

Transforming Growth Factor- β (TGF- β) Inhibits the Expression of Factor VII-activating Protease (FSAP) in Hepatocytes^{*[5]}

Received for publication, June 22, 2016, and in revised form, July 22, 2016. Published, JBC Papers in Press, July 26, 2016, DOI 10.1074/jbc.M116.744631

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Deletion of the *Habp2* gene encoding Factor VII-activating protease (FSAP) increases liver fibrosis in mice. A single nucleotide polymorphism (G534E) in *HABP2* leads to lower enzymatic activity and is associated with enhanced liver fibrosis in humans. Liver fibrosis is associated with a decrease in FSAP expression but, to date, nothing is known about how this might be regulated. Primary mouse hepatocytes or the hepatocyte cell line, AML12, were treated with different factors, and expression of FSAP was determined. Of the various regulatory factors tested, only transforming growth factor- β (TGF- β) demonstrated a concentration- and time-dependent inhibition of FSAP expression at the mRNA and protein level. The TGF- β -Type I receptor (ALK-5) antagonist SB431542 and *Smad2* siRNA, but neither SIS3, which inhibits SMAD3, nor siRNA against *Smad3* could block this effect. Various regions of the *HABP2* promoter region were cloned into reporter constructs, and the promoter activity was determined. Accordingly, the promoter activity, which could phenocopy changes in *Habp2* mRNA in response to TGF- β , was found to be located in the 177-bp region upstream of the transcription start site, and this region did not contain any SMAD binding sites. Mutation analysis of the promoter and chromatin immunoprecipitation assays were performed to identify an important role for the ATF3 binding element. Thus, TGF- β is the most likely mediator responsible for the decrease in FSAP expression in liver fibrosis.

Liver fibrosis arises because of varying etiologies such as viral infections, alcohol abuse, fatty diet, and or ingestion/accumulation of toxins (1). Hepatic damage leads to concomitant pro-inflammatory status leading to an expansion of fibroblast and hepatic stellate cells (HSC).² Furthermore, tissue injury recruits inflammatory cells as well as activates hepatic stellate cells (HSC) that further promote extracellular matrix remodeling (2). In patients with hepatic fibrosis there is a very high risk of

further progression to hepatocellular carcinoma (HCC) (3). Broad specificity tyrosine kinase receptor inhibitors have shown promising results in reversing liver fibrosis (4).

Factor VII-activating protease (FSAP) is a liver-derived circulating plasma serine protease that is involved in the regulation of coagulation and fibrinolysis (5). Human genetic epidemiological studies have shown that a single nucleotide polymorphism (SNP) in the FSAP-encoding gene (*HABP2*), rs7080536 or G534E also called the Marburg I (MI) polymorphism is found in about 5% of the European population and it is associated with the severity of liver fibrosis (6). MI-FSAP has about a 5-fold weaker proteolytic activity compared with wild-type (WT) FSAP toward various substrates *e.g.* platelet-derived growth factor (PDGF)-BB (7). Circulating latent FSAP zymogen is activated by factors that are released from dead/injured or apoptotic cells such as histones and nucleosomes (8, 9). This fits well with the previous experimental results that acute application of a hepatotoxin, carbon tetrachloride (CCl₄), leads to FSAP activation in mice, presumably through induction of cell death in the liver (10). We have demonstrated that CCl₄-mediated liver injury in a mouse model is associated with an up-regulation of FSAP expression in the long term (11). To further define the role of FSAP in liver fibrosis, we have measured FSAP mRNA expression in liver from patients with varying progression of disease and found a significant negative correlation between fibrosis/inflammation and FSAP expression. Furthermore, circulating FSAP concentration was lower in patients with advanced liver cirrhosis (12). Experiments with FSAP^{-/-} mice also confirmed a protective influence of FSAP on inflammation and liver fibrosis. These studies demonstrate an important role for FSAP in regulating the inflammatory and fibrotic adaptation to liver injury (13).

Although hepatocytes are the main source of circulating FSAP, monocytes express low levels of FSAP that is increased by estrogen and progesterone (14) as well as lipopolysaccharide, Interleukin (IL)-6 and IL-1 β (15). There is also ectopic expression of FSAP in some tumors (16, 17). *In silico* analysis of the *HABP2* promoter sequence upstream of the transcription start site indicated the presence of AP-1, SP-1, and HNF1 α transcription factor binding sites but these have never been experimentally verified (18). To date, no other information is available as to how FSAP expression is regulated in hepatocytes at the molecular level. Thus, we have tested a range of factors on FSAP expression in hepatocytes. The only factor that mediated

* This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft (Bonn, Germany) (KA 1468/4-1) as well as Kreftforeningen (Project 3365379). The authors declare that they have no conflicts of interest with the contents of this article.

[5] This article contains supplemental Figs. S1–S6 and Table S1.

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² The abbreviations used are: HSC, hepatic stellate cell; FSAP, factor VII-activating protease; TGF, transforming growth factor; SNP, single nucleotide polymorphism.

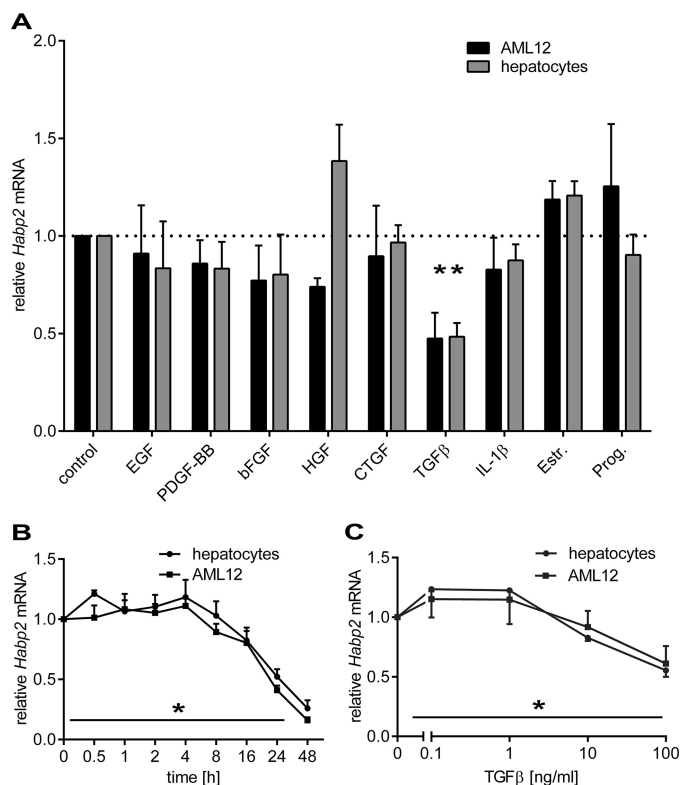


FIGURE 1. Inhibition of *Habp2* mRNA expression by TGF- β . A, murine AML12 hepatocyte cell line (black bars) and murine primary hepatocytes (gray bars) were stimulated with epidermal growth factor (EGF, 50 ng/ml), platelet-derived growth factor (PDGF-BB, 50 ng/ml), basic fibroblast growth factor (bFGF, 10 ng/ml), hepatocyte growth factor (HGF, 10 ng/ml), connective tissue growth factor (CTGF, 50 ng/ml), transforming growth factor- β (TGF- β , 100 ng/ml), interleukin-1 β (IL-1 β , 20 ng/ml), estrogen (Estr., 10 μ g/ml), and progesterone (Prog., 10 μ g/ml) for 24 h. Relative *Habp2* mRNA levels were normalized to *Gapdh* and represent mean \pm S.D. ($n = 3$). B and C, AML12 cells (square) and primary hepatocytes (circles) were treated with 100 ng/ml of TGF- β for increasing time (B), or with increasing concentrations of TGF- β for 24 h (C) and *Habp2* mRNA levels were quantified. Values are mean \pm S.E. ($n = 3$) of a single experiment and similar results were obtained in three independent experiments. Statistically significant results ($p < 0.05$) compared with untreated cells as measured by ANOVA is denoted by *.

a robust down-regulation was transforming growth factor- β 1 (TGF- β). The detailed analysis of how TGF- β regulates FSAP expression completes the description of a circuit that further supports the role of FSAP as an effector protease in liver fibrosis.

Results

TGF- β Is an Inhibitor of *Habp2* Expression in Primary Hepatocytes and AML12 Cells—To identify factors that regulate the expression of *Habp2* in hepatocytes we tested a range of factors on primary mouse hepatocytes and the mouse hepatocyte cell line AML12 cells. The following substances were tested at various concentrations and for various time points and were found not to alter the expression of *Habp2* mRNA in either cell type: epidermal growth factor (EGF), PDGF-BB, basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), connective tissue growth factor (CTGF), IL-1 β , estrogen (Estr.), and progesterone (Prog.) (Fig. 1A). The only factor found to decrease FSAP mRNA expression was TGF- β . The effect of TGF- β was concentration- and time-dependent (Fig. 1, B and C). The response was evident after 24 h and stronger at 48 h.

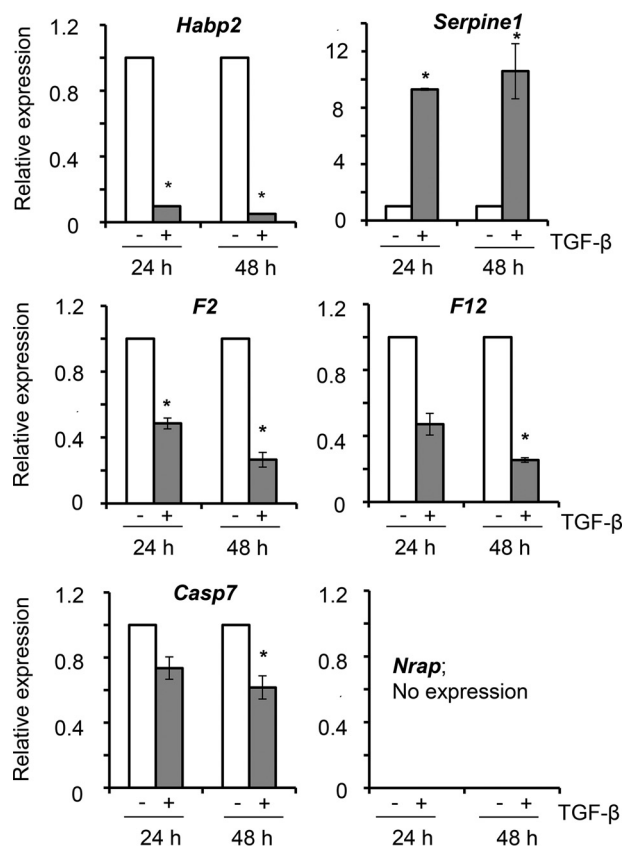


FIGURE 2. Regulation of *Habp2*, *Serpine1*, *F2*, *F12*, *Casp7*, and *Nrap* mRNA expression by TGF- β . Primary hepatocytes were stimulated with TGF- β (10 ng/ml) for 24 or 48 h. Relative mRNA levels were normalized to *Gusb* and represent mean \pm S.D. of three independent experiments performed in duplicate. Student's *t* test: *, $p < 0.05$.

Genes that are functionally related to *HABP2* and also expressed by hepatocytes, such as Factor XII (*F12*) and prothrombin (*F2*), were also down-regulated by TGF- β (Fig. 2). As shown before (19) plasminogen activator inhibitor-1 (*Serpine1*) was up-regulated. Similarly, the neighboring gene at the *HABP2* locus, *Casp7* was not altered in its expression at 24h but there was some inhibition at 48h (Fig. 2). Expression of *Nrap*, another neighboring gene, which overlaps with the *HABP2* sequence on the opposite strand, was not detected in hepatocytes but in the mouse heart (data not shown). Thus, the down-regulation of *Habp2* expression is specific response to TGF- β treatment.

ALK5 Receptor Is Responsible for the Effect of TGF- β on *Habp2* Expression—We then investigated the signal transduction pathway responsible for mediating the down-regulation of *Habp2* expression. TGF- β receptor activation can trigger the canonical pathway including the SMAD2/3 and non-canonical pathways including signaling via p38, JNK, c-Abl, PI3K, and TAK1 (20). Treatment of both cell types with the TGF- β receptor (ALK5) inhibitor SB431542 confirmed the role of ALK5 in the TGF- β -mediated down-regulation of *Habp2* mRNA. This was not the case with the SMAD3 inhibitor SIS3 (Fig. 3, A and B). Western blotting also showed that TGF- β could decrease the amount of FSAP protein and that this was reversed by SB431542 but not by SIS3 (Fig. 3C, densitometric analysis in supplemental Fig. S1). To further characterize the signaling

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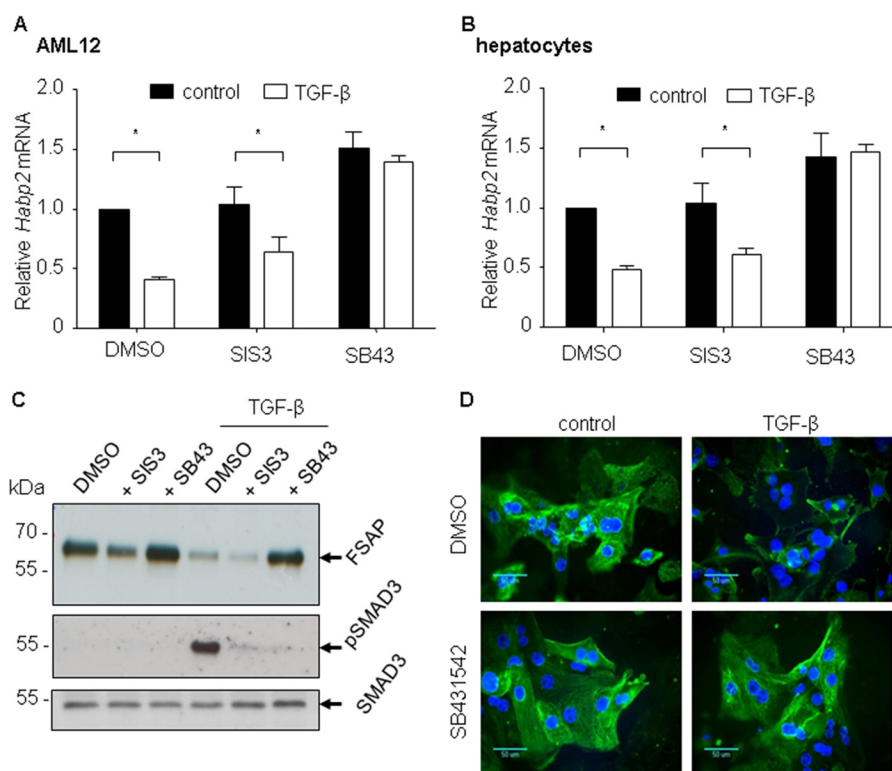


FIGURE 3. Inhibition of TGF- β Type-I receptor (ALK5), but not SMAD3 blocks the effects of TGF- β . AML12 cell line (A) and primary hepatocytes (B) were pre-treated with SB 431542 (10 μ M), SIS3 (10 μ M) or DMSO for 30 min before stimulation with TGF- β (100 ng/ml) for 24 h (white bars) or were left unstimulated (black bars). qPCR analysis of *Habp2* mRNA expression was performed and represent mean \pm S.E. ($n = 3$). Statistically significant results ($p < 0.05$) compared with untreated cells as measured by ANOVA are denoted by *. C, Western blotting analysis of FSAP in the supernatant of AML12 cells as well as phospho SMAD3 and SMAD2/3 in the cell extracts. D, primary hepatocytes were pre-treated with SB 431542 (10 μ M) for 30 min before stimulated with TGF- β (100 ng/ml) for 24 h. Cells were fixed with paraformaldehyde and stained against FSAP (green) and nuclei (DAPI, blue). Similar results were obtained in two independent experiments.

pathway involved, SMAD3 phosphorylation was investigated in AML-12 cells. SB431542 and SIS3 blocked TGF- β -mediated phosphorylation of SMAD3 while, total SMAD2/3 levels were unchanged (Fig. 3C, densitometric analysis in supplemental Fig. S1). Immunostaining of primary hepatocytes for FSAP also confirmed the down-regulation of expression by TGF- β and the reversal of this effect by SB431542 (Fig. 3D).

Because SIS3 was ineffective in blocking this response we further used a siRNA approach to re-examine this. *Smad2* and *Smad3* were separately knocked down in AML-12 cells and its influence on *Habp2* expression was determined. *Smad3* knock-down did not influence the effect of TGF- β but *Smad2* knock-down did (Fig. 4A and supplemental Fig. S2). We also tested whether the, non-canonical pathways, ERK1/2, p38MAPK, or Akt pathways could be involved using specific inhibitors of these pathways. LY294002 (PI3K inhibitor), PD98059 (MEK1 inhibitor) and SB203580 (p38MAPK inhibitor) did not influence the down-regulation of *Habp2* mRNA by TGF- β (Fig. 4B). The effectiveness of LY294002 and PD98059 was tested in respective Western blotting experiments analyzing ERK1/2 and Akt phosphorylation (supplemental Fig. S3). Thus, a clear involvement of SMAD3 and non-canonical pathways was not evident indicating that ALK5 mediated signaling via SMAD2 was responsible.

The TGF- β Response Is Mediated via a 177-bp Promoter Region of the HAPB2 Gene that Is Directly Upstream of the Transcription Start Site—We then determined the nature of the transcriptional regulation of the *HABP2* gene by TGF- β .

Deletion mutations representing fragments of the first 2400 bp of the 5' region upstream of the transcription start site of the human *HABP2* gene were cloned into the luciferase reporter vector pGL4.10[luc2] (Fig. 5A). Basal luciferase activity in untreated AML12 cells was significantly reduced in the case of the sub-fragments -1/-177, -313/-427, and -177/-313 compared with the 500-bp fragment (Fig. 5B). TGF- β induced a decrease in the promoter activity of the -1/-2400, -1/-1800, -1/-1000, -1/-500, and the -1/-177 bp construct but not of other sub-fragments tested. The effect of TGF- β on the FSAP promoter activity was reversed by treatment with SB431542 (Fig. 5C). The direct effect of SB431542 alone on *HABP2* expression may be due to the inhibition of the effect of endogenous TGF- β produced by the cells. For comparison, we also cloned the previously described *Serpine1* (PAI-1) (19) and *c-myc* promoter (21) since these have been shown to be, respectively, up- or down-regulated in hepatocytes after treatment with TGF- β and the expected responses were observed (supplemental Fig. S4) confirming the validity of the test system.

Furthermore, *in silico* analysis of the human and mouse *HABP2* 5' 177-bp sequence upstream of the transcription start site (Fig. 6) showed a relative high conservation of 69% between the species and a similar pattern of putative binding sites for AP-1, SP-1, and HNF1 α . These results are in agreement with earlier reports regarding the FSAP promoter region (18). Since we determined the activity of the human *HABP2* promoter in a

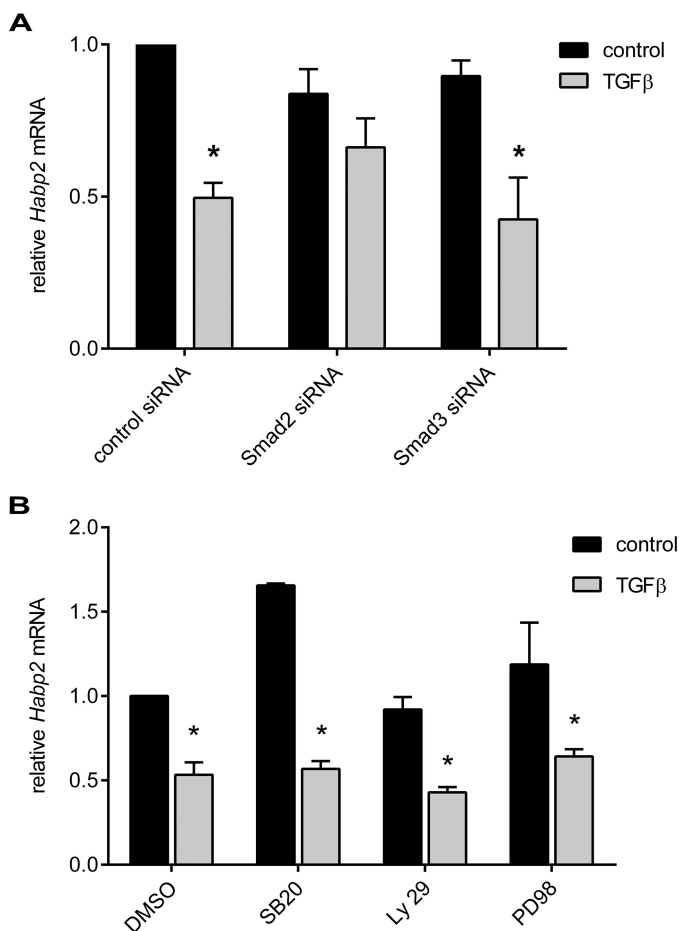


FIGURE 4. Effect of inhibition of signaling pathways on the expression of *Habp2* by TGF β . *A*, AML-12 cells were transfected with control siRNA or *Smad2* and *Smad3* for 24 h. Cells were then stimulated with TGF- β (100 ng/ml) for 24 h before analysis of *Habp2* mRNA. Expression relative to *Gapdh* is shown, and values are mean \pm S.D. ($n = 3$) of a single experiment and similar results were obtained in two independent experiments. *B*, AML12 cell were pre-treated with Ly294002 (10 μ M), PD98059 (10 μ M), SB203580 (10 μ M), and DMSO for 30 min before stimulation with TGF- β (100 ng/ml) for 24 h or were left unstimulated. qPCR analysis of *Habp2* mRNA expression was performed and represent mean \pm S.E. ($n = 3$). Statistically significant results ($p < 0.05$) compared with untreated cells as measured by ANOVA is denoted by *. Similar results were obtained in two independent experiments.

mouse hepatocyte cell line AML12 cells we also performed these studies in the human hepatocellular carcinoma-derived hepatocytic cell line HepG2. The activity of the 500-bp *HABP2* promoter in human cells was down-regulated by TGF- β confirming the inter-species congruence (supplemental Fig. S5).

Transcription Factors Involved in the Regulation of *HABP2*—Because the $-1/-177$ promoter fragment could phenocopy the changes in *Habp2* expression by TGF- β we investigated this region in detail. We performed *in silico* analysis to specifically identify transcription factor binding elements that are unique to this fragment compared with other non-responsive control fragments ($-313/-427$ and $-177/-313$, respectively). This showed that the $-1/-177$ fragment contains binding elements for c-jun, c-fos, AP-1, HNF1 α , HNF3 β , and C/EBP δ (22–24). No predicted SMAD binding sites were found. To determine the role of c-fos and c-jun, we investigated the protein/DNA interaction of both transcription factors and this promoter

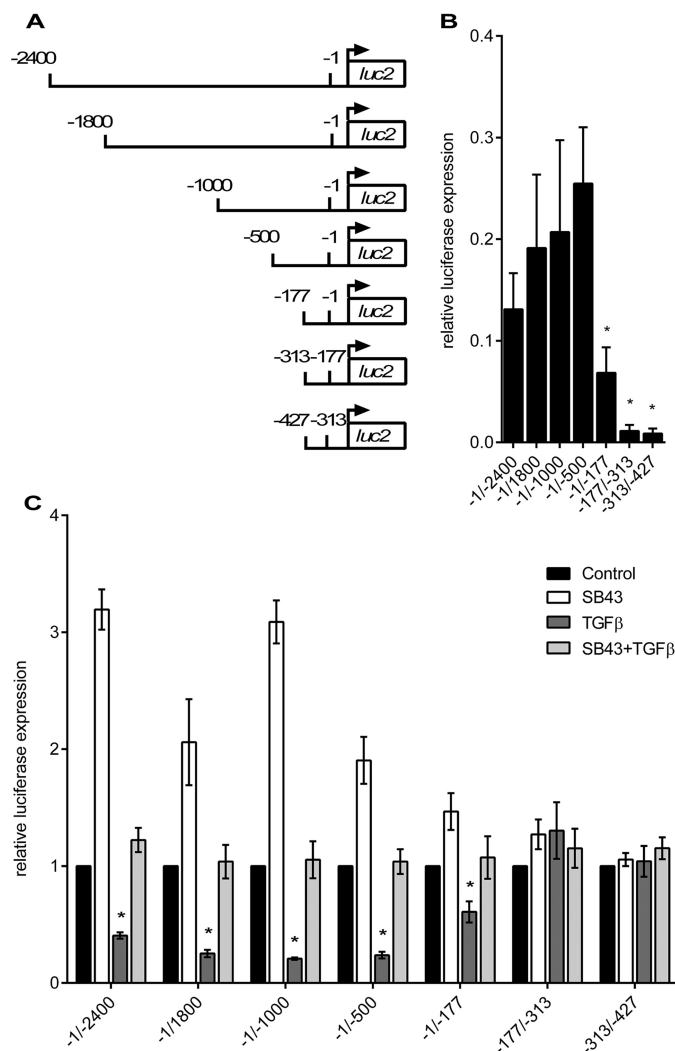


FIGURE 5. Activity of human *HABP2* promoter reporter constructs. *A*, indicates details of the *HABP2* promoter fragments used. *B*, basal promoter activity of the human *HABP2* promoter fragments of various lengths was tested in AML12 hepatocytes. Significant changes relative to the $-1/-500$ fragment are indicated by *. *C*, cells were pre-treated with SB 431542 (10 μ M) or 30 min before stimulated with TGF- β (100 ng/ml) for 24h before lysis. Activity is shown as mean \pm S.E. ($n = 5$). Since the basal expression of the different constructs varied, the activities of the treated samples are normalized to the untreated sample for that particular construct. Statistically significant difference ($p < 0.05$) between control and TGF- β as measured by ANOVA is denoted by *. Similar results were obtained in three independent experiments.

region. Nuclear extracts of AML12 cells were incubated with biotinylated dsDNA fragments of the promoter to capture c-fos and c-jun, which were detected using Western blotting. c-fos binding to the $-1/-177$ promoter fragment was 8-fold and 3-fold higher compared with other control DNA fragments ($-313/-427$ and $-177/-313$, respectively), which showed no TGF- β responsiveness in the reporter assays (Fig. 7A). c-jun binding of the $-1/-177$ promoter fragment was 2.5-fold higher compared with the $-313/-427$ fragment and was identical compared with the control DNA fragment $-177/-313$ (Fig. 7A). We then compared chromatin binding of c-fos in AML12 cells with or without TGF- β treatment. In TGF- β -treated cells c-fos binding to the $-1/-177$ minimal promoter was reduced by 70% compared with no change in the control

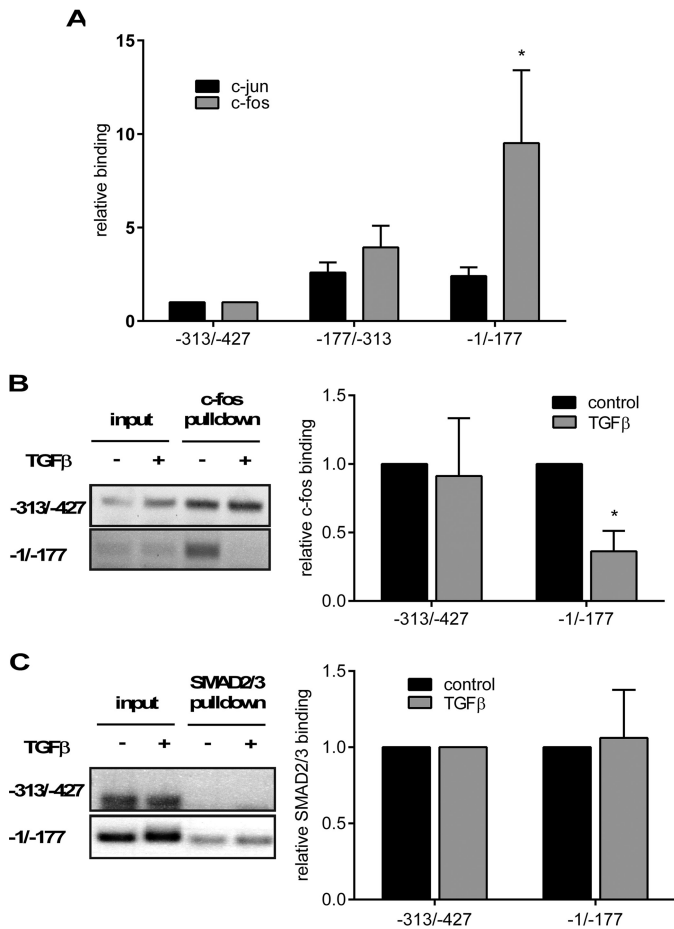


FIGURE 7. c-fos interacts with the minimal *Habp2* promoter. *A*, DNA sequence representing the minimally active and TGF- β -responsive promoter region (-1/-177) and other non-responsive regions (-313/-427 and -177/-313) were biotinylated and incubated with nuclear extracts from AML12 cells. After pull-down with streptavidin magnetic beads the bound fraction was subjected to Western blotting analysis with an anti-c-fos antibody or anti-c-jun antibody. Band density was quantified, and results represent mean \pm S.E. ($n = 3$). *B/C*, ChIP was performed on nuclear extracts of AML12 cells with or without TGF- β stimulation for 24 h. Pull down was performed with an anti-c-fos (*B*) or an anti-SMAD 2/3 (*C*) antibody. Intact extract was used as an input control. PCR was performed to amplify the minimally active and TGF- β -responsive promoter region (-1/-177) and other non-responsive region (-313/-427). Values represent mean \pm S.E. ($n = 3$ independent experiments). Statistically significant results ($p < 0.05$) compared with untreated cells as measured by ANOVA is denoted by*.

canonical and non-canonical pathways to the 177-bp region of the promoter region was characterized.

Hepatocytes are the major producers of all coagulation factors and certainly interesting was the fact that *F2* (prothrombin) and *F12* (FXII) genes were also downregulated by TGF- β . The regulation of these key coagulation factors in hepatocytes by TGF- β has never been studied before and unlike *Habp2* their regulation has not been observed in comparable microarray analysis (31). Partial hepatectomy in mice causes a proliferative/dedifferentiated phenotype in hepatocytes and this was associated with the down-regulation of the expression of many coagulation factors, including *F2* and *F12* (32). This points to a coordinated regulation of coagulation factor expression that may be relevant for the interactions between pathophysiological changes in the liver and their consequences for coagulation pathways.

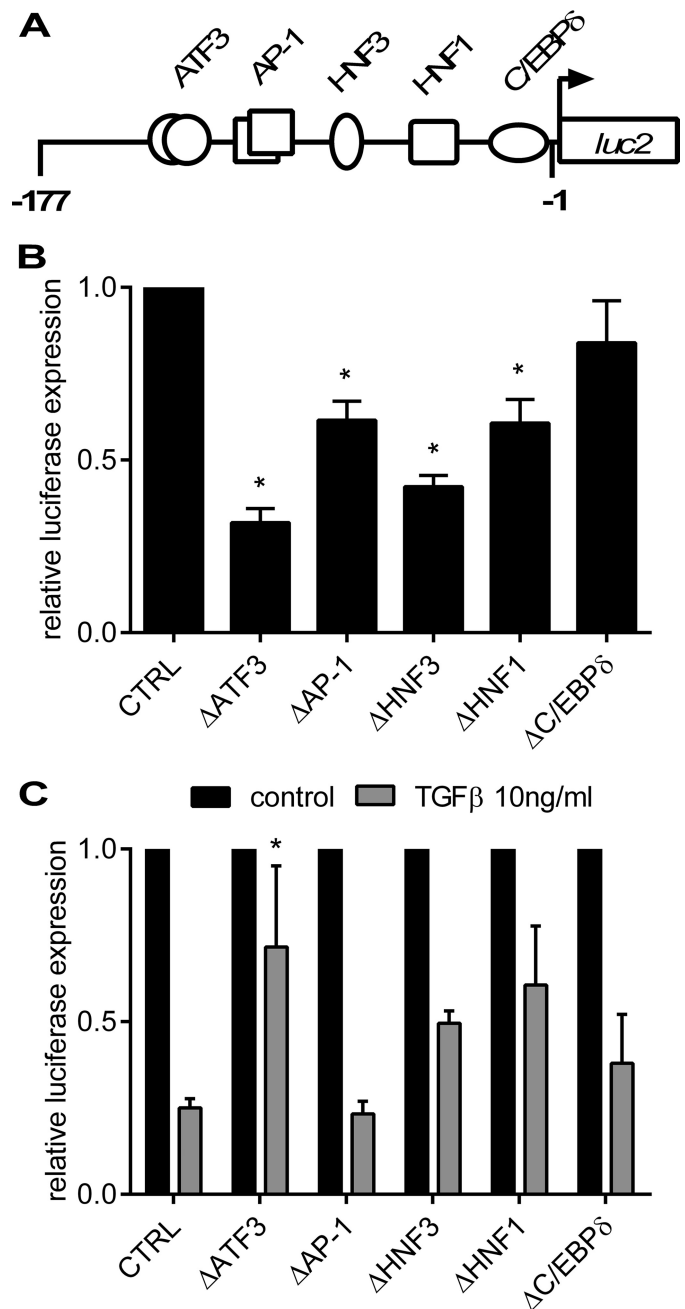


FIGURE 8. Deletion mutations within the 177-bp *HABP2* promoter. *A*, transcription factor binding sites were deleted from the minimally active and TGF- β -responsive promoter region (-1/-177) as indicated in Fig. 6*A*. *B*, basal reporter activity was measured in AML12 cells. *C*, reporter activity was measured in the absence or presence of TGF- β (10 ng/ml) for 24 h. Values represent mean \pm S.E. ($n = 3$). Since the basal expression of the different constructs varied, the activities of the treated samples are normalized to the untreated sample for that particular construct. Statistically significant results ($p < 0.05$) compared with TGF- β -treated cells transfected with the control (-1/-177) construct was determined by ANOVA and is denoted by*.

In the canonical TGF- β pathway the activation of ALK5 leads to activation of signaling via SMAD2/3-SMAD4 complexes and their interaction with the SMAD binding elements (CAGAC) by association with many cofactors leading to the transcriptional regulation of target genes. SMAD2 and SMAD3 are functionally interchangeable but do have distinct functions. Neither siRNA against SMAD3 nor a syn-

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thetic SMAD3 inhibitor could modulate this action of TGF- β . However, knockdown of SMAD2 significantly influenced the down-regulation of *Habp2* by TGF- β . Of the many non-canonical pathways activated by TGF- β (20), we did not find any influence of inhibiting the p38MAPK, ERK1, and Akt pathways. The 177-bp promoter region, that exhibited all the characteristics of the TGF- β response, did not have any predicted SMAD binding sites and ChIP analysis with SMAD2/3 antibody also failed to detect any TGF β response. Thus further studies are required to complete the understanding of the pathway involved.

On the other hand, there was a robust pull down of this fragment with a c-fos antibody that was decreased in the presence of TGF- β . In a corollary experiment this DNA fragment could pull down c-fos protein, but not c-jun, in a specific manner. Further mutation studies suggested a role of c-jun/c-fos, HNF1 α , and HNF3 in the basal expression of the *HABP2* gene, since deletion of any of those caused reduced expression in AML12 cells. When we treated the different mutants with TGF- β only the Δ ATF3 mutant showed a significant loss of responsiveness, indicating an important role of ATF3 binding element in this process. ATF3, also known as liver regeneration factor 1 (LRF-1), plays an important role in the stress-response of the liver after partial hepatectomy or CCl₄ treatment (33, 34). After CCl₄ treatment ATF3 forms a heterodimer with Jun B and represses gene expression by competing with the c-jun/c-fos complex for the same binding sites (33). c-jun, Jun B and ATF3 are up-regulated in three different human epithelial cell lines upon TGF β treatment, while C/EBP δ is down-regulated. On the basis of these results we hypothesize that upon TGF β treatment levels of ATF3 and junB increase, effectively replacing c-jun/c-fos binding to the promoter, which leads to reduced *Habp2* expression (35). A similar model has been proposed for the up-regulation of PAI-1 after TGF β treatment (36).

Human genetic studies as well as the analysis of FSAP knockout mice indicate an important role for FSAP in the pathophysiology of liver fibrosis, thrombosis, stroke and thyroid cancer. We have for the first time performed a comprehensive analysis of factors that may regulate FSAP expression in hepatocytes and have identified TGF- β as a major inhibitory factor. This cytokine is a pleiotropic factor that regulates growth, apoptosis, differentiation, epithelial-mesenchymal transition, deposition of extracellular matrix, and is a strong immune-modulatory factor. It is strongly up-regulated in liver fibrosis and plays a crucial role in the development of liver fibrosis and hepatocellular carcinoma (3). This together with the fact that FSAP can influence the activation of hepatic stellate cells (11), indicates that FSAP could be part of a local paracrine loop in the liver that is regulated by TGF- β .

Experimental Procedures

Cell Culture—Mouse hepatocyte cell line AML12 (ATCC[®] CRL-2254TM) was cultivated in DMEM (Invitrogen, Darmstadt, Germany) with 10% (v/v) fetal calf serum (FCS), ThermoFisher Scientific (Fermentas), Rockford), 10 units/ml penicillin and 10 μ g/ml streptomycin (Invitrogen) on cell culture-treated plastic (Nunc, Wiesbaden, Germany). Mouse primary hepato-

cytes were isolated as described earlier by collagenase perfusion (2) and were cultivated as above. All cells were maintained in a humidified atmosphere of 5% CO₂ at 37 °C. All animal studies performed in this study were approved by the local authority for animal welfare; Regierungspraesidium Giessen, Germany and Mattylsynet, Oslo, Norway. The mice were treated in accordance with the recommendation of the guidelines for the care and use of Laboratory animals of the National Institute of Health (USA) and the local authorities.

SMAD Phosphorylation and Knockdown—Mouse hepatocytes or AML12 cells were cultivated in normal medium and thereafter switched to serum-free medium for 2 h. Cells were stimulated with agonists for 30 min, washed with PBS and lysed in lysis buffer containing 1 mM orthovanadate and 0.5 mM PMSF. Western blotting was performed with anti-phospho SMAD3 antibody. In parallel, total SMAD 2/3 protein was analyzed using anti-SMAD 2/3 (both Cell Signaling Technology, Frankfurt, Germany; anti-SMAD 2/3 8685; anti-phospho-SMAD 3 9520). SMART-Pool siRNA were obtained from Dharmacon (Lafayette, CO); control siRNA D-001206-13-05 (siGENOME Non-Targeting siRNA Pool), Smad2 siRNA M-040707-01-0005, and Smad3 siRNA M-040706-01-0005. They were used exactly as described by the manufacturer.

Western Blotting Analysis—SDS-PAGE was performed, and proteins were transferred to Hybond-P PVDF membrane (GE Healthcare, Frankfurt, Germany). For analysis of Western blotting chemiluminescence plus reagent (GE Healthcare) was used. Polyclonal rabbit antibody against FSAP was kindly provided by Michael Etscheid, Paul Ehrlich Institute, Langen, Germany. SMAD 2/3 was used as a loading control and densitometric analysis was performed to calculate relative expression using the LabImage 1D (Kapelan, Leipzig, Germany) system.

RNA Isolation and qPCR Analysis—Total RNA was extracted using total RNA Miniprep Kit (Sigma Aldrich). Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For real-time PCR, SensiMix SYBR Kit (Bioline GmbH, Luckenwalde, Germany) was used, and analysis was performed on a Step One Plus Real-Time PCR System (Applied Biosystems, Darmstadt). Melting curve was performed at 60 °C for 1 min following 95 °C. Amplification (cDNA denaturation 95 °C for 15 s, primer hybridization 60 °C for 30 s, elongation 72 °C for 30 s) plot was monitored over 40 cycles and continuous fluorescence measurement indicated mRNA expression of analyzed genes. Fluorescent threshold cycles (ct) were set and normalized against ct of reference genes *Gapdh* or *Gusb* (37). Primer sequences are provided in [supplemental Table S1](#).

Construction and Mutations of the *HABP2* Promoter—Various genomic fragments from the human *HABP2* gene were cloned into the luciferase reporter vector pGL4.10[luc2] (Promega, Mannheim, Germany). These constructs served as templates for smaller constructs, deletion mutants and for site-directed mutagenesis. These constructs included up to 2400 bp of the 5' region of the human *HABP2* gene directly upstream of the transcription start site as defined earlier (18). Site-directed mutagenesis was performed using QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). Details of the con-

structs are shown in the results section. DNA sequencing was performed to check for the correct sequence of the resulting clones, deletion, and other mutants.

Transfection and Reporter Assays—AML12 or HepG2 cells were transfected with empty vector as well as the various constructs (500 ng) using Lipofectamine2000 (Invitrogen). Adjustment for the relative transfection efficiency was performed by co-transfecting the *Renilla* vector (pGL4.75[hRluc/CMV]) (Promega). Cells were then switched to serum-free medium with or without TGF- β for 24 or 48 h. After lysis, determination of the firefly and *Renilla* luciferase was performed using a Dual luciferase kit from Promega. Normalized *Renilla* luciferase activity to control firefly activity was calculated and expressed as fold change compared with empty vector.

Protein/DNA Interaction Pull-down Assay—Confluent AML12 cells were untreated or treated with 100 ng/ml TGF- β for 48 h in serum-free medium or regular growth medium. Lysis buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, and 0.5 mM DDT was added to the cells to induce swelling and bursting of cells for 20 min on ice. After centrifugation in a microfuge the pellet was resuspended in a buffer containing 50 mM HEPES pH 7.8, 3 mM MgCl₂, 300 mM NaCl, 1 mM DTT, 0.1 mM PMSF as added to the pellet and subjected to sonification. Streptavidin-conjugated Dynabeads (Thermo Fisher, 11205D) were prepared and used according to the manufacturer's protocol. The immobilization of nucleic acids was performed using biotinylated DNA baits which were amplified from plasmid DNA containing the FSAP promoter region. The same primer sequences as in the ChIP were used (supplemental Table S1).

Chromatin Preparation by Sonication and Immunoprecipitation Assays (ChIP)—The chromatin preparation by sonication has been performed as described before; 10⁷ cells were disrupted by sonication to prepare fragmented chromatin. These were incubated overnight at 4 °C with antibody (anti-cjun SC-1694 and anti-cfos SC-52, both Santa Cruz) pre-coupled for 2 h to magnetic Dynabeads Protein G (Invitrogen) as described before (38).

Immunofluorescence Analysis—Primary hepatocytes were fixed with 4% (w/v) paraformaldehyde for 10 min followed by permeabilization with 0.2% (w/v) Triton X-100. After blocking with 3% (w/v) BSA the cells were stained with the primary antibody followed by an appropriate secondary antibody. Nuclei were stained with (4',6'-diamidino-2-phenylindole) (DAPI), and the slides were mounted in Vectashield™ mounting medium (Biozol Diagnostica). Controls were performed by including an isotype-matched negative control primary antibody. Images were taken on a Leica DMR HC fluorescence microscope (Leica Microsystems, Wetzlar, Germany), and image analysis was performed using Metamorph software, Meta Series 7.0 (Visitron Systems, Puchheim, Germany).

Statistical Analysis of the Data—Statistical significance was tested using Analysis of Variance (ANOVA) and the individual comparisons were made using Bonferroni test using Graphpad Prism5 (version 5.02) (GraphPad Software, Inc, La Jolla, CA). In some cases a Student's *t* test was used. A value of *p* < 0.05 was considered to be statistically significant.

Author Contributions—S. L. performed most of the experiments and analyzed the data and edited the manuscript. S. S. conducted experiments deleting TFBS in the promoter and measured luciferase activity under various conditions, ChIP experiments and edited the manuscript. A. M. P. performed the expression experiments and edited the manuscript. L. M. performed immunofluorescence analysis and edited the manuscript. S. M. K. designed the study, analyzed the data and wrote the manuscript together with S. L.

Acknowledgments—We thank Susanne Tannert-Otto and Thomas Schmidt Wöll for their excellent technical assistance.

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