Suppression of von Hippel-Lindau Protein in Fibroblasts Protects against Bleomycin-Induced Pulmonary Fibrosis

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Abstract

We have reported that von Hippel-Lindau protein (pVHL) expression is elevated in human and mouse fibrotic lungs and that overexpression of pVHL stimulates fibroblast proliferation. We sought to determine whether loss of pVHL in fibroblasts prevents injury and fibrosis in mice that are treated with bleomycin. We generated heterozygous fibroblast-specific pVHL (Fsp-VHL) knockdown mice (Fsp-VHL^{+/-}) and homozygous Fsp-VHL knockout mice (Fsp-VHL^{-/-}) by crossbreeding *vhlh* 2-lox mice (VHL^{fl/fl}) with Fsp-Cre recombinase mice. Our data show that Fsp-VHL^{-/-} mice, but not Fsp-VHL^{+/-} mice, have elevated red blood cell counts, hematocrit, hemoglobin content, and expression of hypoxia-inducible factor (HIF) targets, indicating HIF activation. To examine the role of pVHL in bleomycin-induced lung injury and fibrosis *in vivo*, we administered PBS or bleomycin to age-, sex-, and strain-matched 8-week-old VHL^{fl/fl}, Fsp-VHL^{+/-}, and Fsp-VHL^{-/-} mice. In Fsp-VHL^{+/-} and Fsp-VHL^{-/-} mice, bleomycin-induced collagen accumulation, fibroblast proliferation, differentiation, and matrix protein dysregulation were markedly attenuated. Suppression of pVHL also decreased bleomycin-induced Wnt signaling and prostaglandin E₂ signaling but did not affect bleomycin-induced

initial acute lung injury and lung inflammation. These results indicate that pVHL has a pivotal role in bleomycin-induced pulmonary fibrosis, possibly via an HIF-independent pathway. Paradoxically, pVHL does not affect bleomycin-induced lung injury and inflammation, indicating a separation of the mechanisms involved in injury/inflammation from those involved in pulmonary fibrosis.

Keywords: von Hippel-Lindau protein; hypoxia-inducible factor; pulmonary fibrosis; lung injury; inflammation

Clinical Relevance

This study provides the first evidence that fibroblast-specific von Hippel-Lindau protein (pVHL) is required for bleomycin-induced pulmonary fibrosis but not for bleomycin-induced lung injury and inflammation. pVHL likely regulates fibrogenesis by altering bleomycin-mediated Wnt signaling and prostaglandin E₂ signaling via a hypoxia-inducible factor-independent pathway.

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Idiopathic pulmonary fibrosis (IPF) is a devastating disease associated with progressive and irreversible destruction of the lung architecture (1, 2). The cause of IPF remains largely unknown, and the annual incidence of IPF is between 4.6 and 16.3 per 100,000 people, and is rising (1–4). Patients with IPF have a poor median survival of 2.5–3.5 years after diagnosis, and the treatment options are limited to lung transplantation (1, 3, 4). Hallmarks of pulmonary fibrosis include lung fibroblast proliferation, activation, differentiation, and exaggerated deposition of extracellular matrix (ECM) proteins, especially collagen and fibronectin (Fn) (5, 6). However, the detailed molecular mechanisms underlying altered fibroblast biology during fibrosis remain elusive.

Von Hippel-Lindau protein (pVHL) was first identified as a tumor suppressor, because the mutation of VHL gene was associated with tumors via activation of hypoxia-inducible factor (HIF) (7–11); however, mounting evidence suggests that pVHL also has HIF-independent functions in cytoskeleton dynamics, alveolar epithelial functions (12, 13), epithelial cilia maintenance (14), ECM assembly, and cell proliferation (14–21). Although the function of pVHL in human disease is mostly elucidated in a loss-of-function fashion (22), recent reports have shown that expression levels of the VHL gene and protein are increased in patients with chronic obstructive pulmonary disease and IPF (23, 24).

We have previously reported that lungs from patients with IPF express increased levels of pVHL in fibroblastic foci (24). Bleomycin treatment also induces pVHL in mouse lung fibroblasts (MLFs), but not in alveolar type II cells (24). Overexpression of pVHL increases lung fibroblast proliferation, induces Fn, collagen, and Fn receptor $\alpha 5$ integrin subunit, and increases activation of focal adhesion kinase (FAK) (24). Moreover, suppression of pVHL prevents transforming growth factor (TGF)- $\beta 1$ -induced proliferation of mouse embryonic fibroblasts (24). These observations indicate that elevated expression of pVHL results in the aberrant expression of Fn and collagen, activation of integrin/FAK signaling, fibroblast proliferation, and fibrosis, and suggest that pVHL may have a role in the molecular pathogenesis of IPF.

In this study, we provide novel evidence that fibroblast-specific pVHL (Fsp-VHL) is required for the development of pulmonary fibrosis in mice treated with bleomycin. We generated novel strains of fibroblast-specific knockout or knockdown mice to determine whether suppression of pVHL in fibroblasts prevents bleomycin-induced fibrosis independent of HIF activation. Paradoxically, we found that pVHL does not appear to affect bleomycin-induced lung injury and inflammation, indicating a specific role of pVHL on pulmonary fibrosis only. Moreover, suppression of pVHL inhibits activation of Wnt and rescues the antifibrotic PGE signaling. Together, our results indicate that pVHL plays a role in the development of pulmonary fibrosis.

Materials and Methods

Mice

Homozygous *vhlh* 2-lox mice (provided by Dr. Volker Haase, Vanderbilt University, Nashville, TN) and homozygous fibroblast-specific protein (Fsp)-Cre recombinase mice (provided by Drs. Anthony J. Trimboli from laboratories of Dr. Gustavo Leone and Michael C. Ostrowski, Ohio State University, Columbus, OH) were cross-bred and back-crossed to generate mice with fibroblast-specific knockout of pVHL (25, 26). Tail DNA from offspring was examined by PCR to confirm the genotypes with the primers listed in Table E1 in the online supplement on a Mastercycler epGradient S model (Eppendorf, Hamburg, Germany) (25, 26). Animal studies were conducted according to National Institutes of Health guidelines, and the experimental protocols were approved by the University of Illinois at Chicago (Chicago, IL) Institutional Animal Care and Use Committee.

Delivery of Bleomycin and PBS to Mouse Lungs

Saline or bleomycin (50 μ l, 0.045 units; Sigma-Aldrich, St. Louis, MO) dissolved in saline was delivered intratracheally as described previously (24, 27). Briefly, animals were sedated and secured. A flexible metal wire guided the placement of an angiocath, and bleomycin was administered by direct instillation into the angiocath (two 25- μ l aliquots 2 min apart) using a Hamilton syringe.

Measurements of Prostaglandin E₂ by Mass Spectrometry

Quantitative analysis of prostaglandin (PG) D₂ and PGE₂ were performed by ultra-high-performance liquid chromatography (UHPLC) and mass spectrometry as described previously (28). Briefly, the samples were separated using UHPLC on a Shimadzu (Columbia, MD) Nexera UHPLC system equipped with an Acquity ultra-performance liquid chromatography ethylene bridged hybrid (UPLC BEH) C₁₈ (2.1 mm $\times$ 50 mm, 1.7 μ m) analytical column (Waters, Milford, MA) at 50°C. An isocratic mobile phase was used, consisting of acetonitrile/aqueous 0.1% formic acid (40:60, vol/vol) at a flow rate of 1.2 ml/min. The UHPLC system was interfaced to an LCMS-8040 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) that was operated using negative ion electrospray. Isomeric PGD₂ and PGE₂ were measured using a selected reaction monitoring transition of *m/z* 351 to *m/z* 271, and the transition of *m/z* 355 to *m/z* 275 was selected for the internal standards d4-PGE₂ and d4-PGD₂.

Flow Cytometry Analysis

MLFs were isolated from pVHL wild-type or knockout mice and plated. For each sorting, we collected 4×10^6 cells and washed them with PBS. Cells were then fixed with 4% formaldehyde for 15 minutes at room temperature and then washed with PBS twice. After permeabilizing with ice-cold 90% methanol for 30 minutes on ice, cells were washed twice with PBS and incubated with 5% BSA/PBS for 30 minutes on ice for blocking nonspecific binding. Cells were incubated with primary antibodies against Proliferating cell nuclear antigen (PCNA) (Sigma PLA-0079, rabbit) or Fsp-1 (Sigma F4771, mouse) for 1 hour at room temperature. After three washes with PBS, cells were resuspended and incubated with secondary antibodies, R-phycoerythrin (RPE)-conjugated goat anti-rabbit IgG (H+L) (P2771MP; ThermoFisher Scientific, Grand Island, NY) or allophycocyanin (APC)-conjugated goat anti-mouse IgG (heavy chain [H]+light chain [L]) (A865; ThermoFisher Scientific) for 30 minutes at room temperature. After three washes with PBS, cells were analyzed and sorted at the University of Illinois at Chicago

Research Resources Center Flow Cytometry Service facility.

Statistical Analysis

Results are expressed as mean ($\pm$ SEM). Data were analyzed using one-way ANOVA. When the ANOVA indicated a significant difference, we performed the Bonferroni correction post-test to compare all individual groups. Statistical significance was set at a 0.05 level.

Detailed MATERIALS AND METHODS can be found in the online supplement.

Results

Fibroblast-Specific Knockout of pVHL and Activation of HIF

We reported previously that pVHL is up-regulated in fibroblasts in fibrotic tissues, and that overexpression of pVHL induces fibroblast proliferation (24), suggesting that pVHL may play a role in the development of IPF. To address this, we investigated whether loss of pVHL prevents fibrosis. We generated strains of Fsp-VHL knockdown (Fsp-VHL^{+/-}) and knockout mouse (Fsp-VHL^{-/-}) by cross-

breeding the homozygous *vhlh* 2-lox (VHL^{fl/fl}) mice with homozygous Fsp-Cre mice (provided by Dr. Volker Haase of Vanderbilt University and Dr. Gustavo Leone of the Ohio State University, respectively) (Figure 1A) (25, 26). *vhlh* 2-lox mice contain two loxP sites flanking the VHL promoter and the first exon, and they develop normally. In Fsp-Cre mouse, Cre expression is driven by the Fsp-1 (also known as S100A4) promoter (26, 29). Although other cell types, such as endothelial cells and vascular smooth muscle cells, may also express Fsp-1 (30), in lungs, fibroblasts,

Figure 1. Conditional knockout of von Hippel-Lindau protein (pVHL) in fibroblasts. (A) Diagrammatic scheme of generating heterozygous fibroblast-specific pVHL (Fsp-VHL) knockdown mice (Fsp-VHL^{+/-}) and homozygous pVHL knockout mice (Fsp-VHL^{-/-}) by cross-breeding *vhlh* 2-lox (VHL^{fl/fl}) mice with Fsp-Cre recombinase mice and the subsequent back-crossing. (B) Genotypes of these mice were confirmed by polymerase chain reaction using DNA obtained from mouse tails. (C) Mouse lung fibroblasts (MLFs) were isolated from these mice and were used to measure the mRNA levels of pVHL. (D–F) Mice were anesthetized and blood was drawn to measure the amounts of red blood cells (RBCs), hematocrit (HCT), and hemoglobin (HGB) ($n \geq 5$). (G–I) The MLFs isolated from these mice were used to measure the mRNA levels of vascular endothelial growth factor (G), erythropoietin (H), and Glut 1 (I) ($n = 5$) for each genotype. (J) The MLFs isolated from VHL^{fl/fl} mice were used to perform fluorescence-activated cell sorting (FACS) analysis after Fsp-1 antibody labeling ($n = 8$). Results are expressed as mean ($\pm$ SEM). * $P < 0.05$; ** $P < 0.01$. *Vhlh*, gene symbol for murine VHL; Vhl fl, floxed VHL; Vhl wt, wild-type VHL.

but not macrophages or alveolar epithelial type II cells (ATII) cells, express Fsp-1, and Fsp-1 is expressed in procollagen-positive cells, suggesting that Fsp-1 is relatively fibroblast specific in lungs (31). The genotypes of VHL^{fl/fl}, Fsp-VHL^{+/-}, and Fsp-VHL^{-/-} mice were confirmed by PCR of the DNA samples obtained from mouse tails (Figure 1B). We used a combination of mice with genotypes VHL^{fl/fl}, VHL^{fl/wt}, and VHL^{wt/wt} as wild-type control mice (VHL^{+/+}). We also isolated MLFs from these mice and confirmed the graded knockdown of pVHL mRNA in Fsp-VHL^{+/-} and Fsp-VHL^{-/-} mice (Figure 1C). We tested two commercially available pVHL antibodies to detect pVHL proteins in MLF; however, none of them showed a band even in wild-type MLFs, suggesting a limitation of pVHL antibody on MLFs. pVHL is a known ubiquitin E3 ligase that targets HIF α for proteasome degradation, and loss of pVHL increases HIF activity (22, 32). As expected, Fsp-VHL^{-/-} mice displayed elevated levels of hematocrit, red blood cells, and hemoglobin (Figures 1D–1F). However, Fsp-VHL^{+/-} mice displayed the same levels of red blood cells, hematocrit, and hemoglobin as wild-type mice (Figures 1D–1F). Furthermore, MLFs isolated from Fsp-VHL^{-/-} mice, but not Fsp-VHL^{+/-} mice, exhibited elevated mRNA levels of HIF downstream genes, vascular endothelial growth factor, erythropoietin (EPO), and glucose transporter (Glu) 1 (Figures 1G–1I). These results suggest that Fsp-VHL^{-/-} mice, but not Fsp-VHL^{+/-} mice, displayed activation of the HIF pathway. Thus, Fsp-VHL^{+/-} and Fsp-VHL^{-/-} mice are an ideal model to dissect the HIF-dependent and HIF-independent functions of pVHL during fibrosis. To address the percentage of Fsp-1-positive population in MLFs, we isolated MLFs from VHL^{fl/fl} mice, labeled them with Fsp-1 antibody, and performed fluorescence-activated cell sorting analysis. We found that 93.97 ($\pm$ 3.84) % (mean [$\pm$ SEM]) of MLF were Fsp-1 positive (Figure 1J). In pooled MLFs isolated from five Fsp-VHL^{-/-} mice, 96.21% of cells were Fsp-1 positive. These results suggest that knockout of pVHL does not alter the population of Fsp-1-positive lung fibroblasts.

Suppression of Fsp-VHL Prevents Bleomycin-Induced Fibrosis *In Vivo*

To assess whether pVHL is required for bleomycin-induced fibrosis, we administered 0.045 U bleomycin/mouse intratracheally to VHL^{+/+}, Fsp-VHL^{+/-}, and

Figure 2. Suppression of Fsp-VHL prevents bleomycin (Bleo)-induced fibrosis *in vivo*. Wild-type (VHL^{+/+}) mice, Fsp-VHL^{+/-} mice, and Fsp-VHL^{-/-} mice were administered PBS or bleomycin intratracheally, and 21 days later the lungs of these mice were collected and used for collagen analysis by the Sircol assay (A), real-time quantitative RT-PCR (qRT-PCR) for collagen (col)-1a1 and col1a2 (B and C), and Masson's trichrome staining (D). RPL19 gene was used as the internal control for qRT-PCR ($n \geq 5$). Results are expressed as mean ($\pm$ SEM). * $P < 0.05$; ** $P < 0.01$; * and ** indicate differences between VHL^{+/+}/PBS and other groups. Scale bars: 100 μ m.

Fsp-VHL^{-/-} mice. Mice administered with the same volume of PBS were used as control. At 21 days after administration, we collected the lung tissues of these mice and determined the collagen content, a hallmark of fibrosis. As shown in Figure 2A, despite a slight increase of basal collagen levels in Fsp-VHL^{-/-} mice, bleomycin-induced collagen expression was inhibited in both Fsp-VHL^{+/-} and Fsp-VHL^{-/-} mice. Similarly, elevation of the mRNA levels of collagen (col)-1a1 and col1a2 was inhibited in Fsp-VHL^{+/-} and Fsp-VHL^{-/-} mice (Figures 2B and 2C). We confirmed these observations with Masson's trichrome staining (Figure 2D). These results suggest an HIF-independent function of pVHL in the pathogenesis of pulmonary fibrosis.

Suppression of Fsp-VHL Prevents Bleomycin-Induced Fibroblast Proliferation, Differentiation, and Matrix Protein Dysregulation *In Vivo* and *In Vitro*

Pulmonary fibrosis is associated with fibroblast proliferation, differentiation, and ECM protein dysregulation. To determine

whether suppression of pVHL may prevent fibrosis via inhibition of fibroblast proliferation *in vivo*, we determined the expression levels of PCNA in the lung tissues collected from VHL^{+/+}, Fsp-VHL^{+/-}, and Fsp-VHL^{-/-} mice 21 days after administration of PBS or bleomycin. We found that bleomycin-induced PCNA was inhibited in Fsp-VHL^{+/-} and Fsp-VHL^{-/-} mouse lungs (Figure 3A). We also found that suppression of pVHL inhibited bleomycin-induced α -smooth muscle actin (α -SMA; Acta2; Figure 3B). These data suggest that suppression of Fsp-VHL inhibits fibroblast proliferation and differentiation. We then assessed the population of PCNA-positive MLFs. We found that MLFs isolated from VHL^{fl/fl}, Fsp-VHL^{+/-}, and Fsp-VHL^{-/-} mice contained equal amounts of PCNA-positive (PCNA⁺) cells ($\sim$ 90%; Figure 3C). We collected these PCNA⁺ MLFs and compared the expression levels of proteins that participate in fibrosis. We found that PCNA⁺ MLFs from Fsp-VHL^{-/-} mice expressed lower levels of integrin α 5 and

Figure 3. Suppression of Fsp-VHL prevents bleomycin-induced fibroblast proliferation, differentiation, and matrix protein dysregulation *in vivo*. We harvested lungs of VHL^{+/+}, Fsp-VHL^{+/-}, and Fsp-VHL^{-/-} mice 21 days after administration of PBS and bleomycin. Mouse lungs were homogenized and used to extract total RNA for the measurement of mRNA levels of mouse proliferating cell nuclear antigen (PCNA) (A), α -smooth muscle actin (SMA) (B), integrin α 5 (E), matrix metalloproteinase (MMP) 2 (F), and MMP9 (G) by real-time qRT-PCR. RPL19 gene was used as the internal control for qRT-PCR ($n \geq 5$). (C) We isolated MLFs from VHL^{+/+}, Fsp-VHL^{+/-}, and Fsp-VHL^{-/-} mice and labeled them with PCNA antibody, followed by FACS analysis. (D) PCNA-positive MLFs from sorting in (C) were used to determine the levels of proteins participating in fibrosis. Quantification is shown in the *right panel* ($n = 6$). (H and I) N12 cells were transfected with control small interfering (siRNA) or siRNA against MMP9 and incubated for 2 days. Cells were lysed and cell lysates were used to determine the expression levels of indicated proteins ($n = 4$). Results are expressed as mean ($\pm$ SEM). * and # $P < 0.05$; ** $P < 0.01$. In E–G, * and ** indicate differences between VHL^{+/+}/PBS and other groups. Fn, fibronectin.

Figure 4. The Wnt/Frizzled pathway is essential for the pVHL-mediated extracellular matrix dysregulation during fibrosis. (A) N12 cells infected with adenovirus (Ad)-green fluorescent protein (GFP) (GFP1, GFP2, and GFP4) or Ad-VHL-HA (VHL1, VHL3, VHL4) were used for microarray analysis with Affymetrix GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA). The overexpression of pVHL was validated in our previous publication (24). ANOVA was used to calculate significance of the differential expression. Microarray expression data were analyzed by Pathway Studio (Elsevier, Amsterdam, the Netherlands) to represent all known relationships and potential interactions between the differentially expressed genes. The numerical scale of fold change is shown in the bottom of the heat map. In clustering analysis, Euclidean distance is shown at the upper left corner to define the distance between gene expression levels. (B–D) We measured the mRNA levels of Wnt5b (B), Wnt16 (C), and lipoprotein receptor-related protein 6 (Lrp6) (E) in lungs of VHL $^{+/+}$, Fsp-VHL $^{+/-}$, and Fsp-VHL $^{-/-}$ mice administered PBS and bleomycin by real-time qRT-PCR. RPL19 gene was used as the internal control for qRT-PCR ($n \geq 5$). (E) We infected N12 human normal lung fibroblasts with control Ads (Ad-Null) or Ad encoding Wnt5b (Ad-Wnt5b) or Wnt16 (Ad-Wnt16), and then lysed the cells and determined the expression levels of various proteins ($n = 5$). Quantification is shown in the right panel. Results are expressed as mean ($\pm$ SEM). * $P < 0.05$; ** $P < 0.01$. In E and G, * and ** indicate differences between VHL $^{+/+}$ /PBS and other groups. Fz, frizzled.

matrix metalloproteinase (MMP)9 than those from VHL^{fl/fl} and Fsp-VHL^{+/-} mice. However, levels of α -SMA, collagen, Fn, and PCNA remained the same in these mice (Figure 3D). These results suggest that pVHL is required for the maintenance of integrin α 5 and MMP9 in proliferating fibroblasts.

During fibrosis, expression of ECM proteins is dysregulated. Previously, we have shown that overexpression of pVHL stimulates expression of Fn and integrin α 5 β 1 and activates FAK in lung fibroblasts, leading to fibroblast proliferation (24). We showed that, in mouse lungs collected 21 days after administration of PBS and bleomycin, suppression of pVHL in fibroblasts prevented the expression of integrin α 5 (Figure 3E). MMP2 and MMP9 regulate the dysregulation of ECM during fibrosis (33, 34). Although suppression of pVHL had little effect on bleomycin-induced MMP2, MMP9 expression was inhibited in these mice (Figures 3F and 3G). These results indicate that pVHL may regulate fibroblast ECM deposition via an MMP9-specific pathway. Consistently, suppression of MMP9 in N12 normal human lung fibroblasts did not alter expression levels of MMP2, Fn, collagen, PCNA, or integrin α 4 and α 5, but it was able to inhibit integrin β 1 (Figures 3H and 3I). Thus, pVHL may regulate fibroblast ECM via an integrin α 5 and MMP9/integrin β 1 pathway.

To determine the effect of pVHL on the early fibrotic responses, we determined the expression levels of these genes in the lung tissues from VHL^{+/+}, Fsp-VHL^{+/-}, and Fsp-VHL^{-/-} mice 5 days after administration of PBS or bleomycin. We found that bleomycin-induced PCNA, MMP2, and MMP9 were inhibited in Fsp-VHL^{+/-} and Fsp-VHL^{-/-} mouse lungs, whereas α -SMA (Acta2) levels were similar in these mice (Figure E1). Interestingly, we found that collagen (col1a1 and col1a2) and integrin α 5 were up-regulated in bleomycin-treated Fsp-VHL^{+/-} and Fsp-VHL^{-/-} mice (Figure E1). Therefore, in spite of the differential regulation of fibrotic responses in the early and late stages, the requirement of pVHL for bleomycin-induced MMP9 is preserved in both stages.

The Wnt/Frizzled Pathway Is Essential for the pVHL-Mediated ECM Dysregulation during Fibrosis

Emerging data suggest that Wnt signaling plays a critical role in IPF (35–38). Patients with IPF have elevated levels of Wnt-2b, 5b, and frizzled (Fz)-related proteins, and β -catenin targets are known to promote fibrosis or proliferation (39–41). Although the Wnt ligands are distinct, they share the same core elements in canonical Wnt signaling through β -catenin stabilization and translocation to the nucleus. To study whether pVHL regulates Wnt signaling, we overexpressed pVHL in N12 normal human lung fibroblasts and performed a microarray analysis, observing that overexpression of pVHL increased Wnt signaling (Figure 4A). In Fsp-VHL^{+/-} and Fsp-VHL^{-/-} mouse lungs harvested 21 days after administration of PBS or bleomycin, bleomycin-induced expression of Wnt5b and Wnt16 was inhibited (Figures 4B and 4C). Although bleomycin had little effect on lipoprotein receptor-related protein 6 (Lrp6) expression, suppression of pVHL decreased Lrp6 expression in bleomycin-treated mice (Figure 4D). These results suggest a correlation between Wnt signaling and pVHL expression. Similarly, in mouse lungs harvested 5 days after administration of PBS or bleomycin, we found that deletion of pVHL had limited effects on Wnt2 between PBS- and bleomycin-treated mice; however, deletion of pVHL inhibited bleomycin-induced Wnt2b, Wnt5b, and Wnt16 (see Figure E2 in the online supplement). These results show that the pVHL is required for the bleomycin-induced Wnt5b and Wnt16 in both the early and late stages of fibrosis.

To assess the significance of this pVHL–Wnt link, we overexpressed Wnt5b and Wnt16 in N12 normal human lung fibroblasts by adenovirus infection. We found that overexpression of Wnt5b and Wnt16 increased expression of Fn, integrin α 5, integrin β 1, and collagen, while decreasing expression levels of integrin α 4, MMP2, and MMP9 to various degrees (Figure 4E). However, overexpression of Wnt5b and Wnt16 had little effect on expression levels of PCNA (Figure 4E). Interestingly, overexpression of Wnt16 also induced levels of Wnt5b (Figure 4E). These results suggest that pVHL-mediated expression of integrin α 5 β 1/Fn is

controlled by the Wnt5b/Wnt16 signaling pathway.

Suppression of pVHL Protects against Bleomycin-Mediated Suppression of PGE₂ and PGD₂ in the Fibrotic Stage

Recent studies have indicated that IPF is characterized by a loss in production of PGs, including PGE₂ (42–44). PGE₂ signaling can suppress fibrogenesis by limiting lung myofibroblast differentiation and migration (44, 45). We show that bleomycin-induced fibrotic lungs have decreased PGE₂ levels, whereas Fsp-VHL^{+/-} and Fsp-VHL^{-/-} mice retained PGE₂ and PGD₂ levels after bleomycin administration (Figures 5A and 5B), suggesting that knockdown or knockout of pVHL prevents pulmonary fibrosis via maintaining the homeostasis of PGE₂ signaling.

PGE₂ is synthesized from PGH₂, and this conversion is catalyzed by PG synthase enzymes (e.g., PGE synthases) (44, 46). The conversion of PGH₂ from arachidonic acid is driven by cyclo-oxygenase (COX)-1 or COX-2 enzymes (44, 47). PGE₂ binds E prostanoïd (EP) receptors, particularly EP2 and EP4, during fibrosis (44, 48). To address the mechanism underlying the restoration of PGE₂ signaling in Fsp-VHL^{+/-} and Fsp-VHL^{-/-} mice, we collected lung tissue samples and analyzed the gene expression of COX-1, COX-2, PGE synthase 1, 2, and 3, and EP2 by quantitative RT-PCR. Our data show that bleomycin induced the expression of COX-1, COX-2, PGE synthase 1 and 3, and EP2 (Figures 5C–5H), which is consistent with previous reports that there is compensatory induction of PGE₂ production and COX-2 expression shortly after bleomycin treatment (49, 50). However, PGE synthase2 levels remained unchanged (Figure 5F). Interestingly, Fsp-VHL^{+/-} and Fsp-VHL^{-/-} mice did not appear to have this compensation in the bleomycin-treated group (Figures 5C–5F). These results indicate that suppression of pVHL is sufficient to maintain PGE₂ homeostasis and prevent fibrosis.

Suppression of pVHL in Fibroblasts Has No Effect on Bleomycin-Induced Lung Injury or Inflammation *In Vivo*

Inflammation may precede fibrosis, and inflammatory cells may secrete profibrotic cytokines to activate fibroblasts, contributing to fibrosis (51, 52). In bleomycin-induced lung fibrosis, there is

Figure 5. Suppression of pVHL protects against bleomycin-mediated suppression of prostaglandin (PG) E₂ and PGD₂ in the fibrotic stage. We collected the bronchoalveolar lavage fluid (BALF) from VHL^{+/+}, Fsp-VHL^{+/-}, and Fsp-VHL^{-/-} mice 21 days after administration of PBS and bleomycin and measured the quantity of PGE₂ (A) and PGD₂ (B). (C and H) We measured the mRNA levels of prostaglandin-endoperoxide synthase 1 (PTGS1)/cyclo-oxygenase (COX)-1 (C), PTGS2/COX-2 (D), PGE synthase 1 (E), PGE synthase 2 (F), PGE synthase 3 (G), and prostaglandin E receptor 2/E prostanoic acid 2 (H) in lungs of these mice by real-time qRT-PCR. RPL19 gene was used as the internal control for qRT-PCR ($n \geq 5$). Results are expressed as mean ($\pm$ SEM). * $P < 0.05$; ** $P < 0.01$; * and ** indicate differences between VHL^{+/+}/PBS and other groups.

an initial acute lung injury and inflammatory response (27, 53), and fibrosis is maximal at 21 days (54, 55). To determine whether knockdown or knockout of pVHL prevents bleomycin-mediated injury and inflammation, we collected bronchoalveolar lavage fluid (BALF) from VHL^{+/+}, Fsp-VHL^{+/-}, and Fsp-VHL^{-/-} mice 5 days after PBS or bleomycin treatment, and determined the protein amount, total cell counts, and differential cell counts. Overall, we found that VHL^{+/+}, Fsp-VHL^{+/-}, and Fsp-VHL^{-/-} mice had similar BALF protein amount, total cell counts, and differential cell counts with the exception that Fsp-VHL^{-/-} mice displayed higher protein amount and differential cell counts compared with Fsp-VHL^{+/-} mice (Figures 6A–6C). TGF- β 1 is a potent profibrotic cytokine; however, suppression of pVHL did not alter bleomycin-induced production of TGF- β 1 in BALF (Figure 6D). We also measured the gene expression levels of cytokines that participate in fibrosis in these mice. We found that deletion of pVHL had little effect on IL-1 α , IL-1 β , and IL-10 after bleomycin treatment, but the deletion inhibited bleomycin-induced IL-6 (Figure E2). These results suggest that pVHL has limited effects on bleomycin-mediated lung injury and inflammation.

Discussion

The current paradigm presumes that loss of pVHL enhances HIF, which drives fibrosis via promoting epithelial mesenchymal transition or pulmonary hypertension (22, 32, 56). Intriguingly, we have previously shown that pVHL is up-regulated in fibrotic fibroblasts, and overexpression of pVHL promotes fibroproliferation and expression of collagen and Fn, independently of HIF (24). Consistently, our data show that Fsp-VHL^{+/-} and Fsp-VHL^{-/-} mice are resistant to bleomycin-induced fibrosis (Figure 2). These results suggest that Fsp-VHL is required for fibrosis using mouse models. Moreover, our data show that both Fsp-VHL^{+/-} (HIF is not activated, Figure 1) and Fsp-VHL^{-/-} (HIF is activated, Figure 1) mice are resistant to bleomycin-induced fibrosis (Figure 2); this suggests that an HIF-independent

Figure 6. Suppression of pVHL in fibroblasts has no effect on bleomycin-induced lung injury and inflammation in the early stage. Wild-type (VHL^{+/+}) mice and Fsp-VHL^{+/-} and Fsp-VHL^{-/-} mice were administered PBS or bleomycin intratracheally and, 5 days later, mouse BALF was collected and used to determine the protein concentration (A). Aliquots of 180 μl BALF were applied to cytospin and Wright staining to determine total cell counts (B) and differential cell counts (C) under light microscopy (200× magnification). The cell counts are expressed as numbers of cells/high power field (hpf). Lympha, lymphocyte; Mac, macrophage; Neutro, neutrophil. (D) The BALF was also used to determine the transforming growth factor (TGF)-β1 concentration ($n \geq 5$). Results are expressed as mean ($\pm$ SEM). * and # $P < 0.05$; ** and ## $P < 0.01$; * and ** indicate differences between VHL^{+/+}/PBS and other groups, whereas # and ## indicate differences between bleomycin-treated groups.

function of pVHL is required in fibrogenesis. This notion is consistent with our previous report that overexpression of pVHL mediates fibroblast proliferation and expression of collagen and Fn in an HIF-independent, but Fn/integrin/FAK-dependent, pathway (Figure 7) (24). Therefore, our research provides new evidence supporting a gain-of-function mechanism for pVHL in fibrosis in addition to the use of a loss-of-function model, and indicates that HIF-independent functions of pVHL may also be involved in promoting fibrogenesis.

We would also point out that there is a mild increase of collagen in Fsp-VHL^{-/-} mice, even without treatment of bleomycin, suggesting a spontaneous fibrosis in these

mice. Similarly, Hickey and colleagues (32) have reported that mice with a mutation of pVHL at codon 200 (R200W) develop pulmonary vascular remodeling, hemorrhage, edema, and macrophage infiltration, as well as fibrosis in the later stage, presumably via an HIF2-dependent function. It is possible that, in Fsp-VHL^{-/-} mice, loss of both alleles of VHL stabilizes HIF, which promotes the extravasation of fibrocytes to the injurious sites, resulting in fibrosis (4, 57–59). Therefore, the role of pVHL in fibrosis may be complex and may vary depending on both HIF-independent and HIF-dependent functions.

Fibrosis is characterized by fibroblast proliferation and differentiation to

myofibroblasts, as well as dysregulation of ECM protein. Suppression of fibroblastic pVHL appears to inhibit PCNA and α -SMA expression in bleomycin-treated mice (Figures 3A and 3B), suggesting that fibrotic pVHL is necessary for fibroblast proliferation and differentiation. We have reported that overexpression of pVHL promotes fibroblast proliferation via an integrin $\alpha 5$ -dependent mechanism (24). Consistently, our results show that suppression of fibroblastic pVHL attenuates the induction of bleomycin-induced integrin $\alpha 5$ (Figure 3E). ECM protein synthesis and degradation are regulated by MMPs, among which MMP2 and MMP9 are up-regulated in IPF (60). We have also found that both MMP2 and MMP9 mRNA are up-regulated in bleomycin-treated mice, and that suppression of pVHL only inhibits bleomycin-mediated induction of MMP9 (Figures 3F and 3G), suggesting an MMP9-specific pathway in pVHL-mediated fibrosis, similar to administration of high-dose adenovirus-mediated fibrosis (61).

Patients with IPF have elevated levels of Wnt2, Wnt2b, Wnt5b, and Fz-related protein known to promote fibrosis (39–41). In addition, inhibition of the Wnt/ β -catenin signaling prevents bleomycin-induced lung fibrosis in mice (62). Our results demonstrate that overexpression of pVHL increases Wnt signaling in fibroblasts, whereas suppression of pVHL inhibits bleomycin-induced Wnt5b and Wnt16 and decreases Lrp6 expression in bleomycin-treated mice (Figure 4). Thus, these results provide a link between pVHL–Wnt–fibrosis. However, how pVHL regulates Wnt signaling remains unclear, and warrants further investigation.

During fibrosis, reduced production of PGE₂ promotes fibrogenesis via induction of lung myofibroblast differentiation and migration (42–45). Consistently, we found that bleomycin-induced fibrotic lungs have decreased PGE₂ levels, whereas Fsp-VHL^{+/-} and Fsp-VHL^{-/-} mice retain PGE₂ levels after bleomycin administration (Figures 5B–5F), suggesting a role for pVHL in regulating the homeostasis of PGE₂ signaling. Previous reports suggest that, in bleomycin-treated mice, there is a compensatory induction of PGE signaling genes (49, 50). We found that Fsp-VHL^{+/-} and Fsp-VHL^{-/-} mice did not appear to have this compensatory induction of COX-1, COX-2, PGE synthase1 and 3, and EP2 (Figures 5C–5H), further confirming the role of pVHL in the homeostasis of PGE₂.

Figure 7. A schematic diagram depicting the role of pVHL on fibroblast phenotype and pulmonary fibrosis. pVHL may act at multiple levels, such as Fn/integrin $\alpha 5\beta 1$ /focal adhesion kinase (FAK), PGE_2 , and Wnt/Frizzled pathways to coordinately regulate fibroblast proliferation, differentiation, and migration during fibrogenesis.

signaling. Future studies are warranted to elucidate the molecular mechanisms underlying this regulation.

Bleomycin administration is known to induce acute lung injury and lung inflammation, which is accompanied by elevated levels of proinflammatory cytokines. However, knockout of pVHL had little effect on BALF protein amount, total cell counts, and differential cell counts (Figures 6A–6C), suggesting a limited effect of pVHL on lung injury. Moreover, bleomycin-induced TGF- $\beta 1$ is comparable between wild-type mice, Fsp-VHL $^{+/-}$ mice, and Fsp-VHL $^{-/-}$ mice, suggesting a limited role of pVHL in bleomycin-induced TGF- $\beta 1$ signaling (Figure 6D). Although the gene expression patterns at Days 5 and 21 after bleomycin treatment are not identical, reflecting the difference in gene regulation in the acute injurious stage and the late fibrotic stage, the common features in both stages are the

decreased expression of PCNA, MMP9, Wnt5b, and Wnt16 in bleomycin-treated Fsp-VHL $^{+/-}$ and Fsp-VHL $^{-/-}$ mice. In conjunction with our previous report that loss of pVHL inhibits TGF- β -induced fibroproliferation, and that overexpression of pVHL induces fibroproliferation (24), pVHL likely acts on the proliferative stage in the bleomycin model, and we believe that Fsp-VHL $^{-/-}$ mice have a decrease in the fibroproliferative response to bleomycin. Whether these mice have accelerated resolution of the fibrotic state will need further investigation.

Our studies are limited to the bleomycin-induced fibrosis model, which recapitulates some aspects of IPF. It would be of interest to use other fibrosis models to corroborate these results. Although Fsp-1 has originally been thought to be fibroblast specific, other cell types have been shown to express Fsp-1 as well (30). Therefore, it is

possible that, in our model, there may be other cell types contributing to the phenotype changes in pVHL knockout mice. Likely, Fsp-1-positive fibroblasts only represent a portion of the total fibroblast population in the lung, and thus, the role of Fsp-1-driven knockout of pVHL in fibrosis reflects the significance of this portion of fibroblasts in fibrogenesis. Recent reports also suggest that fibrocytes and epithelial cells experiencing EMT also express Fsp-1 (63–66). Thus, knockout of pVHL with Fsp-Cre may ablate pVHL expression in interstitial fibroblasts and fibroblasts/myofibroblasts derived from epithelial cells and fibrocytes. As such, inhibition of fibrosis in Fsp-VHL $^{+/-}$ and Fsp-VHL $^{-/-}$ mice may reflect the combination of the contribution of all these types of cells.

In summary, our data suggest that pVHL plays a role in pulmonary fibrosis by regulating fibroblast proliferation and differentiation in an HIF-independent pathway, and that pVHL regulates the homeostasis of MMP9, Wnt signaling, and PGE_2 signaling (Figure 7). ■

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

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