

**Renin is an angiotensin-independent profibrotic mediator. Role in pulmonary fibrosis**

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## Abstract

The pathogenesis of idiopathic pulmonary fibrosis (IPF) is likely the result of interplay between cytokines/chemokines and growth factors. The renin-angiotensin system is involved, although its profibrotic effect is attributed to angiotensin II. However, recent studies suggest that renin, through a specific receptor, is implicated in fibrogenesis.

In this study, the expression of renin and renin receptor was examined in normal and IPF lungs and fibroblasts. Normal human lung fibroblasts (NHLF) were stimulated with renin or transfected with renin-siRNA, and the expression of TGF- $\beta$ 1 and  $\alpha$ 1-type I collagen was analyzed.

Normal lungs and NHLF expressed renin which was strongly up-regulated in IPF lungs and fibroblasts ( $\sim$ 10-fold increase;  $p < 0.05$ ). Immunocytochemistry showed intense renin staining in IPF fibroblasts. Renin stimulated NHLF displayed an increase in the expression of TGF- $\beta$ 1 ( $1.8 \pm 0.2 \times 10^3$  versus  $1.2 \pm 0.3 \times 10^3$  mRNA/rR18s;  $p < 0.01$ ) and collagen ( $5.93 \pm 0.66 \times 10^2$  versus  $3.28 \pm 0.5 \times 10^2$  mRNA/rR18s;  $p < 0.01$ ), while knocking-down renin expression by siRNA provoked a strong decrease of both molecules. These effects were independent of angiotensin II since neither losartan nor captopril decreased these effects. Renin also decreased MMP-1 expression and induced TGF $\beta$ 1 activation ( $163 \pm 34$  versus  $110 \pm 15$  pg active TGF $\beta$ 1/mg of total protein).

These findings highlight the possible role of renin as an angiotensin-II-independent profibrotic factor in lung fibrosis.

## **Introduction**

Idiopathic pulmonary fibrosis (IPF) is a progressive and devastating disease characterized by the expansion of the fibroblasts/myofibroblasts population and excessive accumulation of extracellular matrix resulting in progressive and severe distortion of the lung architecture (1-2).

Despite numerous studies performed in human disease and in experimental models, the molecular mechanisms that underlie lung fibrosis are still uncertain. Transforming growth factor beta (TGF- $\beta$ ) seems to play a major profibrotic role inducing fibroblast to myofibroblast differentiation and increasing collagen expression (3, 4). However, the fibrotic lung reaction is likely the final result of a complex interplay between growth factors, cytokines and chemokines (5, 6).

Renin is a protease that unlike other aspartyl proteases has only one known substrate, angiotensinogen which cleaves to form angiotensin I (Ang I) (7). Then, the angiotensin-converting enzyme (ACE) converts this renin-cleaved product into Ang II. The renin-angiotensin system (RAS) is essential for the control of blood pressure and fluid homeostasis. Importantly, it has been recently demonstrated the existence of local autocrine/paracrine RAS that is physiologically active in many tissues (8).

Studies in different organs including the lung indicate that the renin-angiotensin system plays an important role in fibrogenesis, although the effect is primarily attributed to the actions of angiotensin II through the angiotensin type 1 (AT1) receptor (9-12).

Recently however, it was demonstrated that renin can directly induce a marked dose- and time-dependent increase of TGF- $\beta$ 1 in human and rat mesangial cells and consequently, of various extracellular matrix components (13). Importantly, the activation of the renin/prorenin receptor in mesangial cells induced the synthesis of TGF- $\beta$  through angiotensin II-independent mechanisms.

To date there are no studies dealing with renin expression and activities in the lung. The aim of our study was to examine the expression of renin and renin receptor in normal and IPF lungs and fibroblasts and to evaluate the effect of renin on different fibroblast activities. Our results revealed that renin is upregulated in IPF lungs and fibroblasts and increases collagen synthesis and TGF- $\beta$  expression.

## **Methods**

### ***Materials***

Antibodies for renin, ERK 1/2, pERK1/2,  $\beta$ -tubulin,  $\alpha$ SMA and (pro)renin receptor were obtained from Santa Cruz biotechnology (Santa Cruz, CA). Peroxidase-conjugated secondary antibodies were purchased from Invitrogen (Life technologies, Grand Island, NY). Captopril, Losartan, RIPA buffer, protease inhibitor cocktail and phosphatase inhibitors were obtained from Sigma (Sigma, St. Louis, MO). Recombinant human pro-renin was obtained from Cayman Chemical (Ann Arbor, MI).

### ***Cell culture***

Primary human fibroblasts from IPF (n=8) and control lungs (n=4) were obtained as previously described (14). Fibroblasts (passages 5-8) were cultured at 37°C in 5% CO<sub>2</sub>-95% air in 25-cm<sup>2</sup> Falcon flasks containing Ham's F-12 medium supplemented with 10% FBS, 100 U/ml of penicillin, 100  $\mu$ g/ml of streptomycin, and 2.5 mg/ml of amphotericin B.

### ***Western blot***

Cells reaching 80% confluence were cultured in serum-free medium. The conditioned medium were recovered, concentrated 25X and dialyzed in columns with 3000 Da pore of molecular limit (3000 YM Millipore MA, USA). All procedures were performed with inhibitors of proteases AEBSF, EDTA, Bestatin, E-64, Leupeptin, and Aprotinin (Millipore). For extraction of intracellular proteins, cells were lysed in RIPA buffer containing protease inhibitor cocktail (Biorad) and phosphatase inhibitors (Sigma). Eight micrograms were run on 7-12% SDS-PAGE

gel, followed by immunoblotting. Conditions for each antibody were performed according of manufacturer instructions. Protein concentration was determined by Bradford assay (Bio-Rad Hercules, CA).

### ***Gelatin Zymography***

SDS-polyacrylamide gels containing gelatin (1 mg/ml) were used to identify proteins with gelatinolytic activity present in serum-free conditioned media from human lung fibroblasts stimulated with renin. Each lane was loaded with 0.3 µg of protein (15).

### ***Stimulation with human recombinant renin***

Normal lung fibroblasts were plated at sub-confluent density on T-25 flask and grow 24 hours in serum-free medium. Then, the medium was changed by serum-free medium containing 10nM of human recombinant renin and fibroblasts were cultured for 3 hours. Total RNA was extracted with trizol™ (Life technologies, Grand Island, NY), and the cell supernatants were frozen at -70°C until use. In parallel experiments, fibroblasts were pre-incubated with losartan or captopril at concentrations of 10nM 1 hr before the addition of renin. To evaluate the role of the renin receptor, in some experiments fibroblasts were pre-incubated during 1 h at 37°C with a polyclonal anti-renin antibody (16nM; Santa Cruz CA, Sc-67390).

### ***Enzyme-linked immunosorbent assay***

Active TGF-β1 was measured in 3 µg of conditioned media using a commercial ELISA technique, according of manufacturer instructions (Emax; Promega, Madison, WI).

### ***Immunocytochemistry.***

Fibroblasts ( $1 \times 10^4$ ) were incubated on coverslips with serum-free medium for 24h. Fibroblasts were fixed with acetone-methanol (1:1) at  $-20^\circ\text{C}$  for 2 min and incubated with antihuman renin monoclonal antibody (Serotec, Kidlington, UK) at  $37^\circ\text{C}$  for 30 min followed by biotinylated goat anti-mouse IgG for 20 min (Biogenex, San Ramon, CA). 3-amino-9-ethyl-carbazole (BioGenex) in acetate buffer containing 0.05%  $\text{H}_2\text{O}_2$  was used as substrate. Cell nuclei were counterstained with hematoxylin. Coverslips were viewed with Olympus 1X81 microscope, and images were captured using a Evolution MP camera and processed by Adobe PhotoShop software. Image analysis was performed using Image Pro-Plus 4.5 software; quantification was expressed in pixels mean density (pmd).

### ***RT-PCR and quantitative real-time PCR amplification***

Total RNA and proteins were extracted with Trizol™ (Life technologies), according to the specifications of the manufacturer. One  $\mu\text{g}$  of total RNA was reverse transcribed using Advantage RT-for-PCR kit (Clontech, Palo Alto, CA). Quantitative real-time PCR amplification was performed with an i-Cycler iQ detection system (Bio-Rad, Hercules, CA) (14). PCR was performed with a cDNA working mixture in a 25- $\mu\text{l}$  reaction volume containing 3  $\mu\text{l}$  of cDNA, PCR Master mix 20X (Applied Biosystems, Foster City, CA) and the Taqman probes. For TGF- $\beta$ 1,  $\alpha$ - smooth muscle actin ( $\alpha\text{SMA}$ ),  $\alpha$ -1 type I collagen and renin, 1  $\mu\text{l}$  of 20x TaqMan FAM-MGB probes (cat.# Hs99999918\_m1, Hs00426835\_g1, Hs01076780\_g1 and Hs00166915\_m1) (Applied Biosystems, Foster City, CA) were used. For renin receptor we used primers designed from sequence GI:15011917 using Primer-

BLAST. The sequences are: sense: 5'-CATTGTCCATGGGCTTCTCT-3'; antisense: 5'-GCATTCTCCAAAGGGTACGA-3'. For real time PCR we used SYBR® Green PCR Master Mix and 10 pmol of each primer. A dynamic range was built with each product of PCR on copy number serial dilutions of  $1 \times 10^{10}$  to  $1 \times 10^2$ ; all PCRs were performed in triplicate. Results were expressed as the number of copies of the target gene normalized to 18S rRNA (4352930E, Applied Biosystems).

### ***Small interference RNA (siRNA)***

siRNA was designed as described (16, 17). The sequence of oligonucleotides to generate the siRNA for renin was planned using <http://bioinfo2.clontech.com/ARNidesigner/> and the homology was verified in BLAST (Basic Local Alignment Search). Two complementary oligonucleotides were synthesized and cloned in pSIREN-retroQ-Tet (BD Clontech, High Wood, CA). Ligation of oligonucleotide in the vector pSIREN-Retro-Q-Tet was done according to manufacturer instructions. Oligonucleotides were obtained from Applied Biosystems.

### ***Transfection in packings cells PT67***

PT67 cells were seeded into a 6-well plate at 80% confluence ( $1 \times 10^6$  cells/well) 24 hours before transfection; 4 µg DNA and 10 µl lipofectamine 2000 were used for transfection. Retropack PT67 cells were diluted at a ratio of 1:20 and plated at 24 hours posttransfection. The transfected PT67 cells were cultured for 10 days with 2 µg/ml puromycin (Clontech, USA), and the large, healthy colonies were isolated and transferred into individual wells and plates. After 24 hours the medium was replaced and to determine the efficiency of the infection, small cellular



subpopulations were put under treatment with antibiotic. The infected cells were used for experiments or for selection as soon as it was possible, but not before 24 hours post infection. Quantitative PCR was used to verify the inhibition of the expression of renin. Filtered medium containing viral particles of iREN-siRNA or LUC (20  $\mu$ l) was added to fibroblasts in 2 ml low-glucose DMEM for infection. The infected fibroblasts were diluted at a ratio of 1:10 24 hours later and then puromycin was added to a final concentration of 0.5  $\mu$ g/ml. Two weeks later, the large, healthy colonies were isolated and transferred into individual wells and plates.

#### ***Collagen measurement***

Collagen was quantified in cell conditioned medium using the Sircol collagen assay (Biocolor Ltd, UK) (18). For these experiments, one ml of Sirius red dye was added to 100  $\mu$ l conditioned medium and mixed for 30 minutes at room temperature. After centrifugation at 10,000g for 10 minutes, the collagen-bound dye was dissolved with 1 ml of 0.5M NaOH, and absorbance at 540nm was measured by spectrophotometer (Nanodrop 1000, Thermo Scientific, MA).

#### ***Statistical analysis***

Values are presented as mean  $\pm$  SD. Data were analyzed using Student's t test or by one-way analysis of variance followed by Dunnett's test. Analysis of correlation was carried out with spearman test. Values of  $p < 0.05$  were considered statistically significant.

## Results

### ***Renin expression is increased in IPF lungs and IPF fibroblasts***

Eight IPF and 4 normal human lung fibroblast strains were cultured at subconfluence and the levels of renin expression were quantified by real time PCR.

As illustrated in **figure 1A**, fibroblasts from IPF lungs showed a 10-fold increase in their basal levels of renin mRNA compared with normal lung fibroblasts ( $4.7 \pm 1.2 \times 10^6$  versus  $0.4 \pm 0.1 \times 10^6$  copies of renin mRNA/rR18s;  $p < 0.01$ ). No differences were found in the expression of the renin receptor (**Figure 1B**), although a tendency to be higher in IPF fibroblasts was observed at the protein level (**Figure 1C and 1D**). The increased expression of renin by the IPF fibroblasts was confirmed at the protein level by immunocytochemistry. As exemplified in **Figure 2**, IPF fibroblasts showed an intense cytoplasmic staining of renin that appeared to be located in the endoplasmic reticulum and Golgi apparatus. Quantitative analysis demonstrated a significant increase of renin staining in IPF fibroblasts compared to normal lung fibroblasts ( $159 \pm 4.8$  versus  $125 \pm 8.4$  pmd; Figure 2, panel E).

Likewise, the levels of renin mRNA expression were significantly increased in IPF lungs ( $n=7$ ) compared with normal lungs [( $n=5$ ) ( $17.5 \pm 13.8$  versus  $1.9 \pm 0.6$ ;  $p < 0.05$ ; **Figure 3A**)]. Also the levels of renin receptor showed a tendency to be increased although the result did not reach statistical significance ( $185.5 \pm 193.5$  versus  $20.7 \pm 8$  copies of renin receptor mRNA/rR18s;  $p=0.06$ ) (**Figure 3B**). Interestingly, there was a positive correlation between the expression of renin and the receptor, thus the 4 patients with higher expression of renin also had the higher expression of the receptor (Spearman  $r = 0.65$ ;  $p < 0.05$ ).

Renin up-regulates TGF- $\beta$ 1 and collagen expression through an Ang II independent mechanism

To evaluate putative profibrotic effects of renin, the recombinant protein was used to stimulate two different normal human lung fibroblast strains, and the expression of TGF- $\beta$ , and  $\alpha$ -1 type I collagen were measured by real time PCR. As shown in **figure 4**, renin significantly increased the levels of both TGF- $\beta$ 1 ( $1.8 \pm 0.2 \times 10^3$ , versus  $1.2 \pm 0.3 \times 10^3$  copies of TGF- $\beta$ 1 mRNA/rR18s;  $p < 0.01$ ) (**figure 4A**) and collagen ( $5.93 \pm 0.66 \times 10^2$ , versus  $3.28 \pm 0.5 \times 10^2$  copies of  $\alpha$ -1 type I collagen mRNA/rR18s;  $p < 0.01$ ) (**figure 4B**). Pre-treatment of the cells with a specific anti-renin receptor (Ab) abolished renin-induced collagen increase (**figure 4C**). This effect was independent of angiotensin II since losartan, a specific inhibitor of the receptor AT1, and captopril, an inhibitor of angiotensin-converting enzyme, did not decrease the renin-induced over-expression of TGF- $\beta$ 1 or collagen. When fibroblasts were stimulated with Ang II, the levels of expression of TGF and  $\alpha$ -1 type I collagen were also increased- $\beta$ 1 (**Figure S1A and S1B**); however as expected, this increment was abolished by losartan. The stimulation with renin also increases the expression of  $\alpha$ SMA (**figure 4D**), although this result was not confirmed at the protein level (**figure 4E**).

#### ***Renin decreases MMP-1 expression and has not effect on MMP-2***

Human lung fibroblasts were treated with renin and the expression of MMP-1 and MMP-2 two enzymes that have been associated to the pathogenesis of IPF were examined (**19**). Stimulation with renin caused a significant decrease of the

expression of MMP-1 (**figure 5A**), while it showed no effect on MMP-2 expression (**5B**) or activity (**5C**).

On the other hand, It has been suggested that the effect of renin is mediated by extracellular-regulated kinases 1 and 2 (ERK1/2) (**13**). To investigate whether the stimulation of human lung fibroblasts by renin, also activated the ERK1/2 signaling pathway, the expression of total and phosphorylated ERK1/2 was determined in cell extracts by Western blot. As illustrated in **Figure 6A and 6B**, treatment of fibroblasts with renin strongly stimulated phosphorylation of ERK1/2. The temporal profile of ERK1/2 activation showed an increase that reaches a plateau at 20-30 min with a gradual decline thereafter.

Renin induces TGF- $\beta$ 1 activation through an Ang II independent mechanism

*Two different normal human lung fibroblast cell-lines were stimulated with renin and the levels of active TGF $\beta$ 1 were assessed in the conditioned medium by ELISA. As shown in figure 6C, renin stimulation significantly increased the levels of active TGF $\beta$ 1 which was not affected by the ACE inhibitor or the AT1 receptor blocker.*

### ***Silencing renin induces a marked decrease of TGF- $\beta$ and collagen expression***

To determine the significance of loss of renin on fibroblast behavior, human normal lung fibroblasts were transiently transfected with renin small interfering RNA. With the siRNA we achieved ~75% silencing of renin as measured by PCR (**Figure 7A**); this decrease was confirmed at the protein level by Western blot performed on conditioned media (**Figure 7B**). Real-time PCR was used to determine the effect of renin siRNA on TGF- $\beta$ , collagen and  $\alpha$ -SMA expression. As shown in **figure 7C**,

compared to the levels observed in normal fibroblasts and fibroblasts treated with the virus with empty vector (Luc), silencing of renin caused a significant decrease in the expression of TGF- $\beta$ 1 (control:  $1.84 \pm 0.48 \times 10^2$ , Luc:  $1.26 \pm 0.42 \times 10^2$  and iRen:  $0.42 \pm 0.24 \times 10^2$  copies of TGF- $\beta$ 1 mRNA/rR18s;  $p < 0.01$ ).

Collagen expression was also significantly decreased at the mRNA level (control:  $5.2 \pm 0.6 \times 10^2$ , Luc:  $4.7 \pm 0.11 \times 10^2$  and iRen:  $0.74 \pm 0.4 \times 10^2$  copies of  $\alpha$ -1 type I collagen mRNA/rR18s;  $p < 0.01$ ), and at the protein level where the inhibition of the expression of renin provoked ~ 50% decrease of secreted collagen in the fibroblasts conditioned media (**figures 8A and 8B**).  $\alpha$ -SMA expression did not show changes (data not shown).

## Discussion

The development of IPF and other fibrotic lung disorders involves the activation of fibroblasts, their differentiation to myofibroblasts and the exaggerated production of extracellular matrix proteins with the subsequent aberrant architectural remodeling. The pathogenesis of IPF, the most aggressive interstitial lung disease, is believed to be related to a dysregulated cross-talk between epithelial cells and fibroblasts and to be mediated by a complex interplay among various cytokines, chemokines and growth factors, with a central role of TGF- $\beta$ . In this context, several studies have implicated the RAS system in lung fibrogenesis, but its profibrotic effects have been attributed primarily to angiotensin II (20).

Our results demonstrate, for the first time to our knowledge, that human lungs express renin and that this expression is strongly upregulated in IPF lung tissues. Likewise, IPF fibroblasts showed a 10-fold increase of renin expression and displayed an intense cytoplasmic immunoreactive renin signal compared with normal human lung fibroblasts. On the other hand, the results of our in vitro experiments support a profibrotic role of renin since stimulation of human lung fibroblasts with renin induced the over-expression of TGF- $\beta$ , a pivotal fibrogenic factor and of collagen the main component of the fibrotic scar. Our results also showed that normal human fibroblasts stimulated with renin release increased amounts of active TGF $\beta$ 1. Interestingly, renin also provoked a decrease of MMP-1, an effect that is also observed when fibroblasts are treated with TGF- $\beta$  (21). Moreover, knocking down renin expression using siRNA caused an important decrease of the basal expression of TGF- $\beta$ 1 and collagen, however did not affect

basal  $\alpha$ -SMA expression. A similar effect was described in mesangial cells where it was found that the targeting of the renin receptor with siRNA abolish the upregulation of TGF $\beta$ 1 induced by renin, indicating a direct effect through this receptor (13).

Our understanding of the renin as well as of the renin-angiotensin system as evolved considerably over the last years. Thus, RAS which has traditionally been viewed as a circulatory system may also acts locally, and recent evidence indicates that a complete, functional RAS exists within cells (22). However, the physiological role of this system as well as its implication in tissue pathology remains to be determined.

Recent identification of a specific 350 amino-acid protein (pro)renin receptor has increased the complexity of the system. Considered as an enzyme only responsible for the cleavage of angiotensinogen to form angiotensin I, a growing body of evidence indicates that renin and prorenin binding to the receptor trigger intracellular signaling that in turn modifies gene expression (23). Thus, renin exhibits novel receptor-mediated actions, independent of angiotensin II, that appears to be mediated by extracellular signal-regulated kinases 1 and 2 (ERK 1/2) of the mitogen-activated protein kinase (MAPK) system (24-26). In this context, our results also demonstrated that renin stimulation induces a substantial activation of ERK1/2 in human lung fibroblasts corroborating the role of this pathway in the up-regulation of pro-fibrotic genes such as TGF- $\beta$ 1 and collagen. Importantly, recent evidence indicates that (pro)renin receptor binds both renin and its inactive precursor prorenin, and their binding triggers intracellular signaling that

up-regulates the expression of fibrogenic mediators **(27)**. In our study we also measured the levels of the renin receptor in both lungs and fibroblasts, and although we observed a tendency to be increased in the IPF lungs and fibroblasts the results did not reach statistical difference. However, an interesting observation was a strong correlation in the IPF lungs between the expression of renin and of its receptor.

Previous studies have suggested that renin may play a profibrotic role in vivo as it has been demonstrated in the clipped kidney of Goldblatt rats treated for hypertension **(28)**.

However, studies in other organs, including the lungs are scanty. As in other tissues, angiotensin II has been typically considered the main effector of RAS in lung repair and remodeling. Thus, it has been shown that ANG II causes apoptosis of alveolar epithelial cells and is a potent inducer of procollagen production by human lung fibroblasts both effects via activation of the type 1 receptor and, at least in part, via the autocrine action of TGF- $\beta$  **(29-31)**. Nevertheless, our findings demonstrated that renin has a direct effect on human lung fibroblasts since inhibition of angiotensin II by losartan or of angiotensin-converting enzyme by captopril had a minimal or no effect on the renin-induced upregulation of the fibrotic mediator TGF- $\beta$ 1, and of collagen. These results clearly support the notion that in lung mesenchymal cells renin is able to induce the upregulation of profibrotic molecules through an ANG-II independent pathway.

In a recent study, primary human mesangial cells isolated from healthy kidneys were used to evaluate the renin- and prorenin-triggered gene expression profiles. It



was shown that both have a similar transcriptional signature that is independent of angiotensin production. Importantly, the changes in gene expression induced by renin and prorenin were consistent with the development of organ damage and fibrosis, primarily through TGF- $\beta$  mechanisms (32).

Our findings and the mentioned studies in kidney fibrosis indicate that renin by itself may play an important role in extracellular matrix accumulation, and suggest that the inhibition of the (pro)renin/receptor system may decrease the release of fibrillar collagens as well as of fibrotic factors such as TGF- $\beta$ . In this context, it was recently demonstrated that aliskiren, an orally effective direct renin inhibitor, markedly prevented cardiac hypertrophy, inflammation and fibrosis, and renal glomerulosclerosis caused by cuff injury in eNOS-deficient mice (33). In our study, targeted renin gene silencing by small interfering RNA in cultured human lung fibroblasts ameliorated the expression of collagen and TGF- $\beta$ 1 expression.

In summary, our findings highlight the possible role of renin as an angiotensin-II-independent profibrotic factor in lung fibrosis. IPF is by far the most aggressive interstitial lung disease, and most patients die from this disorder few years after diagnosis. While the pathogenic mechanisms are incompletely understood, the disease is likely the result of the abnormal expression and regulation of a variety of genes that arise from a combination of individual susceptibility and environmental triggers. The results of our study indicate that renin can be one of the mediators involved in the pathogenesis of this disorder.

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## Legend for Figures

**Figure 1: Expression of renin and renin receptor in different strains of fibroblasts.** Fibroblasts were cultured at early confluence, and the expression of renin and its receptor was measured by real-time PCR. **Panel A:** Renin was over-expressed in fibroblasts derived from patients with IPF (n=8) compared with fibroblasts derived from human control lungs (n=4) \*p<0.01. **Panel B:** No differences were detected in the expression of the renin receptor. **Panel C:** Cell lysates prepared from normal and IPF lungs were analyzed by western blot for renin expression as described in Materials and Methods. By densitometric analysis no significant differences were detected (**Panel D**).

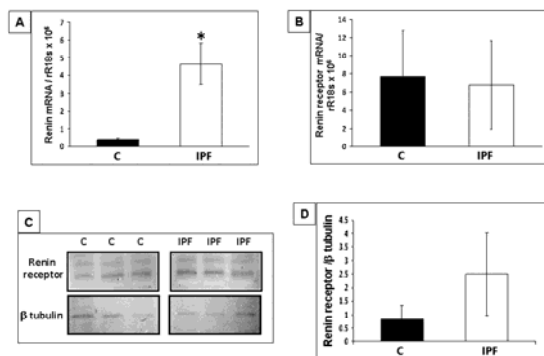


Figure 1

**Figure 2: Immunocytochemical staining of renin.** Fibroblasts from IPF (panels A, 10X and B, 40X) and normal lungs (panel C, 10X) were plated on coverslips and incubated with antihuman renin monoclonal antibody. IPF fibroblasts exhibit intense cytoplasmic labeling as is shown at higher magnification in panel B while human normal lung fibroblasts exhibit a weak staining. **Panel D:** Negative control in

which the primary antibody was replaced by non-immune serum (10X). **Panel E:** Quantification of renin labeling (pixels mean density); C= normal lung fibroblasts; F= IPF fibroblasts; \* $p < 0.01$ . This figure illustrates experiments carried out in 4 different strains.

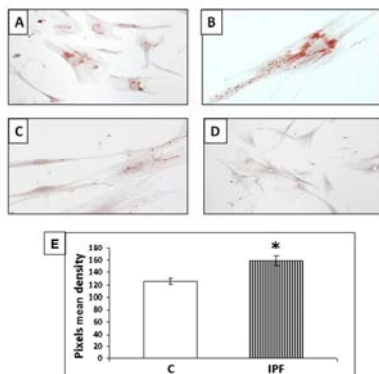


Figure 2

**Figure 3: Gene expression of renin and renin receptor in IPF and normal lungs.** mRNA expression profile of renin and its receptor was assessed by quantitative real-time PCR in lung samples from controls (n = 5) and IPF patients (n = 7). **Panel A:** Renin was over-expressed in the lungs derived from patients with IPF; \*  $p < 0.01$ . **Panel B:** Renin receptor showed a non-significant tendency to be higher in IPF lungs ( $p = 0.06$ ). Black diamonds represent the lung samples and empty squares represent mean.



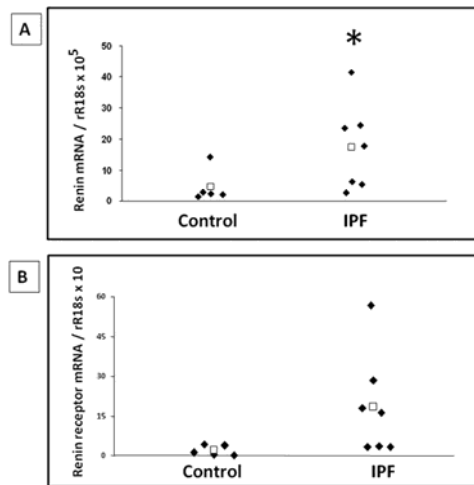


Figure 3

**Figure 4: Renin up-regulates TGF $\beta$ 1 and  $\alpha$ 1 type I collagen gene expression.**

Human lung fibroblasts stimulated with 10nM renin recombinant showed a significant increase of TGF $\beta$ 1 (**panel A**), and collagen (**panel B**). Treatment with Losartan (Los) and Captopril did not revert this effect; \*p<0.01, \*\*p<0.05. The increase of collagen expression was blocked by a specific anti-renin receptor antibody (p<0.01; **panel C**).  $\alpha$ SMA mRNA levels were determined by qRT-PCR analysis (**panel D**) and the protein levels by Western blot analysis (**panel E**). C= non-stimulated fibroblasts; R= fibroblasts treated with renin. Results represent the average of 2 independent experiments with different fibroblast strains, each performed in triplicate.

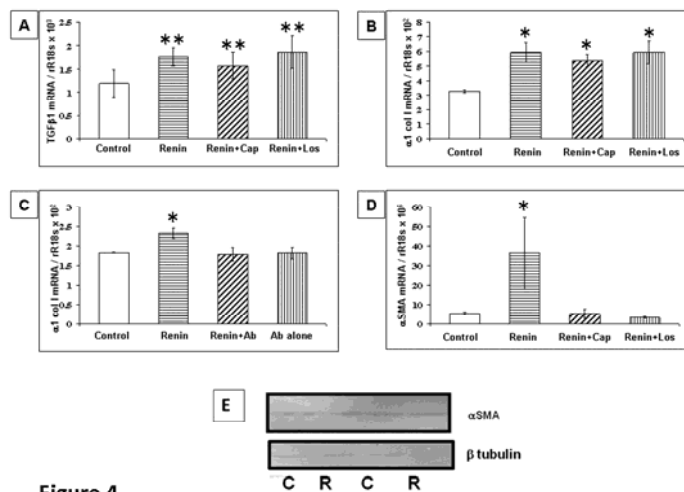


Figure 4

**Figure 5: Renin down-regulates MMP-1 expression while has not effect on MMP-2.** Human lung fibroblasts stimulated with 10nM renin recombinant displayed a significant decrease in the expression of MMP-1 (**panel A**). Gene expression of MMP-2 was not modified (**panel B**). By zymography no changes were observed in the pro-MMP-2 and MMP-2 gelatynolytic activity bands (**panel C**). Results exemplify results of 2 independent experiments with different fibroblast strains, each performed in triplicate.

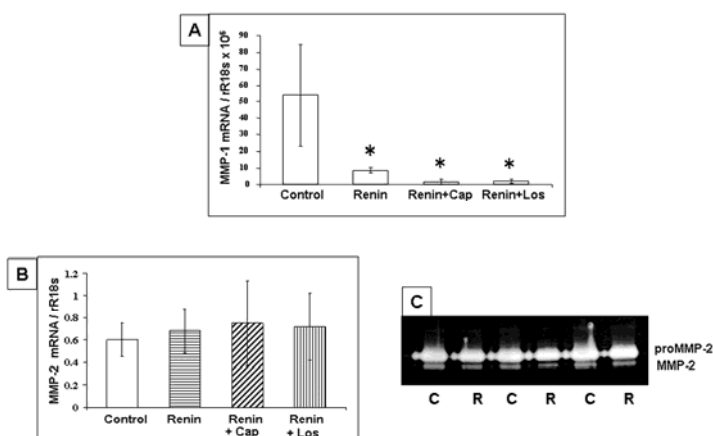


Figure 5

**Figure 6: Renin activates ERK1/2 phosphorylation and increases active TGFβ1.** Human lung fibroblasts were stimulated with 10nM renin recombinant. **Panels A and B** illustrate the Western blotting and densitometric analysis of total and phosphorylated ERK1/2 demonstrating that renin induces ERK ½ phosphorylation. **Panel C** shows a significant increase of TGFβ1 active, effect that was not reverted with captopril or losartan; \*p<0.01, \*\*p<0.05. Results represent the average of 2 independent experiments with different fibroblast strains, each performed in triplicate.

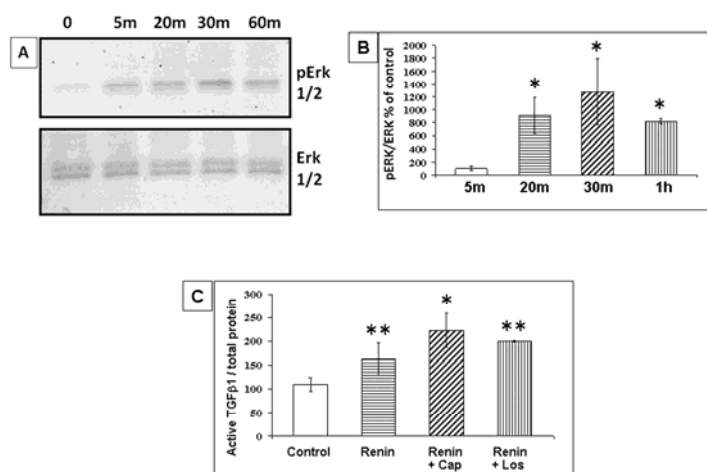


Figure 6

**Figure 7: Renin silencing by small interfering RNA causes down-regulation of TGFβ1.** Human lung fibroblasts were treated with the virus containing renin siRNA (iREN) or with the virus with empty vector (Luc). siRNA caused a significant reduction of renin expression at the gene and protein level (**Panels A and B**). Cells treated with renin siRNA displayed a significant reduction of TGFβ1 (**Panel C**) \*p<0.01. Results represent two independent experiments performed with one fibroblast transfected strain by triplicate.

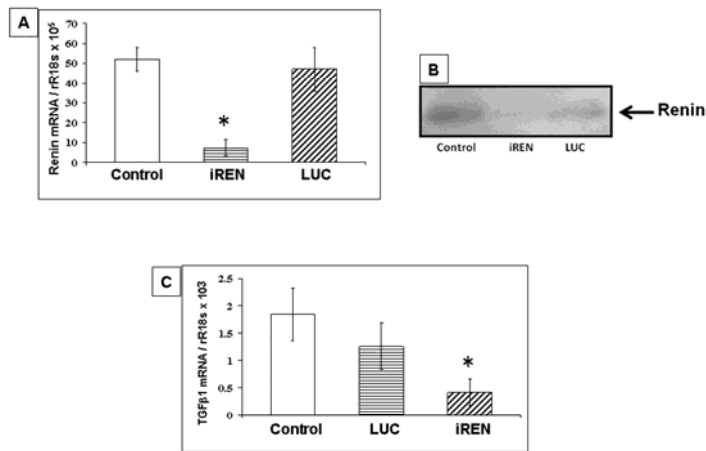


Figure 7

**Figure 8: Effect of down-regulation of renin on collagen production.**

Human lung fibroblasts were treated with renin siRNA and  $\alpha$ 1 type I collagen gene expression was examined by real-time PCR. Collagen protein levels were measured in the conditioned medium by Sircol. Down-regulation of renin caused a significant decrease of collagen synthesis; \*p<0.01. Results represent two independent experiments performed with one transfected fibroblast strain by triplicate.

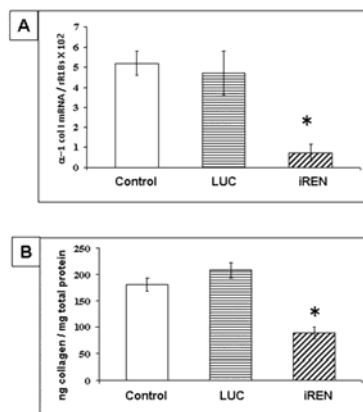


Figure 8



## Renin is an angiotensin-independent profibrotic mediator. Role in pulmonary fibrosis

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Figure for on-line supplement

**Figure S1: *Angiotensin II induces an increase of TGF $\beta$ 1 and  $\alpha$ 1 type I collagen gene expression.*** Human lung fibroblasts stimulated with 10nM of Ang II, displayed a significant over-expression of TGF $\beta$ 1 (**Panel A**) and collagen (**Panel B**), effect that was reverted by Losartan (Los); \*p<0.01. Data represent experiments performed in two different fibroblast cell-lines by triplicate.