Biochemical and Biophysical Research Communications 385 (2009) 55–61

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Profiling of anti-fibrotic signaling by hepatocyte growth factor in renal fibroblasts

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ARTICLE INFO

Article history:
Received 1 May 2009
Available online 6 May 2009

Keywords:
Microarray
Smad
Akt
TGFβ
Fibrogenesis
ECM
CCN family
CTGF
Nov
Renal fibrosis

ABSTRACT

Hepatocyte growth factor (HGF) is a multifunctional growth factor affecting cell proliferation and differentiation. Due to its mitogenic potential, HGF plays an important role in tubular repair and regeneration after acute renal injury. However, recent reports have shown that HGF also acts as an anti-inflammatory and anti-fibrotic factor, affecting various cell types such as renal fibroblasts and triggering tubulointerstitial fibrosis of the kidney.

The present study provides evidence that HGF stimulation of renal fibroblasts results in the activation of both the Erk1/2 and the Akt pathways. As previously shown, Erk1/2 phosphorylation results in Smad-linker phosphorylation, thereby antagonizing cellular signals induced by TGFβ. By siRNA mediated silencing of the Erk1/2-Smad linkage, however, we now demonstrate that Akt signaling acts as an auxiliary pathway responsible for the anti-fibrotic effects of HGF. In order to define the anti-fibrotic function of HGF we performed comprehensive expression profiling of HGF-stimulated renal fibroblasts by microarray hybridization. Functional cluster analyses and quantitative PCR assays indicate that the HGF-stimulated pathways transfer the anti-fibrotic effects in renal interstitial fibroblasts by reducing expression of extracellular matrix proteins, various chemokines, and members of the CCN family.

Introduction

Hepatocyte growth factor (HGF) is a mesenchyme-derived multifunctional polypeptide affecting cellular processes, e.g., cell survival, proliferation, migration, and differentiation in epithelial and endothelial cell types (summarized in [1]). Since HGF is the most potent mitogen that is highly increased after acute injury, it is crucial for organ regeneration [2,3]. The specific receptor for HGF is c-met, which belongs to the receptor tyrosine kinase superfamily [4,5]. After HGF binding, the met-receptor becomes autophosphorylated and functions in its phosphorylated form as a multiple docking site for diverse signal transducers such as Gab1, Grb2, Shc, Src, and phospholipase Cγ. They, in turn, forward signals to the PI3K/Akt, Ras-Raf/MAPK, or Stat3 pathways [6].

Recent studies have shown that in addition to the role of HGF in organ regeneration, HGF is an anti-inflammatory and anti-fibrotic factor. The anti-fibrotic action of HGF was first observed in experimental liver fibrosis of chronically intoxicated rats [7]. Subsequently, the anti-fibrotic function of locally or systemically applied HGF was demonstrated in a variety of experimental systems including models of murine and porcine renal failure. HGF treatment resulted in deceleration of histological fibrotic changes and less extracellular matrix (ECM) deposition [8–10]. However, the molecular and cellular mechanisms underlying the anti-fibrotic activities of HGF are not well understood. Recently, Yang et al. reported that HGF mediates its anti-fibrotic effects by antagonizing the pro-fibrotic function of transforming growth factor (TGF)-β [8]. TGFβ is the central fibrotic mediator, which triggers fibrosis by inducing synthesis of ECM proteins and pro-fibrotic growth factors in mesenchymal cell types. Furthermore, TGFβ down-regulates gene expression of MMPs resulting in decreased ECM degradation [11]. Various pathways including signal transduction by the TAK1/p38, JNK and MAPK pathways, the PI3-kinase/Akt-mTor, the Rho pathways, and most importantly by the Smad pathway, are involved in TGFβ signaling [12]. After TGFβ binding, which results in recruitment and phosphorylation of the type I receptor, the receptor-associated Smad2 or Smad3 are phosphorylated. Smad2

Abbreviations: Akt, protein kinase B; Bambi, BMP and activin membrane-bound inhibitor of TGFβ; CCN, Cyr61/CTGF/Nov; COL1A1, α1 chain of collagen type I; CTGF, connective tissue growth factor; FIGF, c-fos induced growth factor; HPRT, hypoxanthine-guanine phosphoribosyl transferase; MMP, matrix metalloproteinase; Nov, nephroblastoma overexpressed protein; Stat3, signal transducer and activator of transcription 3; Wisp2, wnt-induced secreted protein-2.

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doi:10.1016/j.bbrc.2009.05.010

and Smad3 then interact with the common Smad4 and translocate as a heteromeric complex into the nucleus [13,14]. Stimulation with the anti-fibrotic factor HGF was shown to inhibit TGFβ signal transduction by Erk1/2 initiated phosphorylation and blockade of the Smad2/3 transducers in epithelial cells and fibroblasts [15,16].

In the present study, we demonstrate that HGF stimulation of renal fibroblasts results in the activation of the Erk1/2 and the Akt pathways. Comprehensive expression profiling and functional cluster analyses revealed that HGF-stimulated pathways propagate the anti-fibrotic effects in renal interstitial fibroblasts by repression of ECM production, in addition to down-regulation of various chemokines and members of the CCN family. Blocking the Smad pathway by RNA interference revealed that not only is the Erk1/2/Smad2 interaction involved in down-regulation of fibrotic mediators, it also participates in the HGF-stimulated Akt pathway.

Materials and methods

Antibodies. The rabbit polyclonal phospho-specific antibodies p-Smad2 (Ser245/250/255), p-Akt (Thr308), p-Stat3 (Tyr705), and Erk1/2 (Thr202/Tyr204) as well as the antibodies for the detection of the corresponding unphosphorylated proteins were purchased from Cell Signaling Technology (Danvers, USA) and used in blocking solution (1:1000). Rabbit Smad4 antibody (1:500) and the affinity-purified secondary antibody goat anti-rabbit (1:5000) was obtained from Cruz Biotechnology (Santa Cruz, USA).

Cell culture, transient transfection and stimulation with hHGF. Rat kidney interstitial fibroblasts (NRK49F) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) in 5% CO_2 at 37 °C in a humidified atmosphere. For treatment with hHGF, cells were maintained until 70% confluence. Then, either medium was changed and cells were cultured for 24 h in DMEM with 0.5% FCS followed by stimulation with 40 ng ml^{-1} recombinant hHGF (Dianova, Hamburg, GER) for different time periods as indicated, or stimulation was directly performed in medium containing 10% FCS. Finally, cells were harvested for protein or RNA isolation. For transfection we used Lipofectamine 2000 (Invitrogen, Karlsruhe, GER) according to manufacturer's instructions. Twelve hours after transfection with 20 nM Smad4 Silencer Select siRNA or scrambled control siRNA (ABI, Darmstadt, GER), the transfection medium was changed to DMEM supplemented with 10% FCS for another 12 h incubation. Then cells were stimulated with 40 ng ml^{-1} recombinant hHGF for 24 h and harvested for RNA isolation.

Quantification of transcript levels by real-time PCR. Total RNA was isolated via the NucleoSpin RNA II kit of Macherey und Nagel (Düren, GER) according to manufactureŕs instructions. Total RNA (1 μg) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (ABI, Darmstadt, GER) following the supplier's recommendations. cDNA (10 ng) was applied to real-time PCR using specific primers (Supplemental Table S1) and Power SYBR Green PCR Mastermix (ABI, Darmstadt, GER). All reactions were performed in triplicate. HPRT was used for normalization and transcript levels were evaluated by the ΔΔCt method. All results obtained by real-time PCR were analyzed by paired student's *t* test and $p < 0.05$ was considered significant.

Western blot analyses. Cells were lysed in SDS-lysis buffer (15 mM Tris–HCl pH 6.8, 0.5% SDS, 2.5% glycerol) containing protease inhibitors (Roche Applied Science, Mannheim, GER). Equal protein amounts (10 μg) were separated, incubated with the indicated antibodies and signals were visualized and quantified by enhanced chemiluminescence (ECL) using FLUORCHEM®FC2 Alpha Ease (Biozym, Hess. Oldendorf, GER).

Microarray analyses. Expression analyses of total RNA were performed by the Integrated Functional Genomic of Münster (IZKF, Münster, GER) using an Affymetrix GeneChip system® 3000. The quality of the total RNA used was monitored using an Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, GER). Biotinylated cRNA was hybridized to the Affymetrix Rat 230 2.0 gene chip. The data were converted to numerical data using GeneChip Operating Software (GCOS, Affymetrix) and the output was visually checked for immoderate background and physical conspicuities. Data quality was checked and analyzed with ArrayAssist Software (Agilent) whereas background correction, normalization and probe summarization were performed using the GCRMA algorithm. Data were variance stabilized and transformed on a logarithmic scale. Gene expression differences were determined and statistically interpreted by applying the unpaired Mann–Whitney test. For Gene Ontology (GO) analyses only GO terms with a *p*-value (enrichment score) less than or equal to 0.01 were considered.

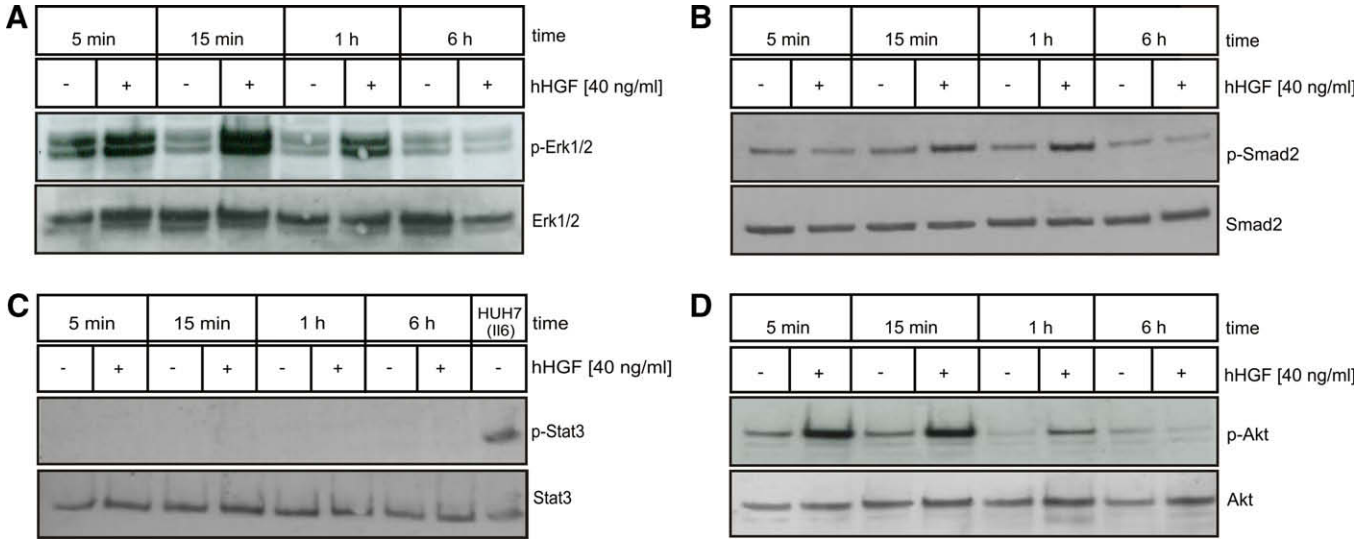
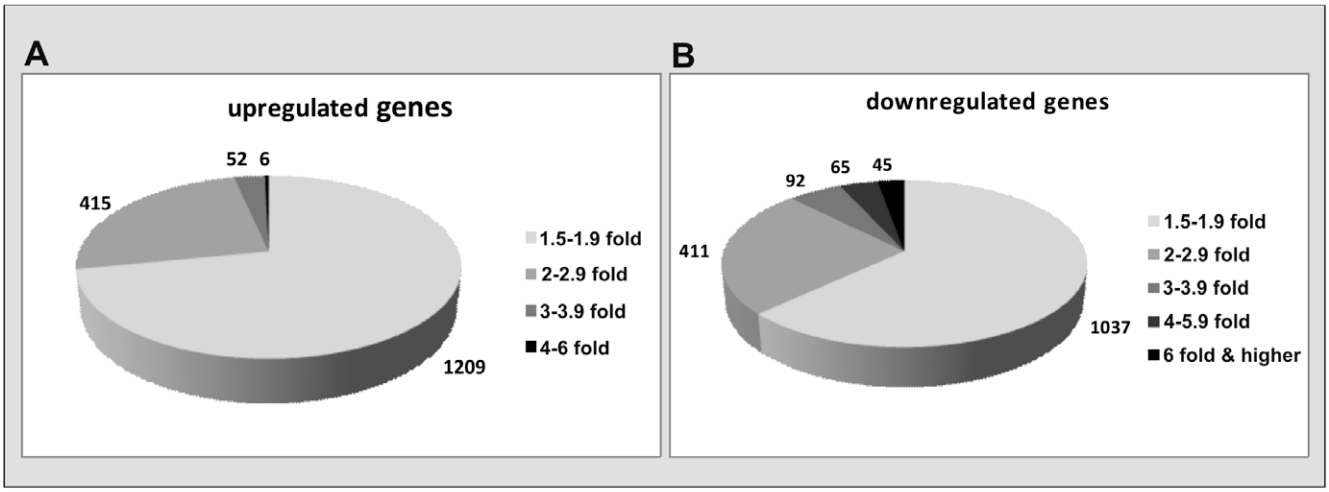
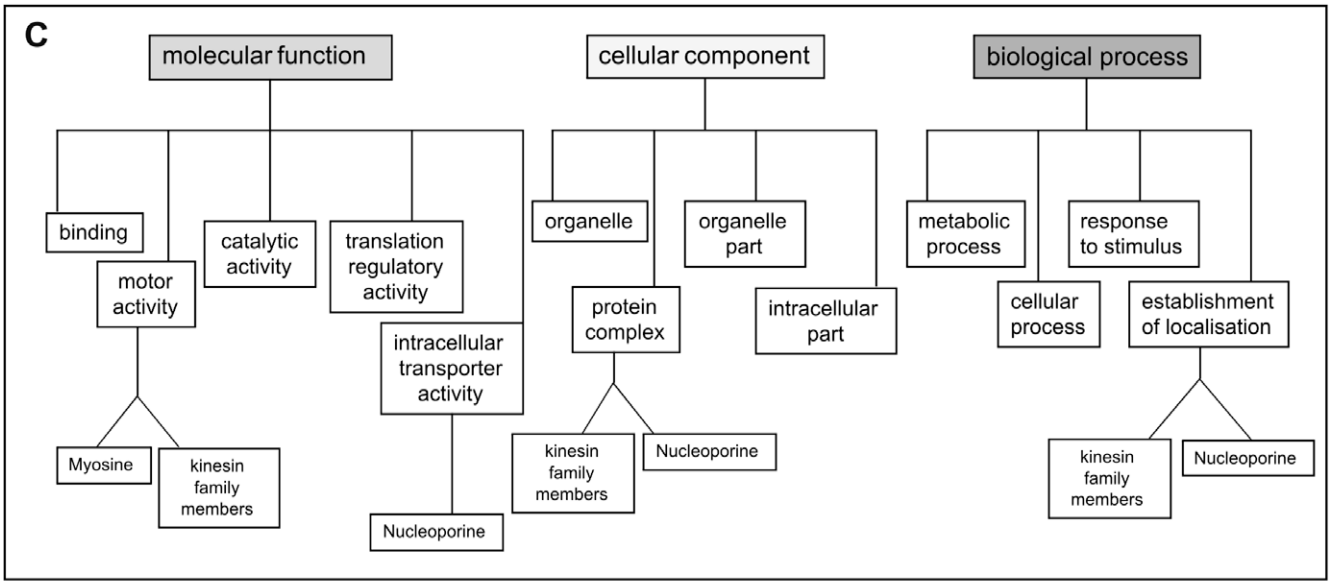


Fig. 1. Western blot analyses of HGF induced signaling cascades in interstitial fibroblasts. NRK49F cells were stimulated with hHGF for the indicated times. Cells stimulated with 40 ng ml^{-1} hHGF are marked by (+), non-stimulated cells by (−). Phosphorylated p-Erk1/2 (A), p-Smad2 (B), p-Stat3 (C), and p-Akt (D) were detected using phospho-specific antibodies. HUH7 cells stimulated with IL-6 served as a positive control for Stat3-phosphorylation (C). Detection of the corresponding unphosphorylated proteins served as loading controls.





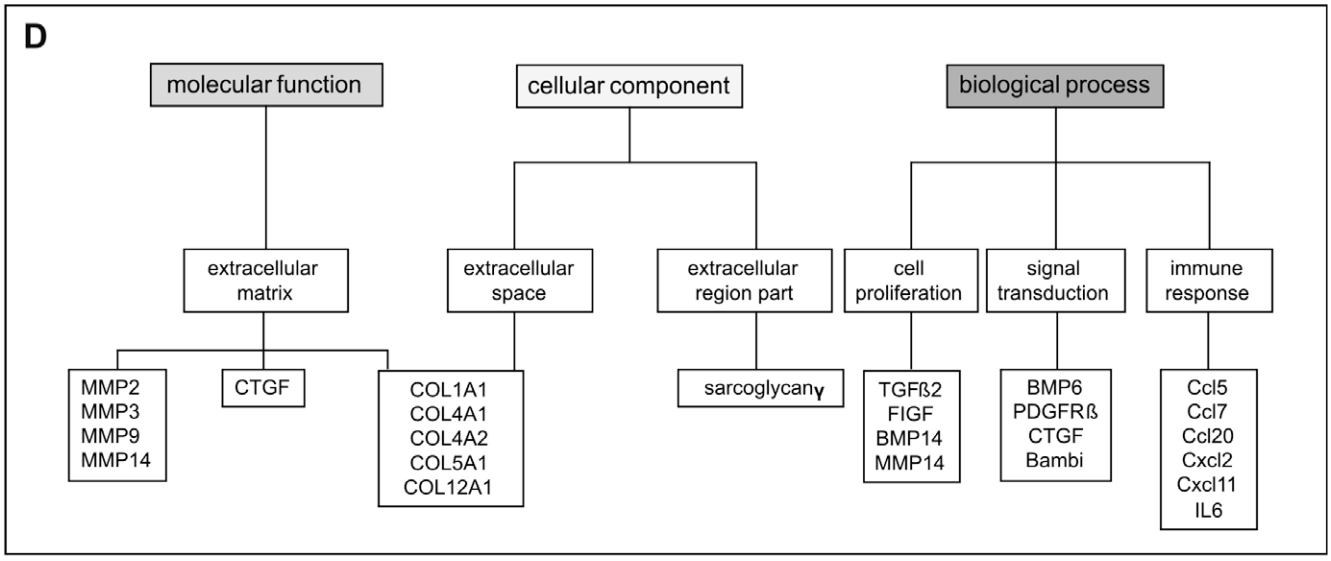


Fig. 2. Expression profiling by microarray analyses of hHGF stimulated NRK49F cells. Up-regulated (A) and down-regulated genes (B) after treatment with hHGF for 24 h. Gene Ontology analyses were performed for functional clustering of up-regulated (C) and down-regulated genes (D) into the three groups: molecular function, cellular component and biological process.

Results

HGF generates anti-fibrotic effects through MAP/ERK- and Akt-signaling

In order to gain insight into the signal transduction of HGF in renal interstitial NRK49F fibroblasts, the phosphorylation status of Erk1/2, Smad2, Akt and Stat3 after hHGF stimulation was analyzed. As illustrated in Fig. 1A, hHGF treatment resulted in phosphorylation of Erk1/2 after only 5 min, reached a peak between 15 and 60 min and then declined. Phosphorylation of Smad2 at the linker region was detectable shortly later than Erk1/2 phosphorylation (Fig. 1B). While Stat3 phosphorylation was not observed (Fig. 1C), the Akt protein was phosphorylated within 5 min after HGF treatment. Thus, activation of the Akt pathway occurs simultaneously with activation of Erk1/2 (Fig. 1D).

Identification of HGF affected genes by microarray analysis

Next, we addressed the question of whether HGF signaling in fibroblasts mediates the anti-fibrotic response by repressing transcript levels of fibrotic mediators. Gene expression profiling identified more than 1600 genes that were either up-regulated or down-regulated by hHGF. Fifty-eight up-regulated genes displayed a fold change higher than 3, however, most of the more than 3-fold differentially expressed genes were down-regulated ($N = 202$) (Fig. 2A). Functional clustering of the genes revealed the up-regulated genes to be preferentially involved in motor and intracellular transporter activity (Fig. 2B). The down-regulated genes, however, were linked to ECM production and degradation, cell proliferation, immune response and signal transduction (Fig. 2C and Supplemental Table S2). In order to identify HGF affected genes that might be involved in fibrogenesis, we selected candidates based on their pronounced divergent expression profile and their potential association with pro-fibrotic processes: (1) genes involved in the synthesis and degradation of ECM, (2) genes of the CCN family, (3) intracellular mediators of TGFβ signals and (4) genes linked to specific signaling cascades.

Repression of collagen type I, SMA and matrix-metalloproteinases by HGF

Both collagen type I, one of the main ECM components of the interstitium upon fibrosis, and SMA, a marker protein for myofibroblastic differentiation, are prominently linked to fibrotic processes. Therefore the transcript levels of the collagen type I α1 subunit and SMA were also analyzed by real-time PCR in hHGF treated renal fibroblasts. We detected a 50% reduction of these transcripts as a result of hHGF treatment (Fig. 3A). Interestingly, in addition to members of the ECM, quantitative PCR analyses confirmed the reduced transcript levels of matrix-metalloproteinases MMP-2, -3, and -9 (Fig. 3A), validating the results obtained by our microarray hybridization (Fig. 2; Supplemental Table S2) .

hHGF inhibits expression of CCN family members and other signal transducers linked to fibrotic processes

Members of the CCN family were dramatically affected by hHGF. CTGF, Wisp2, and Nov displayed significant decreases in their mRNA expression levels with the greatest effect on Nov

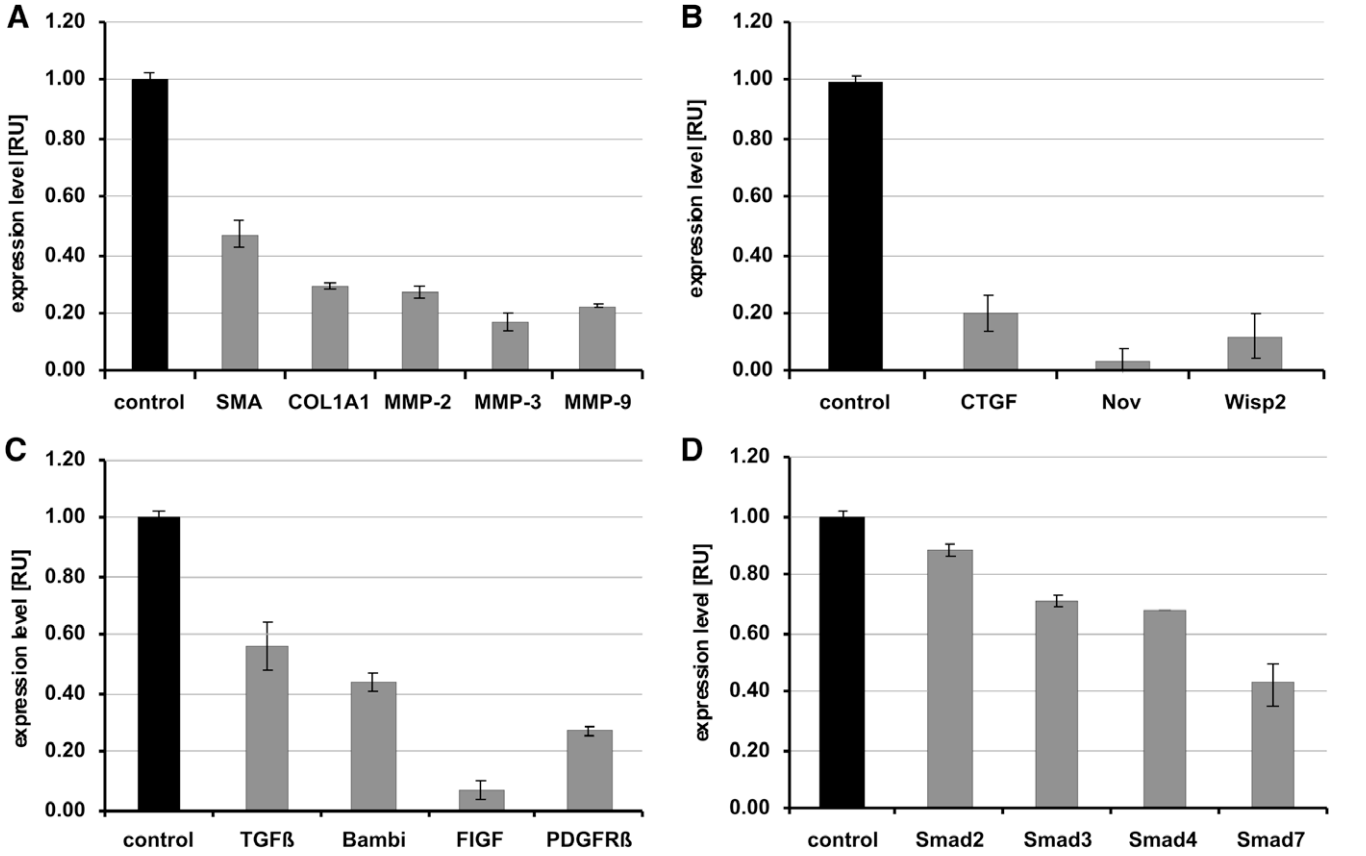


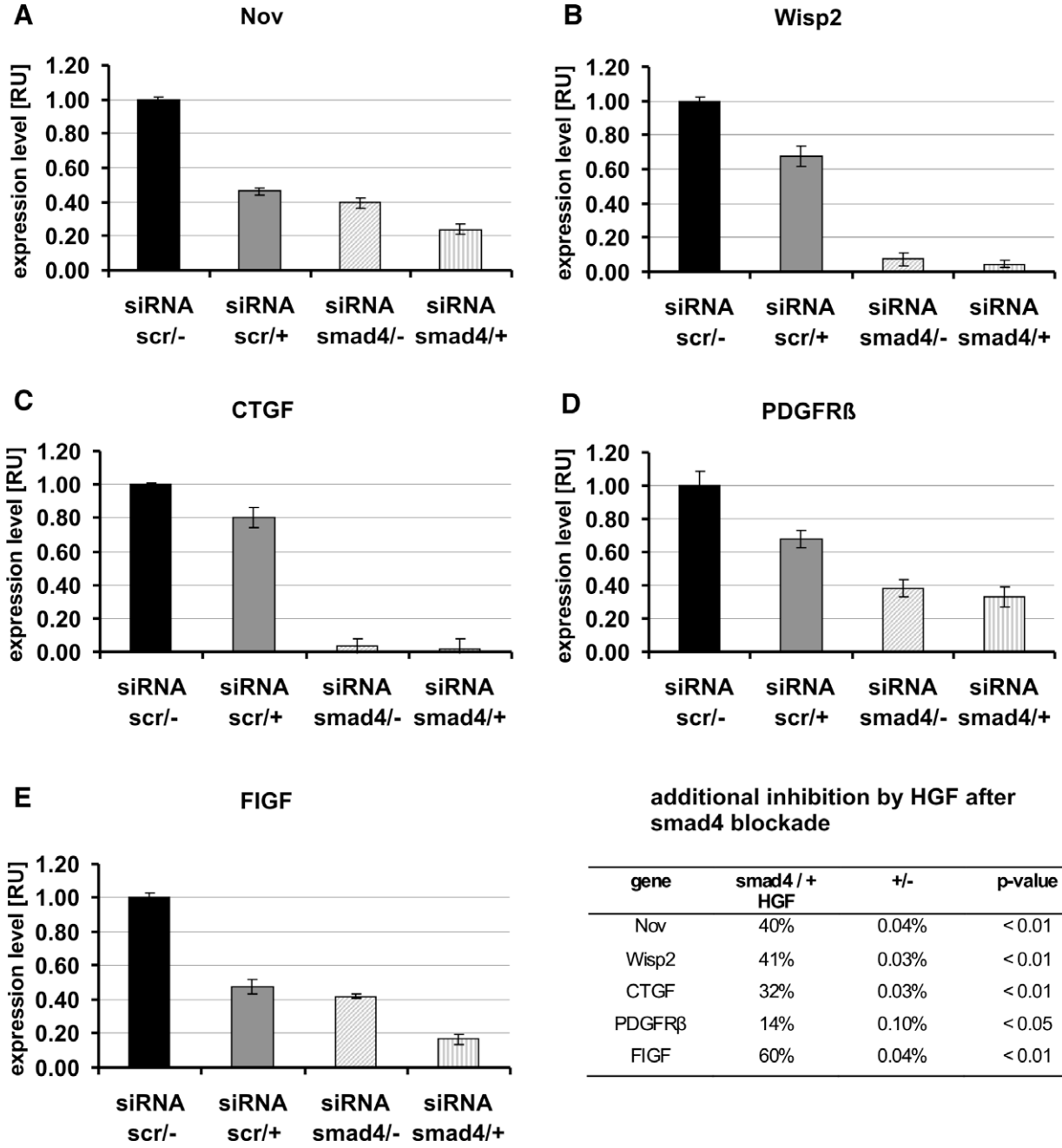
Fig. 3. Expression analysis of hHGF treated NRK49F cells by real-time PCR. PCR was performed with primers specific for SMA, COL1A1, MMP-2, MMP-3, MMP-9 (A), CTGF, Nov, Wisp2 (B), TGFβ, Bambi, FIGF, PDGFRβ (C), and Smad2, Smad3, Smad4, and Smad7 (D). NRK49F cells were cultured in the presence of hHGF [40 ng ml^{-1}] for 24 h (grey bars). The expression levels were normalized to the expression level of HPRT and compared to the untreated cells used as a control (black bars). Each bar represents the mean and SD of three independent experiments, measured in triplicates. The control value was arbitrarily set at 1.

expression (Fig. 3B). The fibrotic mediator TGFβ was more than 40% down-regulated by hHGF. In addition, Bambi and PDGFRβ exhibited 53% and 70% reduced expression levels, respectively. FIGF showed the maximum effect with more than 90% down-regulation (Fig. 3C).

hHGF treatment only had slight effects on the expression levels of Smad2 (10% reduction) or Smad3 and Smad4 (30% reduction). The inhibitor Smad7, however, known to be induced by a feed-back mechanism of Smad2/3 signaling [17], showed a highly reduced mRNA level of almost 60% compared to the control (Fig. 3D).

Smad-independent anti-fibrotic effects of HGF

In order to investigate whether gene expression was repressed in response to HGF, by the inhibition of Smad signaling due to linker phosphorylation after Erk1/2 activation, the common Smad4 was silenced by siRNA in renal fibroblasts which were additionally stimulated with hHGF. Then, 15 of the previously identified HGF target genes were analyzed by real-time PCR. siRNA transfection reduced Smad4 transcript levels to less than 80% of its original levels (Supplemental Fig. S1). While expression of TGFβ and MMP-2 was regulated independently of Smad, Smad7, Bambi,



additional inhibition by HGF after smad4 blockade

gene	smad4 / + HGF	+/-	p-value
Nov	40%	0.04%	< 0.01
Wisp2	41%	0.03%	< 0.01
CTGF	32%	0.03%	< 0.01
PDGFRβ	14%	0.10%	< 0.05
FIGF	60%	0.04%	< 0.01

Fig. 4. Effects of hHGF on the expression levels of different genes in Smad4-inhibited NRK49F cells. The expression levels of Nov (A), Wisp2 (B), CTGF (C), PDGFRβ (D), and FIGF (E) are displayed. Cells were transfected with either Smad4 siRNA (hatched bars) or scrambled (scr) control siRNA (black and grey bars), respectively. Twelve hours after transfection, medium was exchanged with medium containing 10% FCS and cells were cultivated for an additional 12 h. Then the cells were treated with (+) or without hHGF (−) [40 ng ml^{-1}] for another 24 h. RNA was extracted, reverse transcribed and mRNA expression was determined by real-time PCR. All expression levels were normalized to HPRT. Each bar represents the mean and SD of three independent experiments, measured in triplicate. Untreated cells served as a control (black bar) and the value of the control was arbitrarily set to 1. The additional down-regulation by hHGF in Smad4 silenced NRK49F cells compared to un-stimulated Smad4 silenced cells is indicated as a percentage. $p < 0.05$ was considered significant.

SMA, COL1A1, CTGF, Nov, Wisp2, PDGFRβ, and FIGF displayed strongly diminished mRNA expression levels after Smad4-blockade by siRNA. HGF stimulation resulted in a minor auxiliary effect on transcriptional repression of TGFβ and MMP-2, whereas mRNA levels of Nov, Wisp2, PDGFRβ, FIGF, and CTGF were additionally diminished by HGF in Smad-inhibited fibroblasts (Fig. 4A–D). The additive effect of Smad4 inhibition and HGF stimulation in repression of CCN genes, PDGFRβ and FIGF is summarized in Fig. 4.

Discussion

Although it is well accepted that HGF is a central growth factor mediating not only regeneration but also anti-fibrotic processes, little is known about the molecular mechanisms underlying the anti-fibrotic response upon HGF treatment. Here, we show that, consistent with met-signaling in epithelial cells, HGF treatment of renal fibroblasts causes not only Erk1/2 activation but also Akt activation. HGF signaling via Erk1/2 in NRK49F fibroblasts has already been shown by Liu and colleagues [15]. Furthermore, in response to HGF induced Erk1/2 activation, Smad2/3 was phosphorylated at the linker region [15]. Previous data from Kretzschmar et al. [16] have convincingly shown that in epithelial cells linker-phosphorylation of Smad2 and Smad3 due to Erk1/2 activation impedes Smad translocation into the nucleus, thereby abolishing TGFβ mediated responses.

In our study, we demonstrated that, in interstitial fibroblasts, in addition to the phosphorylation of Erk1/2, the Akt protein is also activated by HGF, whereas Stat3 is not affected. Akt is one of the major downstream targets of phophoinositol-3 kinase (PI3 K) [18]. This kinase has been implicated in the regulation of multiple cellular HGF functions including cell growth and survival of epithelial cells [18,19]. However, its role in HGF signaling in renal fibroblasts was unknown. The auxiliary effects of HGF after inhibition of the Erk1/2-Smad linkage by our anti-Smad4 siRNA experiments support the additional function of Akt in anti-fibrotic HGF signaling.

In order to analyze the molecular implications of HGF induced Akt and Erk1/2 signaling in renal fibroblasts we performed a comprehensive expression profiling. Collagen type I and SMA, which are strongly linked to fibrosis, were highly down-regulated upon hHGF stimulation of renal fibroblasts [18] as was also recently shown in dermal and lung fibroblasts [20–22]. However, functional cluster analyses revealed that in addition to these fibrotic markers, HGF treatment affects the transcript levels of a wide panel of mediators involved in fibrosis. Thus, expression levels of other genes encoding ECM components, especially the subunits of various collagens, as well as matrix degrading metalloproteinases, inflammatory mediators including several chemokines, and the members of the CCN family were strikingly reduced upon HGF stimulation (Fig. 2 and Supplemental Table S2).

However, expression of Smad2, -3, and -4, which are the central transducers of the TGFβ induced fibrotic pathways [23], was only slightly affected by HGF. In contrast, the inhibitory Smad7, which antagonizes TGFβ-initiated Smad2/3 signaling through a competitive interaction with receptor type I, is more than 50% reduced after HGF treatment. Although promoter activity of Smad7 was shown to depend on Erk1/2 initiated AP1-binding [24], Smad7 is known to be primarily transcriptionally regulated by TGFβ in a feed-back loop, due to binding of Smad3 to a TGFβ responsive site. Thus, hHGF mediated repression of Smad7 is assumed to occur due to the induced Smad2/3 blockade after hHGF-initiated ERK1/2 phosphorylation. The same has been postulated for other TGFβ induced and Smad-regulated genes such as CTGF [25]. Although the activation of the CTGF promoter by TGFβ is cooperatively mediated via Smad- and Ras/MAPK/Erk signaling [26,27], a TGFβ responsive Smad-binding element in the promoter region seems to be exclusively responsible for direct induction of CTGF by TGFβ [25,28]. CTGF repression in response to HGF associated Smad inhibition is confirmed by our Smad4 knockdown approach, demonstrating a dramatic decrease of the CTGF mRNA level after silencing of Smad4. Two more members of the CCN family, Nov and Wisp2, were also dramatically affected by HGF. Whereas CTGF has been characterized as a mediator of intercellular cell communication involved in inflammatory and fibrotic processes [29], the role of Nov and Wisp2 in fibrogenesis is not yet defined.

Surprisingly, prominent down-regulation by HGF was also observed for various matrix metalloproteinases. During renal interstitial fibrosis these enzymes play an important role in the degradation of ECM. Especially, MMP-2 and MMP-9 take part in the remodeling of ECM in fibrotic lesions. While up-regulated MMP-2 levels are associated with fibrotic processes, MMP-9 is down-regulated in fibrotic disorders and was shown to be up-regulated after HGF treatment in a rat model of 5/6 nephrectomy [30]. MMP-2 has been reported to be induced by TGFβ in epithelial cells in renal fibrosis after ureter obstruction [31]. Furthermore, Cheng and Lovett demonstrated that MMP-2 is absolutely required for the induction of epithelial–mesenchymal transition [32]. A stimulatory effect of CTGF on the expression of MMP-2 in interstitial fibroblasts has been shown by Yang and colleagues [33]. Therefore, the decrease in MMP-2 via HGF could be either a result of inhibited TGFβ signaling or of inhibited CTGF synthesis.

Taken together, our present study reveals that HGF does not only function as an anti-fibrotic factor by its involvement in deceleration of matrix accumulation, but also does so through its participation in delayed matrix turn-over and altered growth factor profiles during fibrosis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.05.010.

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