**Online Supplemental Methods**

# The Low Density Lipoprotein Receptor-Related Protein 1 Regulates Collagen 1 Expression, Proteolysis, and Migration in Human Pleural Mesothelial Cells.

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**Methods.**

**Cell Surface Expression of LRP-1 and uPAR.** MeT5A, HPMCs, REN, MS-1, and M9K cells were examined for the presence of surface uPAR and LRP-1 via FACS analysis. Briefly, cells were cultured in serum-free conditions, 12-15 h prior to analysis. Cells were labeled using uPAR, LRP-1 targeted monoclonal antibodies or an isotype matched mouse and then analyzed using a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA). Histograms were prepared using FlowJo Flow Cytometry Analysis Software.

**Immunohistochemistry.** Immunohistochemical analyses were performed on paraffin embedded lung tissues isolated from normal and pneumonitis lung. Five micrometer sections were cut and analyzed for expression of uPAR (mAb 3936, American Diagnostica), LRP-1 (mAb 8G1, Abcam, Cambridge, MA) or isotype matched mouse IgG control (Jackson ImmunoResearch, West Grove, PA). Labeled sections were then stained using a commercially available kit (Lab Vision, Fremont, CA), as described (1).

**Immunofluorescence confocal microscopy.** To determine the role of LRP-1 in uPA mediated uPAR internalization, immunofluorescence assays were performed. Subconfluent PMCs were serum-starved for 12 h. The cells were then incubated in the presence or absence of 200 nM RAP (2) for 15 minutes at 37°C, then incubated in the presence or absence of 20 nM uPA (American Diagnostica, Stamford CT) for 30 min at 37°C. The cells were fixed in 4% formalin for 30 min on ice and permeabilized with 0.025% Triton X-100/PBS buffer at room temperature. The cells were then immunofluorescence-labeled with a mouse monoclonal antibody against LRP-1 (8G1, Abcam) and a rabbit polyclonal against uPAR (RAb AM3). The cells were then probed with donkey anti-rabbit-rhodamine or donkey anti-mouse Alexa 488 conjugated fluorophores (Invitrogen, Carlsbad, CA). Images were acquired from a field of view at 0.4-μm z-axis increments with the use of the LSM 510 Meta confocal system (Carl Zeiss). The laser setting wavelengths were ± 10 nm excitation and 450 ± 30 nm emission for DAPI, 488 ± 10 nm excitation and 525 ± 10 nm emission for Oregon Green, and 543 ± 10 nm excitation and 575 ± 10 nm emission for Rhodamine Red.

**Reverse Transcription Polymerase Chain Reaction (PCR).** MeT5A and HPMCs were treated with SFM containing PBS, TNF-α (10 ng/ml, R & D), TGF-β (2 ng/ml, R& D), or IL-1β (25 ng/ml) (R & D, Minneapolis MN). RNA was then isolated from the cells as previously described (3). Target mRNA was then transcribed into cDNA using the RNA-to-cDNA High Capacity Master Mix according to manufacturer's instructions (Invitrogen). PCR analysis was then performed on the cDNA using primers against LRP-1 as previously described (4) and β-actin. The amplified DNA fragment was then resolved on 1% agarose gel and imaged using the Bio-Rad Gel Doc Imaging System.

**LRP-1 siRNA Transfection.** Subconfluent MeT5A cells were transfected with 100 nM control and LRP-1 siRNA (Hs_LRP_2, Qiagen, Valencia CA) using LipofectAMINE 2000 (Invitrogen, Carlsbad CA) as previously described (5). The cells were then incubated in complete RPMI media for 24 h. The cells were then placed in SFM for 12-18 h, then subjected to Western blot analysis for LRP-1 expression and β-actin.

**Internalization of Surface Biotinylated uPAR.** Measurement of biotin-labeled uPAR internalization was performed as previously described with some modifications (6, 7). Briefly, serum-starved confluent cells were surface biotinylated with NHS S-S Biotin (1 mg/ml, Thermo Scientific, Waltham, MA) in a PBS buffer (ph 8.0). The cells were then cultured in serum-free RPMI media (SFM) in the presence or absence of 20 nM uPA (American Diagnostica) at 37°C for 0, 0.25, 0.5, 1, and 2 h time points. Two 0 time-point samples were collected. The first was used to establish the total surface biotinylated uPAR and assigned a value of 100%. A second 0 time-point sample was treated with a glutathione (GSH) wash buffer as previously described (7). This time-point was used to determine the efficiency of surface biotin removal and was subtracted from all subsequent time-points for normalization. At the completion of each interval, internalization was stopped by washing the cells with ice-cold PBS. The cells were then subjected to two GSH buffer washes, then washed with ice-cold PBS and lysed using octyl β-D glucopyranoside (OGP) lysis buffer as previously described (3). Biotinylated proteins were then isolated, resolved on SDS-PAGE and Western blotted as previously described. The results were quantified via densitometry using NIH Image J Software.

**Stability of Surface Biotinylated uPAR.** Surface biotinylation analyses were performed as described previously with minor alterations (8). Serum-starved cells were biotinylated as described above. At 0, 3, 6, 12, and 24 h time-points, cells were washed with PBS and lysed in

OGP buffer as previously described (3). Biotinylated proteins were then isolated from lysates resolved, and probed for uPAR as described in the previous section. The results were then quantitated via densitometry using NIH Image J Software.

**Calculation of uPAR protein half-life ($t_{1/2}$).** uPAR Western blots were scanned using an HP Photosmart 3200 Scanner and converted to arbitrary units using NIH Image J software. Densitometry results were then standardized against the Time 0 h biotinylated control which was assigned a value of 100%. Results were converted to a percentage of the control (Time 0 hr), transformed using a $\log_{10}$ scale and graphed as first-order exponential decay ($R>0.95$). uPAR $t_{1/2}$ (i.e. time to 50% remaining) was determined from the respective exponential decay equations.

**Calculation of uPAR Internalization.** Western blots were scanned with a HP Photosmart 3200 and converted to arbitrary units using NIH Image J. Densitometry results were then standardized against the Time 0 h biotinylated control which was assigned a value of 100%. The amount of biotinylated protein detected at Time 0 after GSH buffer treatment was subtracted from all samples for standardization purposes. The percent uPAR internalization of the uPA-treated cells was divided by the percent uPAR internalization of the non-uPA treated cells for each respective time point (0.25, 0.5, 1, and 2 h) to determine the fold increase in internalization above baseline.

**Regulation of cell surface uPA activity via LRP-1**. To determine the role of LRP-1 in the regulation of the durability of cell-associated uPA enzymatic activity, fibrin gel enzymography was performed as described in the text. Acid washed, MeT5A and REN cells were incubated in the presence or absence of LRP-1 neutralizing RAP (200 nM) (2) for 15 min under slight agitation on ice. 10 nM uPA (American Diagnostica) containing SFM was then added to the cells and allowed to incubate on ice for 20 min. The cells were then washed three times in PBS

to remove unbound uPA. The cells were then incubated at 37°C for 0, 3, 6, and 12 h in SFM in the presence or absence of 200 nM RAP at 37°C. Cells were washed with PBS, lysed in OGP buffer, cleared and subjected to 10% SDS PAGE and enzymography.

**Cytokine treatment of PMCs.** Serum-starved MeT5A and primary HPMCs were treated with TNF-α (10 ng/ml, R&D), TGF-β (5 ng/ml, R&D) and IL-1β (25 ng/ml, R&D) for 12-15 h and Western blotted for changes in PAI-1 (RAb, Abcam), uPAR (RAb, AM3) and LRP-1 (85 kDa, Abcam). Cytokine treated cells were also used in uPA-mediated uPAR internalization analyses as detailed above.

**Collagen Western Blotting.** Serum-starved HPMCs and RPMCs were treated with PBS or TGF-β in the presence or absence of uPA (20 nM, American Diagnostica) and/or RAP (200 nM). RPMC were also treated with scuPA (20 nM, a generous gift from Jack Henkin, Abbott Laboratories, Abbott Park, IL) or tPA (20 nM, American Diagnostica). The cells were then incubated at 37°C for 48 h. The conditioned medias were collected and cells lysed using PBX-100, resolved by SDS-PAGE, and transferred to PVDF. The membranes were then probed for collagen 1 (GAb, Southern Biotech, Birmingham, AL), then stripped and probed for LRP-1 and then β-actin or erk.

**TGF-β ELISA.** Serum-starved HPMCs were treated with uPA in the presence or absence of uPA or RAP. The cells were then allowed to incubate for 48 hours in a 37°C incubator. The conditioned media were then collected and assayed for active and total TGF-β via ELISA (Cat. # 437707 and 436707 respectively, BioLegend, San Diego CA) according to manufacturer's instructions.

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