Eur Respir J 2012; 39: 141–148
DOI: 10.1183/09031936.00130310

Renin is an angiotensin-independent profibrotic mediator: role in pulmonary fibrosis

E. Montes*, V. Ruiz*, M. Checa*, V. Maldonado#, J. Melendez-Zajgla¶, M. Montaño*, R. Ordoñez-Razo+, J. Cisneros*, C. García-de-Alba*, A. Pardo§ and M. Selman*

ABSTRACT: The pathogenesis of idiopathic pulmonary fibrosis (IPF) is probably the result of interplay between cytokines/chemokines and growth factors. The renin–angiotensin (Ang) system is involved, although its profibrotic effect is attributed to Ang 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 were stimulated with renin or transfected with renin small interfering RNA (siRNA), and the expression of transforming growth factor (TGF)-β1 and α-1-type I collagen was analysed.

Normal lungs and lung fibroblasts expressed renin, which was strongly upregulated in IPF lungs and fibroblasts (~10-fold increase; $p<0.05$). Immunocytochemistry showed intense renin staining in IPF fibroblasts. Renin-stimulated lung fibroblasts displayed an increase in the expression of TGF-β1 (mean±SD $1.8\times10^3\pm0.2\times10^3$ *versus* $1.2\times10^3\pm0.3\times10^3$ mRNA copies per 18S ribosomal RNA; $p<0.01$) and collagen ($5.93\times10^2\pm0.66\times10^2$ *versus* $3.28\times10^2\pm0.5\times10^2$; $p<0.01$), while knocking down renin expression using siRNA provoked a strong decrease of both molecules. These effects were independent of Ang II, since neither losartan nor captopril decreased these effects. Renin also decreased matrix metalloprotease-1 expression and induced TGF-β1 activation (163±34 *versus* 110±15 pg active TGF-β1 per mg total protein).

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

KEYWORDS: Fibroblasts, fibrosing alveolitis

Idiopathic pulmonary fibrosis (IPF) is a progressive and devastating disease characterised by the expansion of the fibroblast/myofibroblast 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 (TGF)-β seems to play a major profibrotic role, inducing fibroblast to myofibroblast differentiation and increasing collagen expression [3, 4]. However, the fibrotic lung reaction is probably 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 is cleaved by renin to form angiotensin (Ang) I [7]. Then, Ang-converting enzyme (ACE) converts Ang I into Ang II. The renin–Ang system is essential for the control of blood pressure and fluid homeostasis. Importantly, the existence of a local autocrine/paracrine renin–Ang system has been demonstrated, which is physiologically active in many tissues [8].

Studies in different organs, including the lung, indicate that the renin–Ang system plays an important role in fibrogenesis, although the effect is primarily attributed to the actions of Ang II through the Ang 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-β1 in human and rat mesangial cells and, consequently, of various extracellular matrix components [13]. Importantly, the activation of the renin/pro-renin receptor in

AFFILIATIONS
*Instituto Nacional de Enfermedades Respiratorias ''Ismael Cosío Villegas'',
#Instituto Nacional de Cancerología,
¶Instituto Nacional de Medicina Genómica,
+Unidad de Investigación Médica en Genética Humana Centro Médico S. XXI, IMSS, and
§Facultad de Ciencias, Universidad Nacional Autónoma de México, Mexico City, Mexico.

CORRESPONDENCE
V. Ruiz
Instituto Nacional de Enfermedades Respiratorias
Tlalpan 4502
CP 14080
Mexico City
Mexico
E-mail: vicoruz@yahoo.com.mx

Received:
Aug 13 2010
Accepted after revision:
May 24 2011
First published online:
June 09 2011

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

This article has supplementary material available from www.erj.ersjournals.com

mesangial cells induced the synthesis of TGF-β through Ang 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 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-β expression.

METHODS

Materials

Antibodies for renin, extracellular signal-regulated kinase (ERK)1/2, phosphorylated ERK1/2, β-tubulin, smooth muscle α-actin (αSMA) and (pro-)renin receptor were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Peroxidase-conjugated secondary antibodies were purchased from Invitrogen (Life Technologies, Grand Island, NY, USA). Captopril, losartan, radioimmunoprecipitation assay (RIPA) buffer and protease inhibitor cocktail was obtained from Biorad (Hercules, CA, USA), and phosphatase inhibitors were obtained from Sigma (St Louis, MO, USA). Recombinant human pro-renin was obtained from Cayman Chemical (Ann Arbor, MI, USA).

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_2/95% air in 25-cm^2 flasks containing Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 $U \cdot mL^{-1}$ penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin and 2.5 $mg \cdot mL^{-1}$ amphotericin B.

Western blotting

Cells reaching 80% confluence were cultured in serum-free medium. The conditioned media were recovered, concentrated 25-fold and dialysed in columns with a 3,000-Da molecular mass limit (3000 YM; Millipore, Billerica, MA, USA). All procedures were performed in the present of the protease inhibitors 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride, EDTA, bestatin, E-64, leupeptin and aprotinin (Millipore). For extraction of intracellular proteins, cells were lysed in RIPA buffer containing protease inhibitor cocktail and phosphatase inhibitors. 8 μg protein was run on 7–12% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels, followed by immunoblotting. Conditions for each antibody were performed according to the manufacturer's instructions. Protein concentration was determined by Bradford assay (Biorad).

Gelatine zymography

SDS-PAGE gels containing gelatine (1 $mg \cdot mL^{-1}$) 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 protein [15].

Stimulation with human recombinant renin

Normal lung fibroblasts were plated at subconfluent density in 25-cm^2 flasks and grown for 24 h in serum-free medium. The medium was then exchanged for serum-free medium containing 10 nM human recombinant renin and the fibroblasts were cultured for a further 3 h. Total RNA was extracted with Trizol™ (Life Technologies) 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 10 nM, 1 h before the addition of renin. To evaluate the role of the renin receptor, in some experiments, fibroblasts were pre-incubated for 1 h at 37°C with a polyclonal anti-renin antibody (16 nM; Santa Cruz antibody sc67390).

ELISA

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

Immunocytochemistry

Fibroblasts (1×10^4) were incubated on coverslips with serum-free medium for 24 h. Fibroblasts were fixed with acetone–methanol (1:1) at -20°C for 2 min and incubated with anti-human renin monoclonal antibody (Serotec, Kidlington, UK) at 37°C for 30 min followed by biotinylated goat anti-mouse immunoglobulin G for 20 min (Biogenex, San Ramon, CA, USA). 3-amino-9-ethyl-carbazole (Biogenex) in acetate buffer containing 0.05% H_2O_2 was used as substrate. Cell nuclei were counterstained with haematoxylin. Coverslips were viewed with a 1×81 microscope (Olympus, Tokyo, Japan), and images were captured using an Evolution MP camera (Media Cybernetics Inc., Bethesda, MD, USA) and processed using Photoshop (Adobe Systems Inc., San Jose, CA, USA). Image analysis was performed using Image Pro-Plus 4.5 (Media Cybernetics Inc.); quantification was expressed as pixel mean density (pmd).

RT-PCR and quantitative real-time PCR amplification

Total RNA and proteins were extracted with Trizol™, according to the manufacturer's instructions. 1 μg total RNA was reverse transcribed using the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA, USA). Quantitative real-time PCR amplification was performed with an i-Cycler iQ detection system (Biorad) [14]. PCR was performed with a cDNA working mixture in a 25-μL reaction volume containing 3 μL cDNA, PCR Master Mix 20×, Taqman probes for TGF-β1, αSMA, α-1 type I collagen and renin, and 1 μL of 20× Taqman 6-carboxyfluorescein–minor groove binder probes (Applied Biosystems, Foster City, CA, USA). For the renin receptor, we used primers designed from sequence GI:15011917 using Primer-BLAST (Basic Local Alignment Search Tool). The sequences were: sense 5′-CATTGTCCATGGGCTTCTCT-3′; and antisense 5′-GCATTCTCCAAAGGGTACGA-3′. For real-time PCR, we used SYBR® Green PCR Master Mix (Applied Biosystems) and 10 pmol of each primer. The dynamic range was determined for each PCR product by copy number serial dilutions of 1×10^{10} to 1×10^2; all PCRs were performed in triplicate. Results were expressed as the number of copies of the target gene normalised to 18S ribosomal RNA (rRNA) (4352930E; Applied Biosystems).

Small interfering RNA

Small interfering RNA (siRNA) was designed as described previously [16, 17]. The sequences of renin siRNA oligonucleotides were designed using Clontech RNAi Designer (http://bioinfo.clontech.com/rnaidesigner/) and the homology was verified by BLAST. Two complementary oligonucleotides (Applied Biosystems) were synthesised, and were cloned into the pSIREN-retroQ-Tet vector (BD Clontech, High Wood, CA, USA) according to the manufacturer's instructions.

Transfection of packaging cells

RetroPack PT67 cells (Clontech) were seeded into a six-well plate at 80% confluence (1×10^6 cells·well^{-1}) 24 h before transfection; 4 μg DNA and 10 μL lipofectamine 2000 were used for transfection. PT67 cells were diluted at a ratio of 1:20 and plated at 24 h post-transfection. The transfected PT67 cells were cultured for 10 days with 2 μg·mL^{-1} puromycin (Clontech), and large, healthy colonies were isolated and transferred into individual wells and plates. After 24 h, the medium was replaced and to determine the efficiency of the infection, small cellular subpopulations were treated with antibiotic. The infected cells were used for experiments or for selection as soon as possible, but not before 24 h post-infection. Quantitative PCR was used to verify the inhibition of renin expression. Filtered medium containing viral particles harbouring the siRNA or empty vector (Luc) siRNA (20 μL) was added to fibroblasts in 2 mL low-glucose Dulbecco's modified Eagle's medium for infection. The infected fibroblasts were diluted at a ratio of 1:10 24 h later and then puromycin was added to a final concentration of 0.5 μg·mL^{-1}. 2 weeks later, large, healthy colonies were isolated and transferred into individual wells and plates.

Collagen measurement

Collagen was quantified in conditioned medium using the Sircol collagen assay (Biocolor Ltd, Carrickfergus, UK) [18]. For these experiments, 1 mL Sirius red dye was added to 100 μL conditioned medium and mixed for 30 min at room temperature. After centrifugation at 10,000 × *g* for 10 min, the collagen-bound dye was dissolved with 1 mL of 0.5 M NaOH and absorbance at 540 nm was measured by spectrophotometry (Nanodrop 1000; Thermo Scientific, Waltham, MA, USA).

Statistical analysis

Data are presented as mean±SD. Data were analysed using unpaired t-test or by one-way ANOVA followed by Dunnett's test. Analysis of correlation was carried out with Spearman's test. A p-value <0.05 was considered statistically significant.

RESULTS

Renin expression is increased in IPF lungs and IPF fibroblasts

Eight IPF and four 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 ($4.7 \times 10^6 \pm 1.2 \times 10^6$ *versus* $0.4 \times 10^6 \pm 0.1 \times 10^6$ renin mRNA copies per 18S rRNA; $p<0.01$). No differences were found in the expression of the renin receptor (fig. 1b), although a tendency to be higher in IPF fibroblasts was observed at the

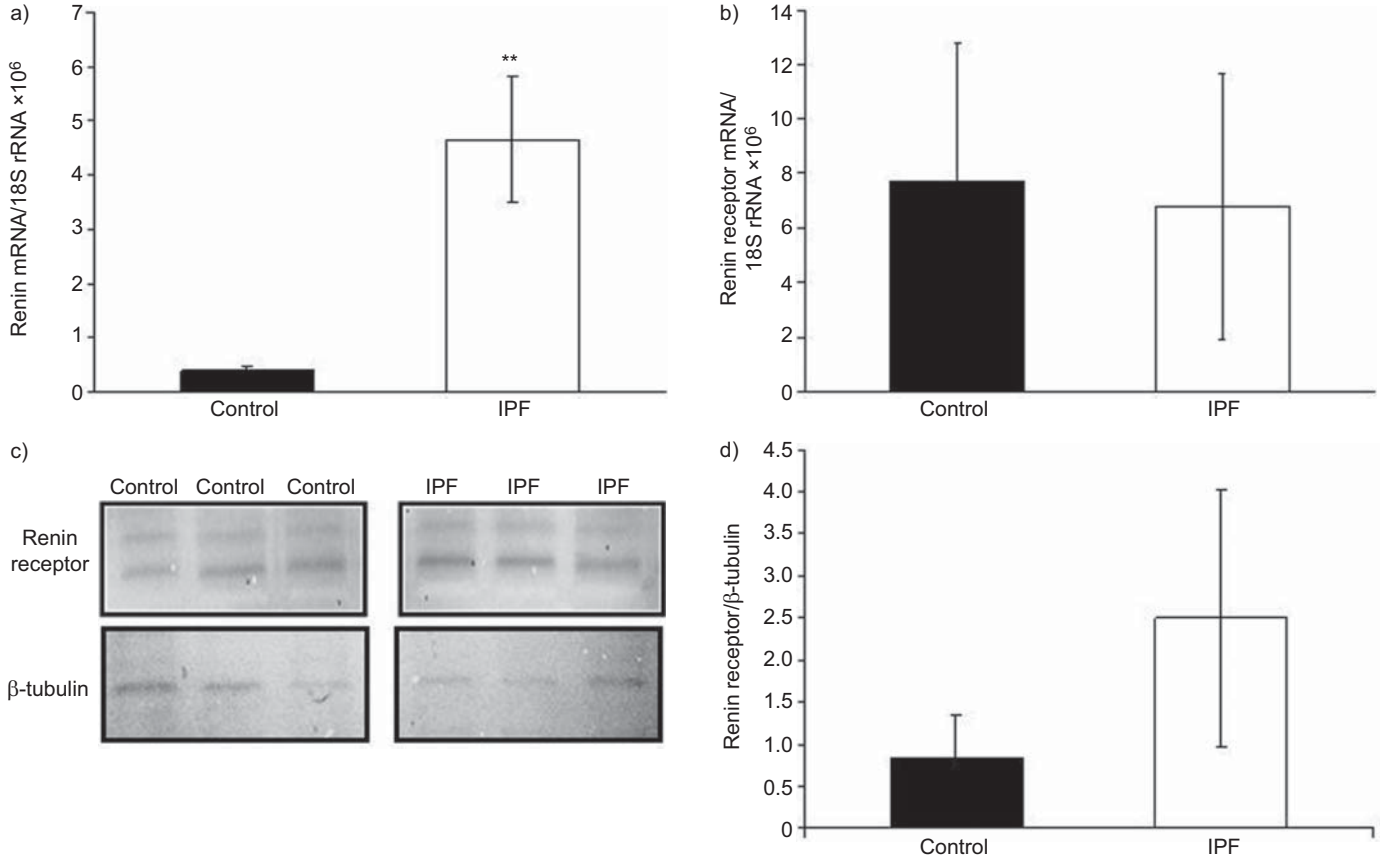

FIGURE 1. Expression of renin and renin receptor in different strains of fibroblasts. Fibroblasts were cultured to early confluence, and the expression of renin and its receptor was measured by real-time PCR. a) Renin was overexpressed in fibroblasts derived from patients with interstitial pulmonary fibrosis (IPF) (n=8) compared with fibroblasts derived from human control lungs (n=4). b) No differences were detected in the expression of the renin receptor. c) Cell lysates prepared from normal and IPF lungs were analysed by Western blotting for renin receptor expression as described in the Methods section. d) No significant differences in protein level were detected by dosimetric analysis. rRNA: ribosomal RNA. **: $p<0.01$.

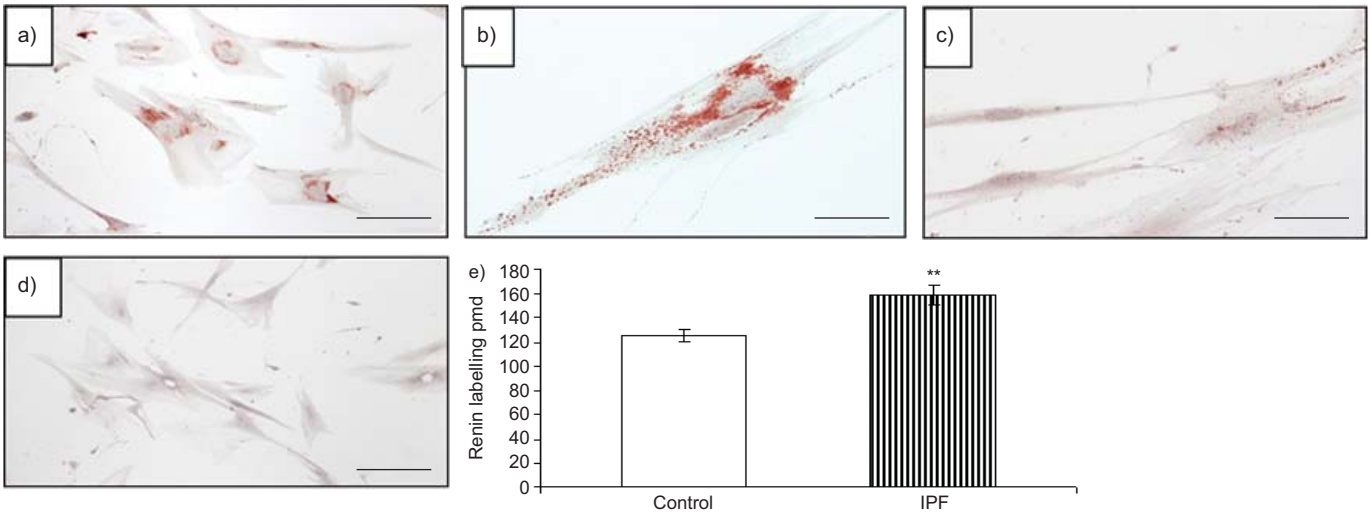

FIGURE 2. Immunocytochemical staining of renin. Fibroblasts from a, b) interstitial pulmonary fibrosis (IPF) and c) control lungs were plated on coverslips and incubated with anti-human renin monoclonal antibody. Scale bars: a) 20 μm; b) 5 μm; c) 5 μm. IPF fibroblasts exhibit intense cytoplasmic labelling, as is shown at higher magnification in b), while human normal lung fibroblasts exhibit a weak staining. d) Negative control in which the primary antibody was replaced by nonimmune serum (scale bar=20 μm). e) Quantification of renin labelling. This figure illustrates experiments carried out in four different strains. pmd: pixel mean density. **: $p<0.01$.

protein level (fig. 1c and d). 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 with normal lung fibroblasts (159 ± 4.8 *versus* 125 ± 8.4 pmd; fig. 2e).

Likewise, the levels of renin mRNA expression were significantly increased in IPF lungs (n=7) compared with normal lungs (n=5) ($17.5\times10^5\pm13.8\times10^5$ *versus* $1.9\times10^5\pm0.6\times10^5$ mRNA copies per 18S rRNA; $p<0.05$; fig. 3a). Also, the levels of renin receptor showed a tendency to be increased, although the result did not reach statistical significance ($185.5\times10^5\pm193.5\times10^5$ *versus* $20.7\times10^5\pm8\times10^5$ renin receptor mRNA copies per 18S rRNA; p=0.06; fig. 3b). Interestingly, there was a positive correlation between the expression of renin and the receptor; thus, the four patients with higher expression of renin also had higher expression of the receptor (Spearman r=0.65, $p<0.05$).

Renin upregulates TGF-β1 and collagen expression through an Ang II-independent mechanism

To evaluate putative profibrotic effects of renin, recombinant renin was used to stimulate two different normal human lung fibroblast strains, and the expression of TGF-β1 and α-1 type I collagen was measured by real-time PCR. As shown in figure 4, renin significantly increased the levels of both TGF-β1 ($1.8\times10^3\pm0.2\times10^3$ *versus* $1.2\times10^3\pm0.3\times10^3$ TGF-β1 mRNA copies per 18S rRNA; $p<0.01$; fig. 4a) and collagen ($5.93\times10^2\pm0.66\times10^2$ *versus* $3.28\times10^2\pm0.5\times10^2$ α-1 type I collagen mRNA copies per 18S rRNA; $p<0.01$; fig. 4b). Pre-treatment of the cells with a specific anti-renin receptor antibody abolished renin-induced collagen increase (fig. 4c). This effect was independent of Ang II, since losartan, a specific inhibitor of the receptor AT1, and captopril, an inhibitor of ACE, did not decrease the renin-induced overexpression of TGF-β1 or collagen. When fibroblasts were stimulated with Ang II, the levels of expression of TGF-β1 and α-1 type I collagen were also increased (online supplementary fig. S1A and B); however, as expected, this increment was abolished by losartan. Stimulation with renin

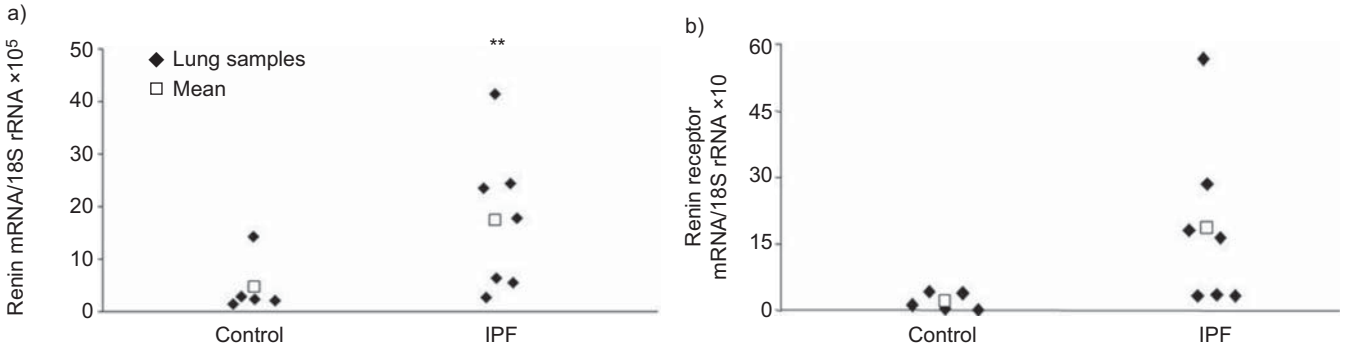

FIGURE 3. Gene expression of renin and renin receptor in interstitial pulmonary fibrosis (IPF) and normal lungs. The mRNA expression profile of renin and its receptor was assessed by quantitative real-time PCR in lung samples from controls (n=5) and IPF patients (n=7). a) Renin was overexpressed in the lungs derived from patients with IPF ($p<0.01$). b) Renin receptor showed a nonsignificant tendency to be higher in IPF lungs (p=0.06). rRNA: ribosomal RNA. **: $p<0.01$.

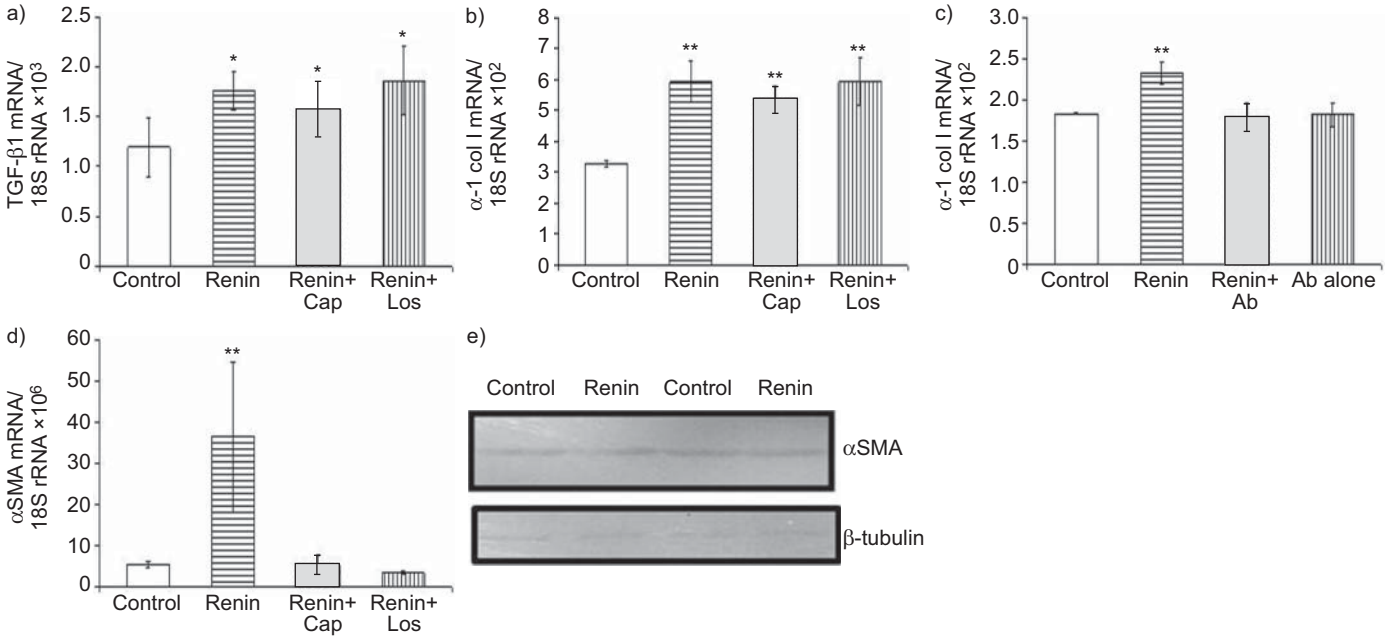

FIGURE 4. Renin upregulates transforming growth factor (TGF)-β1 and α-1 type I collagen (col I) gene expression. Human lung fibroblasts stimulated with 10 nM recombinant renin showed a significant increase in a) TGF-β1 and b) α-1 col I. Treatment with losartan (Los) and captopril (Cap) did not reverse this effect. c) The increase of collagen expression was blocked by a specific anti-renin receptor antibody. d) Smooth muscle α-actin (αSMA) mRNA levels were determined by quantitative RT-PCR analysis and e) the protein levels by Western blotting analysis. Results represent the average of two independent experiments with different fibroblast strains, each performed in triplicate. rRNA: ribosomal RNA. *: $p<0.05$; **: $p<0.01$.

also increased the expression of αSMA (fig. 4d), although this result was not confirmed at the protein level (fig. 4e).

Renin decreases matrix metalloprotease 1 expression but has no effect on matrix metalloprotease 2

Human lung fibroblasts were treated with renin, and the expression of matrix metalloprotease (MMP)-1 and MMP-2, two enzymes that have been associated with the pathogenesis of IPF, was examined [19]. Stimulation with renin caused a significant decrease in the expression of MMP-1 (fig. 5a), while it showed no effect on MMP-2 expression (fig. 5b) or activity (fig. 5c).

It has been suggested that the effect of renin is mediated by ERK1/2 [13]. To investigate whether the stimulation of human lung fibroblasts by renin activated the ERK1/2 signalling pathway, total and phosphorylated ERK1/2 were quantified in cell extracts by Western blot. As illustrated in figure 6a and b, treatment of fibroblasts with renin strongly stimulated phosphorylation of ERK1/2. The temporal profile of ERK1/2 activation showed an increase that reached a plateau at 20–30 min, with a gradual decline thereafter.

Renin induces TGF-β1 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-β1 were assessed in the conditioned medium by ELISA. As shown in figure 6c, renin stimulation significantly increased the levels of

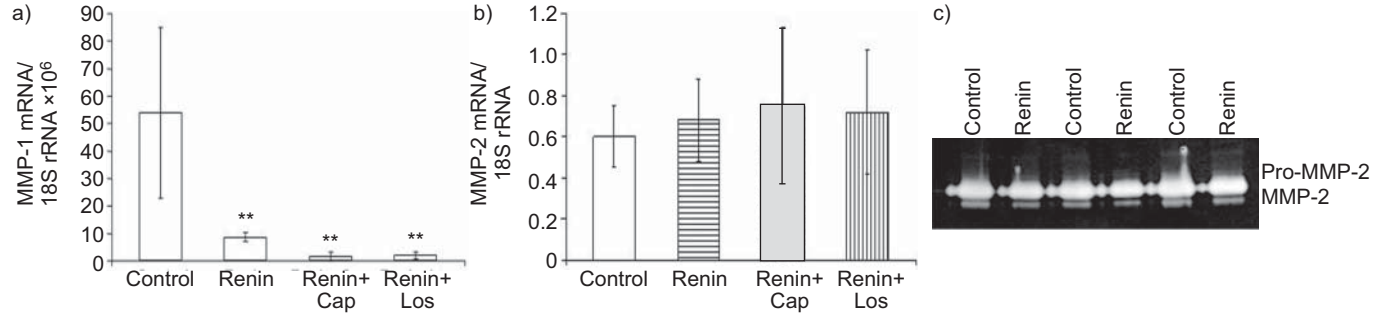

FIGURE 5. Renin downregulates matrix metalloprotease (MMP)-1 expression but has no effect on MMP-2. a) Human lung fibroblasts stimulated with 10 nM recombinant renin displayed a significant decrease in the expression of MMP-1. b) Gene expression of MMP-2 was not modified. c) By zymography, no changes were observed in the pro-MMP-2 and MMP-2 gelatinolytic activity bands. Results represent two independent experiments with different fibroblast strains, each performed in triplicate. rRNA: ribosomal RNA; Cap: captopril; Los: losartan. **: $p<0.01$.

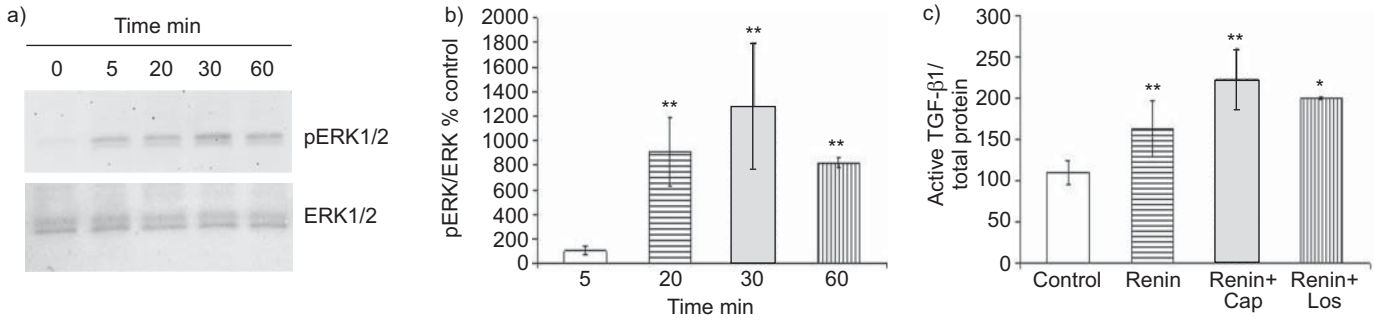

FIGURE 6. Renin activates extracellular signal-regulated kinase (ERK)1/2 phosphorylation and increases active transforming growth factor (TGF)-β1. Human lung fibroblasts were stimulated with 10 nM recombinant renin. a) Western blotting and b) corresponding densitometric analysis of total and phosphorylated ERK1/2 (pERK1/2), demonstrating that renin induces ERK1/2 phosphorylation. c) A significant increase in active TGF-β1 was observed, an effect that was not reversed by captopril (Cap) or losartan (Los). Results represent the average of two independent experiments with different fibroblast strains, each performed in triplicate. *: $p<0.05$; **: $p<0.01$.

active TGF-β1, which was not affected by the ACE inhibitor or the AT1 receptor blocker.

Silencing renin induces a marked decrease of TGF-β and collagen expression

To determine the significance of a loss of renin on fibroblast behaviour, human normal lung fibroblasts were transiently transfected with renin siRNA. With the siRNA, we achieved ~75% silencing of renin, as measured by PCR (fig. 7a); this decrease was confirmed at the protein level by Western blotting of conditioned media (fig. 7b). Real-time PCR was used to determine the effect of renin siRNA on TGF-β, collagen and αSMA expression. As shown in figure 7c, compared with the levels observed in normal fibroblasts and fibroblasts treated with the virus packaged with the empty Luc construct, silencing of renin caused a significant decrease in the expression of TGF-β1 (control $1.84\times10^2\pm0.48\times10^2$, Luc $1.26\times10^2\pm0.42\times10^2$ and renin siRNA $0.42\times10^2\pm0.24\times10^2$ TGF-β1 mRNA copies per 18S rRNA; $p<0.01$).

Collagen expression was also significantly decreased at the mRNA (control $5.2\times10^2\pm0.6\times10^2$, Luc $4.7\times10^2\pm0.11\times10^2$ and renin siRNA $0.74\times10^2\pm0.4\times10^2$ α-1 type I collagen mRNA per 18S rRNA; $p<0.01$) and protein levels, where the inhibition of the expression of renin provoked ~50% decrease of secreted collagen in the fibroblast-conditioned media (fig. 8a and b). αSMA 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 subsequent aberrant architectural remodelling. The pathogenesis of IPF, the most aggressive interstitial lung disease, is believed to be related to dysregulated cross-talk between epithelial cells and fibroblasts, and mediated by a complex interplay among various cytokines, chemokines and growth factors, with a central role of TGF-β. In this context, several studies have implicated the renin–Ang system in lung fibrogenesis, but its profibrotic effects have been attributed primarily to Ang II [20].

Our results demonstrate, for the first time, 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. However, the results of our *in vitro* experiments support a profibrotic role of renin, since stimulation of human lung fibroblasts with renin induced the overexpression of

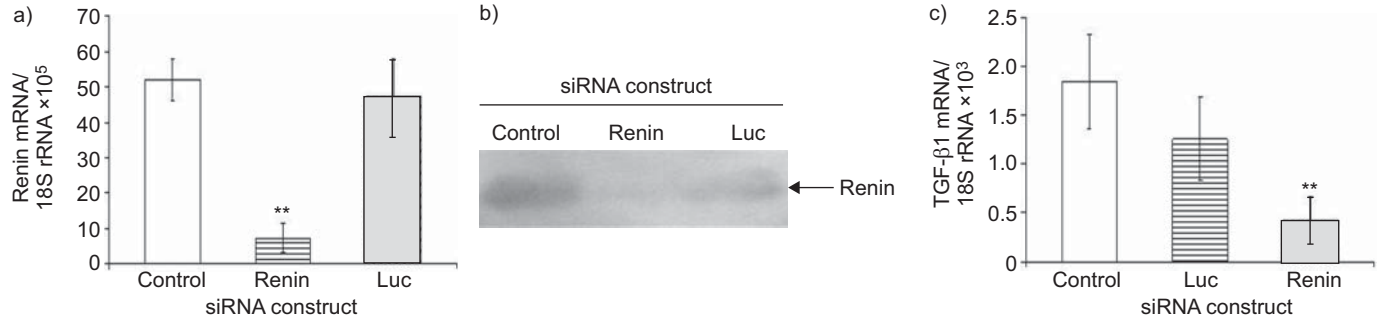

FIGURE 7. Renin silencing by small interfering RNA (siRNA) causes downregulation of transforming growth factor (TGF)-β1. Human lung fibroblasts were treated with virus containing renin siRNA or empty vector (Luc). siRNA caused a significant reduction of renin expression at a) the RNA and b) the protein levels. c) Cells treated with renin siRNA displayed a significant reduction of TGF-β1. Results represent two independent experiments performed with one transfected fibroblast strain in triplicate. rRNA: ribosomal RNA.**: $p<0.01$.

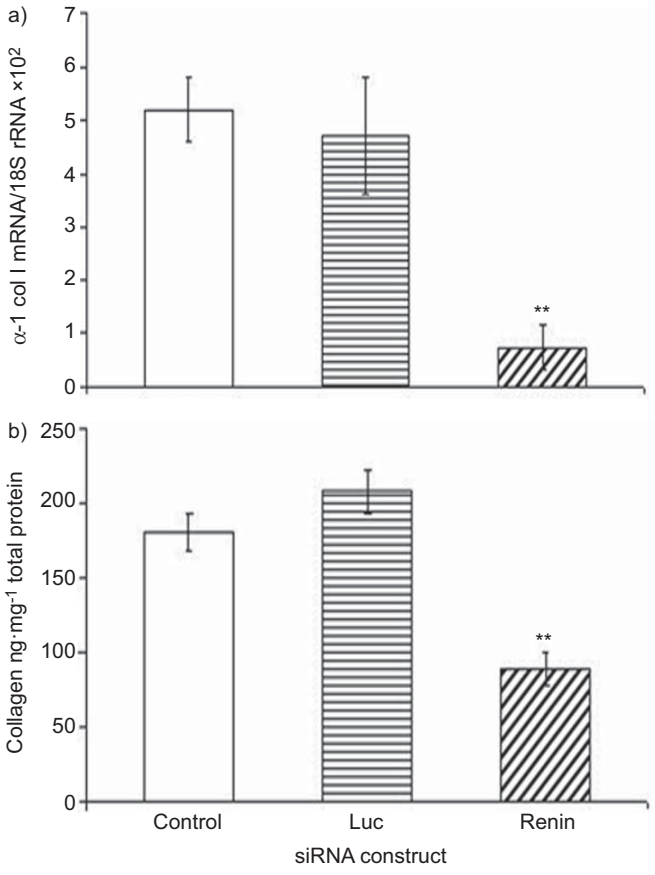

FIGURE 8. Effect of downregulation of renin on collagen production. Human lung fibroblasts were treated with renin small interfering RNA (siRNA), and a) α-1 type I collagen (col I) gene expression was examined by real-time PCR and b) collagen protein levels were measured in the conditioned medium using the Sircol collagen assay (Biocolor Ltd, Carrickfergus, UK). Downregulation of renin caused a significant decrease of collagen synthesis. Results represent two independent experiments performed with one transfected fibroblast strain in triplicate. rRNA: ribosomal RNA; Luc: empty vector. **: $p<0.01$.

TGF-β, 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-β1. Interestingly, renin also provoked a decrease of MMP-1, an effect that is also observed when fibroblasts are treated with TGF-β [21]. Moreover, knocking down renin expression using siRNA caused an important decrease of the basal expression of TGF-β1 and collagen; however, this did not affect basal αSMA expression. A similar effect was described in mesangial cells, where it was found that the targeting of the renin receptor with siRNA abolished renin-induced upregulation of TGF-β1, indicating a direct effect through this receptor [13].

Our understanding of the renin and renin–Ang systems has evolved considerably over the last few years. Thus, the renin–Ang system, which has traditionally been viewed as circulatory system specific, may also act locally, and recent evidence indicates that a complete, functional renin–Ang system exists within cells [22]. However, the physiological role of this system and its implications in tissue pathology remain to be determined. Recently, identification of a specific 350 amino acid protein, (pro-)renin receptor, has increased the complexity of the system. Although renin was previously considered only as an enzyme responsible for the cleavage of angiotensinogen to form Ang I, a growing body of evidence indicates that renin and pro-renin binding to the receptor trigger intracellular signalling that, in turn, modifies gene expression [23]. Thus, renin exhibits novel receptor-mediated actions, independent of Ang II, which appear to be mediated by extracellular ERK1/2 of the mitogen-activated protein kinase pathway [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 upregulation of profibrotic genes, such as those encoding TGF-β1 and collagen. Importantly, recent evidence indicates that the (pro-)renin receptor binds both renin and its inactive precursor pro-renin, and their binding triggers intracellular signalling that upregulates 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 towards increased expression 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 scant. As in other tissues, Ang II has typically been considered the main effector of the renin–Ang system in lung repair and remodelling. Thus, it has been shown that Ang II causes apoptosis of alveolar epithelial cells and is a potent inducer of pro-collagen 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-β [29–31]. Nevertheless, our findings demonstrated that renin has a direct effect on human lung fibroblasts, since inhibition of Ang II by losartan or of ACE by captopril had a minimal or no effect on the renin-induced upregulation of the fibrotic mediators TGF-β1 and 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 pro-renin-triggered gene expression profiles. It was shown that both have a similar transcriptional signature that is independent of Ang production. Importantly, the changes in gene expression induced by renin and pro-renin were consistent with the development of organ damage and fibrosis, primarily through TGF-β mechanisms [32].

Our findings and the aforementioned 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 and fibrotic factors, such as TGF-β. 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 endothelial nitric oxide synthase-deficient mice [33]. In our study, targeted renin gene silencing by siRNA in cultured human lung fibroblasts ameliorated the expression of collagen and TGF-β1 expression.

In summary, our findings highlight the possible role of renin as an Ang II-independent profibrotic factor in lung fibrosis. IPF is by far the most aggressive interstitial lung disease, and most patients die from this disorder within a few years of diagnosis. While the pathogenic mechanisms are incompletely understood, the disease is probably 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.

SUPPORT STATEMENT

This work was partially supported by Consejo Nacional de Ciencia y Tecnología grant III 89442.

STATEMENT OF INTEREST

None declared.

REFERENCES

1 Gross TJ, Hunninghake GW. Idiopathic pulmonary fibrosis. *N Engl J Med* 2001; 345: 517–525.
2 Selman M, King TE, Pardo A. Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. *Ann Intern Med* 2001; 134: 136–151.
3 Willis BC, Borok Z. TGF-β-induced EMT: mechanisms and implications for fibrotic lung disease. *Am J Physiol Lung Cell Mol Physiol* 2007; 293: L525–L534.
4 Gauldie J, Bonniaud P, Sime P, *et al.* TGF-β, Smad3 and the process of progressive fibrosis. *Biochem Soc Trans* 2007; 35: 661–664.
5 Agostini C, Gurrieri C. Chemokine/cytokine cocktail in idiopathic pulmonary fibrosis. *Proc Am Thorac Soc* 2006; 3: 357–363.
6 Strieter RM, Gomperts BN, Keane MP. The role of CXC chemokines in pulmonary fibrosis. *J Clin Invest* 2007; 117: 549–556.
7 Persson PB. Renin: origin, secretion and synthesis. *J Physiol* 2003; 552: 667–671.
8 Lavoie JL, Sigmund CD. Minireview: overview of the renin–angiotensin system – an endocrine and paracrine system. *Endocrinology* 2003; 144: 2179–2183.
9 Hartner A, Porst M, Klanke B, *et al.* Angiotensin II formation in the kidney and nephrosclerosis in Ren-2 hypertensive rats. *Nephrol Dial Transplant* 2006; 21: 1778–1785.
10 Bataller R, Sancho-Bru P, Ginès P, *et al.* Liver fibrogenesis: a new role for the renin-angiotensin system. *Antioxid Redox Signal* 2005; 7: 1346–1355.
11 Li X, Rayford H, Uhal BD. Essential roles for angiotensin receptor AT1a in bleomycin-induced apoptosis and lung fibrosis in mice. *Am J Pathol* 2003; 163: 2523–2530.
12 Marshall RP, Gohlke P, Chambers RC, *et al.* Angiotensin II and the fibroproliferative response to acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2004; 286: L156–L164.
13 Huang Y, Wongamorntham S, Kasting J, *et al.* Renin increases mesangial cell transforming growth factor-β1 and matrix proteins through receptor-mediated, angiotensin II-independent mechanisms. *Kidney Int* 2006; 69: 105–113.
14 Ramos C, Montaño M, Becerril C, *et al.* Acidic fibroblast growth factor decreases α-smooth muscle actin expression and induces apoptosis in human normal lung fibroblasts. *Am J Physiol Lung Cell Mol Physiol* 2006; 291: L871–L879.
15 García-de-Alba C, Becerril C, Ruiz V, *et al.* Expression of matrix metalloproteases by fibrocytes: possible role in migration and homing. *Am J Respir Crit Care Med* 2010; 182: 1144–1152.
16 Liu FY, Xiao L, Peng YM, *et al.* Inhibition effect of small interfering RNA of connective tissue growth factor on the expression of vascular endothelial growth factor and connective tissue growth factor in cultured human peritoneal mesothelial cells. *Chin Med J* 2007; 120: 231–236.
17 Pebernard S, Iggo RD. Determinants of interferon-stimulated gene induction by RNAi vectors. *Differentiation* 2004; 72: 103–111.
18 Distler JH, Jüngel A, Caretto D, *et al.* Monocyte chemoattractant protein 1 released from glycosaminoglycans mediates its profibrotic effects in systemic sclerosis *via* the release of interleukin-4 from T cells. *Arthritis Rheum* 2006; 54: 214–225.
19 Pardo A, Selman M, Kaminski N. Approaching the degradome in idiopathic pulmonary fibrosis. *Int J Biochem Cell Biol* 2008; 40: 1141–1155.
20 Antoniu SA. Targeting the angiotensin pathway in idiopathic pulmonary fibrosis. *Expert Opin Ther Targets* 2008; 12: 1587–1590.
21 Eickelberg O, Köhler E, Reichenberger F, *et al.* Extracellular matrix deposition by primary human lung fibroblasts in response to TGF-β1 and TGF-β3. *Am J Physiol* 1999; 276: L814–L824.
22 Kumar R, Singh VP, Baker KM. The intracellular renin–angiotensin system: a new paradigm. *Trends Endocrinol Metab* 2007; 18: 208–214.
23 Nguyen G, Danser AH. Prorenin and (pro)renin receptor: a review of available data from *in vitro* studies and experimental models in rodents. *Exp Physiol* 2008; 93: 557–563.
24 Huang Y, Noble NA, Zhang J, *et al.* Renin-stimulated TGF-β1 expression is regulated by a mitogen-activated protein kinase in mesangial cells. *Kidney Int* 2007; 72: 45–52.
25 Huang J, Siragy HM. Regulation of (pro)renin receptor expression by glucose-induced mitogen-activated protein kinase, nuclear factor-κB, and activator protein-1 signaling pathways. *Endocrinology* 2010; 151: 3317–3325.
26 Campbell DJ. Critical review of prorenin and (pro)renin receptor research. *Hypertension* 2008; 51: 1251–1264.
27 Cousin C, Bracquart D, Contrepas A, *et al.* Potential role of the (pro)renin receptor in cardiovascular and kidney diseases. *J Nephrol* 2010; 23: 508–513.
28 Krebs C, Hamming I, Sadaghiani S, *et al.* Antihypertensive therapy upregulates renin and (pro)renin receptor in the clipped kidney of Goldblatt hypertensive rats. *Kidney Int* 2007; 72: 725–730.
29 Wang R, Ramos C, Joshi I, *et al.* Human lung myofibroblast-derived inducers of alveolar epithelial apoptosis identified as angiotensin peptides. *Am J Physiol* 1999; 277: L1158–L1164.
30 Marshall RP, Gohlke P, Chambers RC, *et al.* Angiotensin II and the fibroproliferative response to acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2004; 286: L156–L164.
31 Papp M, Li X, Zhuang J, *et al.* Angiotensin receptor subtype AT_1 mediates alveolar epithelial cell apoptosis in response to ANG II. *Am J Physiol Lung Cell Mol Physiol* 2002; 282: L713–L718.
32 Melnyk RA, Tam J, Boie Y, *et al.* Renin and prorenin activate pathways implicated in organ damage in human mesangial cells independent of angiotensin II production. *Am J Nephrol* 2009; 30: 232–243.
33 Yamamoto E, Kataoka K, Dong YF, *et al.* Aliskiren enhances the protective effects of valsartan against cardiovascular and renal injury in endothelial nitric oxide synthase-deficient mice. *Hypertension* 2009; 54: 633–638.