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HGF Regulates the Activation of TGF-β1 in Rat Hepatocytes and Hepatic Stellate Cells

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Hepatocyte growth factor (HGF) ameliorates experimental liver fibrosis through many mechanisms, including degradation of accumulated collagen and decreased expression of fibrotic genes. Investigating an upstream mechanism in which HGF could decrease many fibrotic effectors, we asked whether HGF regulates activation of the fibrotic cytokine transforming growth factor-beta 1 (TGF-β1). Specifically, we tested whether HGF decreases the levels of active TGF-β1, and whether such decrease depends on the predominantly hepatocyte-secreted protease plasmin, and whether it depends on the TGF-β1 activator thrombospondin-1 (TSP-1). With hepatocyte monocultures, we found HGF-induced hepatocyte proliferation did increase total levels of plasmin, while decreasing gene expression of fibrotic markers (PAI-1, TGF-β1, and TIMP-2). With in vitro models of fibrotic liver (HSC-T6 hepatic stellate cells, or co-cultures of HSC-T6 and hepatocytes), we found high levels of fibrosis-associated proteins such as TSP-1, active TGF-β1, and Collagen I. HGF treatment on these fibrotic cultures stimulated plasmin levels; increased TSP-1 protein cleavage; and decreased the levels of active TGF-β1 and Collagen I. When plasmin was blocked by the inhibitor aprotinin, HGF could no longer decrease TGF-β1 activation and Collagen I. Meanwhile, the TSP-1-specific peptide inhibitor, LSKL, reduced TGF-β1 to the same level as in the HGF-treated cultures; combining LSKL and HGF treatments caused no further decrease, suggesting that HGF affects the TSP-1 dependent pathway of TGF-β1 activation. Therefore, HGF can decrease TGF-β1 activation and TGF-β1-dependent fibrotic markers, by stimulating hepatocytes to produce plasmin, and by antagonizing TSP-1-dependent activation of TGF-β1.

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Liver fibrosis is characterized by crucial changes to important cell types in the liver such as hepatocytes that undergo apoptosis and hepatic stellate cells (HSC) that undergo activation and differentiation into myofibroblasts (Iredale, 2001). Hepatocytes comprise up to 80% of liver mass (Blouin et al., 1977) and they have tremendous regenerative capacity in contexts such as hepatectomy (Tilg and Diehl, 2000), but poor survival in the context of liver fibrosis. In fibrosis and chronic liver injury, hepatocytes undergo extensive cell death and are unable to regenerate and repair the damaged liver parenchyma. After injury, an opposite trend occurs in HSCs: they actively proliferate, migrate and differentiate into an activated matrix-secreting myofibroblast phenotype with high levels of alpha-smooth muscle actin (α-SMA) expression (Guyot et al., 2006; Kisseleva and Brenner, 2007). Activated HSCs produce high levels of transforming growth factor-beta 1 (TGF-β1) (Dooley et al., 2000), which in turn cause further activation of HSCs leading to increased deposition of extracellular matrix (ECM) components (mainly Collagen I), excessive accumulation of fibrous tissue, and disruption of the liver vasculature hindering

Abbreviations: α-SMA, alpha-smooth muscle actin; Col 1a1, collagen 1a1; Ctrl, untreated control cultures; ECM, extracellular matrix; HGF, hepatocyte growth factor; HSCs, hepatic stellate cells; PAI-1, plasminogen activator inhibitor-1; PLG, plasminogen; TGF-β1, transforming growth factor-beta 1; TIMP-2, tissue inhibitors of mettaloproteinase-2; TBS, tris-buffered saline; TBST, TBS with 0.01% Tween 20; TSP-1, thrombospondin-1; uPA, urokinase plasminogen activator.

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liver repair. High TGF-β1 also causes further apoptosis of hepatocytes, reducing the regeneration potential of the liver (Bataller and Brenner, 2005). TGF-β1 is thus implicated in multiple causal mechanisms of liver fibrosis progression, and is an important target in the development of anti-fibrotic therapies (Border and Noble, 1994), but therapies seeking a broad block against TGF-β1 signaling could be problematic because TGF-β1 also has beneficial effects in other contexts (Franklin, 1997).

Hepatocyte growth factor (HGF) facilitates hepatocyte regeneration following liver injury and has multiple functionalities including potent hepatotrophic effects (Stella and Comoglio, 1999). HGF levels are abnormally low during fibrosis (Inoue et al., 2006), and therapeutic interventions to overexpress HGF have shown remarkably effective anti-fibrotic effects in liver (Li et al., 2008; Horiguchi et al., 2009), lung, kidney, and heart (Taniyama et al., 2002; Inoue et al., 2003; Kim et al., 2005). Attempting to understand why HGF is so effective, previous studies of liver fibrosis found that HGF causes suppression of hepatocyte apoptosis (Nishino et al., 2008), suppression of TGF-β1 gene expression (Horiguchi et al., 2009); inhibition of α-SMA production, stimulation of apoptosis in activated HSCs (Jiang et al., 2008; Li et al., 2008), and inhibition of Collagen I, III synthesis and promotion of collagen fiber digestion (Jiang et al., 2008; Li et al., 2008). Even though there are many downstream effects, gene expression changes and multiple antagonistic effects on TGF-β1 (Florquin and Rouschop, 2003) that can be influenced by HGF, we hypothesize here that HGF also has an upstream effect on controlling the activation of TGF-β1 (Fig. 1).

TGF-β1 activation occurs in the ECM when stimulated by thrombospondin-1 (TSP-1), integrins, cathepsins, plasmin, reactive oxygen species, heat, and pH changes (Munger et al., 1997; Wipff and Hinz, 2008). In liver fibrosis, activated HSCs secrete high levels of TSP-1, which is a key activator of TGF-β1 (Kondou et al., 2003). TSP-1 participates in a positive feedback loop of fibrosis perpetuation: TSP-1 leads to activation of LTGF-β1, high active TGF-β1 causes an increase in the activation of HSCs, activated HSCs produce more TSP-1, leading to over-activation of TGF-β1 (Breitkopf et al., 2005). Therapies targeting TSP-1 have been effective in experimental models of liver fibrosis (Kondou et al., 2003). Antagonism of hepatic regeneration by elevated levels of TSP-1 in partial hepatectomy models (Hayashi et al., 2012) and suppression of TSP-1 gene expression levels by HGF in thyroid carcinoma cells (Scarpino et al., 2005), suggest potential crosstalk between HGF and TSP-1. We therefore investigated whether the anti-fibrotic effects of HGF in liver cells are mediated in part by inhibition of TSP-1-dependent activation of TGF-β1.

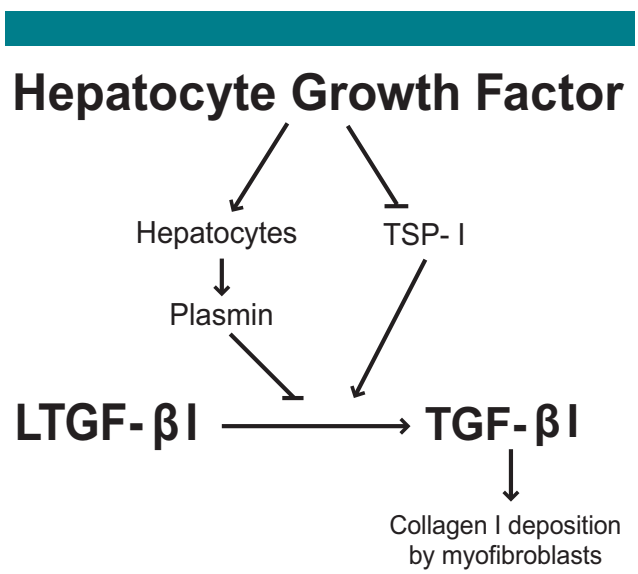

Fig. 1. Schematic diagram of the possible anti-fibrotic effects of HGF on active TGF-β1 during liver fibrosis. HGF is known to act in many ways during liver fibrosis regression in controlling the activation of HSCs, deposition, and accumulation of ECM proteins like Collagens. In this study, we investigated a possible mechanism of HGF-induced fibrosis regression through regulation of the TGF-β1 activation pathway, via the proteins plasmin and TSP-1.

We also investigated the effects of HGF on another important regulator of TGF-β1 activation, plasmin. Plasmin is secreted predominantly by hepatocytes, and during liver fibrosis, there is high degree of hepatocyte apoptosis and a drastic decrease in plasmin levels (Bezerra et al., 1999; Waisman, 2003; Bueno et al., 2006; Wang et al., 2007). Plasmin has a variety of anti-fibrotic effects, aiding the degradation of ECM proteins (Bueno et al., 2006; Martinez-Rizo et al., 2010), and activating matrix metalloproteinases. The effects of plasmin on TGF-β1 activation has been controversial mainly because of its role in TGF-β1 activation observed in vitro while in vivo it inhibits liver fibrosis (Pedrozo et al., 1999; Zheng and Harris, 2004; Hu et al., 2009; Ghosh and Vaughan, 2012); recent work suggests that plasmin might decrease liver fibrosis by inhibiting TGF-β1 signaling (Martinez-Rizo et al., 2010). In animal models of fibrosis, therapies that up-regulate plasmin indirectly, through the plasminogen activation system, have shown improvement of fibrosis markers, and increased clearance of fibrotic matrix proteins (Bueno et al., 2006; Hu et al., 2009). In this study, we investigated whether the anti-fibrotic effects of HGF are mediated by plasmin-dependent regulation of TGF-β1 activation.

One possible strategy to restore normal plasmin levels during liver fibrosis might be hepatocyte transplantation, which is a successful treatment in some clinical studies for liver failure (Hughes et al., 2012), but hepatocytes from healthy donors have poor availability, and hepatocytes derived from stem cells are not yet ready for therapeutic use (Ochiya et al., 2010). Since HGF is known to induce proliferation of endogenous hepatocytes, our study investigated whether HGF could increase the levels of plasmin enough to have significant anti-fibrotic effects, on TGF-β1 activation and fibrotic matrix proteins. Importantly, we combined our study of HGF in hepatocytes with study of HGF in fibrogenic HSCs, to test whether anti-fibrotic effects of HGF in hepatocytes were negated or strengthened by simultaneous effects of HGF in HSCs.

Our study investigated a twofold mechanism of HGF-induced regulation of TGF-β1 activation by plasmin (from hepatocytes) and TSP-1 (from HSCs). In order to simulate liver fibrosis in vitro, we established cell culture models using primary rat hepatocytes and an HSC cell line, HSC-T6, exhibiting high levels of active TGF-β1 and Collagen I. Using these in vitro fibrotic models, we examined the role of HGF in regulating TGF-β1 activation and the expression of downstream fibrotic markers such as Collagen I.

Methods

Cell culture models

Primary rat hepatocytes were isolated from male Wistar rats (250–300 g) by a two-step collagenase perfusion method as described previously (Seglen, 1976). The isolation procedure was approved by the IACUC of National University of Singapore. The isolated hepatocytes were seeded at 2×10^5 cells per 35 mm collagen-coated dishes (IWAKI) in Williams E (Sigma) with 10% fetal bovine serum (FBS; Sigma). After 4 h, media change was carried out with Williams E without serum. After overnight serum starvation, the

cells were treated with HGF (40 ng/ml; 294-HG, R&D Laboratories) and media was collected after 48 h. HSC-T6 cells were seeded at 2×10^5 cells per 35 mm collagen-coated dish and cultured for 3 days in DMEM (without phenol red, Sigma) with 10% FBS to allow for activation. On the fourth day the media of the HSC-T6 monocultures were changed to Williams E without serum and starved overnight. Serum-starved activated HSC-T6 cultures were treated with 40 ng/ml HGF in and media collected 48 hrs after treatment.

Co-cultures were established with hepatocytes and HSC-T6 cells (Vogel et al., 2000) at the ratio of 1:4 (i.e., for every 1 hepatocyte, 4 HSCs are seeded). HSCs were seeded 3 days prior to the co-culture in DMEM with 10% FBS at the density of 2×10^5 cells per 35 mm dish to induce the activation process. Three days later hepatocytes, in an appropriate number according to the above ratio, were seeded in Williams E medium with serum. Four hours later media was changed to Williams E without serum. After overnight serum starvation, the co-cultures were treated with HGF (40 ng/ml).

Inhibitors

In order to study the causal roles of plasmin in the HGF-induced regression of fibrotic markers in both monocultures of HSC-T6 and fibrotic co-cultures of primary hepatocytes and HSC-T6, we simultaneously administered the serine protease inhibitor aprotinin (1 μg/ml; A6103; Sigma–Aldrich, a potent inhibitor of plasmin (Sato and Rifkin, 1989; Longstaff, 1994)) and HGF. Another set of intervention studies included the administration of the small peptide LSKL (5 μM; 60877, Anaspec) to specifically inhibit TSP-1-dependent activation of TGF-β1 (Kondou et al., 2003) in the presence or absence of HGF. Media was collected after 48 h, filtered through a 0.2 μm pore size filter and stored at −20°C for further analysis. All data represented in this study were collected from three or more independent experiments.

Picogreen assay to measure hepatocyte proliferation

Cells from the collagen-coated dishes were collected by treatment with 0.1% SDS and samples were lysed and centrifuged at 11,000 × *g* for 10 min. The supernatant was diluted 10× and the DNA quantity was assayed by incubation with equal amount of Picogreen dsDNA dye for 5 min. The fluorescence was measured at 520 nm and a standard curve was used to calculate the number of cells from the observed DNA quantity.

ELISA

Cell culture supernatants collected and filtered were assayed for active TGF-β1 (Promega TGF-β1 Emax Immunoassay kit) (Budinger et al., 2006), plasmin (1:150 of mouse plasmin, Ab-1 SBF1 antibody, Neomarkers; 1:2,000, Streptavidin-HRP, 540666, BD Pharmingen), TSP-1 (1:150, D4.6, Neomarkers; 1:2,000 Goat anti-mouse IgG-HRP, sc-2005, Santacruz) and Collagen I (1:200 of rabbit anti-rat Collagen I antibody, AB755P, Millipore; 1:1,000 of swine anti-rabbit IgG, P0399, Dako) with the absorbance measured at 450 nm. The concentrations were obtained from a standard graph constructed from internal standards (Human plasmin, P1867, Sigma; Human platelet TSP-1, 605225, Calbiochem; Rat tail collagen, 354236, BD Biosciences).

Western Blot

Protein measurements using Western blot were carried out with cell culture supernatants from 48 h conditioned, serum-free media from the cell cultures; centrifuged at 14,000 rpm for 15 mins at 4°C. Protein content was determined using Bradford assay (Biorad) and equal amounts of protein samples (40 μg) were separated on a 10% SDS–PAGE in 1× Tris Glycine, and transferred onto a 0.22 μm nitrocellulose membrane overnight in 1× Tris-buffered saline (TBS) with 10% methanol. To ascertain equal loading and transfer efficiency, the membranes were stained with Ponceau S. Then the Ponceau S stain was washed off the membrane and the membrane was placed in blocking buffer (2% skimmed milk prepared in 1× TBS with 0.01% Tween 20 (1× TBST)) for 1 h at room temperature with shaking. Later the membrane was washed 3 times in 1× TBST and treated with mouse TSP-1 monoclonal antibody (D4.6, Neomarkers; 1:200 in 1× TBST for 3 h at RT) or mouse plasminogen monoclonal antibody (1:100 in 1× TBST for 2 h at RT, Ab-1 SBF1 antibody, Neomarkers), washed 3 times in 1× TBST and treated with goat anti-mouse IgG-HRP (1:10,000 in blocking buffer, sc-2005, Santacruz) for 1 h at RT. The membrane was developed using SuperSignal West Pico Chemiluminescent solution (Thermo Scientific) and the band intensity was measured using ImageJ (WS Rasband, National Institutes of Health, Bethesda, MD).

Gene expression—RT-PCR

Cell cultures were washed with 1× sterile phosphate buffered saline and mRNA was isolated from the cells using RNeasy mini kit (Qiagen), and its concentration quantified using Nanodrop 2000 UV–Vis Spectrophotometer. one microgram of mRNA from each sample set converted to cDNA (Invitrogen, Superscript Reverse Transcriptase III) and real-time PCR reaction (Roche, Sybr Green Master mix) was carried out for plasminogen (PLG), urokinase plasminogen activator (uPA), plasminogen activator inhibitor-1 (PAI-1), TSP-1, Collagen 1a1 (Col 1a1), alpha-smooth muscle actin (α-SMA), Tissue Inhibitor of Metalloproteinases-2 (TIMP-2), TGF-β1, and β-actin, with in-house primers shown in Table 1. The gene expression values were determined by Del-Del C_T relative quantitation methods (van't Veer et al., 2006); the target C_T values were normalized to the endogenous reference β-actin, and the normalized mRNA was expressed as a fold change relative to the untreated control.

Statistical analysis

The results obtained were from three or more independent set of experiments; the values were expressed as Mean ± SEM and considered significantly different if the *P*-value < 0.05. The *P*-value was calculated from two-tailed unpaired Student's *t*-tests (GraphPad Prism Software, San Diego, CA).

TABLE 1. List of primer sequences for genes probed on quantitative real time PCR

Gene name	Primer sequence (5′–3′)
β-actin	
Sense	ACCCACACTGTGCCCATCTA
Antisense	GCCACAGGATTCCATACCCA
PLG	
Sense	AAGGTGTGCAACCGCGCTGA
Antisense	TTGGGGCGAGCACAGCCAAG
uPA	
Sense	TCGGACAAGAGAGTGCCA
Antisense	TCACAATCCCGCTCAGAG
PAI-1	
Sense	TGGTGAACGCCCTCTATTTC
Antisense	GAGGGGCACATCTTTTTCAA
TSP-1	
Sense	TGCACTGAGTGTCACTGTCAGAA
Antisense	CATTGGAGCAGGGCATGAT
TIMP-2	
Sense	GTTTTGCAATGCAGATGTAG
Antisense	ATGTCGAGAAACTCCTGCTT
LTGF-β1	
Sense	TGCTTCAGCTCCACAGAGAA
Antisense	TGGTTGTAGAGGGCAAGGAC
α-SMA	
Sense	TGC CAT GTA TGT GGC TAT TCA
Antisense	ACC AGT TGT ACG TCC AGA AGC
Collagen 1a1	
Sense	CAAGAATGGCGACCGTGGTGA
Antisense	GATGGCTGCACGAGTCACACC

Results

HGF increased total plasmin levels through hepatocyte proliferation and decreased expression of pro-fibrotic genes

We investigated the relationship between HGF and TGF-β1 activation (Fig. 1) by treating a monoculture of primary rat hepatocytes with 40 ng/ml HGF after overnight serum starvation; and tested the possible link with plasmin by quantifying the plasmin concentrations in the cell culture supernatants by ELISA. The plasmin levels increased significantly in the HGF-treated hepatocytes compared to the untreated control cultures (Fig. 2a, $P < 0.05$). This increase in plasmin was accompanied by a 1.7-fold increase in proliferation (Fig. 2b, $P < 0.05$) of hepatocytes within 48 h of the HGF treatment, compared to the untreated cell culture. When the increased levels of plasmin were normalized to the cell number, the normalized plasmin levels (Fig. 2c) did not show any significant variation between the HGF-treated and the untreated control cultures from four independent trials. Since we did not observe an up-regulation of plasmin protein per hepatocyte we further investigated whether HGF induced an up-regulation in the gene expression of plasminogen (PLG) or its activator, uPA. We observed that PLG and uPA gene expression levels in HGF-treated hepatocyte monocultures and untreated control cultures (Fig. 2d) did not show any significant variation. HGF-induced plasmin increase appears to be the result of hepatocyte proliferation rather than by increasing the production of plasmin per hepatocyte.

We further investigated whether HGF decreased the expression of fibrotic marker genes in liver, such as TGF-β1, as had been found in other culture configurations and fibrotic liver models (Horiguchi et al., 2009). HGF treatment on primary hepatocytes reduced the gene expression of Plasminogen Activator Inhibitor -1 (PAI-1), Tissue Inhibitors of Matrix metalloproteinases-2 (TIMP-2), and TGF-β1 relative to the endogenous reference β-actin in contrast to the untreated control cultures (Fig. 2d). Thus, HGF down-regulated the gene expression levels of important fibrotic markers such as TGF-β1, and also important inhibitors of the plasminogen activation system (PAI-1), and matrix degradation (TIMP-2). The finding of HGF-induced hepatocyte proliferation leading to an increase in plasmin levels, accompanied by a decrease in fibrotic markers, led us to investigate the role of HGF and HGF-induced plasmin, specifically in the context of the TGF-β1 activation system.

Plasmin mediated the HGF-induced decrease of active TGF-β1 and collagen I levels

Previous anti-fibrosis studies show that the plasmin activation pathway may be highly effective at controlling disease progression by regulating the matrix degradation pathway (Bueno et al., 2006; Martinez-Rizo et al., 2010). But other studies indicate a possible link between the plasminogen

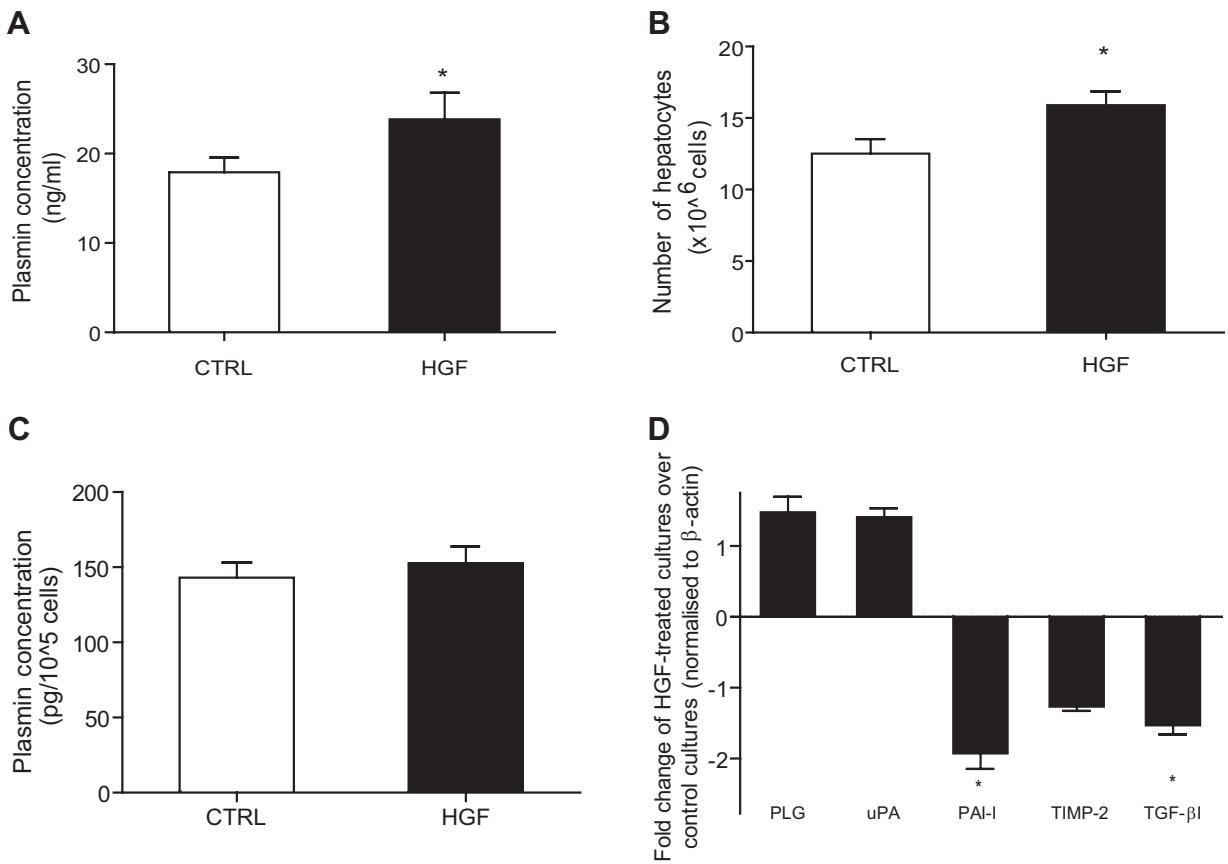

Fig. 2. HGF-induced hepatocyte proliferation increases total plasmin and decreases expression of pro-fibrotic genes. Freshly isolated primary rat hepatocytes treated with 40 ng/ml of HGF for 48 h showed a marked increase in plasmin levels (Fig. 2a) and an increase in the number of hepatocytes as measured by DNA content via picogreen assay (Fig. 2b). Although there was an increase in the total plasmin protein levels, after normalization with the cell number there was no significant change in the plasmin level (Fig. 2c). Real time PCR results showed no significant changes in plasminogen and uPA gene levels whereas pro-fibrotic genes such as PAI-1, TIMP-2, and TGF-β1 were suppressed after HGF treatment (Fig. 2d). This shows that HGF induced an increase in total plasmin and a decrease in pro-fibrotic gene expression. *P-value < 0.05.

activation system and fibrosis regression (Bueno et al., 2006; Hu et al., 2009). The close links of plasmin to the TGF-β1 activation pathway and the positive correlation that we established between HGF-induced hepatocyte proliferation and plasmin levels (Fig. 1), led us to investigate the role of plasmin in the anti-fibrotic effects induced by HGF.

In order to investigate the relationship between HGF, Plasmin and active TGF-β1, we utilized monocultures of activated HSCs (HSC-T6 cells) and co-cultures of primary rat hepatocytes and HSC-T6 cells. Hepatocyte and HSCs are constantly interacting with each other in the liver architecture, and different configurations of this co-culture are common model systems for liver-based studies in vitro (Bhandari et al., 2001; Abu-Absi et al., 2004). A co-culture model is beneficial in our case because it provides an endogenous source of anti-fibrotic proteins such as plasmin, as well as pro-fibrotic proteins such as TSP-1, TGF-β1, and Collagen I. We studied different ratios of primary rat hepatocytes and HSC-T6 cells in co-cultures to identify a co-culture ratio that best represented a fibrotic microenvironment, according to the measured protein levels. During progressive liver fibrosis, plasmin protein levels are low and TSP-1 levels are high. The unequal ratio of 1:4, wherein one hepatocyte was seeded for every four activated HSC-T6 cells, was the configuration at which TSP-1 levels were elevated and plasmin levels were decreased, compared to the lower ratios (Suppl. Fig. 1a). Also, the 1:4 co-cultures provided elevated levels of TGF-β1 (Suppl. Fig. 1b) and Collagen I (Suppl. Fig. 1c) as in a fibrotic microenvironment. We next tested how HGF treatment would affect 1:4 co-cultures and HSC-T6 monocultures.

Treatment of HSC-T6 monocultures with 40 ng/ml of HGF induced plasmin at low levels (Fig. 3a), while it significantly decreased active TGF-β1 (Fig. 3b, $P < 0.01$). Collagen I, an important ECM component, is an indicator of TGF-β1 function and also a marker of fibrotic pathology. HGF-treated HSC-T6 cultures also showed a decrease in Collagen I levels 48 h after treatment (Fig. 3c, $P < 0.05$). The same experiments, performed in 1:4 co-cultures, showed a significant increase in plasmin levels (Fig. 3d,e, $P < 0.05$) in the HGF-treated co-cultures compared to the untreated co-cultures. These effects also corresponded with a decrease in active TGF-β1 levels in the HGF-treated co-cultures (Fig. 3f, solid grey bar; $P < 0.01$). The anti-fibrotic effects of HGF, observed in the HSC-T6 monoculture model, were mirrored in the fibrotic co-culture model as demonstrated by an increase in plasmin levels, and a decline in active TGF-β1 levels.

We further investigated whether the enhanced production of plasmin upon HGF administration (Fig. 2) might be responsible for the decrease in fibrotic markers like TGF-β1 and Collagen I in vitro. To test the causal role of plasmin in these fibrotic co-cultures, we added 10 μg/ml of aprotinin (a serine protease inhibitor (Sato and Rifkin, 1989; Longstaff, 1994) with high specificity against plasmin) to both our fibrotic HSC-T6 monoculture and co-culture configurations, combined with 48 h of HGF treatment (Fig. 4a). HGF-treated cultures (both HSC-T6 monocultures and 1:4 co-cultures) showed significant anti-fibrotic effects such as decreased levels of active TGF-β1 and Collagen I (grey bars in Fig. 4b–e; $P < 0.05$). HSC-T6 monocultures and 1:4 co-cultures treated with aprotinin alone (white bars with horizontal lines in Fig. 4b–e) showed no significant difference in the levels of active TGF-β1 or Collagen I, relative to untreated control cultures (plain white bars in Fig. 4b–e). The anti-fibrotic effects of HGF were reversed with the addition of aprotinin to HGF-treated cultures, as demonstrated by an increase in active TGF-β1 levels and Collagen I levels (grey bars with horizontal lines in Fig. 4b–e; $P < 0.01$). Thus in HGF-treated cells, the addition of aprotinin, a specific inhibitor of plasmin, abrogated the anti-fibrotic effects of HGF (i.e., the decreases in active TGF-β1 and Collagen I levels) in both the HSC-T6 monoculture and the 1:4 co-culture. Treatment with HGF increased the levels of plasmin, and regulated the levels of active TGF-β1. Even in HSC-T6 cells that do not normally produce high levels of plasmin, HGF led to the control of active TGF-β1 and Collagen I levels (Fig. 3a–c). This led us to investigate whether HGF can cause an effect directly on HSCs possibly via other activators such as TSP-1.

HGF antagonized TSP-1-dependent TGF-β1 activation

The TSP-1-dependent activation of TGF-β1 has been shown to be an important pathway during liver fibrosis progression, primarily mediated by activated HSCs (Kondou et al., 2003). To test the effect of HGF on TSP-1, TGF-β1, and TSP-1-dependent activation of TGF-β1, we tested monocultures of HGF-treated HSCs (HSC-T6) with 40 ng/ml HGF for 48 h for TSP-1 gene and protein levels. We observed a significant decrease in TSP-1 and other fibrotic markers' gene expression levels (α-SMA, TGF-β1, Collagen I, PAI-1, and TIMP-2 in Suppl. Fig. 2A) and more than 150-fold increase in TSP-1 protein cleavage (Fig. 5a) in the HGF-treated HSC-T6 monocultures in contrast to the untreated HSC-T6 monocultures (Fig. 5b, white bar, $P < 0.001$). We also found a significant increase in TSP-1 protein cleavage in the HGF-treated fibrotic co-cultures, compared to the untreated co-cultures (Fig. 5c,d, $P < 0.01$). We next investigated whether the HGF-induced decline in TSP-1 protein levels also mediated the HGF-induced anti-fibrotic effects on active TGF-β1.

Since TGF-β1 can be activated by various physiological conditions, we sought the importance of TSP-1-dependent TGF-β1 activation in our fibrotic cell culture configurations. TSP-1 activates TGF-β1 from its latent form via a conformational change, in which the KRFK amino acid sequence on TSP-1 binds the latency associated peptide (LAP) at amino acids LSKL (Murphy-Ullrich et al., 1992; Breitkopf et al., 2005). This conformational change in the latent TGF-β1 molecule is crucial for TSP-1-dependent TGF-β1 activation as demonstrated by the decline in TGF-β1 levels following the addition of synthetic peptide, LSKL both in vitro and in vivo (Kondou et al., 2003).

To test the importance of TSP-1-dependent activation of TGF-β1 we added 5 μM of LSKL to our HSC-T6 cultures and we observed an expected decrease in active TGF-β1 levels (Fig. 5e, white bars with horizontal lines, $P < 0.001$). We use the term "LSKL-vulnerable fraction" to refer to the change in active TGF-β1 concentration that was caused by blocking the TSP-1-dependent activation pathway. The amount of the "LSKL-vulnerable fraction" of active TGF-β1 is similar to the HGF-induced decline in active TGF-β1 levels (Fig. 5e, solid grey bars; $P < 0.001$), which led us to the possibility that the HGF-induced decrease might be mediated by TSP-1. If the HGF-dependent and TSP-1-dependent pathways were mutually exclusive, we would expect an additive decline in active TGF-β1 levels in the presence of HGF and LSKL. In our culture configuration, we observed that when HGF and LSKL were added simultaneously (Fig. 5e, grey bar with horizontal lines; $P < 0.001$), there was no additional decrease beyond the LSKL-vulnerable fraction, implying that HGF is not independent of TSP-1 antagonism. Administering LSKL to the co-cultures produced a decline in active TGF-β1 levels reflecting the LSKL-vulnerable fraction (Fig. 5f, white bar with horizontal lines; $P < 0.01$), and addition of HGF to these LSKL-treated cultures caused a small additional decline in active TGF-β1 (Fig. 5f, grey bar with horizontal lines; $P < 0.01$ compared to LSKL alone). In other words, HGF had little effect on TGF-β1 levels in the presence of LSKL, showing that the HGF-dependent and TSP-1-dependent pathways of regulating TGF-β1 are not independent from each other, and suggesting a

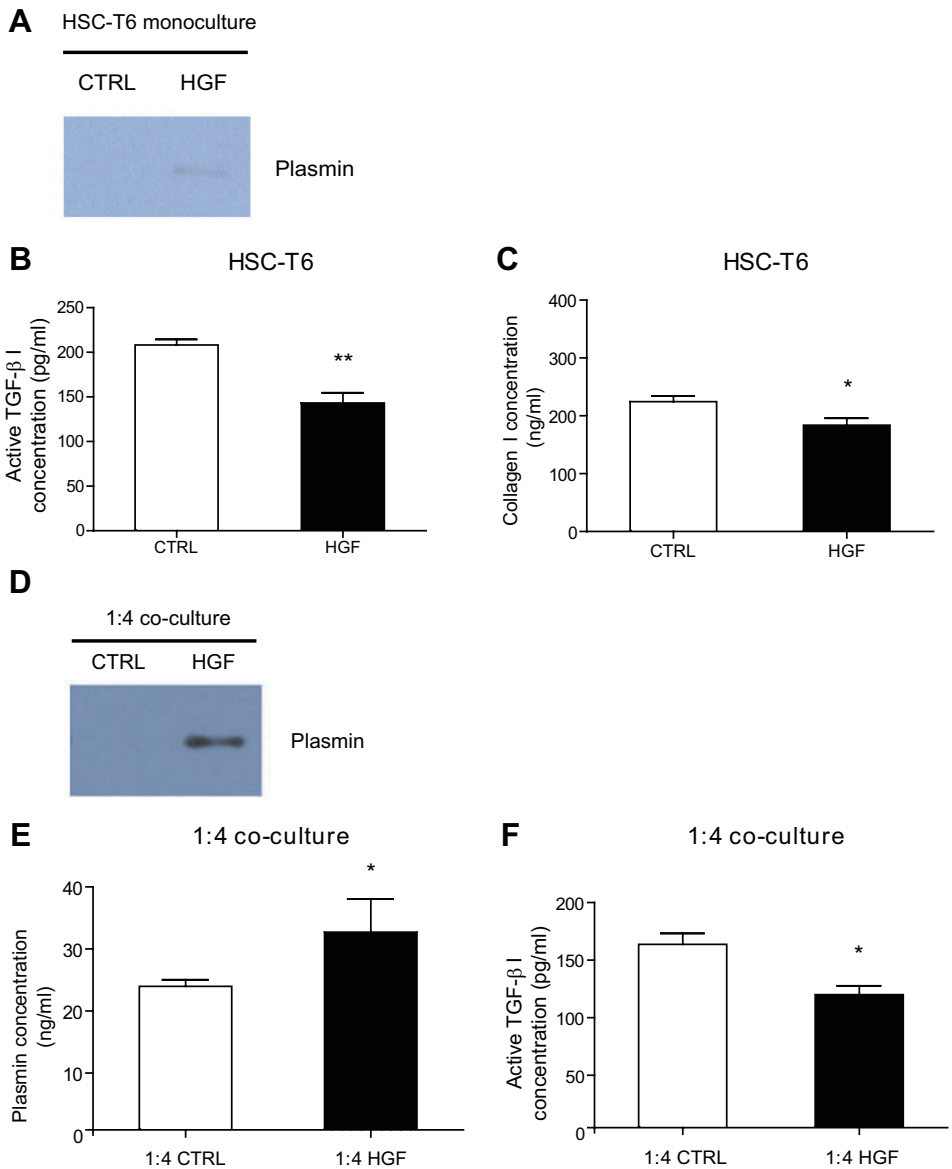

Fig. 3. HGF induced an increase in total plasmin and a decrease in active TGF-β1 in fibrotic cultures in vitro. Monocultures of HSC-T6 (rat HSCs) treated with HGF protein showed an insignificant increase in plasmin levels (Fig. 3a) accompanied by significant decreases in the protein levels of active TGF-β1 and Collagen I (Fig. 3b,c). The anti-fibrotic effects of HGF were mirrored in co-cultures of primary rat hepatocytes and HSC-T6 cells at the ratio of 1:4 with an increase in plasmin protein levels (Fig. 3d,e) and decrease in active TGF-β1 protein levels (Fig. 3f). For Western blots, equal amounts of protein from 48 h conditioned, serum-free media were separated on 10% SDS–PAGE. *P-value < 0.05, **P-value < 0.01.

possible link between HGF-dependent fibrosis regression and antagonism of TSP-1-dependent TGF-β1 activation.

Discussion

TGF-β1 is a major target for liver fibrosis therapeutics and it is causally linked to liver fibrosis through its role in HSC activation, ECM accumulation, and hepatocyte apoptosis. Various anti-TGF-β1 strategies for liver fibrosis antagonize TGF-β1 gene and protein expression, by reducing further HSC activation and hepatocyte apoptosis in an attempt to restore liver health and function. Since hepatocytes are the major cell type in the liver and are severely affected by necrosis and apoptosis during liver fibrosis, repopulation of healthy hepatocytes can be considered essential for restoration of liver function which might also lead to stabilization of TGF-β1 levels. Hepatocytes produce an important serine protease, plasmin but the fibrinolytic function of plasmin makes it unsafe for direct administration. The current experiments tested whether HGF, a potent hepatic mitogen, could cause a decline in TGF-β1 activation via two key players, plasmin and TSP-1.

In our current study, we established that HGF has anti-fibrotic effects on TGF-β1 activation and on the downstream secretion of Collagen I. Hepatocyte monocultures treated with HGF showed an increase in overall plasmin levels; in accordance with the increase in hepatocyte proliferation induced by HGF (Fig. 2a, b) and also suppressed the expression of pro-fibrotic genes such as PAI-1, TIMP-2 and TGF-β1 (Fig. 2d). Previous reports in other systems such as pancreatic cancer cell lines suggested a positive effect of HGF on uPA expression causing an

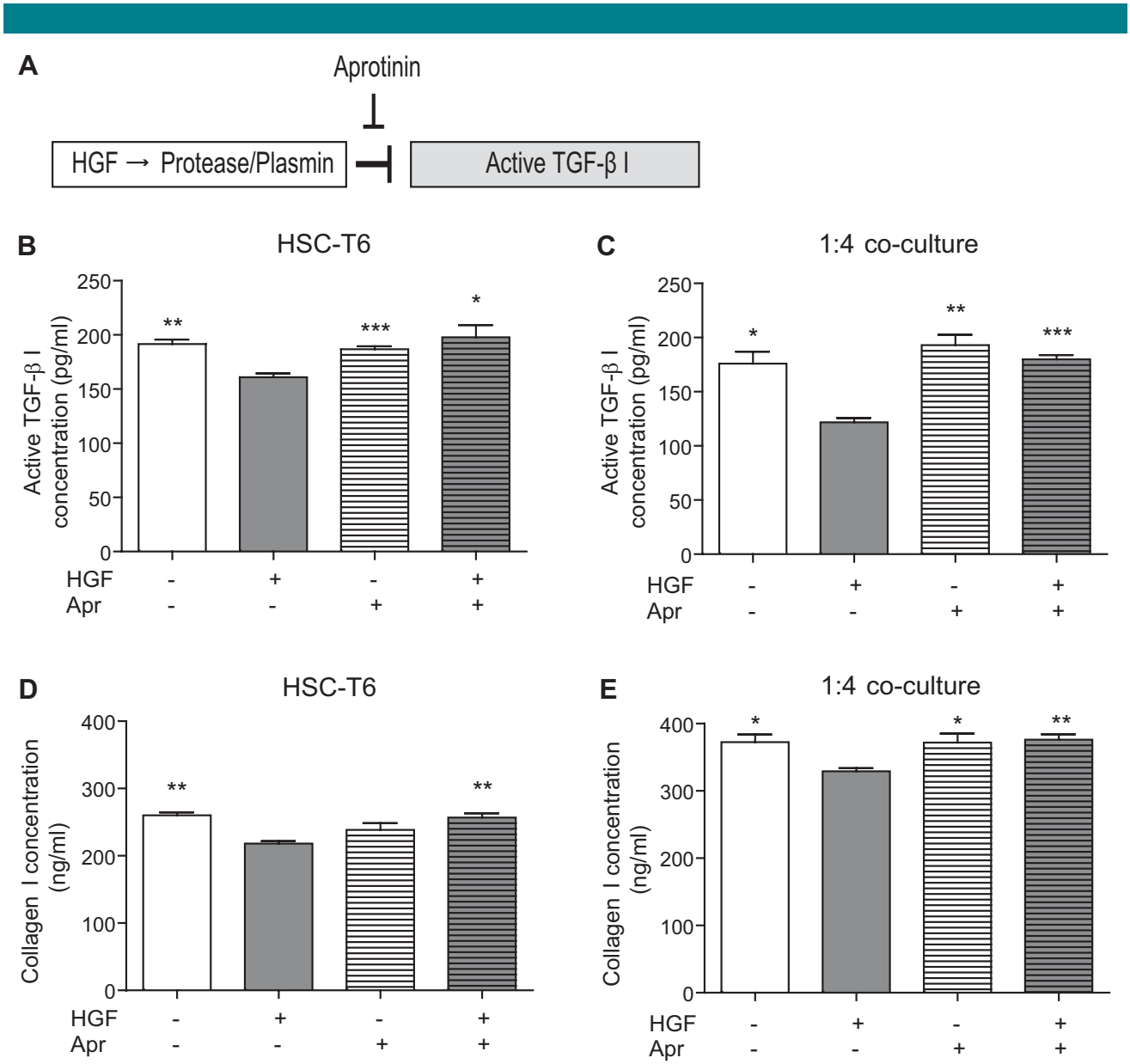

Fig. 4. Plasmin mediated the HGF-induced decrease in active TGF-β1 and Collagen I levels. Addition of aprotinin, a serine protease inhibitor, abrogated the inhibitory effects of HGF on active TGF-β1 (Fig. 4a) and Collagen I protein levels in the cell culture supernatants of monocultures of HSC-T6 (Fig. 4b,d) and fibrotic co-cultures of primary hepatocytes and HSC-T6 (Fig. 4c,e). **P*-value < 0.05, ***P*-value < 0.01, ****P*-value < 0.001.

up-regulation in plasmin production (Nishimura et al., 2003) but this uPA-dependent increase in plasminogen was not observed in our cultures as the gene expression of both plasminogen and uPA did not vary significantly after HGF treatment (Fig. 2d). Our evidence suggests that HGF increased overall plasmin levels via hepatocyte repopulation accompanied by a decrease in fibrotic markers. We further investigated the anti-fibrotic effects of HGF specifically on TGF-β1 activation in a HSC model of activated HSC-T6 cells.

HSC-T6 monocultures, expressing high levels of TGF-β1, Collagen 1a1, and TSP-1, treated with HGF showed an increase in plasmin levels (Fig. 3a) accompanied by a decrease in TSP-1 gene expression levels (Suppl. Fig. 2a) and increased cleavage of TSP-1 protein (Fig. 5a–d). When TSP-1-dependent TGF-β1 activation was selectively blocked using the LSKL peptide, HGF-treated HSC-T6 monocultures did not show further decrease in active TGF-β1 levels (Fig. 5e) compared to LSKL-only treatment, emphasizing the importance of TSP-1-dependent activation of TGF-β1 in our in vitro fibrotic models. This shows that HGF inhibits the TSP-1-dependent activation of TGF-β1, the major pathway active in activated HSCs. To test our idea that HGF exerted multiple anti-fibrotic effects on TGF-β1 in fibrotic models in vitro, we established fibrotic co-cultures of hepatocytes and HSC-T6 cells at 1:4 ratios with high levels of TSP-1, active TGF-β1 and Collagen I (Suppl. Fig. 1). Fibrotic co-cultures treated with HGF showed an increase in plasmin (Fig. 3d–e) accompanied by a significant decrease in active TGF-β1 (Fig. 3f). We hypothesize that this sub-event of plasmin-mediated inhibition of TGF-β1 activation plays a key role in the HGF-induced regression of fibrotic markers in the liver.

There are many studies that examine the potential of HGF for the control of liver fibrosis, and others that specifically study

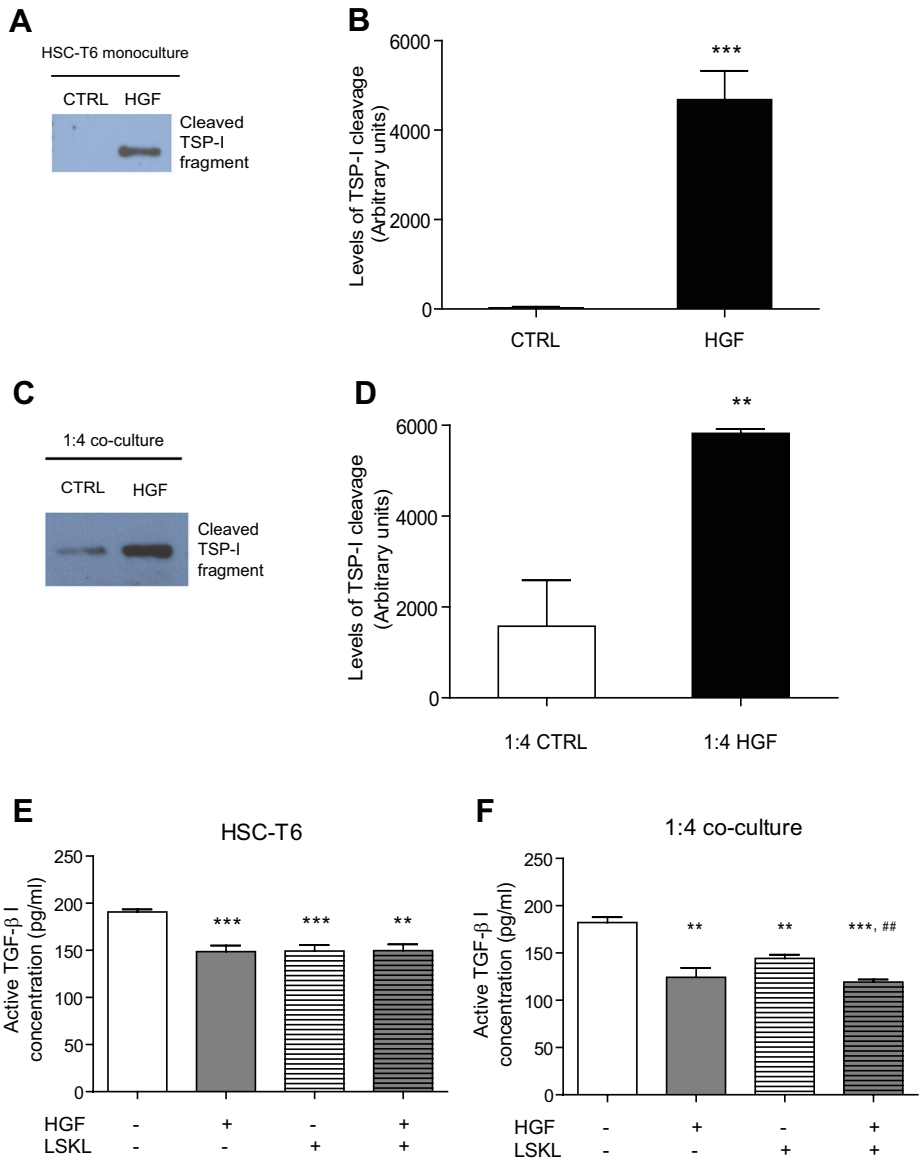

Fig. 5. HGF antagonized TSP-1-dependent TGF-β1 activation. Monocultures of HSC-T6 cells treated with 40 ng/ml HGF showed a significant increase in TSP-1 protein cleavage (Fig. 5a,b). Similarly, co-cultures of primary rat hepatocytes and HSC-T6 cells at 1:4 ratios, treated with HGF increased TSP-1 protein cleavage (Fig. 5c,d). For Western blots, equal amounts of protein from 48 h conditioned, serum-free media were separated on 10% SDS–PAGE. HGF reduced the levels of active TGF-β1 in both monocultures and co-cultures (Fig. 5e,f, solid grey bars). Addition of LSKL reduced the level of active TGF-β1 protein (Fig. 5e,f, white bars with horizontal lines) to the same magnitude as the HGF-treated cultures. Addition of HGF to LSKL-treated cultures showed a further decline compared to the "LSKL-vulnerable fraction", demonstrating that in addition to affecting TSP-1-dependent TGF-β1 activation HGF-induced plasmin can further decrease the active TGF-β1 levels in our culture conditions. **P-value < 0.01 (compared to CTRL), ***P-value < 0.001 (compared to CTRL), $^{\#\#}$P-value < 0.05 (HGF + LSKL compared to LSKL alone).

HGF in parallel with the TGF-β1 pathway. Previously, HGF was shown to inhibit intracellular TGF-β1 signaling and tubular EMT through up-regulation of Smad co-repressor SnoN (Yang et al., 2005). The current study sheds more light on HGF and plasmin-dependent inhibition of TGF-β1 signaling, upstream of TGF-β1 receptor binding, more specifically at the protein activation level. In a larger context, plasmin exerts anti-fibrotic effects on liver fibrosis through its matrix degrading properties. A recent work discovered that plasmin also suppresses TGF-β1 signaling through the up-regulation of SnoN in HSCs (Martinez-Rizo et al., 2010). Those findings that plasmin acts via SnoN, are consistent with the report of HGF and SnoN in tubular cells and suggest a possible parallel or common mechanism of action between plasmin and HGF. We have here demonstrated, through the abrogation of the anti-fibrotic effects of HGF via the simultaneous administration of aprotinin and HGF in our fibrotic models (Fig. 4), that the plasmin-mediated inhibition of TGF-β1 activation is a major event contributing to the anti-fibrotic effects of HGF in the liver.

We also studied the role of HGF on HSCs and HSC-secreted TSP-1 in the control of active TGF-β1. The importance of TSP-1 in TGF-β1/LAP activation was shown in liver fibrosis by specifically blocking the LAP-binding site on TSP-1 with the use of LSKL peptide. In our study, activated HSC-T6 monocultures and co-cultures showed suppression in TGF-β1 activation after TSP-1 blockade with LSKL. In HSC-T6 monocultures, HGF was

unable to exert its anti-fibrotic effects (Fig. 5e,f) in the presence of LSKL. This indicated that the anti-fibrotic properties of HGF occur significantly via inhibition of the TSP-1-dependent activation of TGF-β1. In co-cultures, LSKL treatment dramatically reduced the effects of HGF on active TGF-β1. The remaining LSKL-independent effect of HGF on TGF-β1 in co-cultures might be caused by plasmin. In summary, we have demonstrated multiple anti-fibrotic effects of HGF on TGF-β1 activation, both through an increase in plasmin and also through inhibition of TSP-1-dependent activation.

It would be interesting in further studies to reveal possible links between the HGF-induced plasmin-mediated inhibition and TSP-1-dependent regulation of TGF-β1 activation and the subsequent fibrosis regulation. Earlier studies had suggested a mutual antagonism of plasmin and TSP-1 (Hogg et al., 1992; Anonick et al., 1993; Bonnefoy and Legrand, 2000). Their quantitative relationship in these and other in vivo models need to be established to further understand how HGF regulates TGF-β1 activation and fibrosis via plasmin-mediated inhibition and TSP-1-dependent activation. Such an insight into the importance of HGF-treated suppression of TGF-β1 provides valuable information on the mechanisms of wound healing, fibrosis regression and maybe even TGF-β-induced EMT. In therapy, HGF at stringent doses might be useful to suppress TGF-β1 and reduce fibrosis by modulating the upstream plasmin/TSP-1 levels; and also repopulate the damaged liver parenchyma with healthy hepatocytes leading to functional restoration. Thus, this study elucidates the effects of HGF and HGF-induced plasmin/TSP-1 cleavage on TGF-β1 and fibrosis regulation, which creates new avenues for future designs of new therapeutics for liver diseases.

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