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The antifibrotic effects of relaxin in human renal fibroblasts are mediated in part by inhibition of the Smad2 pathway[2]

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The antifibrotic effects of relaxin in human renal fibroblasts are mediated in part by inhibition of the Smad2 pathway.

Background. The peptide hormone relaxin has been demonstrated to exert antifibrotic effects in renal and extrarenal tissues. The aims of this study were to identify potential antifibrotic effects of relaxin on human renal fibroblasts in vitro and to analyze their mechanisms.

Methods. All experiments were performed in established renal fibroblast cell lines and in primary cortical fibroblasts. Effects of relaxin were analyzed on cell proliferation, apoptosis, activation of renal fibroblasts, synthesis and secretion of collagen type I and fibronectin, as well as on the secretion of matrix metalloproteinases (MMPs). Effects on transforming growth factor-β1 (TGF-β1) receptor binding were analyzed by flow cytometry and on TGF-β1 signal transduction by immunoblot analyses for Smad4 and 7, translocation from cytosol to nucleus for Smad2 and 3 as well as for phosphorylated and unphosphorylated forms of p38, c-Jun NH2 terminal kinase (JNK) and extracellular-regulated protein kinase (ERK). Finally, specific siRNAs for Smad2 and 3 were applied to assess the signal transduction pathway.

Results. After stimulation with relaxin, tyrosine phosphorylation of a 220 kD protein was demonstrated, indicating interaction with the receptor. Relaxin had only modest inhibitory effects on cell proliferation, and no effects on apoptosis. Conversely, relaxin exerted robust effects on TGF-β1–induced fibroblast to myofibroblast transformation as well as on matrix synthesis and secretion even at the smallest dose tested. The secretion of MMP-2 and MMP-9 was induced noticeably by all investigated relaxin concentrations. TGF-β1 receptor binding was not influenced by relaxin; however, it prevented Smad2 phosphorylation, translocation to nucleus, and complex formation between Smad2 and 3 indicating a possible interaction with TGF-β1 signaling. These findings were corroborated by studies using siRNAs to Smad2 and 3 where siRNA to Smad2 but not to Smad3 inhibited the TGF-β1 induction of fibronectin synthesis. There was no influence of relaxin on intracellular Smad3, Smad4, and Smad7 translocation or phosphorylation of mitogen-activated protein (MAP) kinases.

Conclusion. Relaxin is a potent inhibitor of TGF-β1–induced extracellular matrix (ECM) synthesis and secretion as well as fibroblast activation. Furthermore, it induces ECM degradation by induction of MMP-2 and MMP-9. These effects are mediated, at least in part, by inhibition of TGF-β1 signaling.

[1]These two authors contributed equally to this manuscript.

[2]See editorial by Gaedeke and Peters, p. 405.

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The typical morphologic correlate of end-stage renal failure is interstitial fibrosis, tubular atrophy, and dilatation. Renal fibrosis is mainly characterized by an activation and proliferation of interstitial fibroblasts and by an excessive synthesis and accumulation of extracellular matrix (ECM) components, including fibronectin and collagen types I, III, and IV along with a decreased rate of ECM degradation [1]. This ECM degradation is often inhibited during fibrogenesis due to the up-regulation of tissue inhibitor of matrix metalloproteinase 1 (TIMP-1) and/or a decreased activity of matrix metalloproteinase (MMP). Cytokines such as transforming growth factor-β1 (TGF-β1), connective tissue growth factor (CTGF), platelet derived growth factor (PDGF), fibroblast growth factor 2 (FGF-2), and angiotensin II (Ang II) play important roles in the process of fibrogenesis [2]. TGF-β1 is currently viewed as the most important fibrosis-promoting cytokine [3]. It is responsible for several profibrotic events during fibrogenesis, including ECM synthesis in fibroblasts and tubular epithelial cells, differentiation of fibroblasts into myofibroblasts, epithelial-mesenchymal transformation of tubular epithelial cells, and inhibition of matrix degradation by inhibiting the expression of MMPs and up-regulating the expression of TIMPs. Increased expression has been demonstrated in a number of animal models associated with progressive renal disease including anti-Thy1.1 nephritis [4], diabetic nephropathy [5], Heyman nephritis [6], and obstructive nephropathy [7]. TGF-β signal transduction is mainly regulated

via the Smad proteins, the receptor mediated Smad2, Smad3, the common mediator Smad, Smad4, and the inhibitory Smad7 [8]. Furthermore, mitogen-activated protein (MAP) kinase pathways play an important role for TGF-β signal transduction [9].

Interstitial scarring often progresses despite resolution of the primary disease process. Recently, a number of potential factors have emerged with the potential to possibly reverse fibrotic disease in the kidney, including hepatocyte growth factor (HGF) [10] and bone morphogenetic protein-7 (BMP-7) [11]. However, these substances have not been tested in clinical studies and additional approaches are needed. Another potential candidate for a novel therapeutic approach to progressive renal disease is relaxin. It is a peptide hormone with a molecular weight of 6 kD and a member of the insulin-like growth factor (IGF) family. Like insulin, relaxin is composed by two disulfide-linked chains, the A and B chains [12]. It is predominantly produced by the corpus luteum during pregnancy whereby serum levels in pregnant women range from 0.2 to 1.2 ng/mL [13]. The primarily biologic functions of relaxin are the widening of the birth channel in preparation of birth and the renal vasodilation and hyperfiltration during pregnancy [14]. In humans two genes (H1 and H2) code for functional relaxin. However, H2 relaxin is the principal expression product in vivo. Relaxin has already demonstrated promising antifibrotic potential in vitro and in vivo. In dermal and pulmonary as well as in hepatic fibroblasts recombinant human (rh) relaxin prevented TGF-β–induced collagen synthesis [15–17]. Furthermore, in pulmonary fibroblasts, relaxin inhibited the fibronectin synthesis and stimulated MMP secretion [16]. Relaxin deficiency in mice results in an age-related progression of lung fibrosis [18]. In vivo relaxin showed antifibrotic properties in models of pulmonary [15] and renal fibrosis [19, 20]. Furthermore, it reduced skin thickening and improved mobility in a clinical trial in scleroderma patients [21]. However, the mechanisms of its actions are largely unknown.

The aims of this study were to investigate the effects of relaxin on cell growth and activation, matrix synthesis, and degradation in human renal fibroblasts and to identify the intracellular signaling mechanism of these processes. We demonstrate that relaxin influences fibroblast proliferation only modestly but has robust inhibitory effects on fibroblast activation and ECM synthesis while stimulating matrix degradation. These effects are mediated by the prevention of Smad2 phosphorylation and complex formation with Smad3, whereas no interference with the MAP kinase pathway was observed.

METHODS

Materials

rhrelaxin was a generous gift from Dr. Unemori (Connectics Corp., Palo Alto, CA, USA), rhTGF-β1 was purchased from R&D Systems (Minneapolis, MN, USA). The following mouse monoclonal antibodies were used for cell characterization: anticollagen type I [also used for enzyme-linked immunosorbent assay (ELISA)], anticollagen type III (both from Southern Biothechnology, Birmingham, AL, USA), anticytokeratin (Dako, Carpinteria, CA, USA), antivimentin (Boehringer Mannheim, Mannheim, Germany), antifactor VIII (Dako), antihuman leukocyte antigen (anti-HLA-DR) (Dako), anti-α-smooth muscle actin (anti-α-SMA) (Paesel and Lorei, Wiesbaden, Germany), anti-CD44 (Pharmingen, San Diego, CA, USA), anti-CD54, and anti-CD68 (Dako). Rabbit polyclonal antibody to collagen type I from Biotrend (Cologne, Germany) was used for immunoblot analyses; rabbit polyclonal antibody to fibronectin (Sigma Chemical Co., St. Louis, MO, USA) was used for ELISA as well as for immunoblots. Antirabbit IgG alkaline phosphatase (AP) and antisheep/goat IgG AP, Fab fragments (both from Boehringer Mannheim, Mannheim, Germany) were used as secondary antibodies for ELISA. Mouse polyclonal anti-MMP-1 was purchased from Oncogene (Boston, MA, USA) and rabbit polyclonal anti-MT-MMP-1 from Calbiochem-Novabiochem (Nottingham, UK). The rabbit antibodies to extracellular-regulated protein kinase 1/2 (ERK1/2) and c-Jun NH2 terminal kinase 1/2 (JNK1/2), the mouse monoclonal antibodies to phosphorylated JNK1/2, p38 MAP kinase, and phosphorylated p38 MAP kinase as well as the goat polyclonal antibody to phosphorylated ERK1/2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), as was the donkey antigoat-horseradish peroxidase (HRP) secondary antibody for immunoblots. Recombinant antiphosphotyrosine HRP, goat polyclonal Smad7, and mouse polyclonal Smad4/DPC4 were used for immunoblots and purchased from Transduction Laboratories (Lexington, KY, USA). The rabbit polyclonal antibody to phosphorylated Smad2 was a kind gift from Serhiy Souchelnytski (Ludwig Institute for Cancer Research, Uppsala, Sweden) and the rabbit polyclonal antibody to phosphorylated Smad3 was purchased from Cell Signaling (Beverly, MA, USA). The mouse monoclonal antibody to α-SMA was purchased from Progen (Heidelberg, Germany). The mouse antibody to caspase 3 was a friendly gift from Dr. Schweyer (Department of Pathology, Georg-August-University, Goettingen, Germany). As secondary antibodies for immunofluorescence stainings served rabbit antimouse fluorescein isothiocyanate (FITC) and rabbit antimouse Rhodamin from Dianova (Hamburg, Germany). Trypsin ethylenediaminetetraacetic acid (EDTA), Dulbecco's modified Eagle's medium (DMEM), Iscove's modified Eagle's medium (IMDM), and fetal calf serum (FCS) were obtained from Gibco Ltd. (Paisley, Scotland). Cell culture dishes were from Becton Dickinson (Franklin Lakes, NJ, USA).

Cell culture and quantitation of α-SMA–positive cells

Human renal fibroblast cell lines Tk 173 (normal kidney derived) and Tk 188 (from a kidney with tubulointerstitial fibrosis) have been described previously [22]. These cells were cultured in DMEM containing 10% FCS, 1% L-glutamine (both from Gibco) and 5 μg/mL M-plasmocin to avoid mycoplasma contamination (Paesel and Lorei). Primary renal fibroblasts were isolated from human kidney biopsies. Isolation and characterization were performed as described before [23]. Cells that were positive for vimentin, CD44, CD54, α-SMA, and the collagen types I and III, and negative for cytokeratin, factor VIII, and major histocompatability complex (MHC) class II were considered to be fibroblasts and used for induction assays in passages 2 to 8. Additional staining with the Hoechst dye H33258 (Boehringer Mannheim) was performed to facilitate cell counting. Primary cortical fibroblasts Tk 489 were established from a 61-year-old female with membranous gomerulonephritis without interstitial involvement, Tk 461 fibroblasts were cultured from a biopsy of a 25-year-old female with IgA nephritis and tubulointerstitial fibrosis involving 30% of the interstitium.

Quantitation of α-SMA expression was performed as described previously [24]. Briefly, cells were counted and the relative percentage of positive cells determined by a blinded investigator after stimulation with 10 ng/mL TGF-β1 and relaxin in concentrations of 0.1, 1, and 10 ng/mL for 48 hours. Stimulation with TGF-β1 alone served as positive control. Cells with the typical α-SMA structure were considered to be α-SMA–positive, whereas cells with faint green halo were deemed negative.

Tyrosine phosphorylation

Since the exact nature of the relaxin receptors has not been elucidated, we examined a possible specific cellular response of human renal fibroblasts to relaxin stimulation. Fibroblasts were stimulated with relaxin (0.1, 1, and 10 ng/mL) for 15, 30 and 45 minutes and protein was isolated as described before [23]. Briefly, fibroblasts were lysed with a detergent based buffer and 100 μg of cellular protein were run on a 7.5% sodium dodecyl sulphate-polyacrylamide gel elctrophoresis (SDS-PAGE) gel and transferred to a nitrocellulose membrane (Hybond^{TM} ECL^{TM}) (Amersham, Arlington Heights, IL, USA) by wet electroblotting. To prevent degradation of phosphorylated proteins the tyrosine phosphatase inhibitor sodium vanadate (0.5 mmol/L) was added to the lysis buffer. Membranes were stained with Ponceau red to control for equal loading and transfer. After blocking [phosphate-buffered saline (PBS) + 0.1% Tween + 5% dry milk] for 6 hours, the membrane was incubated with the primary antibody (antiphosphotyrosine antibody 1:500) followed by the secondary antibody (antirabbit HRP antibody) (1:3000). Positive reaction products were identified by chemiluminescence detection with enhanced chemiluminescence (ECL)$^{+}$ (Amersham). Antibodies were diluted in PBS containing 3% bovine serum albumin (BSA).

Effects on proliferation, apoptosis, and necrosis

Proliferation studies were performed using cell counts and nonradioactive bromodeoxyuridine (BrdU) incorporation assays (Amersham) as described before [23]. For cell count experiments, Tk 173 and Tk 188 fibroblasts were plated at 1×10^5 cells per well in a 24-well microtiter plate containing DMEM medium with 10% FCS and incubated overnight. Cells were made quiescent by incubation with FCS-free Iscove's medium for 24 hours. Subsequently, cells were incubated with various additives for additional 72 hours. IMDM medium alone served as negative, IMDM medium containing 10% FCS as positive control. After trypsination, cells were stained with trypane blue and counted using a Neubauer chamber. All experiments were repeated three times.

For BrdU incorporation assay, Tk 173 and Tk 188 fibroblasts were plated at 4×10^3 cells per well in 96-well microtiter plates containing DMEM medium with FCS and incubated overnight. Cells were subsequently made quiescent as described above. Medium containing 10% FCS was used as positive control or FCS + relaxin. IMDM without additives served as negative control. Relaxin was added in concentrations of 0.1, 1, and 10 ng/mL. Proliferation was measured after 24, 48, and 72 hours. Optical densities were determined photometrically at 405 nm (Dynatech MR 4000) (Denkendorf, Germany). All experiments were performed in triplicates and repeated five times.

Apoptosis and necrosis rates were investigated in Tk 173 and Tk 188 fibroblasts after incubation with either IMDM (control), TGF-β1, or different concentrations of relaxin for 72 hours. Apoptosis was detected by immunocytochemical staining for caspase 3. After cytospin, cells were air dried, briefly fixed in acetone/methanol (1:1) and stained for caspase 3 using a modified APAAP method as described elsewhere [25]. Using fast red as peroxidase substrate resulted in a red signal in positively stained cells. The number of positively stained cells was counted and results are shown in percent of total cell number.

Necrosis assay was performed by detection of propidium iodide by FACS analysis. After trypsination, cells were counted, and 5×10^5 cells per sample were incubated with phycoerythrin-conjugated antipropidium iodide antibody for 15 minutes, washed twice before they were counted by a FACS Calibur Flow Cytometer (Becton Dickinson). Results are given in percentage of control IMDM.

Matrix secretion and synthesis

ELISAs and immunoblots were performed for collagen I and fibronectin, the main components of the interstitial matrix. ELISAs of supernatants were performed as described before [23]. A total of 8×10^3 cells were plated per well, and cells were again made quiescent by incubation in serum-free IMDM. After 24 hours, IMDM was replaced by medium containing 1 ng/mL TGF-β1 either alone (as positive control) or in combination with relaxin. IMDM without additives served as negative control. Relaxin was again added in concentrations of 0.1, 1, and 10 ng/mL. Ascorbic acid (50 μg/mL) and propionitrile (50 μg/mL) were added in experiments evaluating collagen secretion. After 48 hours of incubation, supernatants were transferred to a Microlite™ plate and incubated overnight at 37°C. Plates were subsequently dried for 2 hours and blocked with PBS/3% dried milk. Incubation with 50 μL/well of the primary antibody (anticollagen type I in a dilution of 1:300 and antifibronectin antibody at 1:5000 in PBS/0.1% BSA) for 2 hours was followed by washing with PBS/0.1% Tween and incubation with the secondary antibody (antirabbit IgG AP and sheep/antigoat IgG AP, both 1:1000 in PBS/BSA). After three final wash steps, 100 μL/well Boehringer Mannheim (BM) chemiluminescence ELISA substrate (Boehringer Mannheim) was added. Chemiluminescence was then measured in a luminometer (Mikrolumat CB 96P) (Berthold, Bad Wildbad, Bermany) using MikroWin™ software (Mikrotek, Overath, Germany). Nonspecific binding was determined by incubation with the secondary antibody only. The value was normally less than 5% of the total chemiluminescence and was subtracted from each assay. All experiments were performed in triplicates and repeated three times. Standardization was obtained for the collagen type I ELISA using human type I collagen (Becton Dickinson). The limit of detection was 1 ng/mL, with a working range of 10 to 1000 ng/mL. Human fibronectin (Sigma Chemical Co.) served as standard for the fibronectin ELISA. The working range of hat ELISA was 0.1 to 10 μg/mL, with a limit of detection of 10 ng/mL.

The influence of relaxin on the matrix synthesis was evaluated by immunoblot analyses for collagen type I and fibronectin. Cells were treated in flasks under conditions as described above and proteins were isolated after 48 hours. Specificity of the antibodies was determined as described [23].

Measurement of TGF-β1 receptor binding

To investigate TGF-β1 receptor binding a Fluorokine™ Cytokine Flow Cytometry Kit was used. Experiments were performed according to the manufacturer's instructions with some modifications. Cells were cultivated as described above. After trypsination, cells were incubated in DMEM for 6 hours on a rocker platform to enable receptor regeneration. The rocker platform was used to prevent reattachment to the substrate. Then cells were washed in PBS and resuspended at 4×10^6 cells per mL in PBS or PBS + relaxin (0.1, 1, and 10 ng/mL, respectively). Twenty-five microliters of each solution was incubated with 12.5 ng of biotinylated TGF-β1 for 60 minutes at 4°C. Then 10 μL avidin FITC reagent was added and incubated for 30 minutes at 4°C. Probes were washed twice, resuspended in 200 μL of RDF1 buffer and measured at 488 nm in a FACS Calibur Flow Cytometer (Becton Dickinson). Biotinylated soy bean trypsin inhibitor served as negative control. Specificity was controlled by incubation of 40 μg antihuman-TGF-β1 with 12.5 ng biotynilated TGF-β1 for 15 minutes at room temperature and identical treatment.

Zymography

Gelatine zymography was used to evaluate the effects of relaxin on gelatinase (MMP-2 and MMP-9) expression. Briefly, cells were plated at a density of 8×10^4/mL and growth-arrested in Iscove's medium for 24 hours before incubation with relaxin (0.1, 1, and 10, ng/mL) in the presence or absence of 10% FCS or neutralizing TGF-β-antibody, respectively, for 48 hours. Cell supernatants were 10× concentrated and equal amounts of total protein were loaded on SDS gel containing 1.0 mg gelatin per mL and electrophoresis on nonreducing SDS-10% polyacrylamide gels (Bio-Rad) was performed. After 30 minutes, incubation in renaturation buffer containing 2.5% Triton X-100, gelatinolytic activity was detected by an 18-hour incubation step in 5 mmol/L $CaCl_2$, 50 mmol/L Tris/HCl, pH 7.5, at 25°C before Coomassie brilliant blue staining of the gels (0.5% Coomassie blue in 40% methanol/10% acetic acid for 1 hour). Destaining was performed in the same buffer devoid of Coomassie blue for 15 minutes. The presence of the MMPs was indicated by an unstained proteolytic zone of the substrate. Molecular sizes of the bands displaying enzymatic activity were identified by comparison to standard proteins and to purified MMPs (both from Calbiochem). Quantification was achieved by densitometric analysis as described above.

Immunoblot analyses for Smad2, 3, 4, 7, phosphorylated Smad2, phosphorylated Smad3, JNK, ERK, p38, phosphorylated JNK, phosphorylated ERK, and phosphorylated p38 MAP kinase

Cells were solubilized and cytosolic proteins were extracted by addition of cytosolic lysis buffer [10 mmol/L Hepes, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 0.1% NP-40, pH 7.9] for 10 minutes on ice. Solubilized proteins were isolated by centrifugation with 3500 U/min for 4 minutes and nuclear proteins were extracted out of the pellet by

adding of nuclear lysis buffer (20 mmol/L Hepes, 420 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, and 25% glycerol, pH 7.9) for 30 minutes on ice. Total cellular proteins were isolated as described before. Solubilized proteins were extracted by additional centrifugation with 10.000 U/min for 10 minutes at 4°C.

The Western blot analyses for Smad2 were performed as described above. For phosphorylated Smad2 analyses, cells were stimulated for 60 minutes with 10 ng/mL TGF-β1 ± relaxin (0.1, 1, and 10 ng/mL). Immunoblots were performed as described above. Rabbit antiphosphorylated Smad2 antibody was diluted 1:600 and rabbit antiphosphorylated Smad3 1:1000, secondary antirabbit-HRP antibody 1:3000. The HRP-conjugated recombinant antiphosphotyrosine antibody was diluted 1:500 in PBS containing 3% BSA.

Immunoprecipitation

Proteins were isolated as described above and 50 μg protein per sample were incubated with anti-Smad2 or anti-Smad3 antibody, respectively, for 1 hour, followed by immunoprecipitation with agarose A overnight at 4°C. After five washes, the immunoprecipitates were boiled for 3 minutes in Laemmli sample buffer. The resulting precipitated complexes were separated on SDS-polyacrylamide gels, blotted and complex-bound Smad3 (Smad2/3 complex) or phosphorylated Smad3 were detected by a specific antibody and quantification was achieved by densitometric analysis as described above.

Smad2 and Smad3 siRNA transfection and immunoblot

Tk 173 and Tk 188 were plated on a 6-well plate in a density of 4×10^5 cells per well and grown for 48 hours. Then the medium was changed and cells were transfected with Smad2 siRNA SMART Pool® (Upstate Cell Signalling Solutions, Waltham, MA, USA) or Smad3 siRNA (Ambion, Austin, TX, USA) using RNAiFect™ Transfection Reagent (Qiagen, Valencia, CA, USA) following manufacturer's instructions. Cells were left untreated (negative control) or stimulated with 10 ng/mL TGF-β1 in the presence or absence of 10 ng/mL relaxin for 48 hours. Cells were lysed and protein isolated as described above. Effective transfection was documented by Western blot analysis for Smad2 or Smad3, respectively. Effects of TGF-β1 on synthesis of fibronectin in the presence or absence of 10 ng/mL relaxin in transfected and untransfected cells were analyzed by immunoblots as described above.

Statistical analysis

All values are expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used to determine statistical differences between growth factor treated groups and controls using Sigma-Stat™-software 2.03 (Jandel Scientific, San Rafael, CA, USA). Bonferroni's method was used to control for multiple testing. P values < 0.05 were considered significant.

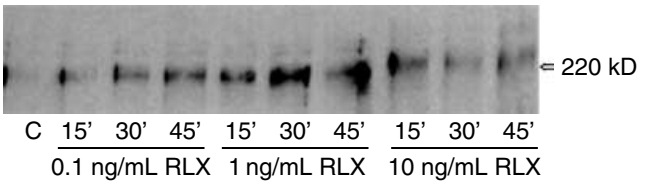

Fig. 1. Relaxin stimulates the tyrosine phosphorylation of a 220 kD protein. After stimulation of Tk 173 fibroblasts with relaxin (RLX) (0.1, 1, and 10 ng/mL) for 15, 30, and 45 minutes a tyrosine phosphorylation of a 220 kD protein was seen. This protein may represent a relaxin receptor. One representative blot is shown.

RESULTS

Stimulation with relaxin results in tyrosine phosphorylation in human renal fibroblasts

Relaxin is an insulin-like hormone. Therefore, its receptor may be, like the insulin receptor, a tyrosine kinase. Relaxin did induce tyrosine phosphorylation in all examined cells. In Tk 173 fibroblasts a significant increase in tyrosine-phosphorylation was seen at relaxin dosages of 1 ng/mL (172.6 ± 16.0% of control after 30 minutes) and 10 ng/mL (151.6 ± 12.2% of control after 45 minutes). Figure 1 displays a characteristic blot.

Relaxin inhibits proliferation of human fibroblasts, but has no effect on either apoptosis or necrosis

The peptide hormone relaxin significantly decreased cell count to 30 ± 13% in Tk 173 or 58 ± 16% in Tk 188 of controls after 72 hours. The effects were reproducible in Tk 188 fibroblasts in the presence of 10% FCS, whereas in Tk 173 they were not (Fig. 2A). Proliferation rate of human fibroblasts were decreased after stimulation with relaxin after 24, 48, and 72 hours at all investigated concentrations (Fig. 2B). However, these effects were not robust and a significant inhibition was only found in Tk 173 cells after 72 hours of stimulation with 0.1 ng/mL relaxin (−10.8 ± 3.7%). The maximum inhibition in Tk 188 was also seen after 72 hours of stimulation with 10 ng/mL relaxin (−22.0 ± 12.6%). There were no effects on apoptosis or necrosis rates (data not shown).

Relaxin inhibits TGF-β1–induced fibroblast activation into myofibroblasts

The differentiation of fibroblasts into myofibroblasts is thought to represent one of the key steps during fibrogenesis. Thus, in order to determine if relaxin influences this process in vitro, primary fibroblasts Tk 461 were incubated with TGF-β1 ± relaxin as described above and the number of α-SMA–positive cells was determined.

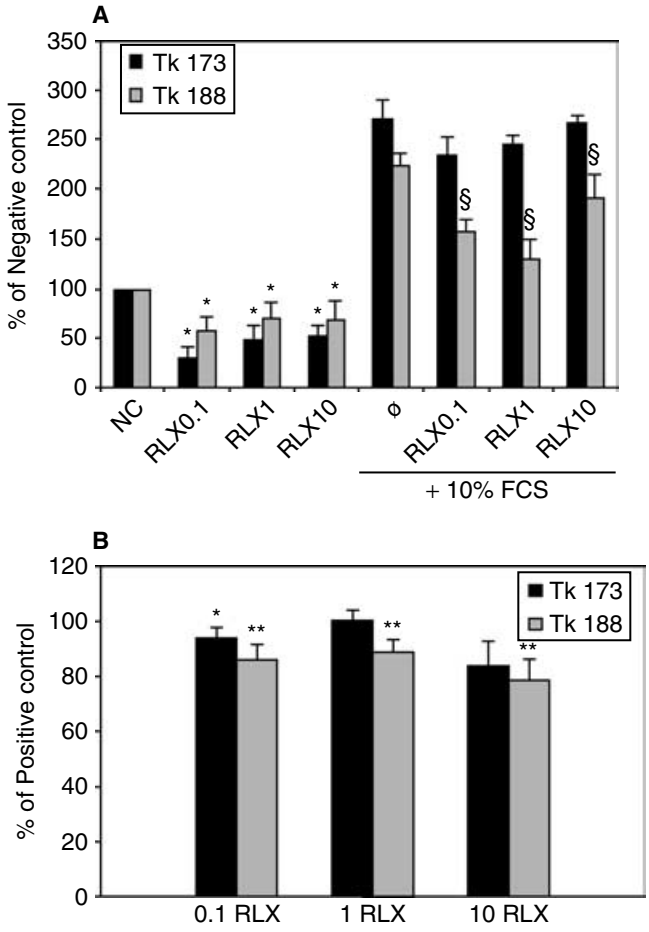

Fig. 2. Effects of human recombinant relaxin (rhRLX) on cell count (*A*) and proliferation (*B*) of immortalized normal kidney-derived (Tk 173) and fibrotic kidney-derived (Tk 188) cortical human renal fibroblasts. Relaxin was added in concentrations of 0.1, 1 and 10 ng/mL for 72 hours. Cell growth was either determined by cell count (*A*) or bromodoxyuridine (BrdU) incorporation (*B*). Relaxin showed antiproliferative effects which were more prominent in Tk 188 fibroblasts. Values are given in percentage of negative control (NC is Iscove's medium alone) (A) or positive control [(PC is 10% fetal calf serum (FCS)] (B) and are the mean of five (Tk 173) and six (Tk 188) experiments. All experiments were performed in triplicates. *P* values were significant against negative control (A) or positive control (B). $^{*}P < 0.05$ vs. negative control; $^{**}P < 0.01$ vs. negative control; §$P < 0.05$ vs. 10% FCS.

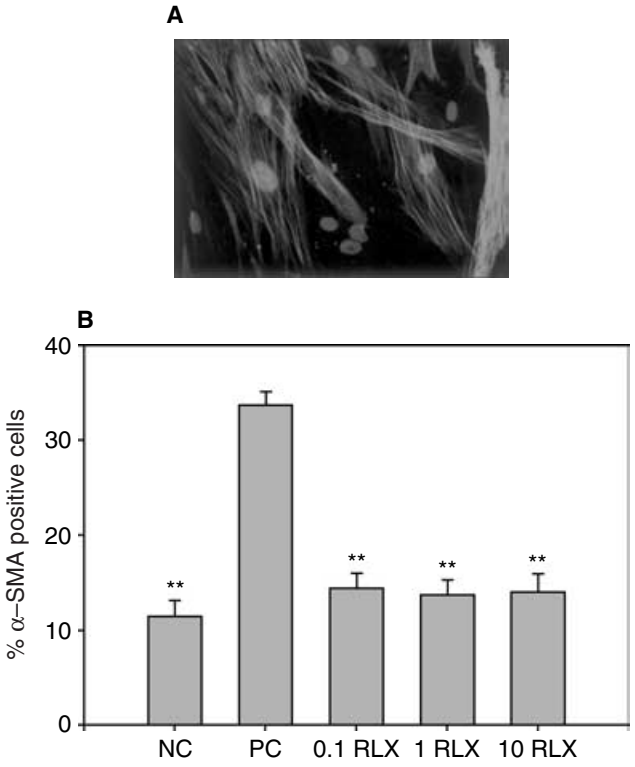

Fig. 3. Expression of α-smooth muscle actin (α-SMA) in primary cortical fibroblasts. The number of α-SMA–positive cells was determined after incubation with 10 ng/mL transforming growth factor-β1 (TGF-β1) and three different concentrations of relaxin (RLX) (0.1, 1, and 10 ng/mL) for 48 hours. Single stimulation with TGF-β1 served as positive control (PC), Iscove's medium as negative control (NC). (A) gives a representative example of the positive control, (B) summarizes the findings graphically. Relaxin reduced α-SMA expression at all investigated concentrations significantly. $^{**}P < 0.01$ vs. positive control.

Under basal conditions (IMDM/1% FCS) the number of α-SMA–positive cells was 11.5 ± 1.7%. Stimulation with 10 ng/mL TGF-β1 raised the number of α-SMA–positive cells to 33.7 ± 1.4%. Relaxin was able to reverse the TGF-β1–induced increase of α-SMA–positive cells in all investigated concentrations. The maximum inhibition was found after stimulation with 1 ng/mL relaxin (13.8 ± 1.6% α-SMA–positive cells). Figure 3 illustrates a typical staining pattern.

Relaxin has robust antifibrotic effects by inhibiting the TGF-β1–induced ECM synthesis and secretion

To study the interactions between relaxin and the TGF-β1–induced ECM synthesis immunoblot analyses for type I collagen and fibronectin were performed. The ECM secretion was investigated by ELISAs for type I collagen and fibronectin. The TGF-β1–induced ECM synthesis was strongly inhibited by relaxin in all investigated cell lines. In Tk 173 the maximum inhibition for collagen I synthesis was found at a dosage of 0.1 ng/mL relaxin (−52.7 ± 6.7%), in Tk 188 at a dosage of 1 ng/mL relaxin (−25.2 ± 8.4%) (Fig. 4). The fibronectin-synthesis was highly significant inhibited at all investigated relaxin doses as well as in all investigated cell lines [Tk 173 and Tk 188 (Fig. 5A) and in primary kidney fibroblasts Tk 489 and Tk 461 (data not shown)]. The maximum inhibition of fibronectin synthesis was seen at dosages of 1 ng/mL relaxin for Tk 461(−34.4 ± 5.6%) and 10 ng/mL relaxin for Tk 173 (−55.0 ± 4.6%), Tk 188 (−51.3 ± 2.4%), and Tk 489 (−40.5 ± 5.7%). However, relaxin alone had no effect on constitutive fibronectin synthesis in neither FCS-starved nor in unstarved Tk 173 and Tk 188 fibroblasts (Fig. 5B).

The TGF-β1–induced secretion of collagen type I was inhibited by relaxin in both cell lines (Fig. 6A and B). In the supernatants of the positive control (1 ng/mL

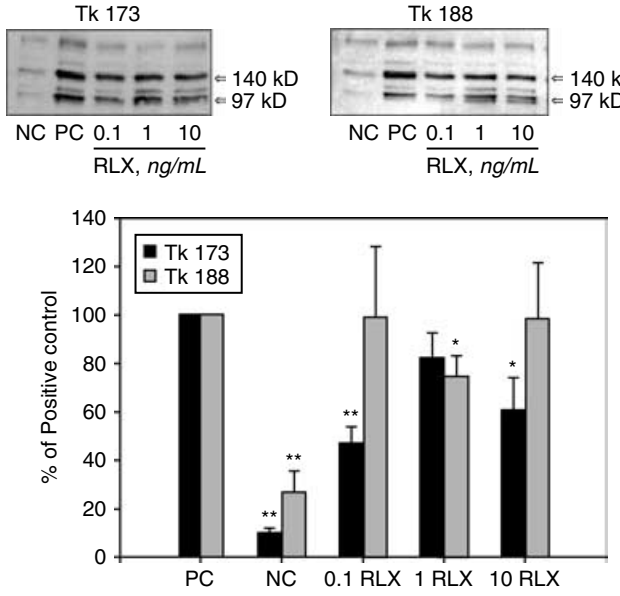

Fig. 4. Inhibition of collagen type I synthesis in Tk 173 (dark shading) and Tk 188 cells (white shading). The effect of relaxin (RLX) on collagen type I synthesis (alpha 1 and alpha 2 chain) was examined by immunoblot analyses of cellular protein after incubation with 1 ng/mL transforming growth factor-β1 (TGF-β1) and three different concentrations of relaxin (0.1, 1, and 10 ng/mL) for 48 hours. Single stimulation with TGF-β1 served as positive control (PC), Iscove's medium as negative control (NC). Values are given in percentage of positive control and are the mean of four independent experiments. At this figure also one representative blot is shown. $^{*}P < 0.05$; $^{**}P < 0.01$.

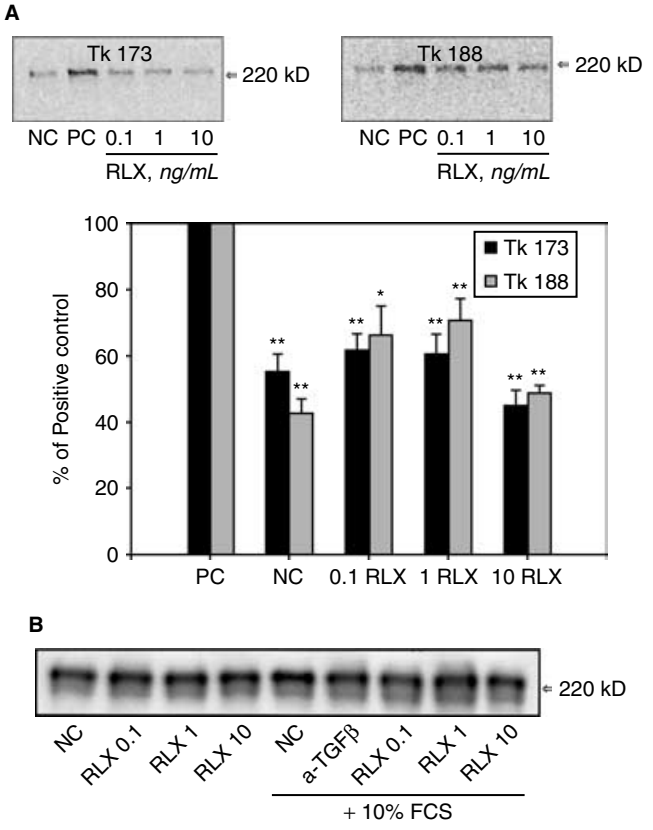

Fig. 5. Inhibition of relaxin (RLX) to transforming growth factor-β1 (TGF-β1)–induced fibronectin synthesis in Tk 173 and Tk 188 human renal fibroblasts. The effect of relaxin on fibronectin synthesis was examined by immunoblot analyses of cellular protein after incubation with 10 ng/mL TGF-β1 and three different concentrations of relaxin (0.1, 1, and 10 ng/mL) for 48 hours. Single stimulation with TGF-β1 (10 ng/mL) served as positive control (PC), Iscove's medium as negative control (NC). Relaxin had robust inhibitory effects on TGF-β1 fibronectin synthesis in both, Tk 173 (dark shading) and Tk 188 (white shading). Values are given in percentage of positive control and are the mean of nine (Tk 173) and seven (Tk 188) independent experiments (*A*). Relaxin alone had no effects on fibronectin synthesis neither in fetal calf serum (FCS)-starved nor in unstarved Tk 173 or Tk 188 fibroblasts (*B*). $^{*}P < 0.05$; $^{**}P < 0.01$.

TGF-β1) 649.9 ± 119.2 ng/mL (Tk 173) and 2072.2 ± 627.8 ng/mL (Tk 188) collagen I were measured. The most visible reduction of secreted collagen I was found at a dosage of 1 ng/mL relaxin (298.5 ± 84.3 ng/mL for Tk 173 and 651.3 ± 310.4 ng/mL for Tk 188). Likewise, the secretion of fibronectin was inhibited by relaxin (Fig. 6C and D). In Tk 173 the amount of fibronectin in the supernatants of the positive control was 1039.6 ± 375.7 ng/mL, in Tk 188 it was 659.59 ± 59.0 ng/mL. The strongest reduction of the fibronectin concentration was found at dosages of 0.1 (Tk 173 460.9 ± 128.6 ng/mL) and 10 ng/mL RLX (Tk 188 372.5 ± 39.8 ng/mL).

Relaxin stimulates secretion of MMP-2 and MMP-9 in renal fibroblasts

The effects of relaxin on matrix-degrading enzymes were studied by gel zymography analyses. Relaxin displayed robust antifibrotic effects by inducing the secretion of MMP-2 and MMP-9 in Tk 173 and Tk 188 fibroblasts (Fig. 7). In both cell lines, MMP-2 secretion was induced highly significantly at all investigated relaxin doses. The most robust increase was seen after stimulation with 10 ng/mL relaxin (131.1 ± 5.7% of control in Tk 173 and 167.0 ± 15.0% of control in Tk 188). Moreover, in Tk 188 incubation with 1 ng/mL relaxin induced a significant induction of the MMP-9 secretion 191.7 ± 22.1% of control. In Tk 173 the strongest increase of MMP-9 secretion was demonstrated by stimulation with 10 ng/mL relaxin (129.7 ± 20.8% of control); however, this effect was not significant.

Additionally, relaxin induced MMP-2 and MMP-9 secretion was determined in unstarved fibroblasts under equal conditions. Relaxin exerted a significant upregulation of both MMP-9 (+68 ± 5.1% vs. control in Tk 173 and +55.5 ± 5.0% vs. control in Tk 188 fibroblasts) ($P < 0.05$) and MMP-2-activity (+107 ± 8.6 vs. control in Tk 188) ($P < 0.05$) after stimulation with 10 ng/mL relaxin (data not shown).

To prove the expression of MMP-1 immunoblot, casein gel zymography, and ELISA analyses were performed. MT-MMP-1 expression was examined by immunoblots. However, both MMP-1and MT-MMP-1 expression could

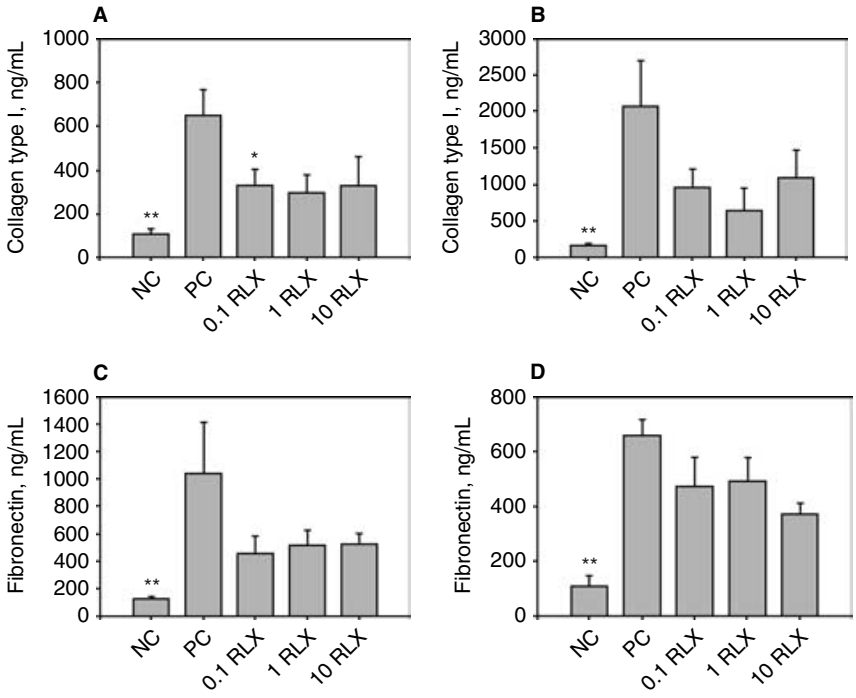

Fig. 6. Influence of relaxin (RLX) on collagen type I and fibronectin secretion of human renal fibroblasts. The effect of relaxin transforming growth factor-β1 (TGF-β1)–induced extracellular matix (ECM) secretion was evaluated by enzyme-linked immunosorbent assay (ELISAs) for collagen type I and fibronectin of cell supernatants. Experiments were performed after stimulation with 10 ng/mL TGF-β1 and three different concentrations of relaxin (0.1, 1, and 10 ng/mL) for 48 hours. Single stimulation with TGF-β1 served as positive control (PC), Iscove's medium as negative control (NC). Relaxin had legible inhibitory effects on collagen type I secretion of Tk 173 (*A*) and Tk 188 (*B*) as well as on fibronectin secretion of Tk 173 (*C*) and Tk 188 (*D*). Values are the mean of six independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

not be demonstrated in the human renal fibroblast cell lines Tk 173 and Tk 188 (data not shown).

Relaxin does not interact with TGF-β1 action by affecting its receptor binding

Possible effects of relaxin on TGF-β1 receptor binding were examined by flow cytometric analyses. Relaxin does not affect TGF-β1 receptor binding in any investigated relaxin dose (data not shown).

In Tk 173 and Tk 188 fibroblasts, relaxin effects the TGF-β1 signal transduction via Smad-proteins by inhibiting Smad2 but not Smad3 pathway

In order to examine the mechanisms of relaxin action, the effects of relaxin on different TGF-β1 signal transduction pathways was investigated. Therefore immunoblot analyses for common Smad4 and inhibitory Smad7 and, moreover, for phosphorylated Smad2, translocation of Smad2 and 3 from cytosol to nucleus, and, for heteromeric formation between Smad2 and 3 after immunoprecipitation were performed. In addition, effects of relaxin on MAP kinase pathway were investigated by immunoblots for the unphosphorylated and phosphorylated forms of the MAP kinases JNK1/2, ERK1/2, and p38.

Smad2 phosphorylation was inhibited by relaxin at all investigated relaxin doses (Fig. 8A). The strongest inhibition was seen at a concentration of 1 ng/mL relaxin ($-24.3 \pm 10.7\%$ vs. positive control). In contrast, relaxin had no effects on Smad3 phosphorylation (Fig. 8B). Since the antiphosphorylation Smad3 antibody cross-reacts with pSmad1, 5, and 8, we precipitated proteins prior to immunoblot analysis with a specific antibody to Smad3. Additionally, relaxin significantly inhibited translocation of Smad2 from cytosol to nucleus, with a maximal inhibition at a dosage of 10 ng/mL relaxin ($-47.3 \pm 7.3\%$ vs. positive control in Tk 173 and $-61.0 \pm 9.2\%$ vs. positive control in Tk 188 fibroblasts) (Fig. 9A). Since phosphorylated Smad2 forms a heteromeric complex with Smad3 and 4, we investigated whether this complex formation was inhibited by relaxin. Consecutively, reduction of Smad2 phosphorylation was associated with a reduction of active complex bound Smad3 as it has been detected by immunoblot after immunoprecipitation for Smad2 in Tk 188 cells (Fig. 9B). The strongest inhibition was seen at the dosage of 0.1 ng/mL relaxin ($-26.3 \pm 14.1\%$ vs. positive control). Conversely, relaxin did have no effect on Smad3 translocation (data not shown) and intracellular Smad7 concentrations (Fig. 9C).

To give further evidence that the effects of relaxin are mediated by Smad2, and not Smad3 inhibition, we compared its effects with those of inhibitory RNA to Smad2 or Smad3. Successful transfection was demonstrated by a reduction of total Smad2 or Smad3, respectively, of more than 80% compared to controls (Fig. 10A). Fibronectin synthesis was chosen as an easy read-out system. Both, relaxin (10 ng/mL) and siRNA to Smad2, significantly blocked TGF-β1–induced fibronectin up-regulation by $-66.33 \pm 18.0\%$ using relaxin ($P < 0.05$ vs. positive control) and $-80.67 \pm 21.1\%$ using siRNA to Smad2 ($P < 0.05$ vs. positive control), but not siRNA to Smad3 (Fig. 10B and C). Representative blots and results are summarized in Figure 10. Conversely, unphosphorylated and phosphorylated forms of MAP kinases JNK1/2,

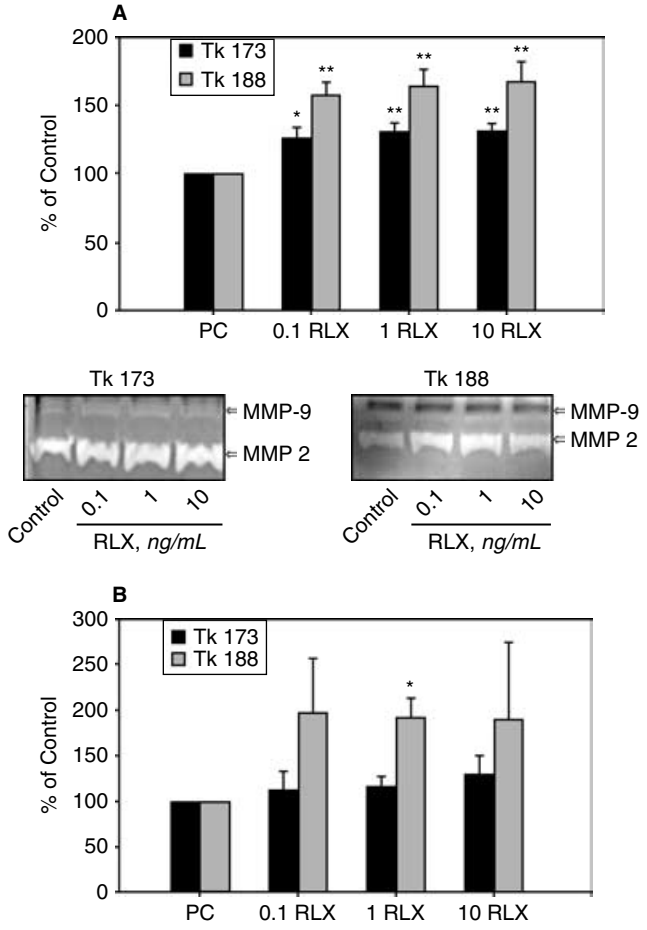

Fig. 7. Effect of relaxin (RLX) on secretion of matrix metalloproteinase 2 (MMP-2) and MMP-9. The effect of relaxin on MMP secretion was evaluated by gel zymography of cell supernatants. Experiments were performed after incubation with three different concentrations of relaxin (0.1, 1, and 10 ng/mL) for 48 hours. Iscove's medium served as control. Relaxin had significant stimulatory effects on MMP-2 secretion (*A*) in both, Tk 173 (dark shading) and 188 (white shading). MMP-9 secretion was also stimulated by relaxin (*B*), where the effect on Tk 188 was more robust than in Tk 173. Values are given in percentage of control and are the mean of six (Tk 173) and three (Tk 188) independent experiments. Also one representative zymography for each cell line is shown. $^{*}P < 0.05$; $^{**}P < 0.01$.

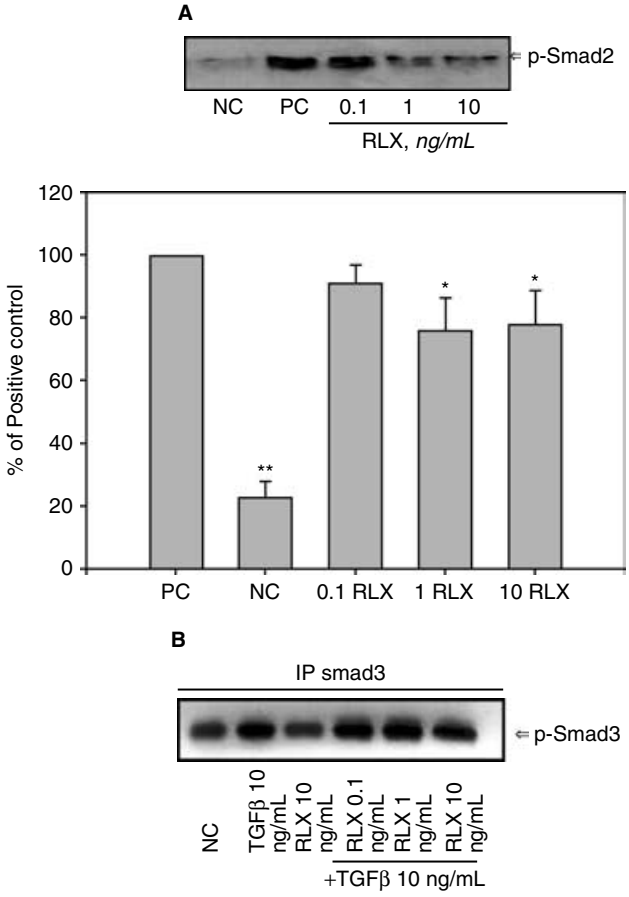

Fig. 8. Influence of relaxin (RLX) on transforming growth factor-β1 (TGF-β1)–induced Smad2 (*A*) and Smad3 phosphorylation (*B*). Human renal fibroblasts were incubated with 10 ng/mL TGF-β1 and three different concentrations of relaxin (0.1, 1, and 10 ng/mL) for 60 minutes. Single stimulation with TGF-β1 served as positive control (PC), Iscove's medium as negative control (NC). In Tk 461 primary human renal fibroblasts, relaxin inhibited Smad2 phosphorylation at all investigated concentrations (A), but there were no effects on Smad3 phosphorylation. Since the antibody against pSmad3 cross-reacts with phosphorylated Smad1, 5, and 8, proteins were immunoprecipitated with a Smad3-specific antibody prior to immunoblot analyses (IP Smad3). Values are given in percentage of positive control and are the mean of four independent experiments. Also one representative blot is shown. $^{*}P < 0.05$ vs. negative control; $^{**}P < 0.05$ vs. positive control.

ERK, and p38 MAP kinase were not affected by stimulation with relaxin in the presence or absence of TGF-β1 (Fig. 11).

DISCUSSION

Tubulointerstitial fibrosis is the final common pathway of almost all forms of chronic progressive renal disease [2]. An effective antifibrotic therapy should inhibit all parts of renal fibrogenesis, including fibroblast proliferation, differentiation into myofibroblasts, ECM synthesis, and matrix degradation. The peptide hormone relaxin has already shown antifibrotic properties on dermal, cardiac, and pulmonary fibroblasts [15, 16]. However, fibroblasts from different origins may display substantial heterogeneity [26].

Our results show that relaxin may inhibit fibroblast proliferation (albeit moderately) and the TGF-β1–induced transformation of these cells to myofibroblasts. Moreover, robust inhibitory effects of relaxin on TGF-β1–induced collagen type I and fibronectin synthesis and secretion could be demonstrated in human lung fibroblasts [16], rat cardiac [27], and very recently in rat renal cortical fibroblasts [28]. Those results implicate strong antifibrotic properties. It has been demonstrated, that fibronectin is able to activate latent TGF-β [29]. Thus, an inhibition of fibronectin synthesis and secretion does inhibit fibrosis

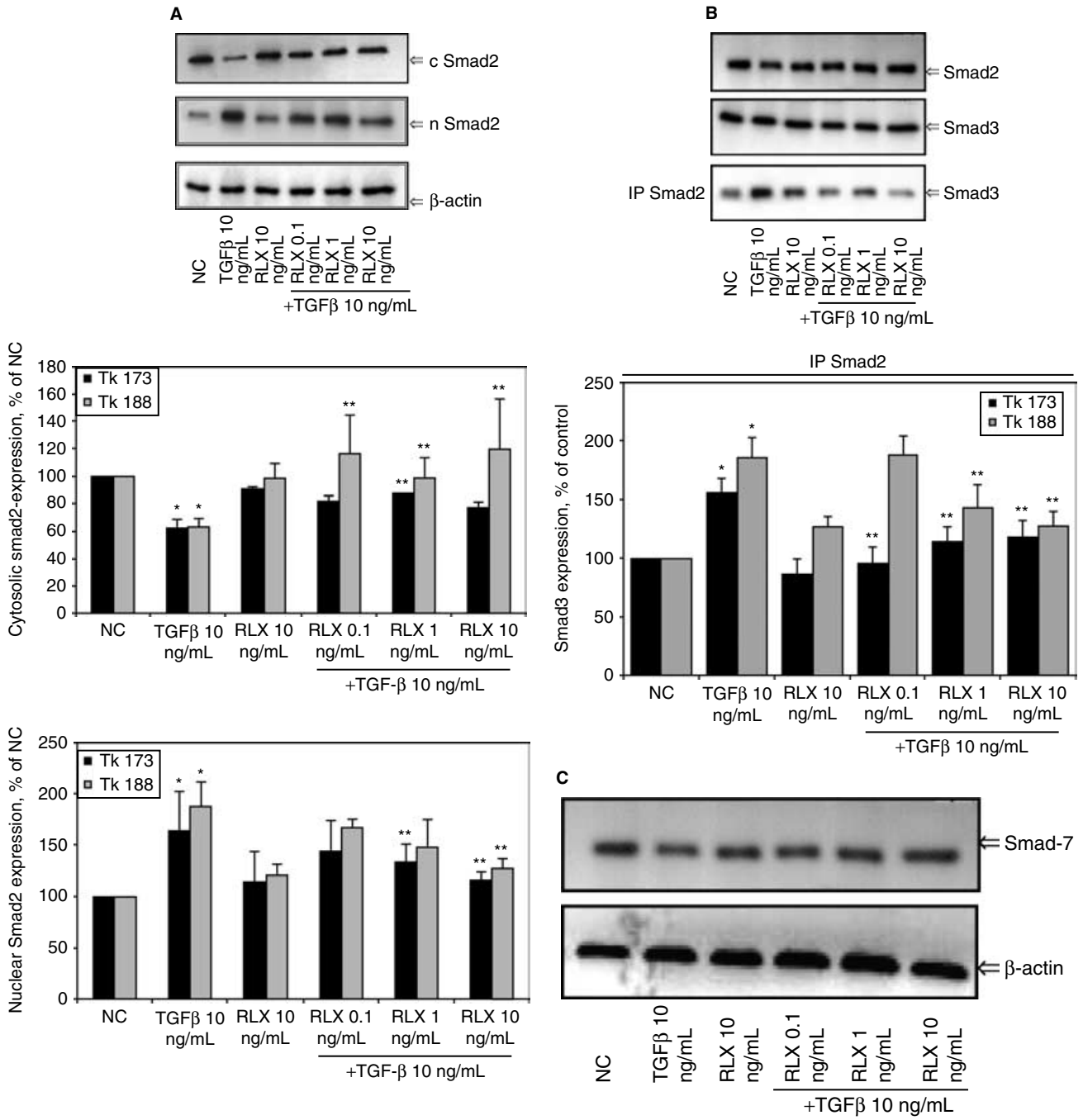

Fig. 9. Influence of relaxin (RLX) on transforming growth factor-β1 (TGF-β1)–induced Smad2 translocation (*A*), heteromer formation between Smad2 and 3 (*B*), and expression of Smad7 (*C*). Human renal fibroblasts (Tk 173 and 188) were incubated with 10 ng/mL TGF-β1 and three different concentrations of relaxin (0.1, 1 and 10 ng/mL) for 60 minutes. Single stimulation with TGF-β1 served as positive control (PC), Iscove's medium as negative control (NC). Cytosolic and nuclear proteins were isolated and cytosolic and nuclear Smad2 was detected by immunoblot analysis. In Tk 188 relaxin inhibited shift of Smad2 from cytosol to nucleus (A). In (B) isolated proteins were immunoprecipitated for Smad2 and immune complex bound Smad3 was detected by immunoblot after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In Tk 188 human renal fibroblasts, relaxin reduced complex formation between Smad2 and 3 at all concentrations. Total cellular Smad2 and 3 expressions were taken as internal controls. Values are given in percentage of negative control and are the mean of four independent experiments. Also one representative blot is shown. $^{*}P < 0.05$ vs. negative control; $^{**}P < 0.05$ vs. positive control. There were no effects of relaxin on inhibitory Smad7 expression detectable (C).

by two mechanisms. First, it decreases ECM deposition and second, it results in reduced matrix expression by diminishing TGF-β activity. However, those effects could mainly prevent the development of renal fibrosis but they are not useful to improve organ function by degrading ECM depositions. Therefore we investigated the influence of relaxin on matrix degrading MMPs. Relaxin was able to raise the secretion of MMP-2 and MMP-9 robustly.

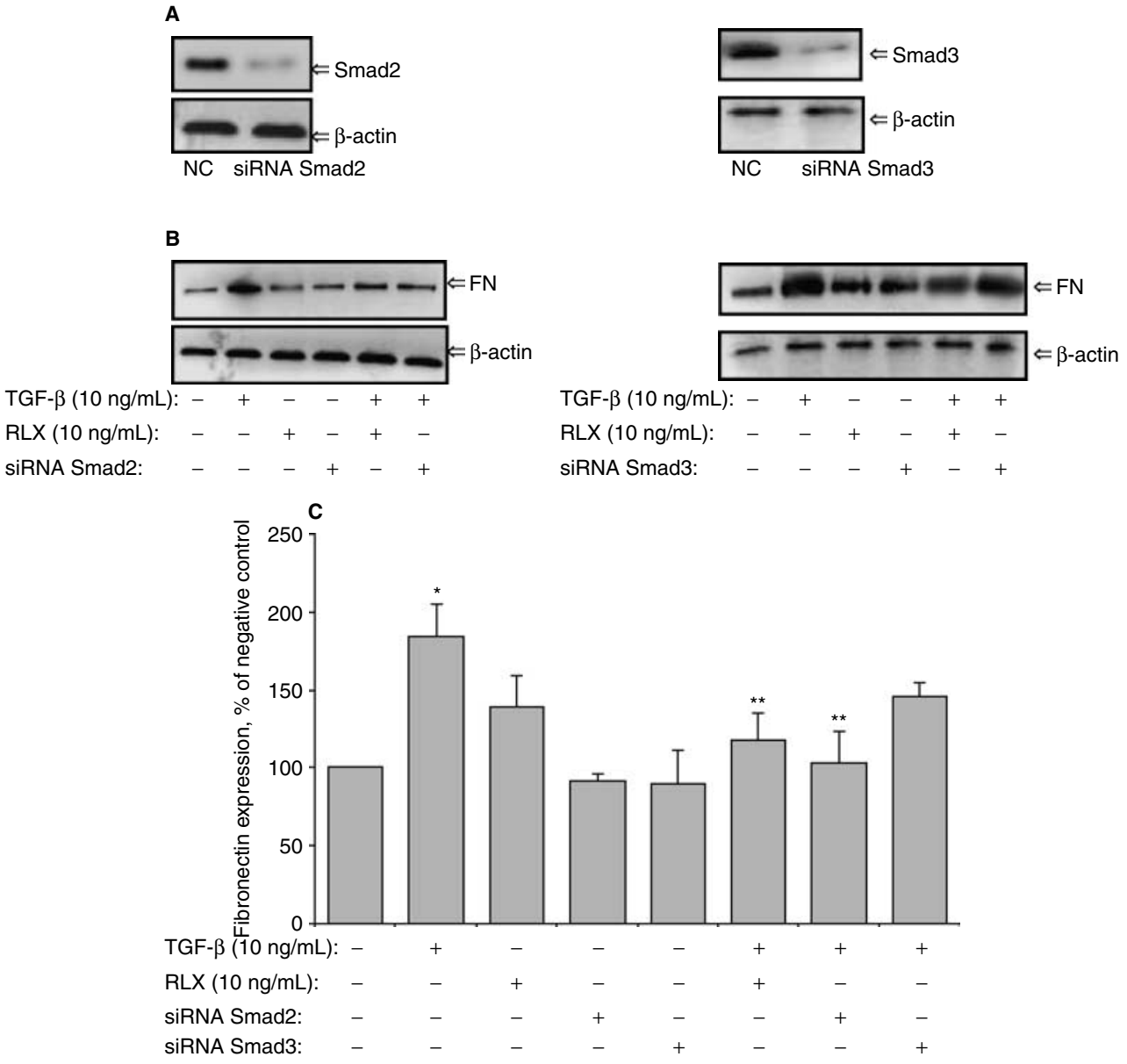

Fig. 10. Relaxin (RLX) as well as siRNA to Smad2, but not siRNA to Smad3 exert comparable inhibitory effects to fibronectin synthesis in Tk 188 renal fibroblasts. (*A*) Effective transfection with inhibitory RNA to Smad2 and Smad3 (siRNA) was demonstrated by a decreased Smad2 or Smad3 expression, respectively, after 48 hours of incubation with siRNA. (*B*) Effects of relaxin in comparison to siRNA to Smad2 or Smad3, respectively, on transforming growth factor-β1 (TGF-β1)–induced fibronectin synthesis was demonstrated. Values are given in percentage of negative control and are the mean of three independent experiments (*C*). $^{*}P < 0.05$ vs. negative control; $^{**}P < 0.05$ vs. positive control.

In murine pulmonary fibroblasts an increase of MMP-1 and MMP-2 has been reported before [16]. Surprisingly, MMP-1 and MT-MMP-1 protein expression could not be demonstrated in any of the investigated human renal fibroblasts indicating that they do not seem to play a role in these cells.

All those results suggest powerful antifibrotic properties of relaxin also in vivo. Relaxin showed robust antifibrotic effects in in vivo models of pulmonary [16] and renal fibrosis [19] as well as well as in a clinical trial of patients with progressive systemic sclerosis [21]. In the treatment of chronic renal failure relaxin might act due to an additional effect. Danielson, Sherwood, and Conrad [14] were able to demonstrate that relaxin attenuates Ang II–induced renal vasoconstriction. Consequently, recently Garber et al [29] noted beneficial effects of relaxin in two models of renal mass reduction. Interestingly, these effects were in part dependent of its effects on blood pressure reduction and in part independent of vasodilation. McDonald et al [20] recently demonstrated that relaxin may increase ubiquitin-dependent fibronectin degradation. In that study, decreased glomerular and interstitial

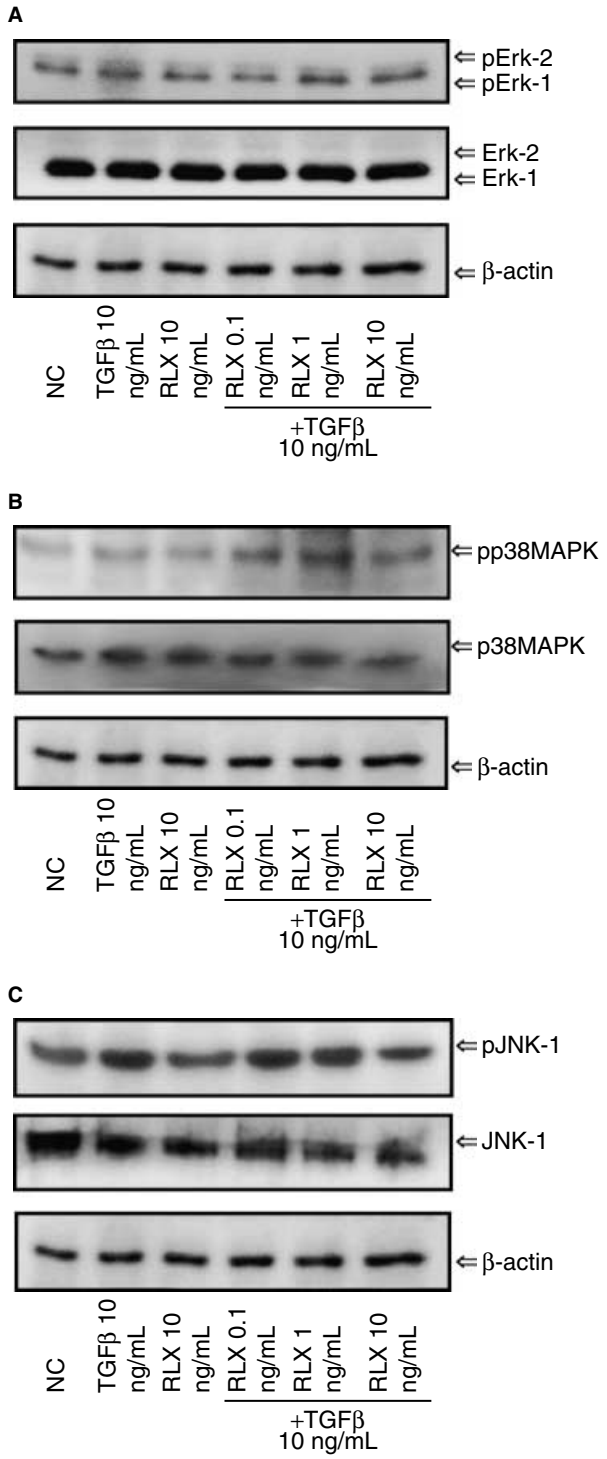

Fig. 11. Relaxin (RLX) has no influence on transforming growth factor-β1 (TGF-β1)–induced phosphorylation of mitogen-activated protein (MAP) kinases, extracellular-regulated protein kinase (ERK1/2) (*A*), p38 MAP kinase (*B*), and c-Jun NH2 terminal kinase (JNK1/2) (*C*). Human renal fibroblasts were incubated with 10 ng/mL TGF-β1 and three different concentrations of relaxin (0.1, 1, and 10 ng/mL) for 60 minutes. Single stimulation with TGF-β1 served as positive control, Iscove's medium as negative control (NC). There were no detectable effects of relaxin on TGF-β1–induced phosphorylation of MAP kinases.

scarring was noted in a rat model of antiglomerular basement membrane disease when the animals were treated with relaxin. Moreover, Samuel et al [18] were able to demonstrate that relaxin−/− mice displayed progression in age-related fibrosis (albeit only in male animals) and that this process could be reversed by addition of relaxin, providing additional evidence for an antifibrotic effect of the hormone.

Interestingly, some of the effects of relaxin we observed were not purely dose-dependent. In most cases the most robust effects were seen at dosages of 0.1 and 1 ng/mL relaxin, the range of physiologic serum concentrations in human pregnancies. These findings correspond to those of Danielson, Sherwood, and Conrad [14] in a clinical trial of relaxin in scleroderma patients and some of the findings by McDonald et al [20]. It is tempting to speculate that they may be explainable by a receptor downregulation or by a relaxin molecule aggregation at higher relaxin concentrations.

As described above, it is not much known about how relaxin exerts its mechanisms since the exact nature of the relaxin-receptor is still unknown. Recently, receptor binding and activation of orphan receptors by relaxin has been demonstrated [30] although the significance of this finding for the effects of relaxin remains to be determined. Moreover, relaxin may bind to glucocorticoid receptors, as was recently shown by Dschietzig and Stangl [31]. In view of these facts, we indirectly demonstrated specific cellular responses of renal fibroblasts. Considering that relaxin is an insulin-like hormone and that the insulin receptor is a tyrosine kinase, we investigated cellular tyrosine phosphorylation in dependence of relaxin stimulation. In fact, we could demonstrate a relaxin-induced tyrosine phosphorylation of a >220 kD protein. Based on these results, we postulate that the phosphorylated >220 kD protein may represent a relaxin receptor. In a next step we investigated the influence of relaxin on TGF-β1 receptor interaction and signal transduction pathways. In a first set of experiments we could demonstrate, that relaxin does not interfere with TGF-β1 receptor binding. The central signal transduction pathway of TGF-β molecules is mediated by Smad proteins. There exists a common mediator Smad, Smad4. Besides Smad4, the most important Smad proteins for the TGF-β1 signal transduction are Smad2 and 3 as well as the inhibitory Smad7. The cytosolic receptor Smad2 and 3 are activated by type I TGF-β receptor and become phosphorylated. Subsequently, they form heteromeric complexes either as Smad2/Smad4, Smad3/Smad4, or as Smad2/Smad3/Smad4. These receptor Smad/costimulatory Smad heteromeric complex translocates to the nucleus where they bind to DNA. Furthermore the TGF-β1 signaltransduction is modulated by MAP kinase pathways via p38, JNK, and ERK. Consequently, the influence of relaxin on all those

proteins was investigated. Relaxin had no influence on cellular amounts of Smad4 and Smad7 as well as on phosphorylated or unphosphorylated forms of MAP kinases JNK1/2, ERK, or p38 MAP kinase. However, relaxin had distinct inhibitory effects on TGF-β1–induced Smad2 phosphorylation resulting in a reduction of translocation of Smad2, but neither on Smad3 phosphorylation nor translocation of Smad3 from cytosol to the nucleus and, moreover, a reduction of heteromeric formation between Smad2 and Smad3 as could be demonstrated by the results of the immunoprecipitation studies. Those results suggest that relaxin affects TGF-β1 signal transduction through an inhibition of receptor regulated Smad2, whereas Smad3, Smad4, or inhibitory Smads were unaffected. Further proof for the specificity of this effect is provided by the fact that inhibition of Smad2 with siRNA resulted in similar results, whereas siRNA to Smad3 did have no noticeable effect on TGF-β1–induced fibronectin synthesis. There is some controversy in the literature regarding the mediation of TGF-β1–mediated fibronectin synthesis and secretion. Whereas our results seem to confirm findings by Isono et al [32] in mesangial cells, others have described that fibronectin synthesis may be independent of the Smad pathway in mouse embryo fibroblasts [33] and a rat fibroblast cell line [34]. Possibly, species differences may explain the differences observed.

CONCLUSION

We conclude that relaxin may represent a potent therapeutic agent to reverse renal fibrosis and remodel renal function. Those properties are at least in part mediated by an attenuation of the profibrotic TGF-β1 effects. Further studies are necessary to delineate the exact nature of the interaction between relaxin and TGF-β1 signal transduction pathways.

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