decreasing again to low levels at the end of alveolization (P30). This developmental expression pattern is consistent with a role for this gene in saccular and alveolar development.

Immunohistochemical Localization of the P311 Protein

To determine the cellular expression of P311, immunohistochemical staining was performed on paraffin sections of human lung tissues from different developmental stages. In human lung development, the beginning of the saccular stage occurs around 24 wk of gestation (26), during which smooth primary septa starts to grow to form channels and saccules (Figure 2A). The beginning of the alveolar stage of human lung development occurs around 36 wk of gestation, during which secondary septa begins to form and subdivides the saccules into smaller airsacs called alveoli (Figure 2B). P311 protein was detected by immunohistochemical staining in both 24- and 39-wk gestation human lung tissues. In the 24-wk gestation lung, almost all cells positive for the P311 protein were found lining the saccular surfaces, consistent with their being epithelial cells (Figures 2C and 2E). There is an apparent increase in the number of P311 expressing cells in 39-wk gestation lungs (Figure 2D). At this stage, P311-expressing cells were found both lining the alveolar surfaces (Figures 2D and 2F, *arrows*), as well as in the mesenchyme (Figures 2D and 2F, *arrowheads*).

Reduced Expression of P311 in Human Lung Samples with Emphysema

Emphysema is a disease of lung destruction associated with cigarette smoking. One of the enigmas of this disease is that not everyone who smokes develops emphysema. The reasons for the difference in the susceptibility to development of emphysema in individuals who smoke are not known. It is possible that the lungs of subjects who develop emphysema are more prone to injury caused by cigarette smoke. Alternatively, it is also possible that individuals who do not develop emphysema are able to repair damaged alveoli or to regenerate alveoli, whereas those who develop emphysema are not able to. Deficiency in the reactivation of developmental pathways, including the inability to induce expression of genes essential for alveolar formation, may be a contributing factor to abnormal lung repair and consequently the development of disease. If P311 is important for alveolar generation, we reasoned that deficiency in its expression might contribute to the pathogenesis of emphysema. We compared P311 mRNA expression in lung samples from subjects with smoking history and with or without emphysema by quantitative real-time RT-PCR. As seen in Figure 3, the expression of P311 mRNA in lung samples from subjects with emphysema was significantly reduced compared with that in lung samples from subjects with smoking history who do not have emphysema (Figure 3A). The decrease in P311 expression in the lung samples from subjects with emphysema was selective, as expression of JST185, a developmentally regulated gene essential for bronchial smooth muscle development (E. J. Yun and coworkers, unpublished observations), showed no significant change (Figure 3B). The aberrant regulation of P311 in emphysema suggests that P311 might be important for the maintenance of adult lung

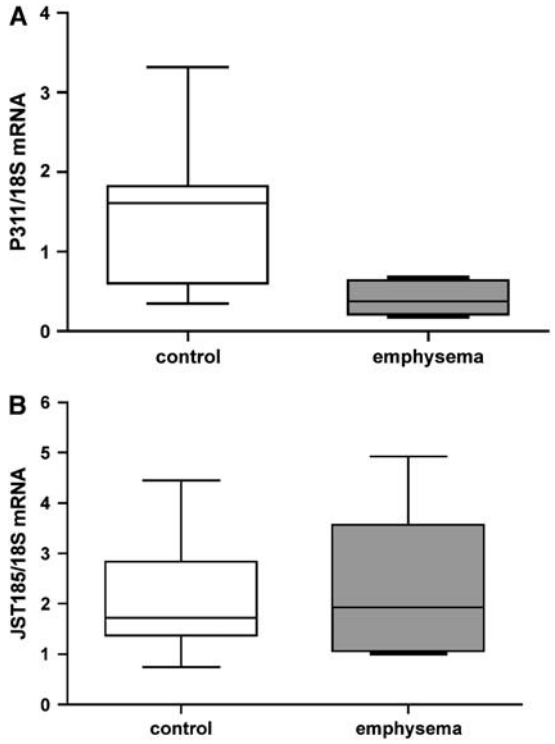

Figure 3. P311 gene expression is downregulated in human lungs with emphysema. (*A*) Quantification of P311 mRNA expression in normal human lungs ($n = 11$) and human lung samples with emphysema ($n = 5$) by quantitative real-time RT-PCR. Results are shown as relative expression of P311 mRNA compared with 18S rRNA in each sample ($P = 0.017$). (*B*) Quantification of JST185 gene expression in the same samples showed no significant change in JST185 gene expression. Means and standard deviation of each group of samples are shown ($P = 1.00$). *P* value was determined by the Mann-Whitney test.

structures or that the induction of P311 expression might be necessary for alveolar regeneration and lung repair.

P311 Is Downregulated in Lungs with Impaired Alveolar Formation

To determine if P311 has a putative role in alveolar formation, we assayed for changes in its expression in an alveolar malformation model. In postnatal animals, dexamethasone (Dex), a synthetic glucocorticoid, inhibits alveolar formation by reducing the outgrowth of new alveolar septa (27, 28). Treatment of newborn mice with Dex resulted in failure of alveolar formation in a dose- and duration-dependent manner (Figures 4A–4F). Treatment of 1-d-old mice with 0.1 mg/kg of Dex for 14 d resulted in enlarged distal airspaces with few secondary septa (Figure 4B) compared with the fine alveolar structure in lungs of littermates treated with saline (Figure 4A). The phenotype is more severe with the higher dose of 2 mg/kg for 14 d (Figure 4C). Shorter duration of treatment with a higher dose also resulted in similar phenotype (Figures 4D–4F). Measurement of P311 gene expression by quantitative real-time RT-PCR showed that the expression of P311 decreased with Dex treatment, and that the magnitude of reduction correlated with Dex dosage and duration and phenotype severity (Figures 4G and 4H). The association between impaired alveolar development and decreased P311 expression is supportive of a role for P311 in alveolar formation.

DISCUSSION

The lung's essential function is to provide sufficient conduit and gas-exchange surface to meet the organism's need for the uptake of oxygen and elimination of carbon dioxide. In human COPD and emphysema, loss of alveoli results in irreversible reduction of lung surface required for proper gas exchange and leads to debilitating physiologic impairments. Alveolar generation is the cornerstone of the formation of the gas-exchange surface, yet the molecular mechanisms of alveolar formation remain largely uncharacterized, despite the important function of this portion of the lung in normal physiology and its involvement in disease.

In the present study, we used SSH to identify genes that may regulate alveolar formation. We found that the P311 gene was differentially expressed during distal lung development and that its expression was significantly downregulated in lung samples from humans with emphysema and in a mouse model of Dex-induced inhibition of alveolar formation. Human subjects included in the study did not receive any oral/systemic corticosteroids during the six months before lung sample collection. The difference in P311 expression between individuals who smoked and who developed emphysema and those who also smoked but did not develop emphysema is intriguing, and suggests that the decreased in P311 expression might contribute to the pathogenesis of emphysema. P311 may play a role in the protection against injury or it may play a role in the repair or regeneration of damaged lungs. The decrease in P311 expression in Dex-treated lungs, which exhibits failure of alveolization, suggests that P311 may indeed function in alveolar formation. It is not clear whether the downregulation of P311 expression is uniform in all the cell types that normally express it in human emphysema lungs or in Dex-treated lungs. Since P311 is expressed in different cell types in the lungs, the decrease in P311 expression in one cell type may cause in a different biological effect than its downregulation in another cell type. The function of P311 in specific lung cell populations, the cell type–specific regulation of its expression, and the cell type–specific contribution of P311 function to normal lung biology and disease are questions of significant interest that remain to be elucidated.

P311, also referred to as pentylenetetrazol (PTZ)-17 (29, 30), is an 8-kD, 68–amino acid, intracellular polypeptide encoded by a 2.2-kb mRNA. This protein is highly conserved among human, mouse, and chicken (25). It is a PEST (unbiquitin/proteasome target) domain containing protein that is rapidly degraded with a very short half-life by both unbiquitin/proteasome system and an unknown metalloprotease (31). It does not belong to any known family of proteins and has no structural motifs that provide clues to its function. In the mouse nervous system, it is expressed in the germinal zones at E17 and in the superficial cortical layer at E20, and in the granular layer of the cerebellum, the hippocampus, and the olfactory bulb in the adult (25). These are thought to be sites either of embryonic neuronal migration or postnatal neurogenesis and synaptic plasticity. P311 was subsequently found to be differentially expressed in several biological processes. Its expression is modulated during chemically induced seizures (29). Transformation of neural and smooth muscle cells by the coexpression of hepatocyte growth factor and its receptor c-Met resulted in downregulation of P311 expression (31). It is overexpressed in glioblastoma cells at the invasive rim of the tumor, and inhibition of P311 expression by antisense oligonucleotides inhibited migration of malignant glioma cells (32). The expression of P311 is either reduced or upregulated in a variety of tissues in the prolactin-null mice, and it is also regulated in the uterus and ovary during pregnancy, suggesting that it is hormonally regulated (33). P311 expression is upregulated in axotomized facial motoneurons and overexpression of

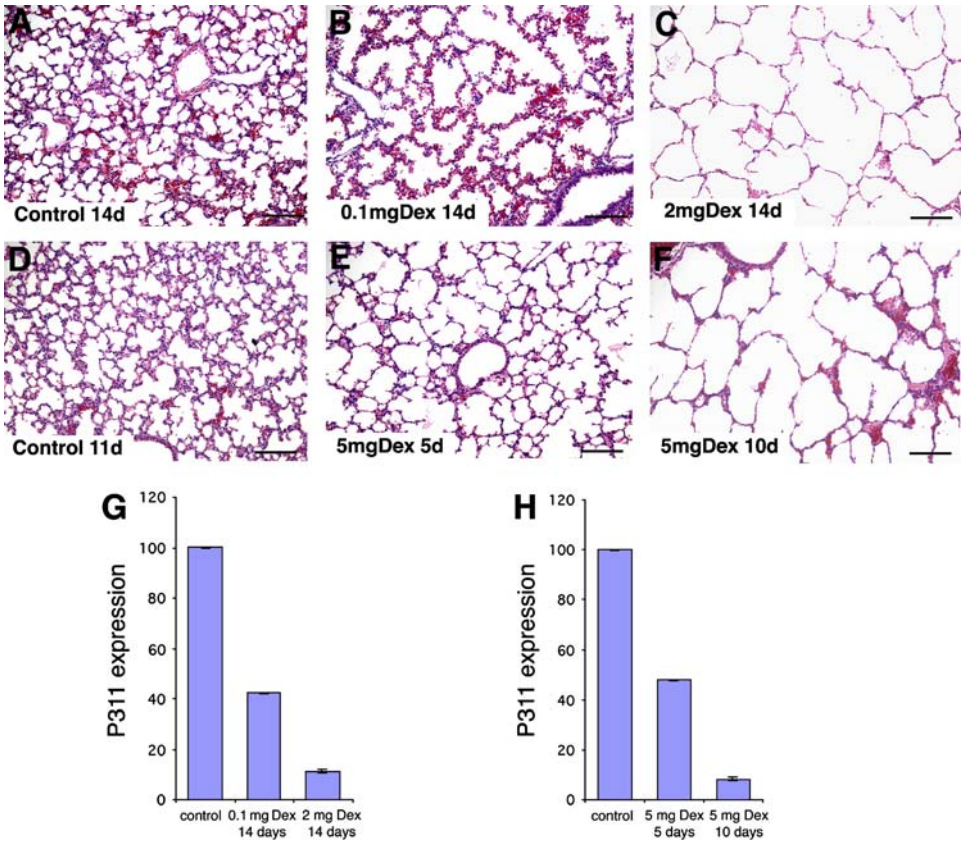

Figure 4. Dex treatment inhibits alveolar formation and downregulates P311 expression. (*A–C*) H&E-stained sections of lungs from 14-d-old mice treated with saline (*A*) or of lungs from 14-d-old mice treated for 14 d with 0.1 mg/ml (*B*) or with 2 mg/ml (*C*) Dex. Dex treatment led to a dose-dependent inhibition of alveolar development, which resulted in enlarged distal airspaces. (*D–F*) H&E-stained sections of lungs from 11-d-old mice treated with saline (*D*) or of lungs from 11-d-old mice treated for 5 d (*E*) or for 10 d (*F*) with 5 mg/ml Dex. Dex treatment led to a duration-dependent inhibition of alveolar development, which resulted in enlarged distal airspaces. *Bar*: 100 μm. (*G* and *H*) Quantification of the expression of P311 in Dex-treated mouse lungs by real-time RT-PCR. P311 expression was decreased in Dex-treated lungs in a dose-dependent manner, which was correlated with phenotype severity. Bar: 100 μm.

P311 promotes neurite outgrowths *in vitro* and nerve regeneration *in vivo* (34).

Interestingly, P311 was found to be differentially expressed during smooth muscle cell differentiation, and overexpression of P311 in fibroblastic cell lines resulted in differentiation of the cells into myofibroblasts and in increased cell proliferation and survival (35). Even though these studies have not shown definitively the function of P311, they implicate this gene in diverse cellular processes, including cell proliferation, differentiation, and migration. The ability of the gene to induce myofibrolastic differentiation is intriguing. Myofibroblasts are thought to be important for pulmonary alveolar septa formation. These cells are believed to be the source of elastin, an extracellular matrix molecule necessary for secondary septation (36). Deficiency in elastin results in defect in alveolar formation. In the platelet-derived growth factor –A null mice, there is deficiency in alveolar myofibroblasts, and alveolar septa do not form. Thus, induction of myofibroblast differentiation might be a mechanism whereby P311 function in alveolization. However, mice treated with Dex showed preferential decrease in P311 gene expression, while the number of myofibroblasts, as identified by α-smooth muscle actin immunostaining, was not affected (L. Zhao, unpublished data). Thus it is unclear if P311 functions in alveolar development through the regulation of myofibroblast development. We found P311-expressing cells at both epithelial and mesenchymal locations. The function of P311 in these cells, and how it relates to alveolar development, are of great interest and will be the subject of further studies.

In summary, our data demonstrated that P311 is a putative gene regulating alveolar formation. It is preferentially expressed during the alveolar stage of lung development. Its expression decreases in a model of impaired alveolar formation and in emphysematous lungs with alveolar destruction and abnormal repair. Further work will be needed to clarify its function *in vivo* and its mechanisms of action. Understanding the function of P311 might provide new therapeutic targets for lung diseases resulting from abnormal development or repair.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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