The Role of Smad3 in Mediating Mouse Hepatic Stellate Cell Activation

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Transforming growth factor β (TGF-β) is the most potent profibrogenic mediator in liver fibrosis. Although Smad proteins have been identified as intracellular mediators in the TGF-β signaling pathway, the function of individual Smad proteins remains poorly understood. The aim of this study was to explore the contribution of Smad3 in mediating TGF-β responses in a model of acute liver injury in vivo and in culture-activated hepatic stellate cells (HSCs). Wild-type, Smad3 heterozygous or Smad3 homozygous knockout mice were treated with a single intragastric administration of CCl₄. After 72 hours, the induction of hepatic collagen α1(I) and α2(I) messenger RNA (mRNA) levels in Smad3 knockout mice was only 42% and 64%, respectively, of the levels induced in wild-type mice. However, smooth muscle α-actin (α-SMA) was expressed at a slightly higher level in livers from knockout mice compared with wild-type mice. In culture-activated HSCs from Smad3 knockout mice, collagen α1(I) mRNA was 73% of wild-type HSCs, but α-SMA expression was the same. HSCs from knockout mice showed a higher proliferation rate than wild-type HSCs. Smad3-deficient HSCs did not form TGF-β1–induced Smad-containing DNA-binding complexes. In conclusion, (1) maximal expression of collagen type 1 in activated HSCs requires Smad3 in vivo and in culture; (2) Smad3 is not necessary for HSC activation as assessed by α-SMA expression; (3) Smad3 is necessary for inhibition of proliferation of HSCs, which might be TGF-β–dependent; and (4) Smad3 is required for TGF-β1–mediated Smad-containing DNA-binding complex formation in cultured HSCs.(HEPATOLOGY 2001; 34:89-100.)

Abbreviations: HSCs, hepatic stellate cells; ECM, extracellular matrix; α-SMA, smooth muscle α-actin; TGF-β, transforming growth factor β; mRNA, messenger RNA; ko, knockout; ht, heterozygous; wt, wild-type; PCR, polymerase chain reaction; cDNA, complementary DNA; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle medium; FCS, fetal calf serum; PDGF, platelet-derived growth factor.

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Activated hepatic stellate cells (HSCs) have a central role as the main extracellular matrix (ECM) protein-producing cell during hepatic fibrogenesis.1 Hepatic injury induces the HSCs to undergo a transdifferentiation or activation process,1 which is characterized by loss of their intracellular vitamin A stores,2 increase in proliferation,3 changes in cellular morphology to a more myofibroblast-like cell type with expression of smooth muscle α-actin (α-SMA),4 and an increase in the production of ECM proteins, including type I collagen.5 In addition, cultured HSCs express all 3 transforming growth factor β (TGF-β) isomers6-7 and TGF-β receptor types I, II, and III on the cell surface.8 Treatment of HSCs in early culture with TGF-β1 stimulates collagen type I messenger RNA (mRNA) expression9,10 and protein synthesis,10,11 inhibits HSC proliferation,11-13 decreases the expression of matrix metalloproteinases, and increases the expression of tissue inhibitors of matrix metalloproteinases.14 No change was observed in the level of α-SMA in TGF-β1–treated HSCs.15

Excessive TGF-β is associated with tissue damage caused by scarring in many diseases.16 Clinical studies have revealed a close correlation between increased TGF-β1 gene expression and the high expression of collagen type I mRNA in the liver tissue of patients with cirrhosis17,18 and in experimental rat models of cirrhosis.19,20 Furthermore, mRNA expression of TGF-β1, TGF-β2, and TGF-β3 is increased in HSCs during fibrosis induced by bile duct ligation in rats.21 Studies from transgenic mice support the etiologic and fibrogenic role for TGF-β in the development of liver fibrosis. Transgenic mice overexpressing mature TGF-β1 under control of hepatocyte-specific promoters develop hepatic fibrosis with increased interstitial deposition of type I collagen.22,23 Liver histology of an inducible transgenic mouse model overexpressing the active form of TGF-β1 showed up-regulation of hepatic collagen type I and III mRNA and activation of HSCs.24 TGF-β signals through its type I and type II receptors, which have serine/threonine kinase activity. The ligand binds to the constitutively active type II receptor, which then recruits and transphosphorylates type I receptor.25 The activated type I receptor transiently associates with and phosphorylates Smad226 and Smad3, which then form heteromeric complexes with Smad4.27-29 These complexes translocate to the nucleus, where the proteins function as transcriptional activators through their interaction with DNA-binding proteins.30,31 Two inhibitory Smad proteins, Smad6 and Smad7, block Smad-mediated signaling in cells.32-34 To date, Smad proteins are the only TGF-β receptor substrates with a demonstrated ability to propagate signals.35 Several other signaling molecules and pathways are also activated by TGF-β, including TGF-β–activated kinase 1 (TAK1)36 and the mito-
gen-activated protein kinase (MAPK) family of extracellular signal-regulated kinase (ERK),\textsuperscript{37} c-Jun N-terminal kinase (JNK),\textsuperscript{38} and p38,\textsuperscript{39} which in turn phosphorylate and activate transcription factors.

In a model of acute liver injury induced by CCl\textsubscript{4}, TGF-β1 knockout mice show only minimal induction of hepatic collagen α1(I) mRNA and α-SMA protein expression compared with wild-type mice.\textsuperscript{40} Since these results indicate the critical role for TGF-β1 in initiating hepatic fibrogenesis, we wanted to assess the role of Smad3 in the same model of CCl\textsubscript{4}-induced acute liver injury. Because an essential role for Smad3 in TGF-β signaling has been shown in Smad3 knockout (ko) mice,\textsuperscript{41} our hypothesis was that some of the TGF-β-mediated effects in liver fibrogenesis are Smad3 dependent. Treatment of Smad3 ko mice with the fibrogenic stimulus CCl\textsubscript{4} provides a unique opportunity to test this hypothesis. We show in vivo and in culture that Smad3 is not required for the activation process of HSCs, but is required for the maximal induction of type I collagen expression.

MATERIALS AND METHODS

Smad3-Deficient Mice. Smad3-deficient mice were generated and characterized previously.\textsuperscript{42} These mice have a deletion in exon1 of the Smad3 gene. Heterozygous (ht) mice were bred to produce Smad3 ko offspring. Wild-type (wt) litter mates served as controls. The genotype of the mice was determined by polymerase chain reaction (PCR) analysis of tail DNA.\textsuperscript{43} Mice were maintained on standard chow and allowed access to food and water ad libitum. Mice (age 7-10 weeks) were treated with a single dose of CCl\textsubscript{4} (7 μL/g body weight; 1:1 dilution with corn oil) by oral gavage. In the control group mice were treated with corn oil alone (7 μL/g body weight). Mice were sacrificed 72 hours later. All animal procedures were performed under the guidelines set by The University of North Carolina Institutional Animal Care and Use Committee and are in accordance with those set by the National Institutes of Health.

RNA Isolation and RNase Protection Assay. Total RNA from mouse livers was extracted with guanidinium thiocyanate and purified by ultracentrifugation through a cesium chloride cushion.\textsuperscript{44} Total RNA from confluent HSCs was isolated on day 14 in culture by use of an RNA extraction kit (Qiagen, Valencia, CA). RNase protection assays were performed as previously described.\textsuperscript{45} Collagen and GAPDH-specific riboprobes were hybridized with the same RNA sample to normalize for the recovery of the RNA. Riboprobes for RNase protection assays are derived from the plasmid pCOLEX5-944 for collagen α1(I), from the plasmid pBSK(+)+Col2a1(45) for collagen α2(I), and from the plasmid pTRI-GAPDH-mouse (Ambion, Austin, TX) for GAPDH. Cytokine mRNA levels were determined by a using mouse cytokine multiprobe template set (mCK-3b; PharMingen, San Diego, CA). Total RNA (50 μg for in vivo studies; 2 μg or 30 μg for detection of collagen α1(I) mRNA or cytokine mRNAs, respectively, in cultured HSCs) was hybridized with 10\textsuperscript{5} cpm of each riboprobe. Two nonspecific bands in the RNase protection assay for collagen α2(I) are protected using transfer RNA or noncollagen-expressing Hela cells (data not shown). The protected riboprobes were visualized by autoradiography and quantitated by phosphorimage analysis (Molecular Dynamics, Sunnyvale, CA).

In situ Hybridization. Complementary (antisense) or anticomplementary (sense, negative control) probes to cellular RNA transcripts were obtained using SP6 or T7 RNA polymerase (GibcoBRL, Grand Island, NY) for run-off transcription of linearized collagen α1(I) complementary DNA (cDNA) plasmid. \textsuperscript{35}S-Labeled nucleotides were used to generate in situ hybridization probes with an average specific activity of 1.2 to 1.4 cpm/μL. Prehybridization, hybridization, washing procedures, RNase digestion of mismatched sequences, and autoradiography were performed as described.\textsuperscript{46} Sections were deparaffinized with xylene and dehydrated in graded ethanol and air dried before hybridization. Hybridization was performed for 18 hours at 50°C using 5 × 10\textsuperscript{5} cpm of \textsuperscript{35}S-labeled RNA probe. Slides were washed for 5 hours at 50°C in modified hybridization buffer and subjected to RNase A digestion (30 minutes at 37°C). After further washing steps, the slides were dehydrated in graded ethanol, air dried, and exposed to ilford K.5F radiographic emulsion (Polysciences, Warrington, PA) at 4°C for 7 days. Slides were developed in Kodak D19 developer (Eastman Kodak Co., Rochester, NY) for 3 minutes, rinsed in 1% acetic acid, and fixed in Kodak Fixer (Eastman Kodak Co., Rochester, NY) for 3 minutes. After extensive washing, the slides were counterstained with hematoxylin, mounted, and photographed under dark and bright-field illumination. All sections were simultaneously processed using the same batches of probes and reagents. Specificity of hybridization signals observed with antisense probes was verified by the absence of hybridization signal with corresponding sense probes. Controls were uniformly negative.

Western Blot Analysis. Frozen liver samples were minced using a razor blade and homogenized in 150 μL of Dignam C buffer\textsuperscript{47} containing protease and phosphatase inhibitors as described.\textsuperscript{48} The homogenized samples were rotated on a shaker for 30 minutes at 4°C, then spun down at 14K RPM for 5 minutes at 4°C. Whole cell extracts were prepared from confluent HSCs on culture day 14 using Dignam C buffer.\textsuperscript{47} Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Proteins (50 μg from whole liver for determination of α-SMA and desmin; 10 μg from HSCs for determination of α-SMA; 20 μg from HSCs for determination of phospho-p44/42 MAPK; 50 to 100 μg from HSCs for detection of p15 and Smad proteins) were separated on a 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted onto a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) in a buffer containing 20 mmol/L Tris, pH 8.3, 150 mmol/L glycine, 0.1% SDS, and 20% methanol. After blocking with 5% nonfat milk in Tris-buffered saline (20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl) containing 0.1% Tween 20 (TBS-T) for 1 hour (12 hours for determination of α-SMA from whole liver extracts), membranes were incubated with the primary antibody, mouse anti-human α-SMA (DAKO, Carpinteria, CA), mouse anti-human desmin (DAKO), rabbit anti-mouse p15 (a kind gift of Dr. Y. Xiong), mouse anti-phospho-p44/42 MAPK (New England Biolabs, Beverly, MA), rabbit anti-human Smad2 (Zymed, South San Francisco, CA), rabbit anti-human Smad3 (Zymed), mouse anti-human Smad4 (B-8; Santa Cruz, Santa Cruz, CA), and mouse anti-actin (ICN-Biomedicals, Costa Mesa, CA), and then washed 3 times in TBS-T. The secondary antibody, horseradish peroxidase–conjugated anti-mouse IgG (Santa Cruz, Santa Cruz, CA) or horseradish peroxidase–conjugated anti-rabbit IgG (Santa Cruz), was incubated on the membrane at a dilution of 1:1,000 in 5% nonfat milk for 30 minutes. After washing the filter 4 times in TBS-T the antibody complexes were detected using the Amersham ECL system. Equal loading of the gel was confirmed by staining the membrane after electrophoretic transfer with Ponceau S.

Immunohistochemistry. Liver tissue was fixed in formalin and embedded in paraffin. Immunohistochemical staining for α-SMA using the DAKO Envision System (DAKO) with the primary antibody mouse anti-human α-SMA (DAKO) (diluted 1:200 in 1% bovine serum albumin in phosphate-buffered saline) was performed according to the manufacturer’s protocol. The specimens were incubated with the peroxidase-labeled polymer conjugated to goat anti-mouse immunoglobulins (diluted 1:2 in phosphate-buffered saline) for 5 minutes. The tissue was counterstained with Aqua Hematoxylin–INNOVEX (INNOVEX BIOSCIENCES, Richmond, CA). Control slides stained with 1% bovine serum albumin instead of the primary antibody did not show any positive staining.

Histologic Assessment. Liver specimens were fixed in formalin and embedded in paraffin. Histologic sections were cut (5 μm) and stained with hematoxylin-eosin to assess necrosis and inflammation. Histopathology of the liver was scored as follows: necrosis: 0, no evidence of necrosis; 1, mild hepatocellular degeneration; 2, minimal necrosis; 3, extensive, confluent necrosis; and 4, fulminating necrosis.
necrosis of centrolobular hepatocytes; 3, obvious centrolobular necrosis; 4, centrolobular necrosis extending toward portal areas; 5, massive diffuse necrosis; inflammation: 0, no evidence of inflammation; 1, minimal inflammatory infiltrate; 2, mild inflammatory infiltrate; 3, moderate inflammatory infiltrate; 4, severe inflammatory infiltrate; 5, diffuse severe inflammatory infiltrate. Histopathology was scored by a blinded veterinarian pathologist, Dr. Scott L. Trasti.

Reverse Transcription–PCR Assays. First-strand cDNA was synthesized using 1 μg total RNA of whole liver samples in a reaction mixture containing 1× first-strand buffer ( Gibco, Grand Island, NY), 10 mmol/L DTT ( Gibco), 0.4 mmol/L of each dNTP (Pharmacia, Piscataway, NJ), 40 U RNasin (Boehringer Mannheim, Indianapolis, IN), 8.7 μmol/L oligo-d(T)$_{16}$, and 200 U Moloney murine leukemia virus reverse transcriptase (Gibco). The reaction was incubated at 42°C for 60 minutes in a final volume of 25 μL. The synthesized cDNA was amplified using specific primer sets for TGF-β2 or TGF-β3 as previously described. The sequence of the primer set for β-actin was 5′-GTGACGAGGCCCAGAGCAAG-3′ and 5′-AGGGCGGACTCATCGTA-3′. The lengths of the amplified products were 396, 360, and 810 bases for TGF-β2, TGF-β3, and β-actin, respectively. PCR reactions contained 3 μL of cDNA, 1× PCR-buffer II (Perkin Elmer, Branchburg, NJ), 1 μmol/L of each primer, 0.25 mmol/L of each dNTP (Pharmacia), and 2.5 U Taq-polymerase (Perkin Elmer) in a total volume of 50 μL. PCR reactions were cycled as follows: initial denaturation at 99°C for 4 minutes; then 35 cycles for TGF-β2, 39 cycles for TGF-β3, and 30 cycles for β-actin at 94°C for 30 seconds, 60°C for 60 seconds, and 72°C for 90 seconds. The final extension was carried out at 72°C for 10 minutes. The cycle number for each amplon used in the PCR was determined to be in the linear range of amplification. Negative controls without cDNA were performed with each PCR reaction. PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining.

TGF-β Bioassay. Liver samples of 2 wt, ht, and ko mice, treated with either CCl$_4$ or corn oil, were combined and the TGF-β bioassay was performed as described. Fifty micromolars of recombinant TGF-β1 resulted in 25,942 ± 367 relative light units.

HSC Isolation and Culture. HSCs were isolated from wt and ko mice essentially as described. Mice were anesthetized with ketamine and acepromazine. The suprahepatic inferior vena cava was ligated and the infraceliac inferior vena cava was cannulated. In situ perfusion of the livers was initiated with 70 mL Hanks’ balanced salt solutions and the portal vein was cut. The perfusion was continued with 40 mL of Hanks’ balanced salt solutions and the livers were initiated with 70 mL Hanks’ balanced salt solutions and the portal vein was cut. The liver was perfused with 70 mL of Dulbecco’s modified Eagle medium F/12 (DMEM F/12; Gibco-BRL) containing 0.5 mg pronase (Boehringer Mannheim) per gram of body weight of the mouse, followed by 70 mL of DMEM F/12 containing 5 mg collagenase type IV (Sigma, St. Louis, MO). The liver was removed and digested in 20 mL DMEM (Gibco-BRL) containing 0.2 mg DNase I (Boehringer Mannheim) at 37°C for 10 minutes. The resulting cell suspension was filtered through a metal sieve. HSCs were washed and purified by arabinogalactan gradient ultracentrifugation as previously described. HSC purity as estimated by the autofluorescence of the cells by ultraviolet-excited fluorescence microscopy was between 90% to 95%. Cells isolated from 2 mice were seeded on one 60-mm uncoated plastic tissue culture dish and cultured in DMEM supplemented with 10% fetal calf serum (FCS) and standard antibiotics in 99% air 5% CO$_2$ humidified atmosphere at 37°C. Growth medium was changed daily. HSCs were passaged after 5 to 7 days and activated HSCs were plated after 10 days in culture at a density of 1.9 × 10$^4$ HSCs/60-mm dish. Total RNA and protein were extracted from confluent cells 14 days after isolation.

3H-Thymidine Incorporation Assay. HSCs from wt and ko mice were plated 11 days after isolation at a density of 1.9 × 10$^4$/well in a 24-well plate in standard medium containing 10% FCS. The next day medium was changed to 0% FCS for 24 hours. Afterwards, the cells were incubated in medium containing either 0% FCS, 10% FCS, or 0% FCS with 20 ng/mL human recombinant PDGF-BB (Roche, Indianapolis, IN) for 24 hours with 1 μCi/mL 3H-thymidine. At the end of the incubation, cells were washed 3 times with cold phosphate-buffered saline, then twice with 10% trichloric acid, solubilized in 0.2 N NaOH, and the radioactivity was measured using a scintillation counter. Experiments were performed in triplicate.

Electrophoretic Mobility Shift Assay. HSCs isolated from wt and Smad3 ko mice were passaged and grown to confluence in 100-mm dishes. Cells were starved for 24 hours in serum-free medium, then treated with 5 ng/mL TGF-β1 (R&D Systems, Minneapolis, MN) for 30 minutes or were left untreated. Nuclear extracts were extracted by the method of Dignam et al. The pellet was resuspended in 20 μL Dignam C buffer, and protein concentration was determined using the Bio-Rad Protein Assay. Extracts were kept at −80°C. A double-strand oligonucleotide representing the consensus binding site for Smad3/Smad4 transcription factor (5′-TCC AGA GCC AGA CAA AAA GCC AGA CAT TTA GCC AGA CAC-3′) served as the probe. Double-stranded oligonucleotides were radiolabeled using T4-Polymerase Kinase (New England Biolabs) and γ-32P adenosine triphosphate (Amersham Pharmacia Biotech, Piscataway, NJ). Five micrograms of nuclear protein extracts were incubated on ice for 20 minutes with a mixture containing 0.1 μL of 5 mg/mL poly(dI-dC) (Boehringer Mannheim), 1 μL of 5 mg/mL single-stranded oligonucleotide, 1× binding buffer (10 mmol/L HEPES, pH 7.8, 50 mmol/L KCl, 0.1 mmol/L EDTA, 2 mmol/L MgCl$_2$, 1 mmol/L DTT, 10% glycerol), and 10$^3$ cpm counts of the 32P-labeled oligonucleotides in a final volume of 20 μL. For supershift analysis, prior to adding the 32P-labeled oligonucleotides extracts were incubated on ice for 30 minutes with 4 μL of antibody or an equal amount of H$_2$O. Antibodies used for supershift assay were goat anti-human Smad2 (S-20; Santa Cruz), rabbit anti-human Smad3 (Zymed), or mouse anti-human Smad4 (B-8; Santa Cruz) antibody. Samples were loaded on a 4% non-denaturing acrylamide gel. The gels were run in 0.25× Tris-borate-EDTA buffer at room temperature. Complexes were assessed by autoradiography and by phosphoimager analysis (Molecular Dynamics).

Statistical Analysis. The results were analyzed for statistical significance according to the Mann-Whitney U-statistic test. For $^{3}$H-thymidine incorporation assay the Student’s t test was used. Statistical values of P ≤ .05 were considered to be significant.

RESULTS

Hepatic Collagen α1(I) and Collagen α2(I) mRNA Is Lower in Smad3 ko Mice Than wt Mice After CCl$_4$ Treatment. To investigate the role of Smad3 in the hepatic expression of collagen α1(I) and collagen α2(I) genes after a fibrogenic stimulus in vivo, mice were treated with a single dose of CCl$_4$ or corn oil as control by oral gavage. Minimal collagen α1(I) and collagen α2(I) mRNA expression was detected in control-treated wt, ht, and ko mice (Fig. 1A). Seventy-two hours after CCl$_4$ treatment, the induction of collagen α1(I) mRNA and collagen α2(I) mRNA in wt animals was 14-fold and 5.6-fold, respectively. The induction of collagen α1(I) mRNA and collagen α2(I) mRNA expression in CCl$_4$-treated Smad3 ko mice was decreased by 58% and 36%, respectively, compared with wt mice, after normalization to GAPDH mRNA levels (Fig. 1B). Smad3 ht mice showed an intermediate level of collagen gene expression (Fig. 1B).

The distribution of collagen α1(I) transcripts was assessed by in situ hybridization. The collagen α1(I) gene was expressed at low levels in control-treated wt, ht, and Smad3 ko mice (Fig. 1C). A single administration of CCl$_4$ increased expression in wt animals predominantly around the central veins and in areas of parenchymal damage. The expression of collagen α1(I) in ht and ko animals was lower than in wt mice. Together, these data show that Smad3 is required for the maximal induction of collagen type I after the fibrogenic stimulus CCl$_4$. 
Expression of α-SMA and Desmin in Liver Tissue Is Slightly Higher in Smad3 ko Mice Compared With wt Mice After CCl₄. To determine the role of Smad3 in the activation of HSCs in vivo, Western blot analysis of α-SMA was performed using protein extracts obtained from whole liver samples. α-SMA was not detectable in control-treated wt, ht, or Smad3 ko mice. Similar levels of α-SMA expression were observed in ht and wt mice after CCl₄ treatment, whereas ko mice expressed α-SMA at a slightly higher level compared with wt mice (Fig. 2A). Quantification of 3 immunoblots revealed a 1.2-fold higher α-SMA expression in ko mice as compared with wt mice. To assess the total HSC population, desmin expression was measured by Western blot analysis of whole liver extracts. Quantification of 3 blots showed a similar expression of desmin in control-treated wt, ht, or Smad3 ko mice. After CCl₄ treatment, desmin expression was 1.3-fold and 1.7-fold higher in ht and Smad3 ko mice, respectively, than in wt mice (Fig. 2A). α-SMA was also determined by immunohistochemistry. No α-SMA–positive staining was observed in control-treated mice (Fig. 2B). α-SMA staining increased after CCl₄ treatment to a similar extent at the site of parenchymal damage in wt, ht, and ko mice (Fig. 2B). Thus, surprisingly, Smad3 is not required for the induction of α-SMA and desmin.

The Extent of Liver Damage and Inflammatory Infiltrate After CCl₄ Are Similar in Smad3 wt, ht, and ko Mice. A single dose of CCl₄ induced a similar parenchymal damage in all mice independent of the genotype, which was characterized by centrolobular necrosis extending toward portal areas (Fig. 3A). A mild inflammatory infiltrate, predominantly neutrophils and lymphocytes, was found in the liver of all mice independent of the genotype after CCl₄ treatment (Fig. 3B). Taken together, our data show that Smad3 deficiency has no consequence on parenchymal damage and inflammatory infiltrate after CCl₄ treatment.

The Level of Activated TGF-β Protein in Liver Tissue Is Higher in Smad3 ko Mice Compared With wt Mice After CCl₄. To assess the levels of endogenous TGF-β mRNA in liver tissue, RNase protection assays were performed using a mouse cytokine multiprobe template set with RNA extracted from whole liver tissue. Quantification of 2 independent experiments showed a similar TGF-β1 mRNA expression in control-treated wt, ht, and Smad3 null mice, after normalization to GAPDH mRNA levels; after CCl₄ treatment TGF-β1 mRNA expression was induced approximately to the same extent in wt, ht, and Smad3 null mice, after normalization to GAPDH mRNA levels (Fig. 4A). Tumor necrosis factor α and interleukin-6 mRNA levels, however, did not increase after the fibrogenic stimulus and showed similar levels in each mouse strain (Fig. 4A). Since TGF-β2 mRNA and TGF-β3 mRNA were not detectable using RNase protection assay, semiquantitative reverse transcription–PCR analysis was performed. Control-treated wt, ht, and Smad3 ko mice showed similar hepatic mRNA levels of...
TGF-β2 and TGF-β3 (Fig. 4B). After CCl4 treatment no induction of TGF-β2 mRNA was observed, and the overall levels of TGF-β2 mRNA were similar in wt, ht, and Smad3 ko mice (Fig. 4B). In contrast, after CCl4 treatment TGF-β3 mRNA levels were induced and increased to a similar extent independent of the genotype (Fig. 4B).

The amount of activated TGF-β protein in the liver was measured by a TGF-β bioassay. This assay does not discriminate between TGF-β1, TGF-β2, and TGF-β3. The amount of activated TGF-β in liver tissue obtained from corn oil (control)-treated mice was approximately 3-fold higher in wt mice than in ht or ko mice (Fig. 4C). CCl4 induced higher levels of active TGF-β in ht and ko mice as compared with wt mice. Taken together, the induction of TGF-β isoforms does not require Smad3.

Culture-Activated HSCs Isolated From Smad3-Deficient Mice Have Decreased Collagen α1(I) mRNA, but Activate Normally in Culture. HSCs isolated from wt and Smad3 ko mice were cultured for 14 days on plastic, and RNase protection assay was performed to quantitate the collagen α1(I) mRNA (Fig. 5A). After normalization to GAPDH mRNA, collagen α1(I) mRNA was nearly 30% lower in culture-activated HSCs isolated from Smad3 ko mice than wt mice (Fig. 5B).

To assess if Smad3 is required for the activation of HSCs in culture, HSCs were isolated from wt and Smad3 ko mice, and grown for 14 days on plastic. Western blot analysis showed that there was no difference in the level of α-SMA expression between HSCs derived from wt and Smad3 ko mice (Fig. 5C). To investigate the requirement of Smad3 for the expression of various cytokines during the in vitro activation process of HSCs, RNase protection assays were performed using RNA isolated from HSCs cultured for 14 days on plastic. After normalization to the GAPDH mRNA level, HSCs from wt and Smad3 ko mice showed similar mRNA levels for TGF-β1, tumor necrosis factor α, and interferon gamma (Fig. 5D). TGF-β2 mRNA and TGF-β3 mRNA were not detected. In
addition, no morphologic differences, including phenotype and loss of vitamin A, were observed between HSCs from wt or Smad3 ko mice (data not shown).

Culture-Activated HSCs Isolated From Smad3 ko Mice Show an Increased Rate of Cellular Proliferation. It has been previously reported that the proliferation rate of Smad3 null mouse embryonic fibroblasts is about 2-fold higher than that of wt mouse embryonic fibroblasts. To investigate whether Smad3 inhibits proliferation of culture-activated HSCs, HSCs were isolated from both wt and Smad3 ko mice, and 3H-thymidine incorporation was assessed 14 days after isolation. After a 24-hour period of serum starvation, proliferation of HSCs was stimulated with 10% FCS or with 20 ng/mL platelet-derived growth factor (PDGF). Serum-starved HSCs isolated from wt and

![Figure 2](image_url)

**Fig. 2.** α-SMA and desmin expression are slightly higher in Smad3 ko mice compared with wt mice after CCl4 treatment. (A) After a single intragastric dose of CCl4 or corn oil vehicle, whole liver protein was extracted 72 hours later, and 50 μg subjected to 12% SDS-PAGE followed by immunoblotting for α-SMA or desmin expression. Shown is a typical experiment, which was performed in triplicate. (B) Activated HSCs were immunohistochemically stained with α-SMA antibody in livers of wt, ht, and Smad3 ko mice 72 hours after CCl4 or corn oil treatment. Shown are photomicrographs of a representative experiment, which was performed in triplicate. (Original magnification ×100.)
Recently it has been shown that the induction of the cyclin-dependent kinase inhibitor p15^INK4B is mediated by a TGF-β-induced complex of Smad2, Smad3, Smad4, and Sp1, and that deficiency of any of the Smad proteins reduced or abolished the TGF-β–dependent p15^INK4B induction. We investigated whether differential expression of p15 in HSCs derived from
wt and ko mice and cultured in 10% FCS might account for the increased proliferation rate observed in Smad3 ko HSCs, but found no difference in p15 protein expression (Fig. 6B).

Smad3 Is Required for the TGF-β1-Mediated Formation of Smad-Containing DNA-Binding Complexes in Culture-Activated HSCs, but not for the Activation of ERK.

TGF-β produces a DNA-binding complex containing Smad3, and this formation is absent in Smad3 null mouse embryonic fibroblasts.41 To investigate this DNA-binding activity in activated HSCs, electrophoretic mobility shift assays were performed using nuclear extracts from wt or Smad3 null HSCs and incubated with a radiolabeled consensus Smad3-Smad4–binding element. Upon TGF-β1 stimulation 2 complexes were induced in HSCs isolated from wt mice (Fig. 7A, lane 2). Incubation with antibodies against Smad2, Smad3, or Smad4 resulted in a supershift of both complexes and in the appearance of 2 complexes (lanes 5-7). That indicates that the TGF-β1–induced complexes contained (at least) Smad2, Smad3, and Smad4. The DNA-binding complex formation was completely absent in HSCs derived from Smad3 ko mice after stimulation with TGF-β1 (lane 4). Hep3B cells were used as a positive control to show the inducibility of the Smad-containing DNA-binding complexes (lanes 8 and 9). The labeled probe alone with or without antibodies resulted in no signal (lanes 10-12 and 14). Unlabeled oligonucleotide probe at 200-fold excess competed with the labeled probe, showing binding specificity (lane 13). Thus, Smad3 is necessary for the TGF-β1–mediated formation of Smad-containing DNA-binding complexes in culture-activated HSCs.

To investigate whether TGF-β mediates signaling in Smad3-deficient HSCs, the activation of ERK by TGF-β1 was explored. Immunoblotting for phospho-p44/42 MAPK (ERK1/2) revealed a similar phosphorylation of ERK1/2 30 minutes after stimulation with TGF-β1 in HSCs derived from wt and Smad3 ko mice (Fig. 7B), suggesting that TGF-β activates ERK independently of Smad3 in HSCs.

Smad3 Deficiency Results in an Increased Protein Expression of Smad2 in Culture-Activated HSCs.

Smad3 might modulate the expression of other Smad proteins. To address this issue, Western blot analysis was performed using Smad-specific antibodies. In activated Smad3-deficient HSCs, the protein levels of Smad2 were increased compared with wt HSCs (Fig. 8). Smad3 was only detected in wt HSCs, and Smad4 protein level was the same in wt and Smad3 ko HSCs (Fig. 8).

**DISCUSSION**

Because TGF-β1 is the critical mediator of hepatic fibrosis, we want to determine the role of individual Smad proteins in the signal transduction pathway. This study provides a novel approach by treating Smad3 ko mice with the fibrogenic stim-
The transcriptional coactivators p300/CBP enhance basal as well as TGF-β– or Smad3-induced collagen α2(I) promoter activity, and stimulate the expression of endogenous type I collagen in human dermal fibroblasts. No comparable element has been identified in the collagen α1(I) gene.

After CCl₄ the level of induced collagen in Smad3 ko mice compared with wt mice is higher than the expression in TGF-β1 ko mice compared with wt mice. Therefore, alternative intracellular pathways must mediate in part the TGF-β signaling after the fibrogenic stimulus that increases type I collagen gene expression. In fact, this interpretation agrees well with a study showing that JNK is activated in response to TGF-β and is required for TGF-β–mediated fibronectin mRNA and protein synthesis. Additionally, fibronectin induction does not require the participation of Smad4. Furthermore, an in vivo study showed that transiently expressed Smad7, which inhibits activation of Smad2 and Smad3, pre-
achieved in nonpassaged 3-day-old rat HSCs, but not in passaged myofibroblast-like HSCs. Furthermore, anti–TGF-β antibody produced only a minimal reduction in the collagen α2(I) mRNA level in passaged myofibroblast-like HSCs. Similar results were observed in our study. Passaged HSCs isolated from wt or ko mice and cultured for 14 days, did not respond with a further up-regulation of collagen α1(I) mRNA upon stimulation with TGF-β1 (data not shown). A suggested mechanism for this unresponsiveness to TGF-β treatment is that despite the expression of TGF-β receptors type I and II on the cell surface of myofibroblast-like HSCs, there is strongly reduced TGF-β ligand-binding activity and no binding of Smad proteins to DNA in electrophoretic mobility shift assays. However, in our study a Smad containing DNA-binding complex forms upon TGF-β1 stimulation in culture-activated HSCs isolated from wt mice, suggesting that there might exist other (perhaps species-specific) mechanisms that make myofibroblast-like HSCs unresponsive to TGF-β treatment.

Activated HSCs isolated from Smad3 ko mice have an increased proliferation rate upon stimulation with FCS or PDGF as compared with wt HSCs. Thus, Smad3 is necessary for inhibition of proliferation of culture-activated HSCs, which might be TGF-β dependent. Consistent with these findings, mouse embryonic fibroblasts isolated from Smad3 null mice also show an increased basal proliferation rate and do not respond with growth inhibition on TGF-β stimulation. To find evidence for an increased proliferation rate of activated HSCs in vivo, we investigated the expression of desmin in the liver. Desmin is considered to represent the total number of HSCs, since freshly isolated rat HSCs express desmin, and the desmin expression remained unchanged after repeated subculturing of activated HSCs. Although we found no difference in the desmin expression in control-treated animals, desmin expression in Smad3 ko mice was 1.7-fold higher than in wt mice, indicating that activated Smad3-deficient HSCs show an increased proliferation rate also in vivo. That might account for the slightly increased α-SMA expression exhibited in the livers of Smad3 ko mice as compared with wt mice after CCl₄ in vivo.

TGF-β plays an important role in regulating the inflammatory response, and TGF-β1 ko mice show excessive infiltration of inflammatory lymphocytes and macrophages in several organs. Smad3-deficient mice show inflammatory lesions in a number of organs; in vitro activated Smad3 null thymocytes and T cells are insensitive to the growth inhibitory effect of TGF-β, and Smad3 null neutrophils are impaired in their chemotactic response toward TGF-β in culture and in vivo. In a model of cutaneous wound healing, Smad3 ko mice had a reduced number of monocytes and neutrophils and reduced local amounts of TGF-β1 compared with wt mice, which contributed to a decreased granulation tissue formation, but to an accelerated wound healing process. Investigating this phenomenon, cultured monocytes derived from Smad3 ko mice had reduced specific chemotaxis to TGF-β1 and showed a failure to up-regulate TGF-β1 expression in an autocrine manner. In our study a similar number of infiltrating inflammatory cells were found at the site of injury in mice independent of the genotype. Moreover, all mouse genotypes had similar areas of necrosis after CCl₄.

One finding in our study was that the gene expression of TGF-β1, TGF-β2, and TGF-β3 was not regulated by Smad3. To our surprise, we found that active TGF-β protein, although lower at baseline, was slightly higher in livers of Smad3 ko mice compared with wt mice after CCl₄ treatment. In general,

Fig. 8  Smad protein expression in culture-activated HSCs isolated from wt and Smad3 ko mice: HSCs were isolated from wt and Smad3 ko mice and were cultured on plastic for 14 days. Cell lysates were prepared and equal amounts (50 μg for detection of Smad2 and Smad3; 100 μg for detection of Smad4) were loaded on a 10% SDS-PAGE. After electrophoresis and transfer on a nitrocellulose membrane, immunoblotting was performed with Smad-specific antibodies. Additionally each membrane was immunoblotted using anti-actin antibody to ensure equal protein loading. Each Western blot was reproduced at least once using different HSC isolations.

vents bleomycin-induced lung fibrosis and significantly reduces, but does not block, pulmonary type I procollagen mRNA expression in mice. The Smad3 deficiency had a more profound effect on inhibiting collagen α1(I) in vivo after CCl₄, than in culture-activated HSCs. Similar results were shown in a study using soluble TGF-β type II receptor, in which fibrogenesis was induced by ligation of the common bile duct in rats. HSCs treated with soluble TGF-β type II receptor in culture, exhibited less reduction in steady state level of collagen α1(I) mRNA (35%-40%) compared with isolated HSCs from rats treated with soluble TGF-β type II receptor at the time of bile duct ligation (74%) or during active fibrogenesis (64%). Thus, the activation of HSCs cultured on plastic has a component that is independent of TGF-β and Smad3.

A recent study indicated that the type I collagen response to TGF-β is modulated in HSCs upon passaging in culture. Stimulation of collagen α2(I) mRNA with TGF-β1 was
the mRNA expression and the protein levels of TGF-β may not correlate.67,68 TGF-β activity is regulated tightly on several levels, including the activation of latent TGF-β complexed with latency-associated peptide and/or latent TGF-β binding protein. The in vivo mechanisms for the activation of TGF-β are complex, including low pH, binding to thrombospondin, or proteolysis involving plasmin. Smad3 null dermal fibroblasts show a greatly reduced TGF-β-mediated induction of endogenous plasminogen activator inhibitor-1.41 One could speculate that after CCl₄, Smad3-deficient cells in the liver produce less plasminogen activator inhibitor-1, resulting in a higher plasmin concentration, which then in turn activates more latent TGF-β. That would explain the 3-fold induction in livers from wt mice versus a 9-fold induction in livers from Smad3 ko mice after CCl₄. Taken together, the phenotype in the Smad3 ko mice did not reflect decreased TGF-β, but a partial block in the signal transduction pathway.

Although Smad2 and Smad3 share 92% homology in their amino acid sequence,69 and both are phosphorylated and activated by TGF-β type I receptor, analysis of targeted disruption of Smad genes have identified functional differences. While Smad2 knockout mice are embryonic lethal because of a lack of anterior-posterior specification and a failure to develop mesoderm,70,71 Smad3 ko mice are viable, show limb malformations and exhibit a smaller phenotype,49 have a defect in immune function,65 and develop colon carcinomas at approximately 4 to 6 months of age.72 Several in vitro studies also indicated that Smad2 and Smad3 are functionally different.73-75 Although a previous study reported that the expression of Smad2 protein in Smad3 ko embryos and in tissues of adult mice is identical to that detected in wt mice,65 Smad3 ko HSCs had slightly increased Smad2 levels. Despite the presence of Smad2, the Smad3 ko HSCs had decreased collagen α1(I) mRNA levels and failed to form any complexes with a Smad binding element.

In conclusion, TGF-β1 is required for HSC activation and type I collagen induction in vivo after CCl₄.60 Smad3 is required for maximal type I collagen gene induction, but surprisingly not for HSC activation. Thus, TGF-β1 is required for nuclear accumulation and signaling. Smad3-dependent and -independent intracellular signaling pathways to induce hepatic fibrogenesis.

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