Liver fibrosis is a progressive pathologic process that involves deposition of excess extracellular matrix leading to distorted architecture and culminating in cirrhosis. The role of transforming growth factor-β (TGF-β) as a key molecule in the development and progression of hepatic fibrosis via the activation of hepatic stellate cells, among other fibroblast populations, is without controversy. We hereby show that TGF-β1 induces an epithelial-to-mesenchymal transition (EMT) state in mature hepatocytes in vitro. EMT state was marked by significant up-regulation of α1(I) collagen mRNA expression and type I collagen deposition. Similar changes were found in a “normal” mouse hepatocyte cell line (AML12), thus confirming that hepatocytes are capable of EMT changes and type I collagen synthesis. We also show that in hepatocytes in the EMT state, TGF-β1 induces the snail-1 transcription factor and activates the Smad2/3 pathway. Evidence for a central role of the TGF-β1/Smad pathway is further supported by the inhibition of EMT by Smad4 silencing using small interference RNA technology. In conclusion, TGF-β1, a known pro-apoptotic cytokine in mature hepatocytes, is capable of mediating phenotypic changes and plasticity in the form of EMT, resulting in collagen deposition. Our findings support a potentially crucial role for EMT in the development and progression of hepatic fibrogenesis.

Liver fibrosis results from increased deposition of type I collagen within the hepatic extracellular space and constitutes a common cardinal signature to all forms of liver injury, regardless of etiology (1). End-stage liver fibrosis is recognized clinically as cirrhosis. Since their initial description, hepatic stellate cells (HSC)3 have dominated the field of liver fibrogenesis—12-Week-old C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were anesthetized with ketamine (50 mg/kg intraperitoneally) and xylazine (10 mg/kg intraperitoneally). Livers were perfused in situ through the portal vein, initially with calcium- and magnesium-free Hanks’ balanced salt solution (Invitrogen) containing 0.5 mM EDTA (Invitrogen) followed by Dulbecco’s modified Eagle’s media (DMEM; Invitrogen) containing collagenase (Sigma) until the liver lost its firm texture. The soft liver was removed and gently shaken in DMEM containing collagenase at 37 °C for 10–15 min. The homogenate was filtered and centrifuged for 2 min. The pellet was washed three times with DMEM and then resuspended in DMEM supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin G, 100 μg/ml streptomycin, 250 ng/ml amphotericin B (Invitrogen), and 5 μg/ml insulin.

EXPERIMENTAL PROCEDURES

Hepatocyte Isolation and Cell Culture—12-Week-old C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were anesthetized with ketamine (50 mg/kg intraperitoneally) and xylazine (10 mg/kg intraperitoneally). Livers were perfused in situ through the portal vein, initially with calcium- and magnesium-free Hanks’ balanced salt solution (Invitrogen) containing 0.5 mM EDTA (Invitrogen) followed by Dulbecco’s modified Eagle’s media (DMEM; Invitrogen) containing collagenase (Sigma) until the liver lost its firm texture. The soft liver was removed and gently shaken in DMEM containing collagenase at 37 °C for 10–15 min. The homogenate was filtered and centrifuged for 2 min. The pellet was washed three times with DMEM and then resuspended in DMEM supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin G, 100 μg/ml streptomycin, 250 ng/ml amphotericin B (Invitrogen), and 5 μg/ml insulin.

α-SMA, α-smooth muscle actin; IP, immunoprecipitation; IF, immunofluorescence; DAPI, 4’,6-diamidino-2-phenylindole; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; IB, immunoblotting.
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(Sigma) (Culture Medium). Cell number and viability were assessed by counting an aliquot in the presence of 0.1% trypan blue. Cells were plated on 60-mm culture plates previously coated with collagen (Cohesion, Palo Alto, CA). Incubation was carried out at 37 °C under 5% CO₂. After 18 h, the culture medium was replaced with serum-free medium (same composition as Culture Medium except that fetal bovine serum was replaced with 9.6 ng/ml dexamethasone (Fujisawa, Deerfeld, IL), 10 μg/liter epidermal growth factor, 0.5 μg/liter transferrin, 5 μg/liter selenium, 0.5 μg/ml linoleic acid, and 0.5 μg/ml fetuin) (serum-free Culture Medium). Recombinant human transforming growth factor-β1 (TGF-β1) (R & D Systems, Minneapolis, MN) was added once, i.e. at time of switching to serum-free medium, at a final concentration of 2 ng/ml. The cells without TGF-β1 (control) and those with TGF-β1 were harvested at 0.5, 1, 6, 24, 48, and 72 h.

Mouse Hepatocyte Cell Line, AML12 Culture and Treatment—A nontumorigenic mouse hepatocyte cell line, i.e. AML12 (CRL-2254), was purchased from ATCC (Manassas, VA). The cell line was maintained in DMEM/F-12 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium (BIO-SOURCE), 40 ng/ml dexamethasone (Fujisawa), and 100 ng/ml amphotericin B (Invitrogen) (Complete Growth Medium). AML12 cells were seeded at ~60% confluence in Complete Growth Medium. The cells were changed to serum-free medium, 18 h later, after washing twice with calcium- and magnesium-free phosphate-buffered saline (PBS). Similar to primary hepatocytes, TGF-β1 (R & D Systems) was added at a final concentration of 2 ng/ml. The cells without TGF-β1 (control) and those with TGF-β1 were harvested at 0.5, 1, 6, 24, 48, and 72 h after TGF-β1 treatment.

Real Time RT-PCR Analysis (qRT-PCR)—qRT-PCR was performed by ABI Prism 7900HT (Applied Biosystem) using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Specific forward and reverse primers were purchased from Qiagen. Data analysis was performed using ABI Prism 7900HT SDS 2.0 software (Applied Biosystem). 2ΔΔCt = 2ΔCtTarget − CTarget calculation was used for relative expression value.

Preparation of Cell Lysate—AML12 cells and mouse primary hepatocytes were lysed with cell lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 μg/ml leupeptin) (Cell Signaling, Beverly, MA) and protease inhibitor mixture (Roche Applied Science) on ice. Cells were scraped and transferred to 1.5-ml Eppendorf tubes and rotated for 1 h at 4 °C, followed by centrifugation at 14,000 × g for 10 min at 4 °C. The resulting supernatants were stored in aliquots at −80 °C until required. Protein concentration in the cell lysate solution was determined using BCA protein assay kit (Pierce).

Western Blot Analysis—The cell lysate was mixed with 6× SDS sample buffer (350 mM Tris, pH 6.8, 30% glycerol, 10% SDS, 0.012% bromphenol blue) supplemented with 5% β-mercaptoethanol (Sigma). Samples were heated at 100 °C for 10 min before loading and being separated on precasted 10% or 4–15% SDS-polyacrylamide gels (Bio-Rad). Proteins were electrotransferred to a nitrocellulose membrane (Millipore, Bedford, MA) in transfer buffer containing 25 mM Tris, 192 mM glycerine, and 20% methanol at 4 °C for 1 h. Nonspecific binding to the membrane was blocked for 1 h at room temperature with 5% nonfat milk in TTBS buffer (20 mM Tris, 500 mM sodium NaCl, and 0.1% Tween 20). Membranes were incubated in 16 h at 4 °C with various primary antibodies in blocking buffer containing 5% nonfat milk at the dilution specified by the manufacturers. The following primary antibodies were used: mouse anti-E cadherin (BD Biosciences), goat anti-vimentin (Abcam, Cambridge, MA), rabbit anti-type I collagen (Calbiochem), rabbit anti-AKT (Cell Signaling), rabbit anti-phospho-AKT (Cell Signaling), rabbit anti-ERK1/2 (Cell Signaling), rabbit anti-phospho-ERK1/2 (Cell Signaling), rabbit anti-phospho-Smad2 (Cell Signaling), mouse anti-Smad4 (Santa Cruz Biotechnology), mouse anti-α-tubulin (Sigma) and mouse anti-GAPDH (Abcam). After washing in TTBS buffer, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Pierce) for 1 h at room temperature in 5% nonfat milk dissolved in TTBS. Membranes were then washed with TTBS...
buffer extensively, and the signals were visualized using the enhanced chemiluminescence system (ECL, Amersham Biosciences). Bands were quantified with Image-J software (National Institutes of Health, Bethesda). Relative protein abundance in each sample was normalized to that of GAPDH or β-tubulin.

**Immunoprecipitation (IP)**—2 μg of rabbit anti-Smad2/3 (Upstate Biotechnologies, Inc., Lake Placid, NY) was added to 510 μg of whole protein cell lysate, and the mixture was incubated with rotation for 3 h at 4 °C. After the addition of 50 μl of protein G-Sepharose beads (Amersham Biosciences), the mixture was incubated with rotation overnight at 4 °C. Sample mixture was centrifuged for 5 min at 3000 rpm at 4 °C, and the beads were then washed three times with cell lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin) which included protease inhibitor mixture (Roche Applied Science). The pellet was resuspended in 1× SDS sample buffer (50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.1% bromphenol blue, 5% β-mercaptoethanol) and boiled for 5 min. Samples were loaded on precasted 4–15% SDS-polyacrylamide gels and analyzed by Western blotting.

**Immunofluorescence (IF) Staining**—Mouse primary hepatocytes for immunofluorescence staining of E-cadherin and vimentin were plated on collagen-coated coverslips in 6-well culture dishes and treated in the same method as above described. Mouse primary hepatocytes for immunofluorescence staining of procollagen/type I collagen were plated on positively charged, noncollagen-coated, coverslips seeded in 6-well culture dishes (to confirm de novo synthesis of type I collagen). Incubation was carried out at 37 °C under 5% CO2. After 18 h, culture medium was replaced with serum-free medium. TGF-β1 was added at the onset of the experiment (i.e. time of change to serum-free medium) at a final concentration of 2 ng/ml.

![FIGURE 2. TGF-β1 down-regulates E-cadherin and up-regulates vimentin in hepatocytes.](image)

![FIGURE 3. Panels i–iii, TGF-β1 induces EMT-type changes in primary hepatocytes. Immunofluorescence staining for E-cadherin (green) and vimentin (red) in primary hepatocytes were evaluated by confocal laser microscopy. DAPI stained nuclei blue. There was apparent near loss of cell membrane-bound E-cadherin in TGF-β1-treated cells in a time-dependent manner. Diffuse vimentin staining was observed in control cells, and TGF-β1-treated hepatocytes exhibited a fibrillar vimentin pattern most noted at 48 and 72 h. Also note that TGF-β1 caused hepatocytes to assume a spindle-shaped morphology, which was not observed in controls. Scale bar, 50 μm.](image)
AML12 cells for immunofluorescence staining were plated on noncollagen-coated (as recommended by ATCC) positively charged coverslips seeded in 6-well culture dishes and treated using the same methods as described above. At 0.5, 1, 24, 48, and 72 h after changing to serum-free medium, cells without TGF-β1 (control) and those with TGF-β1 were washed with PBS three times and fixed with cold methanol:acetone (1:1) for 10 min on ice. Cells were fixed with 3.8% paraformaldehyde in PBS for 10 min at room temperature and permeabilized in 0.5% Triton X-100/PBS for 5 min at room temperature. After washing with PBS for 5 min (three times), cells were blocked with ProBlock (Dako Cytomation, Carpinteria, CA) for 30 min at room temperature, then with 5% serum (from same species as secondary antibody) in PBS for 60 min, and then incubated with the specific primary antibodies. The following primary antibodies were used for immunostaining: goat anti-mouse albumin (Immunology Consultants Laboratory), rat anti-E-cadherin (clone ECCD-2) (Zymed Laboratories Inc.), goat anti-vimentin (Abcam), and rabbit anti-mouse antiprolactin/myoel type I collagen (Chemicon, Temecula, CA). Alexa fluor-488, Alexa fluor-594 (Molecular probes, Eugene, OR), or Texas Red (Abcam)-conjugated secondary antibodies were used. For F-actin staining, we used Alexa fluor-488 phalloidin (Molecular Probes). Cells were co-stained with 4',6-diamidino-2-phenylindole (DAPI), HCl (Molecular Probes), to visualize the nuclei. Stained cells were mounted with fluorescent mounting medium (Dako Cytomation) and viewed by confocal laser microscopy (PerkinElmer Life Sciences). All exposure gains and rates are consistent among samples.

**Small Interference RNA (siRNA) Experiment**—Culture medium was changed 4 h after the isolation of primary hepatocytes, and the cells were cultured in the presence of negative control siRNA (Qiagen) or Smad4 siRNA (Qiagen) for 48 h. Transfection of siRNA was performed according to the manufacturer’s instructions (Qiagen). 10 μM was determined to be the most effective siRNA concentration for Smad4 silencing. Hence, 10 μM siRNA with HiPerFect transfection reagent (Qiagen) in serum-free culture medium was incubated for 10 min at room temperature (transfection mixtures) and added directly to the cultured cells. The AML12 cells were cultured in the absence (control) or presence of 2 ng/ml TGF-β1 for 72 h in serum-free medium. A, levels of E-cadherin (panel i) and vimentin (panel ii) mRNA in AML12 cells were quantified by qRT-PCR. Error bars represent mean ± S.E. of triplicate wells from at least three separate experiments. **, p < 0.05 versus control at same time point (Student’s t test). B, panel i, whole cell protein lysates were immunoblotted with specific anti-E-cadherin antibodies. The same blot was reprobed with -tubulin to ensure equal loading of each lane. Results are representative of at least three independently performed experiments. Panel ii, Western blots were quantified and normalized to -tubulin loading control. E-cadherin/-tubulin showed the relative amounts of E-cadherin. The immunoreactivity at time 0 was arbitrarily set to 1. The error bars represent means ± S.E. from at least three independent experiments. *, p < 0.05 versus control at same time point (Student’s t test). C, immunofluorescence for E-cadherin (green) and vimentin (red) in AML12 cells in the absence (control) or presence of 2 ng/ml TGF-β1 for 72 h in serum-free medium were examined by confocal laser microscopy. DAPI stained nuclei blue. The expression of cell membrane-bound E-cadherin was reduced in TGF-β1-treated cells. Vimentin filament assumed a fibrillar rearrangement in TGF-β1-treated cells, as opposed to a homogeneous cytoplasmic distribution in controls. Note the spindle-shaped morphology assumed by hepatocytes 72 h after TGF-β1. Scale bar, 50 μm. R.E.V., relative expression value.

**FIGURE 4.** TGF-β1 suppresses E-cadherin and up-regulates vimentin in a normal mouse hepatocyte cell line (AML12). AML12 cells were cultured in the absence (control) or presence of 2 ng/ml TGF-β1 for 72 h in serum-free medium. A, levels of E-cadherin (panel i) and vimentin (panel ii) mRNA in AML12 cells were quantified by qRT-PCR. Error bars represent mean ± S.E. of triplicate wells from at least three separate experiments. **, p < 0.05 versus control at same time point (Student’s t test). B, panel i, whole cell protein lysates were immunoblotted with specific anti-E-cadherin antibodies. The same blot was reprobed with -tubulin to ensure equal loading of each lane. Results are representative of at least three independently performed experiments. Panel ii, Western blots were quantified and normalized to -tubulin loading control. E-cadherin/-tubulin showed the relative amounts of E-cadherin. The immunoreactivity at time 0 was arbitrarily set to 1. The error bars represent mean ± S.E. of triplicate wells from at least three separate experiments. **, p < 0.05 versus control at same time point (Student’s t test). C, immunofluorescence for E-cadherin (green) and vimentin (red) in AML12 cells in the absence (control) or presence of 2 ng/ml TGF-β1 for 72 h in serum-free medium were examined by confocal laser microscopy. DAPI stained nuclei blue. The expression of cell membrane-bound E-cadherin was reduced in TGF-β1-treated cells. Vimentin filament assumed a fibrillar rearrangement in TGF-β1-treated cells, as opposed to a homogeneous cytoplasmic distribution in controls. Note the spindle-shaped morphology assumed by hepatocytes 72 h after TGF-β1. Scale bar, 50 μm. R.E.V., relative expression value.
hepatocyte cells were plated with transfection mixtures and cultured for 48 h.

The primary hepatocytes and the AML12 cells with the transfection mixture were changed to serum-free medium after washing twice with PBS. TGF-β1 (R & D Systems) was added at a final concentration of 2 ng/ml. The cells without TGF-β1 (control) and those with TGF-β1 were harvested 1 h and 72 h later.

Statistical Analysis—Values are expressed as means ± S.E. All values were derived from measurements of at least three independently performed experiments. Statistical analyses for comparisons over time course were performed with repeated measure analysis of variance (ANOVA) and post hoc Bonferroni/Dunn’s correction. Student’s t test was used for the comparison of control and TGF-β1 treatment groups at the same time point. A p value <0.05 was considered to indicate statistical significance. ANOVA and Student’s t test were performed using StatView software package (Abacus Concepts Inc., Berkeley, CA).

RESULTS

TGF-β1 Induces Cytoskeletal Rearrangement in Hepatocytes in Vitro—Primary adult (12-week-old) mouse hepatocytes were cultured in serum-free medium with or without TGF-β1 (2 ng/ml). After 72 h in culture, hepatocytes were co-immunostained for F-actin and albumin and examined by confocal microscopy. In control hepatocytes, F-actin was detected in the cell-cell junction with a peri-cell membrane distribution, and TGF-β1-treated cells acquired a spindle-shaped morphology with polarization of the F-actin stress fibers throughout the cell (Fig. 1A). This phenotypic appearance suggested that mature hepatocytes can acquire a mesenchymal phenotype. Co-expression of albumin, in both controls and TGF-β1-treated cells, indicates that the cells are hepatocytes (Fig. 1A). Moreover, we confirmed the lack of expression of α-smooth muscle actin (α-SMA), a marker of activated hepatic stellate cells (HSC), in both control and TGF-β1-treated hepatocytes using Western blot analysis (Fig. 1B). Furthermore, to confirm that our results are not because of HSC or fibroblast contamination of our primary culture, the experiments were reproduced using a “normal” mouse hepatocyte cell line (AML12); TGF-β1-treated cells, but not controls, exhibited a similar pattern of F-actin rearrangement to primary hepatocytes after TGF-β1 treatment (Fig. 1C).

TGF-β1 Induces an EMT State in Hepatocytes—E-cadherin is a universal epithelial marker that plays a key role in the maintenance of cellular integrity, and down-regulation of its expression marks early EMT (16, 17). We evaluated the pattern of E-cadherin expression in response to TGF-β1 treatment; E-cadherin mRNA levels were decreased in the TGF-β1-treated hepatocytes as compared with controls, p < 0.05 (Fig. 2A, panel i). Similarly, Western blot analysis showed that E-cadherin protein levels were reduced in the TGF-β1-treated hepatocytes, p < 0.05 (Fig. 2B).

To demonstrate whether the TGF-β1-induced phenotypic changes and down-regulation of epithelial markers were accompanied by acquisition/up-regulation of mesenchymal makers, we evaluated the expression of vimentin, a filament used to identify mesenchymal cells, as well as cells in EMT (10). Both groups of hepatocytes (i.e. controls and TGF-β1-treated) expressed vimentin mRNA, which increased over time. However, the expression of vimentin mRNA was significantly increased in the TGF-β1-treated hepatocytes as compared with controls, p < 0.05 (Fig. 2A, panel ii). Co-immunofluorescence staining for E-cadherin and vimentin, evaluated by confocal microscopy, revealed near loss of cell membrane-bound E-cadherin by 72 h after TGF-β1. Down-regulation of the membrane-bound E-cadherin occurs in parallel with fibrillar rearrangement of vimentin filament in the cytoplasm hepatocytes 48 and 72 h after TGF-β1 exposure. These changes were absent in the controls (Fig. 3).

TGF β1 Induces an EMT State in the AML12 Hepatocytes—To further test our hypothesis, we examined whether the EMT changes observed in primary hepatocytes could be confirmed in the AML12 mouse hepatocyte cell line. E-cadherin mRNA levels were repressed by TGF-β1 treatment, p < 0.05 (Fig. 4A, panel i). Western blot analysis showed that E-cadherin protein levels were also reduced in the AML12 cells after TGF-β1 treat-
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ment, \( p < 0.05 \) (Fig. 4B). Similar to primary hepatocytes, vimentin mRNA levels were up-regulated in TGF-\( \beta \)-1-treated AML12 cells at 48 and 72 h, \( p < 0.05 \) (Fig. 4A, panel ii).

Phenotypic examination by immunohistochemistry using confocal microscopy was also performed on AML12 cells in order to further delineate the distribution pattern of these epithelial and mesenchymal markers (Fig. 4C). The findings are consistent with those observed in primary hepatocytes, and provide further proof that our results are due to EMT and not to potential fibroblast contamination and overgrowth.

**FIGURE 6.** Panels i–iii, immunofluorescence staining for type I collagen in primary hepatocytes. Co-immunostaining for albumin (red) and procollagen/type I collagen (green) in adult primary mouse hepatocytes examined by confocal microscopy. Cells were cultured in the absence (control) or presence of 2 ng/ml TGF-\( \beta \)-1 for 24, 48, and 72 h in serum-free medium. DAPI stained nuclei blue. TGF-\( \beta \)-1-treated hepatocytes increased de novo type I collagen synthesis 48 and 72 h after treatment. Note the cytoplasmic staining of procollagen. Albumin co-staining confirms the hepatocyte identity of the collagen-producing cells. Scale bar, 20 \( \mu m \).

**FIGURE 7.** TGF-\( \beta \)-1 induces expression of type I collagen in AML12 hepatocyte cell line. A, \( \alpha_1 \) collagen mRNA was examined by qRT-PCR. Error bars represent mean \( \pm \) S.E. of triplicate wells from three independent experiments. **, \( p < 0.05 \) versus control at same time point (Student’s t test). B, panel i, whole protein cell lysates were immunoblotted with anti-type I collagen antibody. Blots were reprobed with \( \alpha \)-tubulin to ensure equal loading of each lane. Panel ii, collagen \( \alpha \)-tubulin was quantitated in each lane. The immunoreactivity at time 0 cells is set to 1. The error bars represent means \( \pm \) S.E. of three independent experiments. *, \( p < 0.05 \) versus baseline collagen \( \alpha \) in time 0 cells (ANOVA); **, \( p < 0.05 \) versus collagen \( \alpha \) in same time point control cells (Student’s t test). R.E.V., relative expression value.

**TGF-\( \beta \)-1 Induces Type I Collagen Expression in Primary Hepatocytes**—To evaluate whether the acquisition of a mesenchymal phenotype by hepatocytes (as suggested by changes in morphology and cytoskeleton rearrangement) is accompanied by mesenchymal defining function(s), we determined whether primary hepatocytes could contribute to the production of extracellular matrix (18). For this purpose, we examined the expression of \( \alpha_1 \) collagen mRNA by hepatocytes. TGF-\( \beta \)-1 induced \( \alpha_1 \) collagen mRNA 72 h after treatment (Fig. 5A, panel i). Next, we evaluated the expression of fibroblast-specific protein (FSP1), a member of the S100 family of calcium-binding proteins (S1004A) that is specific to fibroblasts (16). Lack of FSP1 up-regulation after TGF-\( \beta \)-1 further supports that increased \( \alpha_1 \) collagen mRNA expression by TGF-\( \beta \)-1 is not because of fibroblast contamination/overgrowth (Fig. 5A, panel ii).

Subsequently, we examined the type I collagen protein expression in whole cell lysates, and as shown in Fig. 5B, type I collagen was produced by hepatocytes. Levels of type I collagen were higher in the TGF-\( \beta \)-1-treated hepatocytes than in their
control counterparts. Furthermore, the primary hepatocytes were immunostained with anti-procollagen/type I collagen antibody. As shown in Fig. 6, collagen fibrils were clearly detected in the extracellular space 48 and 72 h after TGF-\(\beta\)-1 treatment. Moreover, the intracellular detection of procollagen in albumin-positive cells supports collagen production by hepatocytes.

**TGF-\(\beta\)-1 Treatment Stimulates Type I Collagen Production by AML12 Hepatocytes**—To further confirm that the source of type I collagen was the primary hepatocytes, rather than fibroblasts or other unaccounted cell types that may have potentially contaminated our primary hepatocyte preparation, the AML12 cell line was evaluated for type I collagen production. \(\alpha_1(I)\) collagen mRNA and type I collagen protein were up-regulated after TGF-\(\beta\)-1 treatment (Fig. 7). Immunohistochemistry staining confirmed that AML12 hepatocytes synthesize procollagen/type I collagen in response to TGF-\(\beta\)-1 treatment (Fig. 8).

**FIGURE 8. Immunofluorescence for type I collagen in AML12 hepatocytes after TGF-\(\beta\)-1 treatment.** Co-immunostaining for albumin (red) and procollagen/type I collagen (green) in AML12 cells in the absence (control) or presence of 2 ng/ml TGF-\(\beta\)-1 at 24, 48, and 72 h (shown in panels i, ii, and iii, respectively) in serum-free medium, examined by confocal laser microscopy. Cell nuclei are demonstrated by DAPI (blue). Assembly of type I collagen is demonstrated in TGF-\(\beta\)-1-treated AML12 cells at 72 h. Note the intracellular procollagen staining in TGF-\(\beta\)-1-treated cells. Albumin staining is consistent with the hepatocyte identity of the cells. Scale bar, 50 \(\mu\)m.

**FIGURE 9. TGF-\(\beta\)-1 induces snail-1 in hepatocytes in EMT state and lack of evidence for Akt/Erk1/2 activation.** A, qRT-PCR reveals increased snail-1 mRNA levels in primary (panel i) and AML12 hepatocytes (panel ii) after TGF-\(\beta\)-1 treatment. \(\beta\), whole cell lysates were obtained and analyzed by Western blotting for total and phosphorylated forms of Akt and ERK1/2 proteins using appropriate specific antibodies in primary hepatocytes (panel i) and AML12 cells (panel ii). GAPDH and \(\alpha\)-tubulin were used as loading controls. There was no notable TGF-\(\beta\)-1-mediated up-regulation of Akt and/or Erk1/2 in either primary or AML12 cells, thus suggesting that the non-Smad pathway was not the likely pathway for TGF-\(\beta\)-1 induced EMT changes. R.E.V., relative expression value.
These observations suggested that Smad-independent TGF-β signaling is not significantly involved in mediating EMT. IP assays confirmed binding of Smad2/3 to Smad4, hence their activation (Fig. 10, panel ii). Antibody is shown as a control; increased Smad4 binding to Smad2/3 and increased P-Smad2 levels were not because of increased Smad2/3 levels in TGF-β1-treated hepatocytes. B, co-immunostaining for albumin (red) and Smad2/3 (green) in hepatocytes were examined by confocal microscopy. Cell nuclei are demonstrated by DAPI (blue). At 0.5 h, Smad2/3 translocates into the nucleus of TGF-β1-treated primary hepatocytes: a sine qua non of its activation. No appreciable translocation of Smad2/3 was noted in control cells. Co-staining for albumin confirms the hepatocyte identity of the cells. Scale bar, 50 μm.

P-ERK1/2 had similar patterns and levels of expression in both control and TGF-β1-treated AML12 cells (Fig. 9B, panel ii). These observations suggested that Smad-independent TGF-β signaling is not significantly involved in mediating EMT.

Next, we examined the Smad-dependent signaling pathway, i.e. Smad2/3 and Smad4 activation. IP assays confirmed binding of Smad2/3 to Smad4, hence their activation (Fig. 10A, panel i). P-Smad2 levels increased after TGF-β1 treatment (Fig. 10A, panel ii), and immunohistochemistry staining confirmed the translocation (i.e. activation) of the Smad2/3 complex to the nucleus of hepatocytes 0.5 h after TGF-β1 treatment (Fig. 10B).

AML12 cells were similarly examined for evidence of Smad2/3 activation after TGF-β treatment. IP (Fig. 11A) and immunohistochemistry (Fig. 11B) studies were consistent with Smad2/3 and Smad4 binding and activation.

Taken together, the Smad-dependent signaling pathway was clearly activated in TGF-β1-treated primary and AML12 hepatocytes and not in their control counterparts. This suggests that TGF-β1-induced EMT changes in hepatocytes were predominately mediated via Smad signaling and not via the non-Smad, Akt, and/or ERK1/2 pathway.

**Smad4-siRNA Reverses EMT in Hepatocytes in Vitro**—To demonstrate the central role of the TGF-β1/Smad signal on EMT induction, siRNA technology was used for smad4 gene silencing in both primary and AML12 hepatocytes (24, 25). The efficiency of siRNA transfection and resultant smad4 gene silencing in primary and AML12 hepatocytes was evaluated by IP, qRT-PCR, and IF. An additional arm using control-siRNA (i.e. nonsilencing, negative control-siRNA) was added to all experiments to rule out nonspecific off-target effects that may be unrelated to smad4 gene silencing.

In primary hepatocytes, the activation of the Smad signal was abrogated after transfection with Smad4-siRNA; the TGF-β1-induced binding of Smad4 to Smad2/3, a sine qua non of the TGF-β1/Smad activation, was repressed, as confirmed by IP (Fig. 12A). Gene silencing of Smad4 was confirmed by qRT-PCR. The Smad4 mRNA levels were significantly decreased up to 72 h after Smad4-siRNA transfection in control hepatocytes; consistent with Smad4 gene silencing. TGF-β1 treatment failed to induce Smad4, as evidenced by comparable Smad4 mRNA levels to the untreated (control) Smad4-siRNA-transfected hepatocytes. This effect was not observed in the control siRNA group (Fig. 12B).
Whether Smad4-siRNA could reverse the TGF-β1-induced EMT was then examined. In fact, the suppression of E-cadherin mRNA expression by TGF-β1 treatment was partly, but significantly, preserved after Smad4-siRNA treatment (Fig. 12B). snail-1, a repressor of E-cadherin transcription, was shown to be induced 1 h after TGF-β1 treatment (Fig. 9A). As shown in Fig. 12B, panel ii, snail-1 mRNA was significantly repressed by Smad4-siRNA in TGF-β1-treated hepatocytes. Vimentin mRNA expression was decreased slightly, but not significantly, by Smad4-siRNA in TGF-β1-treated primary hepatocytes (Fig. 12B). On the other hand, the reduction of albumin mRNA expression by TGF-β1 was prevented after Smad4-siRNA transfection. Most importantly, the upregulation of α(I) collagen mRNA by TGF-β1 was significantly suppressed by Smad4-siRNA (Fig. 12B).

The effect of Smad4-siRNA on the expression of E-cadherin and vimentin was then confirmed by IF. Consistent with qRT-PCR results, E-cadherin was partly recovered after Smad4 RNA knockdown in TGF-β1-treated primary hepatocytes. Also, vimentin staining was equally intense after siRNA transfection, hence consistent with the nonsignificant decrease in vimentin mRNA after Smad4-siRNA treatment. Notably, however, vimentin assumed a less fibrillar pattern and exhibited a more diffuse, granular cytoplasmic distribution (Fig. 13). Next, to confirm the reduction of de novo α(I) collagen production by Smad4-siRNA in primary hepatocytes, slides were immunostained with anti-procollagen/type I collagen antibody. As shown in Fig. 14, TGF-β1-treated hepatocytes that were transfected with Smad4-siRNA exhibited a significant decrease in type I collagen. This effect was not seen in the control-siRNA-treated hepatocytes. Furthermore, cellular morphology of TGF-β1-treated hepatocytes reverted from a spindle shape to a more cuboidal/hexagonal shape, after smad4 silencing. This effect was not observed in control-siRNA-transfected and TGF-β1-treated hepatocytes that preserved a spindle-shaped morphology.

Consistent with the results obtained with primary hepatocytes, Smad4-siRNA repressed the binding of Smad4 to Smad2/3 in TGF-β1-treated AML12 cells, as confirmed by IP assay (Fig. 15A). This lack of binding was because of Smad4
silencing, as reflected by a significant decrease in Smad4 mRNA expression in the non-TGF-β1-treated (control) AML12 hepatocytes. This effect was not seen after control-siRNA transfection. Similarly, TGF-β1-treated AML12 cells that were transfected with Smad4-siRNA failed to induce the Smad4 gene (Fig. 15B).

The TGF-β1-induced suppression of E-cadherin mRNA was inhibited after Smad4-siRNA treatment (Fig. 15B). Consistently, snail-1 mRNA expression was repressed by Smad4-siRNA in TGF-β1-treated AML12 cells (Fig. 15B). Increased expression of vimentin mRNA after TGF-β1 treatment was slightly, but significantly, reduced after siRNA-induced smad4 silencing (Fig. 15B). Also, the reduction in albumin mRNA by TGF-β1 was partly, but significantly, prevented by Smad4-siRNA. Moreover, the induction of α1(I) collagen mRNA by TGF-β1 was clearly suppressed by Smad4 silencing (Fig. 15B).

Immunofluorescence confirmed the above qRT-PCR results; membrane-bound E-cadherin was preserved by Smad4 silencing in TGF-β1-treated AML12 cells (Fig. 16). Similar to primary hepatocytes, the pattern of vimentin staining reverted from fibrillar to granular in TGF-β1-treated, Smad4-siRNA-transfected AML12 cells (Fig. 16). Also, as shown in Fig. 17, Smad4 silencing in AML12 hepatocytes suppressed the TGFβ1-induced type I collagen production. Overall, our results support a central role of the TGFβ1/Smad pathway in EMT induction.

**DISCUSSION**

In this study, we demonstrate that hepatocytes synthesize type I collagen in response to low dose TGF-β1 (2 ng/ml), one of the most ubiquitous and powerful mediators of fibrosis across organ systems. Our results revive the seminal work by Chojkier (26) that hepatocytes are able to produce collagen. Those early findings were eclipsed by multiple criticisms that undermined the ability of hepatocytes to contribute to hepatic fibrosis (27). Currently, it is postulated that only HSC, portal myofibroblasts, and mesenchymal cells of bone marrow origin have a fibrotic potential in the liver (28, 29). Although other studies have also shown that hepatocytes may synthesize collagen, the notion persists that hepatocytes have an insignificant role in the perpetuation of liver fibrosis, and hence progression to cirrhosis. We suspect that hepatocytes play a potentially important role in the genesis of liver fibrosis and progression to cirrhosis.

Yet the contribution of epithelial cells to “fibrogenesis” is not a novel concept. In fact, EMT refers to that very ability, which in its “strict” definition implies that epithelial cells are at the origin of local formation of interstitial fibroblasts. EMT implies that
epithelial cells lose their “characteristic” epithelial phenotype and markers in favor of acquiring mesenchymal markers. This latter phenomenon has been studied and proven to occur in many epithelial cells as follows: kidney tubular epithelia, mammary glands, alveoli, and prostate (8, 17, 30, 31). Controversy in EMT nomenclature remains, however, and in part stems from the fact that mesenchymal markers lack specificity. To avoid such confusion, we prefer the term “EMT state” and better describe the phenotypic changes that hepatocytes undergo in our in vitro system, namely that TGF-β1-treated hepatocytes down-regulate epithelial markers in favor of up-regulating mesenchymal markers and inducing type I collagen synthesis, a known fibroblast function. Hence, we do not imply that hepatocytes transform into fibroblasts but that they assume a fibroblast-like phenotype and function (collagen synthesis), while down-regulating but preserving to a certain extent the epithelial marker E-cadherin. In the liver, EMT was first noted to involve the hematopoietic lineage, i.e. stromal cells, in the fetal liver and was later recognized in fetal and neonatal hepatocytes when treated with TGF-β1 or upon serum starvation (20, 32). Although it has been concluded that EMT is not a major phenomenon in mature hepatocytes because of terminal differentiation, no studies have been conducted to elucidate the potential role of EMT in hepatic fibrogenesis. Since its demonstration in other organs, namely lung, kidney, prostate, and mammary glands, little attention has been given to EMT in mature hepatocytes and its potential implication in liver fibrogenesis (8, 18, 23, 30, 31, 33). Lately, however, a few studies have alluded to EMT potential in liver cellular subpopulation (34, 35).

Several concerns arise when trying to study EMT in a primary hepatocyte culture, the most critical being contamination with resident fibroblasts and/or stellate cells/myofibroblasts. Moreover, TGF-β1, a known inducer of apoptosis in hepatocytes, may in fact favor the overgrowth of such contaminating cells. To address these major concerns, we resorted to multiple strategies as follows: (a) co-labeling with hepatocellular/epithelial specific and mesenchymal-type markers. Thus, immunofluorescence has shown co-localization of E-cadherin and vimentin, as well as collagen and albumin. Type I collagen was detected intracellularly and extracellularly in albumin-positive cells, hence providing further proof that hepatocytes are the source of synthesized collagen. This is expected because collagen synthesis starts in the form of procollagen, which then matures in the form of interlacing fibrils once secreted to extracellular space. The next strategy is as follows: (b) assay for fibroblast-specific marker and a stellate cell-specific marker. To rule out contamination with HSC and interstitial fibroblasts, we

![FIGURE 15. Smad4 silencing inhibits TGF-β1-induced EMT in AML12 cells. A, AML12 cells were transfected with control-siRNA or Smad4-siRNA. Whole protein cell lysates from TGF-β1-treated cells for 1 or 6 h and their corresponding controls (i.e. non-TGF-β1-treated) were immunoprecipitated (IP) for Smad2/3 and protein G-Sepharose. Panel i, IB for Smad4 reveals decreased Smad4 binding to Smad2/3 in Smad4-siRNA-transfected hepatocytes. Panel ii, IB with anti-Smad2/3 antibody is shown as loading control. B, AML12 cells were transfected with control-siRNA or Smad4-siRNA. mRNA was extracted at 72 h (panel i) and 1 h (panel ii) from TGF-β1-treated (black bars) and non-TGF-β1-treated cells (control, white bars). qRT-PCR results are shown as follows. Panel i, E-cadherin, vimentin, albumin, α(I)collagen; panel ii, snail-1. Smad4-siRNA prevented E-cadherin loss, decreased vimentin expression, and abrogated α(I)collagen and snail-1 expression and hence consistent with EMT inhibition with Smad4 silencing. qRT-PCR for Smad4 was performed to confirm suppression of Smad4 mRNA expression by Smad4 siRNA. Error bars represent mean ± S.E. of triplicate wells from three representative experiments. *, p < 0.05; Smad4-siRNA versus control-siRNA (student’s t test). R.E.V., relative expression value.]

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assayed for α-SMA and FSP-1, two specific markers with high specificity to both types of cells, respectively. α-SMA was not detected, therefore ruling out HSC contamination. Furthermore, qRT-PCR for FSP-1 did not show up-regulation after TGF-β1 treatment, thus indicating that our results were because of EMT in hepatocytes and not to contamination with, or overgrowth of, fibroblasts. We then resorted to a “normal” nontransformed hepatocyte cell line, i.e. AML12. EMT changes were also confirmed in the AML12 mouse hepatocyte cell line, thus confirming that the observed changes in primary hepatocytes were indeed because of EMT changes rather than to potential HSC, fibroblast, or other mesenchymal cell contamination. Yet another concern arises when trying to address EMT of hepatocytes in vitro, which is that hepatocytes dedifferentiate in culture. Hence, hepatocytes take a more elongated and flattened morphology. Although our cells may have dedifferentiated to a certain degree (as expected when grown in culture), this is controlled for in the nontreated group. Changes reported after TGF-β1 treatment are beyond what we may expect from dedifferentiation alone, which by itself does not account for collagen production.

Our results further support the general “EMT hypothesis” and have two important potential implications. First, EMT may be a continuum rather than an all or none phenomenon, namely that further testing in vitro with a different experimen-tal design is warranted to study the spectrum. Second, hepatocytes are to be viewed as perpetuators of hepatic fibrogenesis, rather than as victims.

As another mean of evaluating our results, we examined known mediators of the EMT mechanism reported in other cellular types (kidney tubular epithelial and fetal hepatocytes). To date, it is thought that the sole effect of TGF-β1 on “mature” hepatocytes is to induce apoptosis (10, 12, 36). It is evident, however, that some hepatocytes escape this fate by undergoing EMT (32). Mechanistically, snail transcription factor is central to EMT (14, 20, 21). snail-1 is activated by TGF-β and is implicated in the direct suppression of E-cadherin transcription. Indeed, snail is known to trigger EMT during embryonic development and tumor progression; hence, snail mutant mice die at gastrulation because of a defective EMT (21). Moreover, snail is upstream of mesenchymal markers such as vimentin (37). TGF-β1 exerts its effects by binding to the TGF-β type II receptor (TβRII) and subsequently recruiting the TGF-β type I receptor (TβRI) (38). Smad2/3 and Smad4 are known intracellular mediators of TGF-β1. Once phosphorylated by the activated TGF-β1 receptor, Smad2 and/or Smad3 complex with Smad4 and translocate to the nucleus where they regulate TGF-β1 target genes (15, 38, 39). It has been shown that Smad2/3 activation is induced during EMT. Numerous reports indicate a central role for Smads as mediators of TGF-β-induced EMT and demonstrate their important role in fibrosis (18, 40–43). Smad-independent pathways have also been implicated in the induction of EMT in certain cell types (23, 44). In Madin-
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Darby canine kidney cells, TGF-β1 promotes EMT via mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) and PI3K pathways that up-regulate snail expression (19, 45, 46). In NMuMG mammary cells, PI3K pathway was found to be essential for TGF-β1-mediated EMT (47). Moreover, Valdes et al. (48) reported activation of the PI3K pathway in fetal rat hepatocytes that escape TGF-β1-induced apoptosis.

We have also shown that TGF-β1 mediates EMT in hepatocytes by the induction of snail-1 and activation of Smad4. This is further supported by the abrogation of EMT after Smad4 RNA interference-mediated silencing (49). In fact, transfection of primary and AML12 hepatocytes with Smad4-siRNA inhibits TGF-β1-induced EMT as follows: preserving the epithelial phenotype (E-cadherin) and function (albumin), and most importantly, inhibiting TGF-β1-induced type I collagen expression. Even though vimentin was not significantly down-regulated in primary hepatocytes, there was a notable reversal of its intracellular polymerization. Smad4-siRNA transfection regulated in primary hepatocytes, there was a notable reversal.

Although specific therapies to inhibit the progression of liver fibrosis are still not available, we propose that our findings may open new avenues to the understanding of the development and progression of hepatic fibrogenesis. This may lead to new therapeutic options for hepatic fibrosis.

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