

# Transforming growth factor $\beta$ signal transduction in hepatic stellate cells via Smad2/3 phosphorylation, a pathway that is abrogated during in vitro progression to myofibroblasts

## TGF $\beta$ signal transduction during transdifferentiation of hepatic stellate cells

Steven Dooley<sup>a,\*</sup>, Bert Delvoux<sup>a</sup>, Maike Streckert<sup>a</sup>, Linda Bonzel<sup>a</sup>, Marcin Stopa<sup>a</sup>, Peter ten Dijke<sup>b</sup>, Axel M. Gressner<sup>a</sup>

<sup>a</sup>Institut für Klinische Chemie und Pathobiochemie, RWTH-Universitätsklinikum, Pauwelsstr. 30, 52074 Aachen, Germany

<sup>b</sup>The Netherlands Cancer Institute, Division of Cellular Biochemistry, H3, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

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**Abstract** To current knowledge, transforming growth factor  $\beta$  (TGF $\beta$ ) signaling is mandatory to establish liver fibrosis and various molecular interventions designed to affect the TGF $\beta$  system were successfully used to inhibit fibrogenesis. Activated hepatic stellate cells (HSC), which are one important source of TGF $\beta$ , are the major producers of extracellular matrix proteins in liver injury. We have previously shown that the TGF $\beta$  response of this cell type is modulated during the transdifferentiation process. This work delineates the activation of TGF $\beta$  downstream mediators, the Smads, in quiescent HSC and transdifferentiated myofibroblasts (MFB). The expression level of all Smads remained largely unchanged during this process. The response of HSC to TGF $\beta$ , leading to, e.g., induction of  $\alpha 2$  (I) collagen expression, is mediated by phosphorylation of Smad2 and Smad3 and subsequent nuclear translocation of a Smad containing complex. Neither TGF $\beta$ -dependent nor endogenously phosphorylated Smad2/3 was detectable in comparable amounts in transdifferentiated MFB, indicating loss of TGF $\beta$  sensitivity. Ectopic expression of Smad7 in HSC led to inhibition of Smad2 phosphorylation and abrogated TGF $\beta$  response. In transdifferentiated MFB, expression of a constitutively active TGF $\beta$  receptor I, but not treatment with TGF $\beta$ 1, resulted in transcriptional activation of a TGF $\beta$  responsive promoter, thereby demonstrating completely restored TGF $\beta$  signal transduction. Our data indicate that in contrast to a postulated mechanism of enduring autocrine TGF $\beta$  signal transduction, early and late stages of HSC activation have to be distinguished, which is of importance for antifibrotic therapies. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Fibrogenesis; Transforming growth factor  $\beta$  signal transduction; Smad

### 1. Introduction

Members of the transforming growth factor  $\beta$  (TGF $\beta$ ) family of peptide growth factors, which include TGF $\beta$ , bone morphogenetic proteins and activins, regulate a broad range of cellular processes, reaching from cell growth and differentiation to apoptosis [1–3]. The signaling responses to TGF $\beta$  and other family members are mediated by a heteromeric complex of two types of transmembrane serine/threonine kinase receptors at the cell surface and their intracellular substrates, the Smad proteins [1–4]. Following ligand binding, the type II receptor kinases phosphorylate and thereby activate the type I receptor cytoplasmic domains. The Smads then act as type I receptor-activated signaling effectors, which, following receptor-induced phosphorylation, move into the nucleus to activate transcription of a select set of target genes [5,6].

TGF $\beta$  is thought to be an important cytokine in the regulation of the production, degradation, and accumulation of extracellular matrix (ECM) proteins, and it may play a pivotal role in fibroproliferative changes that occur following tissue damage in liver fibrosis. TGF $\beta$  is synthesized by several mesenchymal liver cells and is also released by hepatocytes [7] and generated by infiltrating cells such as lymphocytes, monocytes/macrophages, and platelets. Following injury or inflammation, all these cells are potential sources of TGF $\beta$ .

In liver, hepatic stellate cells (HSC), comprising 15% of the total number of resident cells, are the key fibrogenic progenitor cell type [8,9]. In normal liver, they are the principal storage site for retinoids [10]. Subsequent to liver injury, HSC undergo a process known as activation, which is the transition of quiescent cells into proliferative, fibrogenic, and contractile myofibroblasts (MFB). Characteristic features of activated HSC are a variety of interactions with hepatocytes, endothelial cells, Kupffer cells and inflammatory phagocytes [11–13], involving cytokines, chemokines [14,15], oxidants [16], and products of ECM [17]. The potential profibrogenic role of TGF $\beta$  has been impressively shown in numerous descriptive studies and in experimental models [18–20]. These findings, together with TGF $\beta$ 's ability to regulate the expression of many ECM protein genes, and its increased expression and release during activation of HSC, have led to a widely accepted model, in which autocrine stimulation of MFB by

\*Corresponding author. Fax: (49)-241-8888 512.

E-mail address: steven.dooley@post.rwth-aachen.de (S. Dooley).

**Abbreviations:** BDL, bile duct ligation; CA, constitutive active; DMEM, Dulbecco's modified Eagle medium; ECM, extracellular matrix; FCS, fetal calf serum; HSC, hepatic stellate cell(s); MFB, myofibroblast(s); TBS, Tris-buffered saline; TGF $\beta$ , transforming growth factor  $\beta$ ; T $\beta$ RI, TGF $\beta$  receptor type I; T $\beta$ RII, TGF $\beta$  receptor type II

endogenously produced TGF $\beta$  is the dominant stimulus of fibrogenic ECM production and perpetuation in diseased liver. In contrast to this, we have previously shown that quiescent HSC, activated HSC and MFB behave differently to treatment with TGF $\beta$ 1 [21]. In quiescent HSC, addition of TGF $\beta$ 1 resulted in proliferation inhibition, transcriptional induction of different TGF $\beta$  target genes, hyaluronan release and formation of Smad binding element interacting complexes. In contrast, transdifferentiated MFB were at least partially resistant to the aforementioned TGF $\beta$  effects. TGF $\beta$  receptor types I and II (T $\beta$ RI and T $\beta$ RII) were expressed with similar transcript amounts in both cell types. However, strongly reduced radiolabeled ligand binding activity to surface receptors was detected in MFB, suggesting modulation of TGF $\beta$  responsiveness during activation and transdifferentiation of HSC.

In recent years, antifibrotic strategies, based on a general suppression of TGF $\beta$  signaling, have been developed. However, due to many essential roles of TGF $\beta$  in important cellular processes, anti-TGF $\beta$  therapies have to be carefully focused to specific cell types and physiological stages. Therefore, it is important to get further insight into the role of TGF $\beta$  in different stages of HSC activation during liver fibrogenesis. In the current report, we have analyzed molecular details of intracellular TGF $\beta$  signaling to the nucleus comparatively in HSC and MFB. Based on our findings with primary cultured cells, we suggest a refined model of profibrogenic TGF $\beta$  signal transduction, in which transdifferentiated HSC become partially TGF $\beta$  insensitive.

## 2. Materials and methods

### 2.1. Materials

Restriction enzymes and modifying enzymes (T4 DNA ligase, Klenow fragment of DNA polymerase I) were purchased from Roche or Gibco-BRL. Taq polymerase and [ $\alpha$ -<sup>32</sup>P]dCTP were from Amersham/Pharmacia. All reagents for cell culture (Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), trypsin-EDTA and phosphate-buffered saline) were from Gibco-BRL. The monoclonal anti-Flag antibody was from Sigma (M5, F-4042). Oligonucleotides were from MWG Biotech or Genset. The transfection reagent Fugene-6 was from Roche. The luciferase assay system was from Promega. Antisera for Smad2, 3, 4 and 7 were raised against the peptides described in [22] or purchased from Santa Cruz Biotechnology or Zymed Laboratories Inc., as indicated. The anti-mouse horseradish peroxidase-conjugated secondary antibody was from Santa Cruz or Zymed. All other chemicals were obtained from commercial sources at the purest grade available.

### 2.2. Experimental induction of fibrosis in rats

Male Sprague–Dawley rats (about 300 g body weight) were purchased from Harlan-Winkelmann. The animals were kept in a temperature-controlled environment (22°C) with a 12 h light–dark cycle and fed ad libitum with standard rat chow. Animals had free access to tap water. HSC were isolated from bile duct ligated (BDL) rats as described below. Before each procedure, animals were anesthetized with 100 mg/kg ketamine (Sanofi-ZEVA) and 50  $\mu$ l 2% Rompun (Bayer) intraperitoneally. Study protocols were performed in compliance with the institution guidelines. Experiments were performed after an overnight fast. BDL was performed as previously described [23,24]. Briefly, the common bile duct was located through a midline incision and double ligated near the liver hilus with transection between the ligatures. Controls underwent a sham operation that consisted of exposure, but no ligation of the common bile duct.

### 2.3. Preparation of cells

Isolation and culture of liver HSC from male Sprague–Dawley rats (500–600 g body weight) were performed as described previously

[25,26]. All animals received human care in compliance with the German Animal Protection Act, which is in accordance with the National Research Council's criteria. In brief, non-parenchymal liver cells were isolated by the pronase-collagenase method. HSC were purified by a single-step density gradient centrifugation with Nycodenz (8.1%; w/v) in Hank's balanced salt solution without NaCl (Nyegaard Co. AS, Oslo, Norway) and identified by their typical light-microscopic appearance and vitamin A-specific autofluorescence. The mean purity of freshly isolated cells was 85–95%, cell viability was 95%, and the yield ranged from 20 to 40  $\times$  10<sup>6</sup> cells/liver. HSC were seeded with densities of 0.2  $\times$  10<sup>6</sup> cells/10 cm<sup>2</sup> for immunostainings and 3  $\times$  10<sup>6</sup> in a 75 cm<sup>2</sup> flask for metabolic labeling or RNA preparations. They were cultured in DMEM supplemented with 4 mmol/l L-glutamine, 10% FCS, 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. The first change of medium was made approximately 20 h after seeding, after which the purity of HSC was 97%. The second change of medium was done about 18 h later, during which FCS supplementation was reduced to 0.5%. MFB were obtained by secondary culture of 7 day old HSC, and cells were kept in general for 48 h after initiation of secondary culture. Cultured HSC were checked for contaminating endothelial cells and Kupffer cells with diacetylated low-density lipoprotein (Harbor Bioproducts) and Fluoresbrite latex beads (1 mm; Polysciences Inc.), respectively. HEK293 human embryonic kidney carcinoma cells were cultured in DMEM, 4 mM L-glutamine, including 10% FCS. Additionally, all culture media were supplemented with penicillin (100 IU/ml)/streptomycin (100  $\mu$ g/ml). All cultures were maintained at 37°C, 5% CO<sub>2</sub> in a humidified atmosphere.

### 2.4. Preparation of total cell lysate, nuclear extracts and immunoblot analysis

Total lysates from 64 mm<sup>2</sup> cell culture plates were prepared with 0.9 ml RIPA buffer (1  $\times$  Tris-buffered saline (TBS), 1% Nonidet P-40 (Amresco), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)); protease inhibitors were provided as Protease Inhibitor Mix (Roche) just before use: for preparation of nuclear extracts, 10  $\mu$ l/ml PMSF (10 mg/ml in isopropanol) and 10  $\mu$ l/ml 100 mM sodium orthovanadate were added. To inhibit protein dephosphorylation, Phosphatase Inhibitor Mix (Sigma) was added. Using a syringe fitted with a 21 gauge needle to shear DNA, lysates were transferred to a microcentrifuge tube. 10  $\mu$ l of a 10 mg/ml PMSF stock was added followed by a 30–60 min incubation on ice. Subsequently, cell lysates were cleared by centrifugation at 15 000  $\times$  g for 20 min at 4°C. For nuclear extract preparation, cells were harvested and processed according to the protocol of Dennler et al. [27] with minor modifications. Briefly, confluent cells from four dishes (100 mm<sup>2</sup>) were washed with TBS and scraped. After another washing, cells were suspended in 400  $\mu$ l/dish of ice cold buffer A (10 mM HEPES-KOH pH 7.9, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.5 mM dithiothreitol (DTT), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.2 mM PMSF). The cells were allowed to swell on ice for 10 min and then lysed by 30 strokes of a Dounce all glass homogenizer. Nuclei were pelleted by centrifugation and resuspended in 50  $\mu$ l/dish of ice cold buffer C (20 mM HEPES pH 7.9, 20 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 0.13  $\mu$ M okadaic acid, 1 mM EDTA, 1 mM EGTA, 0.4 mM ammonium molybdate, 420 mM NaCl, 20% glycerol, 1 mM DTT, 0.5 mM PMSF and 1  $\mu$ g/ml each leupeptin, aprotinin and pepstatin). At this step Phosphatase Inhibitor Mix (Sigma) was added. The nucleus membrane was lysed by 15 strokes of a Dounce all glass homogenizer. The resulting suspension was stirred for 30 min at 4°C. The clear supernatant was aliquoted and frozen at –80°C.

40  $\mu$ l of lysates or nuclear extracts were separated by SDS–8% polyacrylamide gel electrophoresis (PAGE) and transferred to a 0.45  $\mu$ m nitrocellulose membrane (Protran BA 85; Schleicher&Schüll). Non-specific binding was blocked by 5% non-fat milk powder in TBS overnight at 4°C followed by incubation with the primary antibodies (diluted 1:2000 in 2.5% non-fat milk powder in TBS for 1 h at room temperature). Blots were washed two times in TBS/0.05% Tween-20 (Bio-Rad, Munich, Germany) and subsequently three times in TBS for 5–10 min each. The secondary antibody anti-rabbit horseradish peroxidase (stock solution: 400  $\mu$ g/ml; Santa Cruz Biotechnology Inc.) was incubated at a dilution of 1:50 000 for an additional hour at room temperature followed by five washes as described above. Bound antibodies were detected by developing the membrane in Supersignal Ultra (Pierce) for 5 min and subsequent evaluation with a Lumi Imager (Roche). Specific antisera against Smad2, Smad3,

Smad4 and Smad7 were described in [22] or purchased by Santa Cruz Biotechnology Inc. and Zymed as indicated. The antiserum PS2, which was raised against the phosphorylated synthetic peptide SS(P)MS(P) as previously described [28], was used to detect phosphorylated Smad2. The antiserum recognizing phosphorylated Smad1 [28] crossreacts with phosphorylated Smad3 and was used to detect Smad3 activation. As a control for specificity and to facilitate identification of the protein of interest, detection was performed in the absence or the presence of the peptide, to which each of the antisera was originally raised. Furthermore, extracts of cells ectopically expressing Flag-tagged Smad proteins were used as positive controls in Western blots. Their expression was checked before by an anti-Flag antibody (Sigma).

### 2.5. Immunostainings

Immunostainings of HSC were performed using an immunofluorescence method. TGF $\beta$ -treated HSC or MFB and untreated controls were seeded on glass precoated with FCS in six well plates. The cells were washed three times with TBS and fixed with 4% (w/v) paraformaldehyde in TBS for 15 min at room temperature. After fixation, cells were rinsed with TBS and then permeabilized with 0.1% (v/v) Triton X-100 plus 0.1% (w/v) sodium citrate for 2 min on ice. Unspecific binding sites were blocked with 50% (v/v) FCS in TBS plus 0.1% (w/v) bovine serum albumin (BSA). Cells were then incubated for 2 h at room temperature with the polyclonal anti-PS2 serum (1:500) in TBS plus 0.1% BSA. After extensive washing (five times with TBS), the appropriate fluorochrome-linked secondary antibody (Cy3-conjugated anti-rabbit IgG, Zymed 62-6115) was added for 1 h. Finally, cells were washed three times in TBS, one time in H<sub>2</sub>O and embedded in antifade. Staining was documented by an LSM 510 Laser Scanning Microscope (Zeiss).

### 2.6. Adenoviral infections

Adenoviral stocks for (CAGA)<sub>9</sub>-MLP-Luc were obtained using the Adeasy cloning and recombination procedure (Quantum, Appligene, www.quantum-appligene.com) and infections were performed as described previously [29]. Under optimal conditions, more than 90% of HSC and MFB were infected as determined by the green fluorescent autofluorescence of an adenoviral green fluorescent protein (GFP) construct. Routine infections were performed at a multiplicity of infection (m.o.i.) of 50 with single virus clones. After infection, cells were cultured for 2 days in DMEM with 10% FCS, serum-starved for 8 h with medium containing 0.5% FCS and stimulated with 5 ng/ml TGF $\beta$ 1. Lysates for luciferase detection were prepared 14 h later. Adenoviral stocks for constitutively active T $\beta$ R1 (CA-T $\beta$ R1) and

Smad7 were kindly provided by A. Moustakas, Ludwig Institute for Cancer Research, Uppsala, Sweden.

## 3. Results

### 3.1. Activation of HSC and transdifferentiation to MFB

Freshly prepared, highly purified rat HSC were seeded on uncoated plastic. In an early and quiescent state, HSC showed perinuclear droplets, containing vitamin A. During growth, the cells became spontaneously activated and developed features of fibrogenically activated HSC (Fig. 1). The morphological changes of transdifferentiated HSC, here termed MFB, are preceded by, e.g., increased expression of collagen,  $\alpha$ -smooth muscle actin, and TGF $\beta$  [30]. We have investigated these known biochemical markers to confirm fibrogenesis like progression of in vitro cultured HSC. The expression of  $\alpha$ -smooth muscle actin and TGF $\beta$ 1 increased during ongoing activation (data not shown), supporting the widely accepted paradigm of HSC activation, including autocrine TGF $\beta$  signaling as key event of ECM production and fibrogenesis. After 7 days, activated HSC were passaged and subsequently displayed the fully transdifferentiated phenotype (MFB, Fig. 1). We found that HSC- and MFB-produced TGF $\beta$  in conditioned medium was present almost entirely in the latent and not in its active form (data not shown). Therefore, we used overnight serum starvation and subsequent addition of active TGF $\beta$ 1 into the medium to test TGF $\beta$  responsiveness in HSC and in MFB.

### 3.2. TGF $\beta$ signal transduction by activation of Smad2

To study intracellular TGF $\beta$  signal transduction, we analyzed activation of endogenously expressed Smad2 using an antiserum specifically detecting phosphorylated Smad2 (Fig. 2). In HSC, an impressive TGF $\beta$ -dependent increase in Smad2 phosphorylation occurred. MFB, in contrast, did not respond to TGF $\beta$ 1 with an elevated P-Smad2 band. Furthermore, Smad2 was not constitutively phosphorylated in MFB

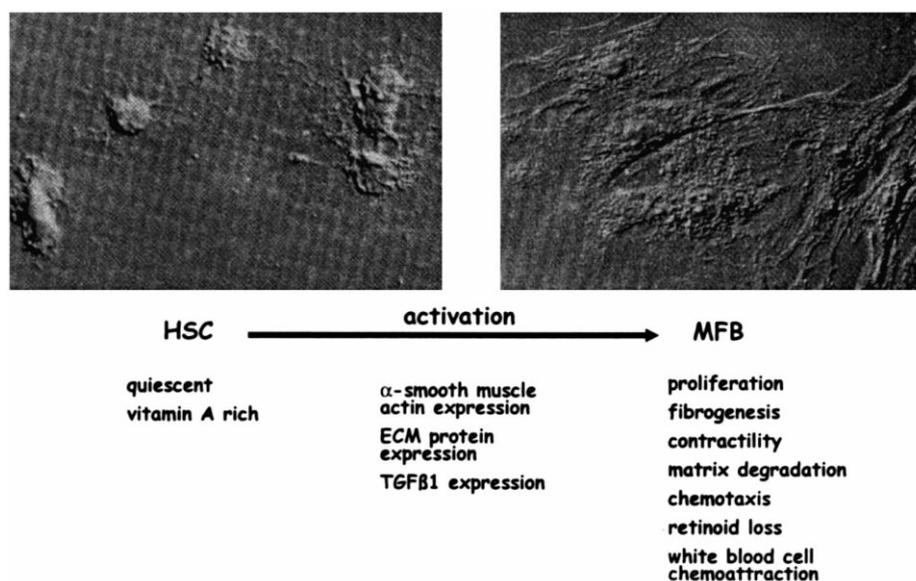


Fig. 1. Phenotypic features of HSC activation. During cultivation of HSC in plastic dishes, cells become spontaneously activated and transdifferentiate from quiescent vitamin A storing cells to proliferative fibrogenic MFB, thereby representing a cell culture model of liver fibrogenesis. Early molecular markers of HSC activation are TGF $\beta$ 1 and  $\alpha$ -smooth muscle actin expression, followed by induction of ECM genes. The resulting phenotypic changes, which characterize MFB, are indicated.

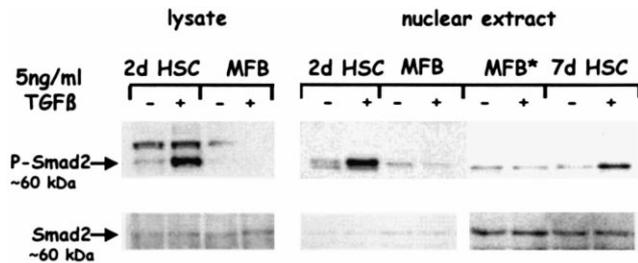


Fig. 2. Analysis of TGF $\beta$ 1-induced Smad2 phosphorylation in HSC and MFB. Cells were grown to confluence and serum-starved overnight with 0.5% FCS, followed by stimulation with 5 ng/ml TGF $\beta$ 1 (+). As controls, untreated cells were analyzed (–). Cell lysates were prepared from whole cells or from isolated nuclei, as indicated, analyzed by SDS–PAGE, followed by Western blotting and immunodetection using a specific antiserum against phosphorylated Smad2 (PS2). Parallel blots were probed in the presence of blocking peptides, to which the PS2 antiserum was originally raised, resulting in blank lanes (not shown), or with a Smad2-specific antibody, to confirm equal protein expression. The position of the P-Smad2 band is indicated by the arrow. MFB\* indicates lysate from primary cultured activated HSC, which were isolated from BDL fibrotic rats. The results are representative for at least three independent experiments.

(at least not in amounts comparable to stimulated HSC), which would be expected as intermediate of autocrine stimulation by endogenously produced TGF $\beta$ . Phosphorylated Smad2 was also investigated in nuclear extracts from 2 and 7 day old HSC, transdifferentiated MFB and from activated HSC, which were isolated from experimentally induced fibrotic rat liver (MFB\*). The results reflect the data from total cell lysate. Two day old HSC respond stronger to TGF $\beta$ 1 than 7 day old HSC. MFB, cultured subsequent isolation from BDL rats, displayed a slightly increased P-Smad2 band compared to in vitro transdifferentiated MFB. Activated Smad2 forms heterodimeric complexes with Smad4 or heterotrimeric complexes with Smad3 and Smad4, which were trans-

located into the nucleus, where they function as regulators of target gene transcription. We studied nuclear translocation of activated Smad complexes in 3 day old HSC and MFB, in the presence and absence of TGF $\beta$ 1 by immunohistochemistry (Fig. 3). As expected from Western blot data, strong TGF $\beta$ -dependent nuclear staining occurred in HSC. TGF $\beta$ 1-treated MFB displayed a similar background staining as untreated controls. No cytoplasmic staining was observed, confirming that only the phosphorylated form of Smad2 is detected efficiently with anti-PS2. Treatment of anti-PS2 serum with the peptide, against which the serum was raised, prior to immunostaining as a control resulted in cells without fluorescent staining (data not shown).

### 3.3. Proteasome degradation of activated Smad2

One possibility to explain reduced P-Smad2 staining in MFB is a diminished lifetime of the activated protein. It has been shown previously that following receptor-mediated activation of Smad2, multi ubiquitination and subsequent degradation of Smad2 by the proteasome occur [31,32]. In 2 day old HSC, maximal amounts of P-Smad2 were detectable 1 h after TGF $\beta$  treatment, which declined thereafter to background levels (data not shown). To exclude an accelerated P-Smad2 degradation mechanism to be responsible for the absence of a TGF $\beta$ -dependent increase of P-Smad2 in MFB, we treated the cells with two different 26S proteasome inhibitors, MG-132 and lactacystin (Fig. 4). Both inhibitors had the potential to extend the lifetime of phosphorylated Smad2 in HSC, similarly as previously shown for several other cell types (HaCaT, COS-1, HepG2, HeLa, EpH4, EpRas; [31,32]). Six hours after treatment of MFB with TGF $\beta$ 1, the faint band, which was detectable with similar intensities in untreated and TGF $\beta$ -treated MFB (see Figs. 2 and 4, lanes 1–4), is further decreased below this basal level (Fig. 4, lane 5). Addition of lactacystin or MG-132 for 6 h led to conservation of this weak P-Smad2 signal (Fig. 4, lanes 6 and 7), suggesting that a basal TGF $\beta$ -Smad2 signaling is present in MFB, which

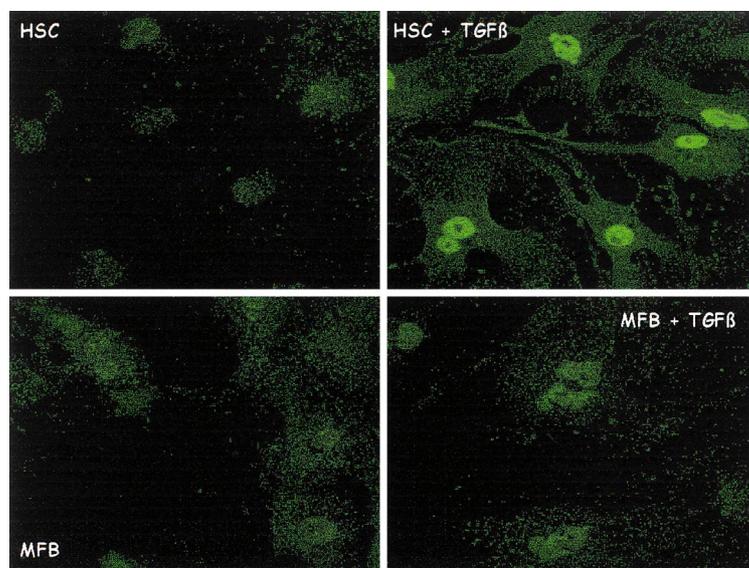


Fig. 3. TGF $\beta$ 1-dependent nuclear translocation of phosphorylated Smad2 in HSC. Three day old HSC and transdifferentiated MFB were subjected to immunostaining with an antiserum against phosphorylated Smad2 (PS2) and subsequent analysis by laser scanning microscopy. TGF $\beta$ 1-treated HSC display a strong nuclear staining, which is absent in TGF $\beta$ 1-treated MFB and untreated controls. TGF $\beta$ 1-treated HSC, which were probed in the presence of blocking peptides to which the PS2 antiserum was originally raised, were used as a negative control (not shown).

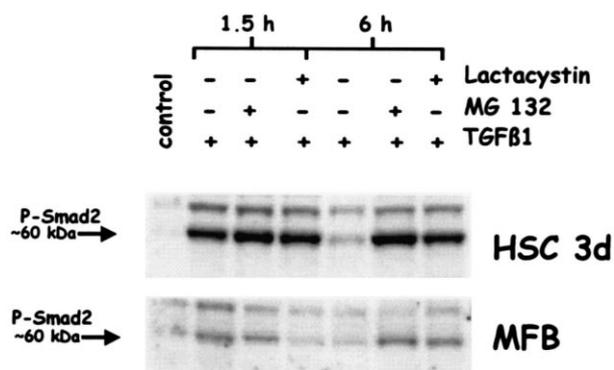


Fig. 4. Proteasome degradation of phosphorylated Smad2 in 3 day old HSC and in MFB. All lysates were analyzed by immunoblotting using the antibody specific to C-terminally phosphorylated Smad2 (PS2). Upon continuous TGF $\beta$ 1 stimulation in HSC, the level of phosphorylated Smad2 peaks at about 1 h and declines thereafter, reaching background staining after 6 h. The proteasome inhibitors MG-132 and lactacystin block time-dependent loss of Smad2 phosphorylation. Both proteasome inhibitors did not display remarkable effects on P-Smad2 availability in MFB preparations.

however is far below the amount in stimulated HSC. These data indicate that reduced TGF $\beta$  signal transduction via Smad2 instead of a more rapid degradation of P-Smad2 takes place in MFB.

#### 3.4. Lack of Smad3 phosphorylation in MFB

To investigate Smad3 activation in HSC and MFB, we used an antiserum, PS1, which was raised against a phosphorylated C-terminal peptide of Smad1 and which displays crossreactivity with phosphorylated Smad3. Due to a faster mobility of Smad3 in PAGE, it can be easily differentiated between P-Smad1 and P-Smad3. Similar to our findings concerning Smad2, HSC showed significant TGF $\beta$ -dependent Smad3 phosphorylation. Like Smad2, Smad3 as well is not activated in MFB, neither by treatment with TGF $\beta$ 1 nor constitutively as a result of a suggested autocrine stimulation (Fig. 5). This suggests that loss of TGF $\beta$ -dependent Smad phosphorylation during fibrogenic progression of HSC is not restricted to a Smad2 containing pathway, but affects Smad3 as well.

#### 3.5. Ectopic expression of Smad7 and CA-T $\beta$ RI in HSC and MFB leads to inhibition/restoration of TGF $\beta$ signaling, respectively

To further investigate TGF $\beta$  effects on HSC and MFB, we used a very sensitive TGF $\beta$  reporter construct, (CAGA) $_9$ -MLP-Luc, containing exclusively nine copies of the Smad binding site, derived from the PAI-1 promoter. (CAGA) $_9$ -MLP-Luc was shown to confer a several hundred-fold TGF $\beta$ -mediated luciferase induction in HepG2, Mv1Lu and NIH3T3 cells. To improve introduction of the construct in HSC and MFB, we generated adenoviruses containing (CAGA) $_9$ -MLP-Luc. Adenovirus-mediated gene transfer is very efficient (80–95% infection rate), and high expression levels of encoded proteins were obtained. In different preparations of HSC, luciferase activity was stimulated between 3- and 10-fold, depending on the activation stage of the cells (Fig. 6A and data not shown). In contrast, MFB did not respond to TGF $\beta$ 1 and no stimulation of the reporter gene was observed (Fig. 6B).

To stimulate the TGF $\beta$  pathway in MFB, we next utilized

an adenoviral vector encoding the CA-T $\beta$ RI (also termed activin receptor like kinase 5). In HSC, expression of CA-T $\beta$ RI, similarly as treatment of the cells with TGF $\beta$ 1, led to Smad2 phosphorylation and activation of a coinfecting (CAGA) $_9$ -MLP-Luc containing virus (Fig. 6A). Additional infection with an adenovirus encoding Smad7 inhibited CA-T $\beta$ RI signal transduction. In MFB, in contrast to TGF $\beta$ 1 treatment, expression of CA-T $\beta$ RI resulted in Smad2 phosphorylation and (CAGA)-box stimulation, comparable to HSC (Fig. 6B). Again, Smad7 was able to antagonize this effect.

#### 4. Discussion

In cirrhotic liver, HSC are responsible for increased production and deposition of ECM. Activation of HSC in vivo and in culture includes, e.g., increased expression of type I collagen, expression of cytoskeletal markers like  $\alpha$ -smooth muscle actin, and increased proliferation [10,30,33]. After liver injury, HSC rapidly are induced to express and secrete TGF $\beta$ . The detailed role of TGF $\beta$  signal transduction during different stages of HSC transdifferentiation remains to be identified. Critical downstream targets of TGF $\beta$  signal transduction are type II and type I receptors and members of the Smad family. In a recent paper [21], we have compared the effect of TGF $\beta$  in different stages of HSC activation and found striking differences between quiescent HSC and transdifferentiated MFB. Whereas HSC display typical TGF $\beta$ -dependent proliferation inhibition and target gene activation, MFB did not respond. Both receptors were expressed with similar amounts in HSC and MFB. However, ligand binding to TGF $\beta$  receptors was strongly decreased in transdifferentiated MFB compared to HSC. In contrast to this, it was reported that isolated HSC from CCl $_4$ -treated rats displayed constant surface expression

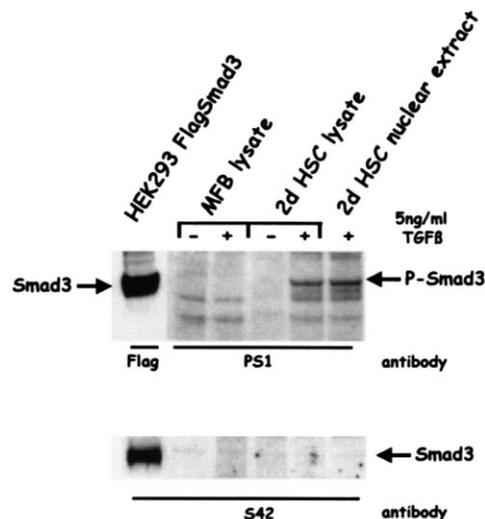


Fig. 5. Analysis of TGF $\beta$ 1-induced Smad3 phosphorylation in HSC and MFB. Protein extracts as described in Fig. 2 were used. Immunodetection was performed using an antiserum, which was originally raised to detect phosphorylated Smad1 (PS1). This antiserum displays crossreactivity with phosphorylated Smad3, which has a faster mobility during PAGE than Smad1 and Smad2. To confirm Smad3 specificity of the bands, a parallel blot of Flag-tagged Smad3 expressing HEK293 cells was probed with an anti-Flag antibody. The position of the P-Smad3 band is indicated by the arrow. The blot was reprobbed with a Smad3-specific antibody to confirm equal expression (lower panel).

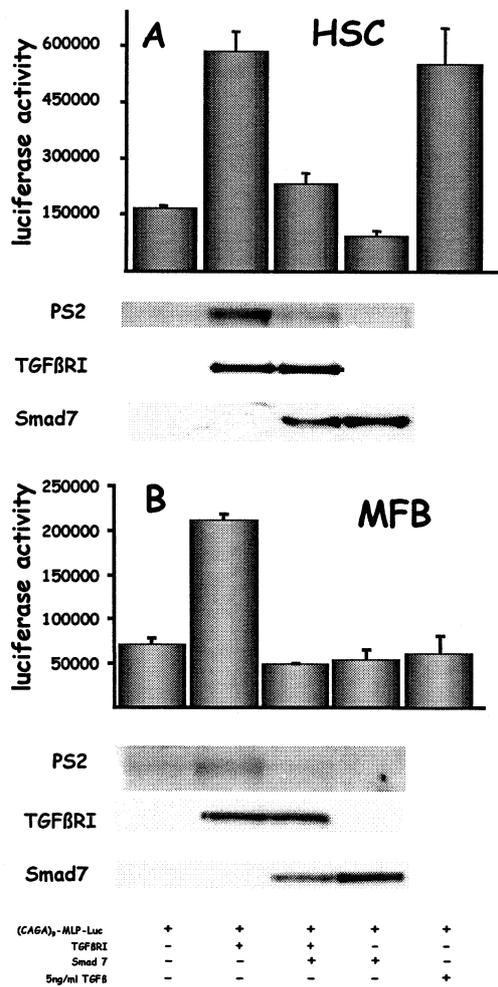


Fig. 6. Modulation of TGF $\beta$  signal transduction by ectopic expression of Smad7 and CA-T $\beta$ RI in HSC and MFB. HSC (A) and MFB (B) were infected with adenoviruses carrying CA forms of T $\beta$ RI, Smad7 and a (CAGA)<sub>9</sub>-MLP-Luc reporter DNA at an m.o.i. of 50 each. Expression of T $\beta$ RI and Smad7 proteins was confirmed by Western blot experiments using specific antibodies as described in Section 2 and in [28]. The effect of protein expression on TGF $\beta$  signal transduction was measured by PS2 immunoblot and (CAGA)<sub>9</sub>-MLP-Luc reporter gene analysis. For TGF $\beta$ -dependent (CAGA)<sub>9</sub>-MLP-Luc stimulation, cells were serum-starved for 8 h with 0.5% FCS prior to addition of TGF $\beta$ 1, and subjected to lysate preparation 14 h later. The presented data are representative for at least three independent investigations.

of all TGF $\beta$  receptors [34], suggesting possible differences in the transdifferentiation mechanisms in spontaneous or ligation-induced versus CCl<sub>4</sub>-induced HSC activation. A possible explanation for reduced binding of recombinant TGF $\beta$ 1 to surface receptors in MFB may be high level autocrine signaling of endogenously synthesized TGF $\beta$ . Therefore, we focused our advertence to TGF $\beta$  downstream signaling and examined the activation of Smads in different stages of HSC activation. Smad expression was largely unchanged during *in vitro* activation of HSC (data not shown). Rapid TGF $\beta$ 1-dependent phosphorylation of Smad2 was present in quiescent HSC (2 days cultured), decreased during activation and was absent in fully transdifferentiated MFB. MFB, isolated from BDL rat liver, similarly did not show significant Smad2 phosphorylation. These data were confirmed by examining TGF $\beta$ -dependent transport of activated Smad complexes into the nucleus,

which was found exclusively in HSC. To detect possible differences in the turnover rate of activated Smad2, we investigated its proteasome degradation as previously described [31]. The results, displaying only a minimal influence of two different proteasome inhibitors on the availability of phosphorylated Smad2 in MFB, further suggested that a strongly reduced signaling via Smad2 is present in transdifferentiated cells. The faint P-Smad2 band preserved from degradation in MFB may be based on the presence of a few cells, that are not fully transdifferentiated and therefore are still reactive to TGF $\beta$  or alternatively to a residual sensitivity of MFB.

Besides Smad2, TGF $\beta$  may use Smad3 or a different signaling cascade involving mitogen-activated protein kinase pathways to transmit an intracellular signal. To investigate the Smad pathway more generally, we infected HSC and MFB with adenoviruses containing (CAGA)<sub>9</sub>-MLP-Luc, which recruits activated Smad3 and/or Smad4 containing complexes. Significant TGF $\beta$ 1-dependent reporter gene activation in HSC was shown, which was completely absent in MFB. Smad-dependent TGF $\beta$  insensitivity was further confirmed by experiments, showing lack of TGF $\beta$ -dependent or constitutive Smad3 activation in MFB.

Substantiated by these results, we have developed a working hypothesis, which is different from the widely suggested model of liver fibrosis, based on continuous autocrine fibrogenic stimulation of transdifferentiated MFB by endogenously produced TGF $\beta$ . We propose that TGF $\beta$  action is an important early and initiating step during HSC activation. Quiescent HSC are very sensitive for TGF $\beta$  and transmit intracellular signals to the nucleus, where numerous genes involved in HSC activation and/or ECM protein expression, including e.g. collagen, are the targets. TGF $\beta$ -dependent transdifferentiation leads to phenotypic changes, which include TGF $\beta$ -independent ECM synthesis [35–38] and loss of TGF $\beta$ -dependent growth control as well as Smad-dependent signal transduction.

TGF $\beta$  insensitivity of MFB does not seem to be based on high levels of endogenous TGF $\beta$  expression and secretion, since 3 day old HSC, which are able to transmit a clearly detectable TGF $\beta$  signal, produce similarly high amounts of TGF $\beta$  as MFB, which are insensitive. TGF $\beta$  receptor surface downregulation/internalization seems to be an important subject involved in reduced TGF $\beta$  signal transduction in MFB. FKBP12 has recently been reported as a negative regulator of TGF $\beta$  receptor internalization [39] and its role in HSC during fibrogenesis has not yet been investigated. Furthermore, in line with our finding that TGF $\beta$  receptor protein expression was high in MFB, but surface localization was low, Zwaagstra et al. have shown a predominant intracellular localization of T $\beta$ RI and its increased nuclear accumulation after growth arrest in Mv1Lu and A549 cells [40,41]. Preliminary data show a similar nuclear staining for T $\beta$ RI in HSC and MFB, and we currently use an adenoviral T $\beta$ RII-GFP fusion construct to identify its subcellular localization in HSC and MFB.

In various experimental models, it was impressively shown that anti-TGF $\beta$  therapeutic strategies are able to significantly reduce or abolish fibrogenesis or ongoing fibrosis [18–20]. Ectopic expression of Smad7 led to a block of TGF $\beta$  signal transduction in HSC and therefore may be a further potential antifibrotic agent, more specifically interrupting Smad-dependent signaling. By expression of a constitutively activated T $\beta$ RI, we were able to restore TGF $\beta$  signaling in MFB, which

also may have therapeutic consequences with respect to its proliferation inhibitory effect.

In summary, our data display that fully transdifferentiated MFB are almost insensitive to TGF $\beta$ , mainly shown by the lack of Smad activation. As a consequence, the profibrogenic effect of TGF $\beta$ , produced in and secreted from MFB, may be paracrine and may target to the environment, thereby activating neighboring quiescent HSC and inducing apoptosis in hepatocytes. In a homeostatic stage, this contributes to wound healing and tissue repair. In a deregulated stage with excess TGF $\beta$ , this may lead to reduced hepatocyte regeneration, proliferation of activated HSC and finally fibrogenesis.

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