

## **MATERIALS AND METHODS**

### **Mice**

Homozygous *vhlh* 2-lox mice (provided by Dr. Volker Haase of Vanderbilt University) and homozygous Fsp-Cre mice (provided by Dr. Anthony J. Trimboli from laboratories of Drs. Gustavo Leone and Michael C. Ostrowski of the Ohio State University) were crossbred and backcrossed to generate mice with fibroblast-specific knockout of pVHL (1, 2). Tail DNA from offspring was examined by PCR to confirm the genotypes with the primers listed in Table S1 on a Mastercycler epGradient S model (Eppendorf, Hamburg, Germany) (1, 2). Animal studies were conducted according to National Institutes of Health guidelines and the experimental protocols were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee.

### **Delivery of bleomycin and PBS to mouse lungs**

Fifty microliters of saline or bleomycin (0.045 units, Sigma-Aldrich, St. Louis, MO) dissolved in saline was delivered intratracheally as described previously (3, 4). 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- $\mu$ L aliquots 2 minutes apart) using a Hamilton syringe.

### **Isolation and culture of mouse lung fibroblasts**

Mouse lung fibroblasts were isolated from mice following published procedures (3). Mouse lung fibroblasts were identified based on the morphology and expression of vimentin and collagen. Cell viability was checked by Trypan blue exclusion assay.

### **Histological analysis**

The mouse lungs were removed *en bloc*, fixed in 10% paraformaldehyde, embedded in paraffin and sectioned. These lung sections were used for staining with H&E and Masson's Trichrome staining (3).

### **Bronchoalveolar lavage (BAL) analysis**

BAL fluid (BALF) was collected through a 20-gauge angiocath ligated into the trachea as described (4) and 1 ml PBS/mouse was injected to collect the BALF. A 200- $\mu$ l aliquot of the BALF was placed in a cytospin and used for Wright staining to determine cell counts and cell differential analysis. The supernatant of the remaining BALF was used for the measurement of BALF protein (Bradford) (3).

### **Sircol assay**

Mouse lungs were homogenized and aliquots of lung homogenates were assayed for total lung collagen levels using the Sircol collagen dye binding assay (Bicolor Ltd., Northern Ireland, UK) according to the manufacturer's directions.

### **Measurements of PGE<sub>2</sub> by mass spectrometry**

Quantitative analysis of PGD<sub>2</sub> and PGE<sub>2</sub> were carried out by UHPLC and mass spectrometry as described previously (5). Briefly, the samples were separated using UHPLC on a Shimadzu (Columbia, MD) Nexera UHPLC system equipped with an Acquity (Waters, Milford, MA) UPLC BEH C<sub>18</sub> (2.1 mm  $\times$  50 mm, 1.7  $\mu$ m) analytical column at 50°C. An isocratic mobile phase was used consisting of acetonitrile/aqueous 0.1% formic acid (40:60, v/v) at a flow rate of 1.2 ml/min. The UHPLC system was interfaced to a Shimadzu (Kyoto, Japan) LCMS-8040 triple quadrupole mass spectrometer that was operated using negative ion electrospray. Isomeric PGD<sub>2</sub> and PGE<sub>2</sub> 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-PGE<sub>2</sub> and d4-PGD<sub>2</sub>.

### **Quantitative real-time RT-PCR and microarray analysis**

Total RNA was extracted using the miRNeasy mini kit (Qiagen, Valencia, CA) and quantified with Nanodrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA). Synthesis of complementary

DNA (cDNA) was performed using the ABI High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA). Quantitative real-time RT-PCR (qRT-PCR) was carried out on the ABI StepOnePlus real-time PCR system using the ABI SYBR Green PCR master mix (Applied Biosystems Inc.). The amplification of the genes was normalized to the amplification of the mitochondrial ribosomal protein L19 (RPL19). The sequences of the primers used in the real time qRT-PCR are shown in Table S1. We overexpressed pVHL in N12 normal human lung fibroblast by infection of pAd-VHL-HA (pAd-GFP was used as control infection) (3), extracted the total RNA, and performed microarray analysis with Affymetrix GeneChip Human Genome U133 Plus 2.0 Array. Data were analyzed in Partek Genomics statistical package from Partek, Inc. (St. Louis, Missouri). Hybridization signal intensities were normalized by quantiles and summarized using the Robust Multi-array Average (RMA) (6).

### **Measurement of TGF- $\beta$ 1 in BALF**

Levels of TGF- $\beta$  (R&D Systems, Minneapolis, MN) were measured using an ELISA kit following the manufacturer's protocol. The optical density of each well was read using a GloMax-Multi Detection System (Promega, Madison, WI) set to 450 nm with wavelength correction set to 540 nm.

### **Western blot analysis**

Western blot analysis was carried out as previously described (3) using the following primary antibodies: integrin  $\beta$ 1 (Millipore, Temecula, CA), integrin  $\alpha$ 4 (Chemicon, Temecula, CA), integrin  $\alpha$ 5,  $\alpha$ v, and  $\beta$ 1 subunits (Santa Cruz Biotechnology, Santa Cruz, CA), fibronectin (Millipore, Temecula, CA), collagen I (Southern Biotech, Birmingham, AL),  $\alpha$ -tubulin,  $\beta$ -actin, Fsp-1, Wnt5b, Wnt16 (Sigma-Aldrich),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA)(R&D Systems, Minneapolis, MN), MMP2, and MMP9 (Cell signaling, Danvers, MA).

### **Overexpression of Wnt5b and Wnt16 in N12 normal human lung fibroblasts**

N12 normal human lung fibroblasts (as described in (3)) were infected with adenoviruses encoding Wnt5b (Ad-Wnt5b) and Wnt16 (Ad-Wnt16) (provided by Dr. Tong-Chuan He, Molecular Oncology Laboratory, The University of Chicago Medical Center, Chicago, IL) at the dose 100 ifu/cells as described previously (3). The coding regions of mouse Wnt5b and Wnt16 were PCR-amplified and subcloned into adenoviral shuttle vectors. The recombinant adenoviruses were generated using the AdEasy technology (7, 8). Null viruses (Ad-null, vector BioLabs, Philadelphia, PA) were used as control.

### **Small interfering RNA (siRNA) suppression of MMP9**

N12 were plated in 60-mm dishes at ~ 70-80% confluence and transfected with MMP9 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) using Lipofectamine2000 (Invitrogen, Grand Island, NY) as described (9). Negative control siRNA (siNeg, Santa Cruz Biotechnology) was used as a negative control. Two days after transfection, cells were lysed for Western blot analysis.

### **Flow cytometry analysis**

Mouse lung fibroblasts (MLF) were isolated from pVHL wild type or knockout mice and plated. For each sorting, we collected  $4 \times 10^6$  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 non-specific binding. Cells were incubated with primary antibodies against PCNA (Sigma PLA-0079, rabbit) or Fsp-1 (Sigma F4771, mouse) for 1 hour at room temperature. After three washes with PBS, cells were re-suspended and incubated with secondary antibodies, RPE-conjugated goat anti-Rabbit IgG (H+L) (P2771MP, ThermoFisher Scientific, Grand Island, NY) or APC-conjugated goat anti-Mouse IgG (H+L) (A865) (ThermoFisher Scientific) for 30 minutes at room temperature. After three washes with PBS, cells were analyzed and sorted at the UIC Research Resources Center Flow Cytometry Service facility.

**Statistical analysis**

Results were expressed as mean  $\pm$  SEM. Data were analyzed using one-way analysis of variance (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.

Table S1: Primers for PCR

Gene	Forward primer (5'to 3')	Reverse primer (5'to 3')	Application
VHL	CTCAGGTCATCTTCTGCAACC	TCTGTCTTGGCCTCCTGAGT	genotyping
Cre	TCCTGCCCTTAGGTCTCAAC	CCTGTTTTGCACGTTCAACG	genotyping
VHL (mouse)	GCTCCTGCTGTAGTCTG	CTTCTCTGCTGTAAGTGTCTG	qRT-PCR
Col1a1 (mouse)	GCACGAGTCACACCGGAAC	AAGGGAGCCACATCGATGAT	qRT-PCR
Col1a2 (mouse)	CTACTGGTGAAACCTGCATCCA	GGGCGCGGCTGTATGAG	qRT-PCR
PCNA (mouse)	GCCAGACCTCGTTCCTCTTAGA	TCAGGCGTGCCTCAAACAT	qRT-PCR
Acta2 (mouse)	CGGGAGAAAATGACCCAGATT	GGACAGCACAGCCTGAATAGC	qRT-PCR
Integrin $\alpha$ 5 (mouse)	AGGTGGGCAGGGTCTACATCT	CGAATCGGCTGAACTCATCTT	qRT-PCR
MMP2 (mouse)	GGACAGTGACACCACGTGACA	GGCCTCATACACAGCGTCAAT	qRT-PCR
MMP9 (mouse)	GGACGACGTGGGCTACGT	CACGGTTGAAGCAAAGAAGGA	qRT-PCR
Wnt5b (mouse)	CCAAGACGGGCATCAGAGA	CACGGTGCTGCAGTTCCA	qRT-PCR
Wnt16 (mouse)	TGTATGGTCGCCACTACCACTT	CGCTACTCAGCTCATAGCCAAA	qRT-PCR
Lrp6 (mouse)	TGGCTTGGCGGTGTGAT	TGCCCCGCTGGCACACT	qRT-PCR
PTGS1/Cox1 (mouse)	TTCCGAGCCCAGTCCAATA	GGATGCCAGTGATAGAGATGGTT	qRT-PCR
PTGS2/Cox2 (mouse)	CAGGTCATTGGTGGAGAGGTGTA	GGATGTGAGGAGGGTAGATCATCT	qRT-PCR
PGE synthase 1 (mouse)	TGGAGCGCTGCCTCAGA	AAGCCGAGGAAGAGGAAAGG	qRT-PCR
PGE synthase 2 (mouse)	AGGCCTTCGACGACCTGAT	TCAATGGCCCCGCTCCAT	qRT-PCR
PGE synthase 3 (mouse)	CGCCCACCCGTTTGTCT	TCGATCGTACCACTTTGCAGAA	qRT-PCR
Ptger2/EP2 (mouse)	GCAACATCAGCGTTATCCTCAA	AATCCGCAGCGGCTTCTT	qRT-PCR
Glut 1 (mouse)	CGAGGGACAGCCGATGTG	TGCCGACCCTCTTCTTTCAT	qRT-PCR
VEGF (mouse)	TACCTCCACCATGCCAAGTG	TCATGGGACTTCTGCTCTCCTT	qRT-PCR
Il-1b (mouse)	CTACAGGCTCCGAGATGAACAAC	TCCATTGAGGTGGAGAGCTTTC	qRT-PCR
Il-1a (mouse)	GCCCGTGTTGCTGAAGGA	AGAAGAAAATGAGGTCGGTCTCA	qRT-PCR
Il-6 (mouse)	CGTATGAAGTTCCTCTCTGCAA	GGAAGGCCGTGGTTGTCA	qRT-PCR
Il-10 (mouse)	CAGCCGGGAAGACAATAACTG	CGCAGCTCTAGGAGCATGTG	qRT-PCR
Wnt2b (mouse)	TGCCAAAGAGAAGAGGCTTAAGG	CGACCACAGCGGTTGTTGT	qRT-PCR
Wnt 2 (mouse)	CACCAGTTCGCCAGCAT	GAGGACCCGGCCAAAGA	qRT-PCR
Integrin $\alpha$ V (mouse)	AGGATGGGCTTTTTCAAACGT	TTCCTTCGCCATTCTCATGAG	qRT-PCR
EPO (mouse)	GTGTCCACCTGGGCATATCC	GGGAGGGTGTGGCACAAG	qRT-PCR

## References:

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