Cardiovascular, Pulmonary and Renal Pathology

The Divergent Roles of Secreted Frizzled Related Protein-1 (SFRP1) in Lung Morphogenesis and Emphysema

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Developmentally expressed genes are believed to play a central role in tissue repair after injury; however, in lung disease their role has not been established. This study demonstrates that SFRP1, an inhibitor of Wnt signaling normally expressed during lung embryogenesis, is induced in the lungs of emphysema patients and in two murine models of the disease. SFRP1 was found to be essential for alveolar formation as *Sfrp1*^{-/-} mice exhibited aberrant Wnt signaling, mesenchymal proliferation, and impaired alveoli formation. In contrast, SFRP1 activated ERK and up-regulated MMP1 and MMP9 without altering TIMP1 production when expressed in human lung epithelial cells. These findings demonstrate that SFRP1 promotes normal alveolar formation in lung development, although its expression in the adult up-regulates proteins that can cause tissue destruction. Thus, SFRP1 induction during tissue injury is unlikely to contribute to the repair response but rather is a participatory factor in the pathogenesis of emphysema and tissue destruction. (Am J Pathol 2010, 177:598–607; DOI: 10.2353/ajpath.2010.090803)

Lung morphogenesis and repair involve complex interactions between cells of endodermal and mesodermal origin. The ultimate goal of these interactions is to generate or restore an alveolus that effectively exchanges gases between the circulation and alveolar space. Importantly, the induction of genes linked to development has been observed in postinjury repair responses in several adult organs.^{1–3} It is theorized that mechanisms of repair use these genes to recapitulate fundamental strategies of normal development and thus restore proper organ function.⁴ In con-

flict with this theory, studies have shown that liver regeneration does not require transcription factors known to be important in liver development.⁵ Furthermore, a study indicated that the reversion to a developmental pattern in the adult gastrointestinal tract could actually trigger dysfunction of the digestive system.⁶ Therefore, the beneficial role of the re-expression of developmentally regulated genes in tissue repair is likely organ specific.

Wnt proteins, critical molecules in lung development, are also up-regulated in adult diseases of the lung.⁷ Wnt signals through the canonical pathway involving β -catenin and through noncanonical pathways involving protein kinase C (PKC)⁸ and mitogen-activated protein kinase (MAPK)8 (also known as JNK1).⁹ Through stimulation of these signaling pathways, the WNTs modulate important processes involved in lung morphogenesis and repair including cell proliferation,¹⁰ migration,¹¹ and differentiation.¹² Secreted frizzled related proteins (SFRPs) bind soluble Wnts and inhibit interaction with the frizzled receptor¹³ thereby antagonizing their action. Though SFRPs are expressed in the lung during embryogenesis,¹⁴ their contribution to lung development has not been determined. Moreover, there are virtually no data regarding the role of SFRPs on noncancer disease states of the adult lung despite the assumption that these molecules are central to the repair of the tissue.¹⁵ The present study identified the selective induction of SFRP1 in the lungs of emphysema patients. Given the known role of Wnt signaling on lung development and repair, analyses were conducted to determine the specific role of SFRP1 on lung morphogenesis and to evaluate how SFRP1 influenced biological responses linked to the development of cigarette smoke-induced emphysema.

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Table 1. PCR Primers

Species	Genes		Primer Sequence	Annealing temperature (°C)
Human	<i>SFRP1</i>	Forward	5'-TACAAGAAGATGGTGCTGCC-3'	52
		Reverse	5'-AGATGTTCAATGATGGCCTC-3'	
	<i>SFRP2</i>	Forward	5'-TCTTCCTCTTTGGCCAGCCC-3'	58
		Reverse	5'-TCACATCAATTGGAGCTTC-3'	
	<i>SFRP3</i>	Forward	5'-TCTGCACCATTGACTTCCAG-3'	52
		Reverse	5'-TTAGAATCTCCTTCACCTCC-3'	
	<i>SFRP4</i>	Forward	5'-TCCTGGCCATCGAGCAGTAC-3'	58
		Reverse	5'-GATGAGGACTTGAAGATCTC-3'	
	<i>SFRP5</i>	Forward	5'-ACTCGGATACGCAGGTCTTC-3'	58
		Reverse	5'-TTCTTGTCCTCCAGCGTAGAC-3'	
Mouse	<i>GAPDH</i>	Forward	5'-TTCCACCCATGGCAAATTCC-3'	50
		Reverse	5'-TTTCTAGACGGCAGGTCAGG-3'	
	<i>Sfrp1</i>	Forward	5'-AGACCATGGCAGAGGTGAAG-3'	58
		Reverse	5'-CCGCCCCAGTATCACTTAAA-3'	
	<i>Wnt5a</i>	Forward	5'-GGTGCCATGTCTTCCAAGTT-3'	51
		Reverse	5'-CAGCACGTCTTGAGGCTACA-3'	
	<i>Wnt10b</i>	Forward	5'-TGCTTTCTCCTTCTCCATGC-3'	57
		Reverse	5'-GATTTACCTGACCCCTGAA-3'	
	<i>Gapdh</i>	Forward	5'-ATGCATCCTGTACCACCAAC-3'	56
		Reverse	5'-TGGTCCTCTGTGTAAGCAAG-3'	

Materials and Methods

Human Lung Tissue Samples

Lung tissue explants from six unrelated patients with emphysema were obtained under consent at Columbia Presbyterian Medical Center from recipient lungs during transplant or lung volume reduction procedures. Normal samples were from accidental death victims or unused donor lungs harvested for transplant. All of the samples were obtained from New York–Presbyterian Hospital with the approval by Institutional Review Board of Columbia University.

Sfrp1^{-/-} Mice

The generation of the *Sfrp1* knockout (*Sfrp1*^{-/-}) mice in the C57BL/6(albino)-129SvEv^{brd} background has been previously reported.¹⁶

Cells

Human small airway epithelial (SAE) cells were purchased from Lonza Walkersville Inc. (Walkersville, MD). A549 cells were obtained from ATCC (American Tissue Culture Collection, Manassas, VA).

Wnt Signaling Analyses

RT-PCR was performed on human and mouse samples using specific primer sets (Table 1). After the reverse transcription reaction of total RNA using SuperScript II (Invitrogen Corporation, Carlsbad, CA), PCR reaction was performed at the appropriate annealing temperature. Amplicons were analyzed on a 2% agarose gel. For Northern analyses of mouse lung, 10 μ g of lung tissue RNA was separated on a 1.2% agarose-formaldehyde gel and transferred to nylon membrane which was then exposed to radiolabeled *Sfrp1* and glyceraldehyde-3-

phosphate dehydrogenase (*Gapdh*) cDNA probes. Membranes were hybridized with a 28S rRNA oligonucleotide probe labeled with (γ -³²P) ATP to control for loading. Using specific primers and probe (Applied Biosystems Inc., Foster City, CA), quantitative PCR for mouse *Wnt5a*, *Wnt7b*, *MMP9*, and *Actin* was performed on cDNA prepared from the lung mRNA of wild-type and *Sfrp1*^{-/-} mice at 14.5, 16.5, and 18.5 days postcoitum (dpc) ($n = 4$ in each group). Western blot for WNT5A (SC-23698), WNT10B (SC-7432), SFRP1 (SC-7425; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), MAPK3 and MAPK1 (also known as ERK1 and ERK2), phospho-ERK1/2 and phospho-JNK1 (Cell Signaling Technology, Inc., Danvers, MA) was performed on the lung tissue homogenates of wild-type mice at 14.5 dpc. Western blot for β -catenin (Cell Signaling Technology, Inc.) was performed on nuclear protein fractions from the embryonic and newborn mice. Nucleophosmin (Invitrogen Corporation) was used as a loading control. Western blot for SFRP1 was performed on the human lung tissue homogenates of six emphysema and five normal lungs as per standard protocol.

Mouse Lung Injury Models

All of the experiments were conducted according to Institutional Animal Care and Use Committee-approved protocol.

Acute Bacterial Inflammation Model

Three-month-old C57BL/6 mice were injected intraperitoneally with 50 μ g of lipopolysaccharide (LPS; Sigma-Aldrich Corp., St. Louis, MO) in sterile PBS. Control littermates were treated with sterile PBS alone. After 16 hours, animals were sacrificed by isoflurane overdose followed by cervical dislocation.

Chronic Smoke Exposure Model

C57BL/6 mice were exposed to air (controls) or cigarette smoke (6 hours/day, 5 days/week) in a specially designed full-body smoke exposure chamber (Teague Enterprises, Woodland, CA) for one day, two weeks, or four months as per standard laboratory protocol.¹⁷ The mice were sacrificed twelve hours after smoke exposure.

Collagenase Transgenic Emphysema Model

Generation of the *MMP1 transgenic* mice has been previously reported.¹⁸

Hyperoxia Lung Injury Model

C57BL/6 mice were exposed to 85% oxygen for three days in a plexiglass container. The mice were provided food and water *ad libitum*.

SFRP1 Administration to Human SAE Cells

SAE cells in six-well plates were grown to 70 to 80% confluence and then treated with recombinant SFRP1 protein (R&D Systems, Inc., Minneapolis, MN) at a concentration of 0, 20, and 100 ng/ml for 3 hours ($n = 6$) or 10 ng/ml for 48 hours ($n = 6$). The control cells were treated with 10 μ g/ml of bovine serum albumin (Sigma-Aldrich Corp.) for 3 or 48 hours ($n = 6$). Each experiment was performed in duplicate. Matrix metalloproteinase (MMP)1, MMP9, and TIMP1 protein levels were determined by ELISA of the media (Millipore, Billerica, MA). Western blots for basal and active ERK-1/2 (Cell Signaling Technology, Inc.) and actin (Santa Cruz Biotechnology, Inc.) were performed on protein from SAE and A549 cell monolayers with or without treatment with human SFRP1 protein.

SFRP1 Adeno-Associated Viral Vectors

The rAAV2 vector plasmids carrying the expression cassettes of *Sfrp1*, *Wnt10b*, and GFP under the influence of the SV40 promoter were constructed as per established protocols.¹⁹ rAAV2 vectors were added at concentrations of 10^8 to 10^{10} vector genome (vg) to T-25 flasks with SAE cells grown to 60% confluency. Twenty-four hours after transduction this media was exchanged with fresh growth media. After 24 hours, MMP1 protein levels in the media were determined by ELISA (Millipore).

Lung Histological Analyses

Immunofluorescent studies for *Sfrp1* (SC-13939), *WNT5A* (SC-30224), and *WNT10B* (SC-25524; Santa Cruz Biotechnology, Inc.) and immunohistochemistry for Clara Cell 10 protein (SC-25555; Santa Cruz Biotechnology, Inc.) were conducted on lung tissue sections as per standard laboratory protocol.²⁰ Proliferating cells were detected using a PCNA monoclonal antibody (PCNA staining kit, Invitrogen Corporation). For smooth muscle actin staining, anti- α -smooth muscle actin antibody (Dako North America Inc., Carpinteria, CA) were used.

Lung Morphometry and Compliance

Morphometric analyses were conducted on H&E-stained slides of eight mice in each group as previously outlined by our laboratory.²⁰ Compliance was determined using the flexiVent apparatus (SCIREQ Scientific Respiratory Equipment Inc., Montreal, Canada) as per our previously published protocol.¹⁷

Gelatin Zymography

Equal amounts of protein were loaded and gelatin zymography was performed on this lung homogenate as previously described.²¹

Lung Lavage Cellularity

Lung lavage cell count and differentials were performed on control, smoke-exposed, and LPS-treated mice as per standard laboratory protocol.²⁰

Statistical Analyses

Data are expressed as the mean $\pm$ SD. One- and two-way analysis of variance measures were conducted using commercially available software (Prism 5, Graphpad Software Inc., La Jolla, CA).

Results

SFRP Expression in Human Emphysema

The lung expression pattern of the SFRP family members in human emphysema and normal lung tissue was determined through RT-PCR analysis. SFRP1 was expressed in the lungs of emphysema patients but not in normal lung tissue (Figure 1A). SFRP2 and SFRP5 (data not shown) were not detected in any of the samples, and FRZB (also known as SFRP3) was detected in only one of four emphysema lungs and in none of the normal lung samples (Figure 1A). SFRP4 was detected in all of the emphysema and normal lung samples (data not shown). SFRP1 was not detectable by Western blot in normal lung tissue; however, it was present in lung tissue samples from four emphysema patients (Figure 1B). Immunohistochemistry studies identified expression of *Sfrp1* in the epithelial cell of the emphysema lung (Figure 1C).

Sfrp1 Is Expressed in Mice during Hyperoxic Lung Injury and Emphysema, but not During Acute Inflammation

Because SFRP1 was expressed in human emphysema we then investigated whether *Sfrp1* could be up-regulated under several distinct conditions of lung injury in mice using Northern blot analysis (Figure 1D). The expression of *Sfrp1* was significantly increased under hyperoxic conditions (85% oxygen inhalation for 3 to 5 days), in a collagenase-induced mouse model of emphy-

Figure 1. SFRP1 is up-regulated in human emphysema and in animal models of the disease. **A:** Using specific primers for human SFRP1–5, RT-PCR analysis was performed on RNA isolated from the lung tissue of human normal controls (N1–5) and emphysema subjects (E1–4). RT-PCR for GAPDH was used as an internal control. SFRP1 was expressed only in the emphysema samples. **B:** Western blot analysis for human SFRP1 was performed on lung tissue protein from normal controls (N1–4) and emphysema subjects (E1–5). Actin Western blot was performed to confirm equal protein loading. SFRP1 was expressed only in the emphysema samples. **C:** Immunohistochemistry studies exhibited negligible staining in the normal human lung samples with the anti-SFRP1 antibodies. In contrast, the epithelial cells stained positively in the human emphysematous lung samples. **Inset:** High-power view of emphysema tissue stained with anti-SFRP1 antibodies; original magnification, $\times 40$. **D:** Northern blot of *Sfrp1* expression in distinct mouse models of lung injury: *MMP1* transgenic mice (Tg1 and Tg2), hyperoxia (three or five days of exposure to 85% oxygen; 3D and 5D, respectively), mice exposed to chronic cigarette smoke for six months (SM), mice injected in the peritoneum with 50 μ g of LPS and a positive control which was RNA isolated from human HEK cells stably transfected with mouse *Sfrp1* cDNA (*Sfrp1*+). The membrane was stripped and reprobed with a probe for GAPDH (middle). The bottom panel shows the ethidium bromide-stained 1.5% agarose formaldehyde gel. *Sfrp1* was expressed in the diseased models (emphysema and hyperoxia) but not in LPS-treated lungs.

sema and after cigarette smoke exposure. In contrast, when the mice were treated with an intraperitoneal injection of LPS, *Sfrp1* was not induced in the lung (data not shown). This was despite the fact that LPS caused an acute increase in lung lavage cellularity (25.8 ± 5.87 control versus 48.8 ± 5.31 LPS-treated; $n = 5$, $P = 0.0161$). This indicates that *Sfrp1* expression is not a generalized reaction to lung inflammation.

SFRP1 Increases MMP Expression in Lung Epithelial Cells

The tissue destruction in emphysema ultimately results from an increase in protease production.²² Studies in mice^{18,23} and human²⁴ have implicated the proteases MMP1 and MMP9 in emphysema and shown the aberrant expression of these enzymes destroy the lung over time. To determine whether SFRP1 modulates MMP production, we measured MMP levels in human SAE cells treated with SFRP1. As shown in Figure 2, A and B, treatment with purified SFRP1 increased MMP1 and MMP9 protein levels in SAE cell media but did not affect TIMP1 (the natural inhibitor) protein levels. The treatment of SAE cells with SFRP1 activated ERK1/2, which is known to regulate MMP expression in SAE cells treated with cigarette smoke (Figure 2C)²³ and MAPK8. Thus, SFRP1 replicates a biological process linked to emphysema formation.¹⁸ Consistent with our results for SFRP1 protein treatment, administration of AAV2 vectors ex-

pressing SFRP1 significantly increased MMP1 protein level compared with AAV2-*Wnt10b* and AAV2-*gfp* treated cells (Figure 2D). In addition, mice treated with AAV2 expressing SFRP1 exhibited increased expression of MMPs in lung tissue homogenates (Figure 2E). These animals did not develop emphysema (data not shown) because the vector was only expressed for one month and MMP-induced emphysema develops over a long period of time.^{18,24}

SFRP1 and WNTs Expression during Lung Embryogenesis

The above data demonstrate a link between SFRP1, MMPs, and emphysema, but Wnt signaling and MMP expression are also known to play a fundamental role in normal development. Therefore, we undertook the following experiments to better understand the role of SFRP1 during lung development so as to potentially gain insight into its function during disease. We first examined the gene expression pattern of *Wnt5a*, *Wnt10b*, and *Sfrp1* during lung morphogenesis in wild-type mice (Figure 3A) as RT-PCR analysis demonstrated these proteins to be expressed during the same embryonic time point. Both WNT5A and WNT10B play a known role in development^{25,26} and are likely regulated by SFRP1.²⁷ All three genes were amplified by RT-PCR in the samples from the embryonic lung tissues between 14.5 to 18.5 dpc; however, their ex-

Figure 2. SFRP1 increases the level of MMP1 and MMP9 and activates ERK in lung epithelial cells. **A** and **B**: MMP ELISAs performed on culture media harvested from SAE cells treated with SFRP1 or BSA protein for three hours (**A**) or 48 hours with 10 ng/ml of either SFRP1 or BSA control protein (**B**), as indicated. Error bars indicate SD (* $P < 0.05$, $n = 12$ in all groups). The y axis represents ng of protein per ml of media. MMP-1 and MMP-9 levels increased with SFRP1 treatment. **C**: Human SAE and A549 cells were treated with varying concentrations of human SFRP1 protein. Western blot analyses for phospho-ERK, total ERK, phospho-JNK1, and actin were conducted on the cell lysate. SFRP1 treatment induced the phosphorylation of ERK and JNK. **D**: Human SAE cells were treated with AAV2 coding *Sfrp1*, *Wnt10b*, or *gfp* at concentrations of 10^8 to 10^{10} vector genome (vg). Protein levels for MMP1 in the media were determined by ELISA. Data are expressed as a percentage of AAV2-*gfp* control. **E**: Gelatin zymography of lung tissues isolated from AAV2-*gfp* and *Sfrp1* transfected mice lung. Both MMP2 and MMP9 were up-regulated in *Sfrp1* transfected lung.

pression was not observed in the postnatal lungs (newborn, 5 days postpartum and 14 weeks) (Figure 3A). Protein levels of these WNT5A, WNT10B, and *Sfrp1* were assessed by Western blot analysis. A single protein band of WNT5A, WNT10B, and *Sfrp1* was seen in the 14.5-dpc lung extracts but not in the 5-day-postpartum lung extracts (Figure 3B).

Localization of WNT5A, WNT10B, and *Sfrp1* in the Developing Lung

To better understand the relationship between *Sfrp1*, WNT5A, and WNT10B, immunolocalization studies were undertaken in developing lung tissue samples of wild-type mice. During the early pseudoglandular stage (10.5 dpc), WNT5A and WNT10B were immunolocalized to the epithelium and the surrounding mesenchymal cells. By 12.5 dpc, detection of WNT5A persisted in the epithelium and mesenchyme while WNT10B became far more prominent within the epithelial region of the lung. In contrast, *Sfrp1* was not detected in the lung during this early pseudoglandular stage. In the mid-pseudoglandular stage, WNT5A staining was prominent in the epithelium and the neighboring mesenchyme at 13.5 dpc. Staining became restricted to the distal lung epithelium and mesenchyme by 14.5 dpc. In contrast, WNT10B localized primarily to the epithelial cells at 13.5 dpc and 14.5 dpc with scant detection noted in the mesenchymal regions of the lung. Expression of *Sfrp1* was first detected within this time period in a pattern that correlated with WNT5A. During the late pseudoglandular stage, WNT5A was detected within the distal epithelium and the surrounding mesenchymal cells at 15.5 dpc. This staining became localized primarily to the mesenchyme between the distal epithelial buds by 16.5 dpc. WNT10B was ob-

served almost exclusively in the distal epithelial cells at 15.5 dpc and 16.5 dpc. As seen with WNT5A, *Sfrp1* was detected in the distal epithelial cells and the surrounding mesenchymal cells at 15.5 dpc and 16.5 dpc. WNT5A, WNT10B, and *Sfrp1* were not observed in the late stage of lung development (17.5 and 18.5 dpc, and neonates) (Figure 3C). The known interaction of *Sfrp1* with WNT and the correlation of *Sfrp1* staining with WNT5A and WNT10B suggest that *Sfrp1* is an important regulator of the signaling effects mediated by this protein during embryogenesis.

Impaired Distal Lung Development in the *Sfrp1* Knockout Mice

The detection of *Sfrp1* in the distal lung solely between 13.5 and 16.5 dpc and its subsequent absence in the later stages of lung morphogenesis suggested that *Sfrp1* could be particularly important for distal lung formation. To determine the role of *Sfrp1* on lung morphogenesis, we carefully analyzed developing lung tissue harvested from *Sfrp1*^{-/-} mice. Gross examination revealed no obvious abnormality in adult lung tissue between these wild-type and *Sfrp1*^{-/-} mice (data not shown). Further, the knockout animals appeared normal from birth through adulthood (data not shown). However, microscopic examination of adult lung tissue sections revealed that *Sfrp1*^{-/-} mice developed marked dilation of the alveolar duct of the lung (Figure 4A). Morphometric analysis revealed a significant increase ($P = 0.0028$) in mean linear intercept (MLI) at two months of age in the lungs of knockout mice (61.0 ± 11.0 ; $n = 8$) compared with wild-type animals (49.0 ± 3.0 ; $n = 8$) (Figure 4B). MLI measurements conducted on animals at six and twelve months of age showed that this abnormality was nonpro-

Figure 3. WNT5A, WNT10B, and SFRP1 expression and protein levels during lung development. **A:** RT-PCR for *Wnt5a*, *Wnt10b*, *Sfrp1* and GAPDH reveals expression of *Wnt5a*, *Wnt10b*, and *Sfrp1* at embryonic stages (14.5, 16.5, and 18.5 dpc) but not later including newborn, five days post partum (5 pp), or fourteen weeks of age. **B:** Western blot analyses for WNT5A, WNT10B, and SFRP1 on mouse lung tissue protein reveals bands of appropriate molecular weight (43 kDa for WNT5A; 40 kDa for WNT10B; 36 kDa for SFRP1) present at 14.5 dpc, but not at 5 days post partum (5 pp). **C:** Using specific antibodies for WNT5A, WNT10B, and SFRP1, immunofluorescent analyses were performed on lung tissue sections from the lungs of mice during early lung development (10.5 to 18.5 dpc). H&E staining was performed for comparison.

gressive; physiological measurements demonstrated that these architectural changes were not associated with changes in lung compliance (data not shown). Therefore, these findings in the knockout mice are consistent with a development defect and not with an ongoing destructive process.

SFRP1 Regulates Mesenchymal Proliferation During Lung Embryogenesis

Due to the general role SFRP1 is known to play in mesenchymal proliferation and differentiation,¹⁶ we hypothesized that the observed ductal dilatation in the adult mice

Figure 4. *Sfrp1* alters distal lung development without affecting lung differentiation. **A:** At two months of age, the wild-type (WT) mice (**upper**) exhibited normal lung architecture. In contrast, the *Sfrp1*^{-/-} mice (**lower**) exhibited dilatation of the alveolar duct. Similar findings were observed at the six-month and one-year time points. **B:** MLI of wild-type and *Sfrp1*^{-/-} mice ($P = 0.0028$).

may have resulted from a defect early in lung development in the mesenchymal component surrounding the forming ductal units. We therefore assessed the state of the mesenchymal component in the developing lung of *Sfrp1*^{-/-} mice compared with wild-type. Morphologically the lungs of the *Sfrp1*^{-/-} mice at 16.5 dpc exhibit dilatation at the terminal ducts. Greater ductal dilatation continues to be seen in the lungs of the *Sfrp1*^{-/-} mice at 18.5 dpc, however as alveolar formation is initiated the difference between wild-type and *Sfrp1*^{-/-} mice is grossly less obvious. Staining for smooth muscle actin was detected around the developing airways and blood vessels at 14.5 dpc in both the wild-type and the *Sfrp1*^{-/-} mice. This staining intensified in the wild-type mice at 16.5 dpc; however, a marked decrease in staining was observed in the knockout mice at this time point (Figure 5). Of note, no difference in staining for elastin was detected between the groups at any of the time points tested (data not shown). We performed immunohistochemistry on lung tissue sections from wild-type and *Sfrp1*^{-/-} mice using

antibodies raised against proximal (SCGB1A1; also known as CC10) and distal (SFTPA1; also known as SP-A and SFTPC; also known as SP-C) markers of lung epithelium. No differences were detected in staining between wild-type and *Sfrp1*^{-/-} lung tissue for the proximal marker, CC10 (data not shown) or the distal lung markers, proteins SP-A and SP-C (data not shown). These results indicate that loss of *Sfrp1* does not alter epithelial cell differentiation in proximal or distal structures in the developing lung despite the abnormality seen in smooth muscle cell content. Therefore, the loss of *Sfrp1* alters normal lung development as early as 16.5 dpc subsequently leading to abnormal formation of the alveolar duct.

Increased β -Catenin in the *Sfrp1*^{-/-} Mice During Lung Development

As shown above, expression of *Sfrp1* is tightly regulated during lung development between 14.5 dpc and 18.5 dpc; therefore, we examined β -catenin levels during these time points to determine whether loss of *Sfrp1* affected the canonical Wnt signaling pathway. Western blot analyses conducted on the lung tissue protein of embryonic and newborn mice demonstrated significantly enhanced nuclear translocation of β -catenin in the knockout mice at both 16.5 and 18.5 dpc (Figure 6A). This increase was not seen in the newborn or adult lung (data not shown) as would be expected because *Sfrp1* is not expressed in the adult. These results demonstrate that loss of *Sfrp1* increased Wnt signaling through the canonical pathway during this embryonic time period leading to a defect in distal lung formation. Though the wild-type and *Sfrp1*^{-/-} lungs were morphologically similar at E18.5, we assert that the impairments in cell signaling that occurred in the knockout mice during this time interval set the stage for the subsequent impairments in distal lung formation. Indeed, the defect in alveoli was evident as early as two weeks after birth.

Altered Wnt Signaling in the *Sfrp1* Knockout Mice During Lung Development

To identify whether loss of *Sfrp1* led to changes in Wnt signaling during embryogenesis, the expression of Wnt5a

Figure 5. Altered lung formation in *Sfrp1*^{-/-} mice. Lung sections of wild-type (WT) and *Sfrp1*^{-/-} mice at 14.5, 16.5, and 18.5 dpc were examined and stained for SMA. Morphologically the lungs of the *Sfrp1*^{-/-} mice at 16.5 dpc exhibit dilatation at the terminal ducts (T). Greater ductal dilatation continues to be seen in the lungs of the *Sfrp1*^{-/-} mice at 18.5 dpc compared with wild-type. Immunostaining for SMA demonstrated marked reduction of SMA-positive cells in the *Sfrp1*^{-/-} mice. The **arrowheads** indicates peribronchial smooth muscle cells. B indicates bronchus; V, large vessel.

Figure 6. SFRP1 regulates Wnt signaling and mesenchymal development in the lung. **A:** Western blot for β -catenin was performed on the nuclear protein extracts of lung tissue obtained from 14.5, 16.5, and 18.5 dpc wild-type (WT) and $Sfrp1^{-/-}$ mice. Western blot for nucleophosmin was used as a protein loading control. β -catenin levels accumulated in the nucleus of $Sfrp1^{-/-}$ mice when compared with wild-type. **B:** Using specific primers for mouse Wnt5a, Wnt7b, and β -actin, SYBR Green quantitative PCR was performed on the RNA isolated from the lung tissue of wild-type (white bars) and $Sfrp1$ knockout mice (black bars) at embryonic day 14.5, 16.5, and 18.5. Expression levels for Wnt5a and Wnt7b were normalized for β -actin (y axis; $n = 4$ in each group, $*P < 0.05$). Expression of Wnt5a and Wnt7b are up-regulated in the $Sfrp1^{-/-}$ mice at 18.5 dpc. **C:** Real-time RT-PCR analysis of mRNA from mice embryos. Compared with wild-type mice (white bar), $Sfrp1^{-/-}$ mice exhibited significant reduction of MMP9 mRNA expression. **D:** The number of inflammatory cells isolated from the lung lavage was determined for wild-type and $Sfrp1^{-/-}$ mice under control conditions or after 1 day or 2 weeks of cigarette smoke exposure. There is no difference between wild-type and knockout mice at all three time points.

and Wnt7b was examined. These Wnts were chosen because they are known to exert key effects on lung development.²⁸ Wnt5a and Wnt7b expression were significantly increased by RT-PCR in the knockout mice at embryonic day 18.5 (Figure 6B). This increased expression in the knockout mice occurs during a period when Wnt expression is normally down-regulated in the developing lung (see Figure 3C).

Sfrp1^{-/-} Mice Decreases MMP Expression in Lung

Since the re-expression of SFRP1 in the adult tissue leads to up-regulation of MMP expression (Figure 2E), we examined whether Sfrp1 modulated MMP production during development. MMP9 levels were measured in mouse lungs from wild-type and $Sfrp1^{-/-}$ mice. As shown in Figure 6C, $Sfrp1^{-/-}$ mice exhibited decreased MMP9 expression at 16.5 and 18.5 dpc when it was normally highly expressed in developing lung of the wild-type mice ($P = 0.0011$; wild-type versus $Sfrp1^{-/-}$). There was no significant difference in TIMP1 expression levels (data not shown). Thus, SFRP1 correlates positively with MMP expression in both the adult and developing lung. While the presence of SFRP1 expression in adult SAE cells up-regulated lung proteases, the lack of Sfrp1 expression during embryogenesis in the knockout mice resulted in inappropriately low MMP lung levels. These findings denote a previously unknown role of SFRP1 in the regulation of lung protease expression.

SFRP1 Does Not Alter Inflammation in Response to Cigarette Smoke Exposure

Given its induction in emphysema, we aimed to determine whether SFRP1 altered the inflammatory response of the lung to acute or subacute smoke exposure. To do so, we exposed wild-type and $Sfrp1^{-/-}$ mice to one day (acute) and two weeks (subacute) of cigarette smoke exposure. These studies demonstrated that cigarette smoke generated an equivalent increase in lung lavage cellularity in the wild-type and $Sfrp1^{-/-}$ mice (Figure 6D). Similar findings were seen after four months (chronic) of smoke exposure (data not shown).

Discussion

The expression of developmental genes in the adult lung is theorized to be part of a well-orchestrated maintenance program to preserve structural integrity. Specifically, the repair/regeneration process initiated in response to lung injury is believed to recapitulate mechanisms involved in normal lung morphogenesis.^{29,30} The findings from the present study suggest, however, that the developmental gene *Sfrp1* exerts distinct effects in the adult lung from those that occur during lung morphogenesis. Here we demonstrate that loss of *Sfrp1* disrupts proper alveolar formation by regulating Wnt signaling and mesenchymal growth and turnover during lung development, its expression in the adult lung induces proteases known to lead to tissue destruction and emphysema formation.^{18,31,32}

SFRP1 up-regulated MMP1 and MMP9 production in both pneumocytes and small airway epithelial cells. This is significant because the small airways are the site of an intense inflammatory and remodeling process that results in severe airway obstruction in emphysema.³³ Furthermore, MMP1 and MMP9 have been linked to emphysema in both animal models^{24,31} and patients.^{34,35} The induction of MMP1 by cigarette smoke is dependent on ERK activation.²³ In this study, SFRP1 activated ERK and induced MMP1 expression within the lung epithelium. Thus, it reproduces biological processes that are linked to the development of cigarette smoke-induced emphysema.²³ Indeed, this is the first study to demonstrate that SFRPs, inhibitors of WNT, directly up-regulate the expression of MMPs. Prior research had shown that WNTs and nuclear translocation of β -catenin stimulate MMP7 production,^{36,37} while the WNT antagonists SFRP3 and DKK1 inhibit MMP expression.^{38,39} In addition, it was recently discovered that SFRPs directly inhibit the activity of the metalloproteinase Xoloid-related (Xlr).⁴⁰ Our data coupled with these previous findings indicate that the regulation of individual MMPs by the WNT signaling pathways is complex. Our study demonstrates that proteases known to play a key role in the pathogenesis of emphysema are up-regulated by SFRP1. While in the adult lung this would lead to lung tissue destruction, the induction of lung proteases during development likely allows proper alveolar formation to occur. Therefore, the absence of SFRP1 during embryogenesis would impair protease-dependent tissue remodeling, thus resulting in alveolar ductal dilation. This is consistent with our finding of decreased MMP expression in the lung during embryonic development in the *Sfrp1*^{-/-} mice.

As early as two weeks of age, the deficiency in *Sfrp1* resulted in dilated alveolar ducts in the knockout mice when compared with wild-type littermate mice. This defect did not progress over time, indicating that it resulted from events that occurred specifically during lung morphogenesis. Indeed, *Sfrp1* expression was detected in the lung only during a defined period of embryogenesis and most predominantly in the mesenchyme and distal lung structures beginning at 16.5 dpc and ending 18.5 dpc. The absence of *Sfrp1* expression in the *Sfrp1*^{-/-} mice enhanced nuclear translocation of β -catenin, resulting in dysregulation of the mesenchyme and deficient alveolar formation. β -catenin, the central target of the canonical pathway, is detected in the lung epithelium and surrounding mesenchyme during lung development.⁴¹ Recently, the loss of β -catenin expression during lung morphogenesis was shown to severely impair the formation of distal airway structures.⁴² Furthermore, aberrant β -catenin activation in pulmonary fibrosis was associated with cellular hyperplasia and honeycombing of the lung.⁷ Given the central role of β -catenin in distal lung development, the changes in β -catenin nuclear translocation observed in the *Sfrp1*^{-/-} mice likely exerted an important role in the aberrant mesenchymal growth and deficient alveolar formation that occurred in these mice.

Multiple components of the Wnt signaling pathway have been implicated to play a role in lung formation.^{43,44} The loss of *Wnt5a* expression in mice causes overexpan-

sion of the distal epithelial airways and thickening of the mesenchymal interstitium.²⁵ Conversely, the transgenic expression of *Wnt5a* during early lung development resulted in decreased epithelial branching and dilation of the distal airways.⁴⁵ *Wnt7b* knockout mice exhibited mesenchymal proliferation that led to the development of hypoplastic lungs.¹⁰ This is the first description of a lung development phenotype attributable to the loss of an SFRP. Given the known effects of WNTs on lung morphogenesis, it is conceivable that the disruption of the distal lung formation in the knockout mice was a consequence of aberrant *Wnt* expression. Our immunofluorescence studies demonstrated that *Sfrp1* and WNT5A colocalize to the developing mesenchyme during embryogenesis. Thus, the dysregulation of *Wnt5a* in the knockout mice may well have contributed to the abnormal mesenchymal proliferation and deficient actin synthesis observed in the lung.

The loss of alveolar tissue in emphysema greatly impairs gas exchange and physiological function in affected patients. Retinoic acid, a dietary element that is crucial in lung formation, reverses elastase-induced emphysema in rodents.⁴⁶ These findings have sparked interest in treatment strategies aimed at inducing developmental processes to reverse the destructive changes seen in emphysema.⁴⁷ We identified the selective up-regulation of SFRP1 in human emphysema. However, rather than serving a reparative role, our results indicate that expression of this developmental gene in the adult lung triggers destructive mechanisms that results in the loss of lung tissue. Future studies will explore whether long-term expression of SFRP1 *in vivo* leads to matrix loss and emphysema formation. If so, targeting SFRP1 with pharmacotherapy may counteract the damaging processes that are a central component of this disease.

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