MC4R-KO mice fed WD supplemented with 5% (wt/wt) EPA for 4 weeks after the development of NASH. (D) Liver triglyceride (TG) content at each time point. †† $P < 0.01$ vs. MC4R-control at 20 weeks; ** $P < 0.01$ vs. MC4R-control at 24 weeks; n.s., not significant. MC4R-control at 20 weeks, $n = 9$; MC4R-control at 24 weeks, $n = 7$; MC4R-EPA Tx, $n = 10$.
(TIF)

S2 Fig. Hepatic mRNA expression in MC4R-KO mice in the therapeutic study. Hepatic mRNA expression levels after 4-week EPA treatment. mRNA expression of *de novo* lipogenesis (FAS and SCD-1) (fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD-1)) and β-oxidation (PPARα, CPT1A) (A), inflammatory markers (F4/80 and tumor necrosis factor α (TNFα) (B) and fibrogenic factors (transforming growth factor β1 (TGFβ1), collagen α1(I) (COL1A1), tissue inhibitor of metalloproteinase-1 (TIMP1), and matrix metalloproteinase-2 (MMP2)) (C). * $P < 0.05$; ** $P < 0.01$; n.s., not significant.
(TIF)

S3 Fig. hCLS formation and TGFβ activation in the liver of MC4R-KO mice in the therapeutic study. (A) F4/80 immunostaining. Arrows indicate hepatic crown-like structures (hCLS). (B) Quantification of hCLS number after EPA treatment. (C) Immunofluorescent analysis for F4/80 and CD11c. (D) Hepatic mRNA expression of CD11c. Quantification of the TUNEL-positive cell number (E) and R58 LAP-DP-positive area (F). (G) Hepatic mRNA expression of urokinase-type plasminogen activator receptor (uPAR). (H) Active TGFβ1 protein levels in the liver. Scale bars, 50 μm. * $P < 0.05$; ** $P < 0.01$.
(TIF)

S1 Table. Dietary composition of standard diet (CE-2) and Western diet (D12079B) used in this study.
(DOCX)

S2 Table. Primers used in this study.
(DOCX)

S3 Table. Fatty acid composition of the liver from MC4R-KO treated with EPA for 24 weeks.
(DOCX)

S4 Table. Serological parameters of MC4R-KO mice in the therapeutic study.
(DOCX)

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Author Contributions

Conceived and designed the experiments: MI T. Suganami. Performed the experiments: KK MI S. Kanai NN T. Sakai. Analyzed the data: KK MI. Contributed reagents/materials/analysis tools: HK MH S. Kojima. Wrote the paper: MI T. Suganami YO. Provided expertise and contributed discussion: YI.

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GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Mechanisms of Action of Acetaldehyde in the Up-Regulation of the Human α2(I) Collagen Gene in Hepatic Stellate Cells

Key Roles of Ski, SMAD3, SMAD4, and SMAD7

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Alcohol-induced liver fibrosis and eventually cirrhosis is a leading cause of death. Acetaldehyde, the first metabolite of ethanol, up-regulates expression of the human α2(I) collagen gene (*COL1A2*). Early acetaldehyde-mediated effects involve phosphorylation and nuclear translocation of SMAD3/4–containing complexes that bind to *COL1A2* promoter to induce fibrogenesis. We used human and mouse hepatic stellate cells to elucidate the mechanisms whereby acetaldehyde up-regulates *COL1A2* by modulating the role of Ski and the expression of SMADs 3, 4, and 7. Acetaldehyde induced up-regulation of *COL1A2* by 3.5-fold, with concomitant increases in the mRNA (threefold) and protein (4.2- and 3.5-fold) levels of SMAD3 and SMAD4, respectively. It also caused a 60% decrease in SMAD7 expression. Ski, a member of the Ski/Sno oncogene family, is colocalized in the nucleus with SMAD4. Acetaldehyde induces translocation of Ski and SMAD4 to the cytoplasm, where Ski undergoes proteasomal degradation, as confirmed by the ability of the proteasomal inhibitor lactacystin to blunt up-regulation of acetaldehyde-dependent *COL1A2*, but not of the nonspecific fibronectin gene (*FN1*). We conclude that acetaldehyde up-regulates *COL1A2* by enhancing expression of the transactivators SMAD3 and SMAD4 while inhibiting the repressor SMAD7, along with promoting Ski translocation from the nucleus to cytoplasm. We speculate that drugs that prevent proteasomal degradation of repressors targeting *COL1A2* may have antifibrogenic properties. *(Am J Pathol 2014, 184: 1458–1467; http://dx.doi.org/10.1016/j.ajpath.2014.01.020)*

Alcohol-induced liver fibrosis is a multifactorial event characterized by increased collagen production as a result of up-regulation of α2(I) collagen (*COL1A2*) gene.[1] This is induced primarily by its immediate oxidation product, acetaldehyde, as well as by other events occurring during alcohol metabolism, such as changes in redox state,[2] formation of free radicals and generation of reactive oxygen species,[2–4] and depletion of antioxidant defenses and generation of aliphatic aldehydes derived from lipid peroxidation (namely, 4-hydroxy-nonenal and malonyldialdehyde).[5] Because ethanol

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also increases the circulation of bacterial-derived endotoxin,[6–8] and this in turn up-regulates expression of inflammatory cytokines, the aforementioned events also play a role in the inflammatory response and therefore in fibrogenesis and complications resulting from chronic alcoholic liver disease.[7,9]

To unravel key molecular mechanisms involved in acetaldehyde-mediated up-regulation of type I collagen, we have investigated the key roles played by this ethanol metabolite in up-regulation of type I collagen genes in hepatic stellate cells (HSCs).[1] We have shown that several different transcription factors are involved in acetaldehyde-dependent up-regulation of the type I collagen genes. Although CAAT/enhancing binding protein p35C (p35C/EBPβ) is required for expression of the α1(I) collagen mRNA,[10] Sp1 and SMAD3 are essential for up-regulation of *COL1A2* gene.[11] We and others have also shown that reactive oxygen species in general,[2–4] and H_2O_2 in particular, play a key role in the acetaldehyde-elicited response and that H_2O_2 acts as a second messenger in both acetaldehyde-dependent and transforming growth factor β1 (TGF-β1)–dependent up-regulation of type I collagen genes.[2,10]

Acetaldehyde up-regulates expression of *COL1A2* gene via a *de novo* protein synthesis–independent, PI3K-dependent mechanism.[12] In addition, the early acetaldehyde-mediated effects, occurring during the first 6 to 12 hours after acetaldehyde treatment, are independent of TGF-β1.[12] However, the mechanisms whereby acetaldehyde modulates expression and activity of members of the SMAD family, including SMAD3, SMAD4, and SMAD7, leading to *COL1A2* up-regulation are not well understood. Here, we show that SMAD3 and SMAD4 are the limiting factors in *COL1A2* gene up-regulation and that this ethanol metabolite enhances expression of SMAD3 and SMAD4 at the mRNA and protein levels.

c-Ski, a homolog of v-Ski in cells and a versatile transcriptional regulator that is widely distributed in different tissues, has been reported to be a corepressor of TGF-β/SMAD signaling.[13] Binding of TGF-β to its receptor serine/threonine kinases results in the regulation of SMAD2 and SMAD3 proteins. The phosphorylated SMADs then form heteromeric complexes with a common mediator SMAD4 (co-SMAD).[14] Together, they translocate into the nucleus, where they bind to DNA and activate transcription of the target genes.[15] We further show that Ski, a member of the Ski/Sno family of oncogenes, is colocalized in the nucleus with SMAD4 and that acetaldehyde induces their cotranslocation to the cytosol, where Ski is degraded by proteasomes. We also demonstrate that inhibiting proteasomal degradation of Ski by lactacystin blunts the acetaldehyde-dependent up-regulation of *COL1A2* gene, but has no effect on expression of the nonspecific protein fibronectin.

Materials and Methods

Plasmids and Reagents

TGF-β1 was purchased from Roche Diagnostics (Indianapolis, IN). Lactacystin was purchased from Calbiochem–Novabiochem (Millipore, Billerica, MA). Acetaldehyde was purchased from Thermo Fisher Scientific (Waltham, MA). The construction of the −378COL1A2LUC chimeric plasmid containing the −378 to +54 region of *COL1A2* linked to the firefly luciferase gene has been described previously.[12] SMAD3, -4, and -7 expression plasmids cloned into the pcDNA3 cytomegalovirus expression vector (Life Technologies, Carlsbad, CA) have been described previously.[11,16] The cDNAs of COL1A2, fibronectin, and TGF-β1 have been described previously.[17–19] S14 ribosomal protein cDNA was obtained from ATCC (Manassas, VA). cDNA fragments of human SMAD3 (nucleotides 501 to 878) and SMAD4 (nucleotides 601 to 1050) were used to determine steady-state levels of SMAD3 and SMAD4 mRNAs. Nonspecific IgG rabbit, mouse, and goat polyclonal antibodies against Ski (sc-9140), SMAD3 (sc-101154), and SMAD4 (sc-1909), respectively, were obtained from Santa Cruz Biotechnology (Dallas, TX). Neutralizing antibody to TGF-β1 was obtained from Promega (Madison, WI).

HHSC Isolation and Culture

Human HSCs (HHSCs) were isolated from a consenting study subject with clinically proven normal healthy liver during gastric bypass surgery for morbid obesity as described previously.[1] Informed consent in writing was obtained from the study patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the Institutional Review Committee. For some experiments, mouse HSCs were isolated as described previously.[20] Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone; Thermo Fisher Scientific, Waltham, MA) and antibiotics. Experiments were performed in triplicate, using cells obtained from at least three different patients cultured for 2 to 8 passages.

Northern Blot Hybridization and Run-On Transcription Assay

Confluent HHSCs were placed in a serum-free medium containing glutamine, nonessential amino acids, and antibiotics. Approximately 14 hours later, acetaldehyde was added at a final concentration of 200 μmol/L. Viability of cells was estimated using the trypan blue exclusion test. In all cases, cellular viability was greater than 90%. Total RNA was extracted at 30 minutes and 1, 3, 24, and 48 hours and was processed for Northern blot hybridizations according to standard protocols.[12] Likewise, a standard protocol was used to determine rates of COL1A2, fibronectin, and TGF-β1 transcription in control and acetaldehyde-treated cells, using S14 and pBR322 DNA as controls.[11]

In some run-on transcription experiments, cells were preincubated with 30 μmol/L lactacystin for 2 hours before acetaldehyde administration; in others, cells were preincubated for 2 hours with a neutralizing antibody to TGF-β1

(Promega) or an unrelated IgG (Santa Cruz Biotechnology) at a final concentration of 5 μg/mL. To determine the effectiveness of the anti–TGF-β1 antibody, some HHSCs were also incubated with 8 ng/mL of recombinant TGF-β1 in the presence or absence of the corresponding neutralizing antibody, or an unrelated IgG, according to a protocol described previously.[21] Nuclei were isolated at 15 and 30 minutes and were used in run-on transcription assays as described previously.[12] Relative intensity of the signals was determined by laser densitometric analysis of the radiographic films. Data were corrected for loading differences, using S14 as control.

RT-PCR Analysis

Transcript levels of SMADs 3, 4, and 7 in HHSCs were measured using a quantitative RT-PCR technique (RT-qPCR). Experiments were performed as described previously.[22] Primer sequences for qPCR amplification were as follows: SMAD3 mRNA , forward 5′-GAGGGCAGGCTTGGGGAAAATG-3′ and reverse 5′-GGGAGGGTGCCGGTGGTGTAATAC-3′; SMAD4 mRNA, forward 5′-AAAGGTGAAGGTGATGTTTGGGTC-3′ and reverse 5′-CTGGAGCTATTCCACCTACTGATCC-3′; SMAD7 mRNA, forward 5′-CGAGACCCTTCTCACTCCTG-3′ and reverse 5′-GCATCCTTGGTTAGGGTCAA-3′; and GAPDH mRNA, forward 5′-GGCCTCCAAGGAGTAAGACC-3′ and reverse 5′-CTGTGAGGAGGGGAGATTTCA-3′. All reagents were purchased from Life Technologies. Relative gene expression was calculated as

$$2^{-\Delta Ct}\left[C_{T(\text{target})} - C_{T(\text{housekeeping})}\right]_{\text{time } X} - \left[C_{T(\text{target})} - C_{T(\text{housekeeping})}\right]_{\text{time } 0.} \quad (1)$$

Western Blot Analysis

Total, nuclear, and cytosolic extracts were prepared from control and acetaldehyde-treated HHSCs as described previously.[1,12] In some experiments, HHSCs were treated with 8 ng/mL recombinant TGF-β1; in other experiments, cells were preincubated for 60 minutes with 30 μmol/L lactacystin before administration of acetaldehyde or TGF-β1. Total extracts (20 μg) were separated by SDS-PAGE on 10% gels, transferred onto a nitrocellulose membrane, and probed with SMAD3 or SMAD4 goat antibodies (1:1000; Santa Cruz Biotechnology), followed by incubation with horseradish peroxidase–conjugated rabbit anti-goat IgG (1:5000; Santa Cruz Biotechnology). Nuclear and cytosolic extracts were separated by SDS-PAGE on 4% to 12% gels, transferred onto polyvinylidene difluoride membranes, and probed with Ski rabbit antibody (1:1000; Santa Cruz Biotechnology), followed by incubation with horseradish peroxidase–conjugated chicken anti-rabbit secondary antibody (1:2000; Santa Cruz Biotechnology). Proteins were detected with a NEN Life Science Products Renaissance enhanced chemiluminescence system (PerkinElmer, Waltham, MA), according to the manufacturer's recommendations.

Coimmunoprecipitation of Ski with SMAD4 and 20S Proteasome

The coimmunoprecipitation experiment was performed as described previously.[23] In brief, SMAD4 was immunoprecipitated with a SMAD4 antibody (Santa Cruz Biotechnology) bound to protein L-agarose beads (Santa Cruz Biotechnology), and the amount of Ski coimmunoprecipitated with SMAD4 was quantified by Western analysis using Ski antibody (1:1000; Santa Cruz Biotechnology).

Cell Transfections

Conditions for the preparation and transfection of plasmids into HHSCs by the calcium phosphate procedure have been described previously.[12,16] HHSCs were treated with 10% glycerol for 90 seconds, at 6 hours after transfection, and then were placed in medium containing 0.1% fetal bovine serum. Twelve hours later, acetaldehyde was added at the final concentration of 200 μmol/L, unless otherwise indicated. For some experiments, 8 ng/mL TGF-β1 (unless otherwise indicated) was added, alone or in combination with acetaldehyde. Cells were procured at 36 hours after the addition of acetaldehyde and were used to determine luciferase activity as described previously.

In some experiments, cells were preincubated for 60 minutes with 30 μmol/L lactacystin before administration of acetaldehyde. Transcriptional activity of acetaldehyde of the chimeric constructs was normalized against cotransfected pSV2CAT. Transfections were performed multiple times and in duplicate. For some experiments, HHSCs were cotransfected with 7.0 μg of the −378COL1A2LUC reporter construct and 2.5 μg of a SMAD4 vector, followed by treatment with acetaldehyde using the conditions described above. For other experiments, HHSCs were cotransfected with 7.0

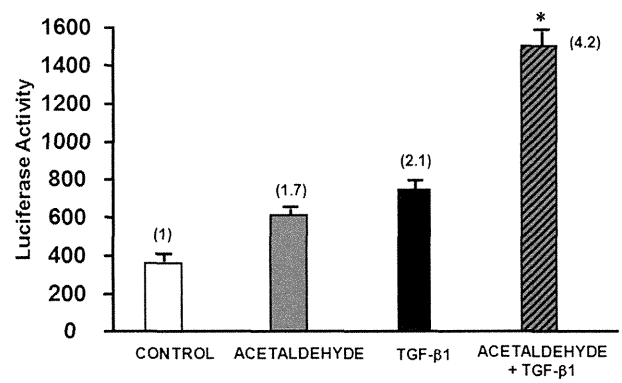

Figure 1 Effect of acetaldehyde, TGF-β1, or the two in combination on the expression of a reporter vector driven by the −378COL1A2 promoter in HSCs. First, HHSCs were transfected with the reporter vector. Next, 12 hours later, cells were incubated with either 100 μmol/L acetaldehyde, 4 ng/mL TGF-β1, or both. Then, 36 hours later, cells were harvested and luciferase activity was determined. Cells treated with the combination of acetaldehyde and TGF-β1 showed a 4.2-fold increase in reporter activity. Controls were transfected cells without treatment. Data are expressed as means ± SEM. *$P < 0.05$ versus control.

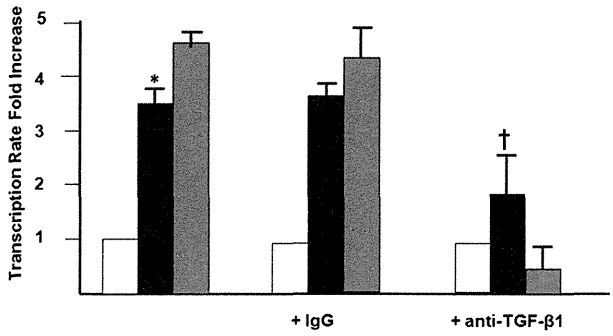

Figure 2 TGF-β1 is involved in late acetaldehyde-dependent up-regulation of *COL1A2*. Run-on transcription assays of control (white bars; untreated cells in serum-free medium) and acetaldehyde-treated (black bars) HHSCs for 24 hours in the presence or absence of a nonspecific IgG or a neutralizing antibody to TGF-β1 (gray bars; anti–TGF-β1). As a control for these experiments, some HSCs were treated with 2 ng/mL of TGF-β1 in the absence or presence of the neutralizing antibody to the cytokine. *COL1A2* gene transcription was up-regulated 3.5-fold by acetaldehyde, whereas the neutralizing antibody to TGF-β1 strongly inhibited *COL1A2* gene transcription in cells treated with acetaldehyde by 60%. The nonspecific immunoglobulin had no effect. Data are expressed as means ± SEM. $^{*}P < 0.05$ versus control. $^{\dagger}P < 0.05$ versus acetaldehyde-treated cells.

μg of the −378COL1A2LUC reporter construct and 2.5 μg of vectors overexpressing SMAD3, SMAD4, both SMAD3 and SMAD4, or SMAD7, followed by treatment with acetaldehyde using the conditions described above.

Immunostaining

Mouse HSCs plated on coverslips were fixed with 4% paraformaldehyde for 10 minutes, permeabilized with −20°C cold acetone for 30 minutes, rinsed with PBS, and blocked with 3% bovine serum albumin–PBS for 1 hour. After blocking, cells were incubated overnight with a goat polyclonal antibody against Ski (1:10) and rabbit polyclonal antibody against SMAD4 (1:10), followed by the appropriate fluorophore-conjugated secondary antibody for 1 hour (1:300); between acetaldehyde procedures, coverslips were washed with 0.1% Tween 20–PBS and mounted on glass slides with Gel-Mount medium (BioMeda, Foster City, CA). Images were captured using an AX70 light microscope (Olympus, Tokyo, Japan) and were processed with Photoshop software version 5.0.2 (Adobe Systems, San Jose, CA).

Statistical Analysis

Statistical differences between experimental groups were analyzed by Student's *t*-test (normally distributed data with equal variances) or *U* test (normally distributed data with different variances). All *P* values of ≤ 0.05 were considered significant. Data represent three to six independent experiments using HHSCs obtained from a single patient. Data are expressed as means ± SEM.

Results

Acetaldehyde and TGF-β1 Have an Additive Effect on Expression of a Reporter Vector Driven by the Acetaldehyde-Responsive Element −378COL1A2LUC

Because TGF-β1 also induced expression of SMADs 3 and 4, and given that the acetaldehyde and TGF-β1 responsive elements are localized in the same region of the *COL1A2* promoter, it was important to determine whether acetaldehyde and TGF-β1 up-regulation of *COL1A2* gene expression is additive or synergistic. To this end, we transfected HSCs with the −378COL1A2LUC reporter vector and treated these cells with 100 μmol/L acetaldehyde, 4 ng/mL TGF-β1, or both. It is important to note that the doses used were half of those normally used in our previous experiments,[12] and we therefore expected a weaker individual response. Neither acetaldehyde nor TGF-β1 alone was sufficient to up-regulate expression of the *COL1A2* reporter vector to previously observed levels (Figure 1).[12] However, cells treated with the combination of acetaldehyde and TGF-β1 responded with a 4.2-fold increase in reporter activity ($P < 0.05$), indicating that acetaldehyde and TGF-β1 have an additive stimulatory effect on the activity of *COL1A2* reporter vector.

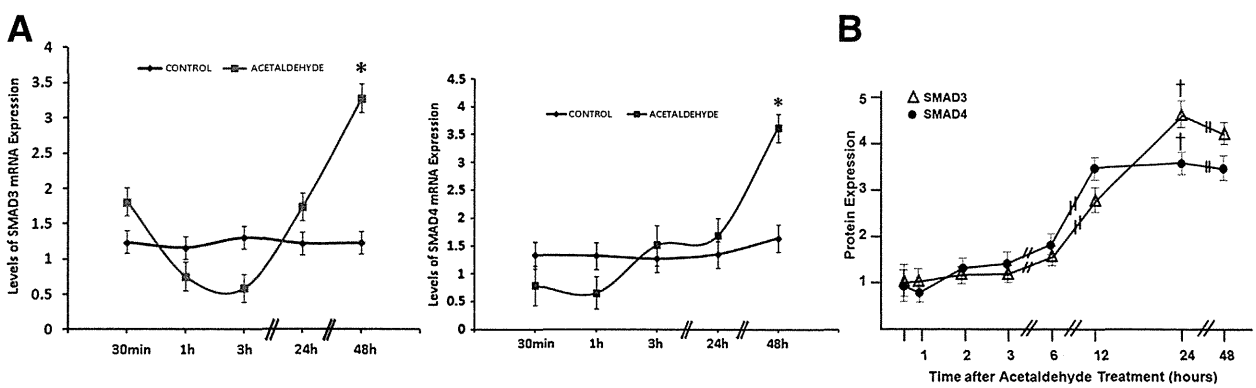

Figure 3 Increase in SMAD3 and SMAD4 mRNA and protein is delayed after acetaldehyde treatment. **A:** Time-course analysis of SMAD3 and SMAD4 mRNA expression levels after acetaldehyde treatment. Total RNA obtained from HHSCs cultured in the absence or presence of 200 μmol/L acetaldehyde for times ranging from 30 minutes to 48 hours was used to determine steady-state levels of SMAD3 and SMAD4 mRNAs by using RT-PCR analysis. **B:** Replica dishes were used to obtain cell extracts and to determine levels of SMAD3 and SMAD4 protein by Western analysis. Data are expressed as means ± SEM. $^{*}P < 0.05$ versus control (**A**); $^{\dagger}P < 0.05$ versus 30-minute time point (**B**).

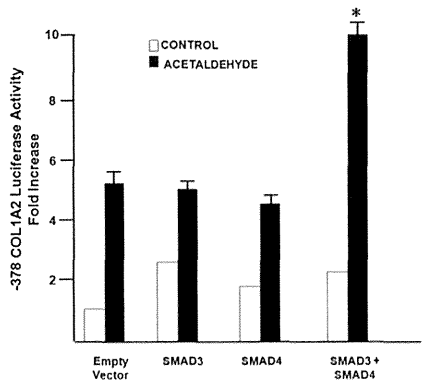

Figure 4 SMAD3 and SMAD4 are limiting factors for acetaldehyde-mediated *COL1A2* up-regulation in HSCs. Effect of acetaldehyde on luciferase activity of HSCs transiently cotransfected with 7 μg −378COL1A2LUC reporter vector and either a control empty cytomegalovirus vector or 2.5 μg expression vectors for SMAD3 or SMAD4. At 12 hours after transfection, cells were treated with 200 μmol/L acetaldehyde for 36 hours. Cells were harvested and used to measure luciferase activity. Up-regulation of either SMAD3 or SMAD4 alone does not significantly enhance reporter activity induced by acetaldehyde; however, in HHSCs cotransfected with both the reporter vector and the two vectors expressing SMAD3 and SMAD4, reporter activity increased twofold, compared with that induced by acetaldehyde alone or cells expressing either SMAD3 or SMAD4. Data are expressed as means ± SEM. $*P < 0.05$.

The Late Acetaldehyde-Dependent Up-Regulation of COL1A2 Transcription Is Mediated by TGF-β1

We have shown that acetaldehyde is directly responsible for the early expression of α2(I) collagen mRNA and for changes in cell signaling occurring early (before 6 to 12 hours). However, the late events appear to be associated with acetaldehyde-induced expression of TGF-β1. To test this possibility, we measured the late acetaldehyde-dependent transcription of *COL1A2* gene in the presence of a TGF-β1 neutralizing antibody. We used a nonspecific immunoglobulin as a control for these experiments. Transcription of *COL1A2* was up-regulated 3.5-fold by acetaldehyde ($P < 0.05$) (Figure 2). Whereas the neutralizing antibody to TGF-β1 strongly inhibited *COL1A2* gene transcription of cells treated with acetaldehyde by 60% ($P < 0.05$), the nonspecific immunoglobulin had no effect.

Acetaldehyde Induces the Late Expression of SMAD3 and SMAD4 mRNAs in HSCs

We have already shown that acetaldehyde induces formation of SMAD3/4 complexes that bind to the *COL1A2* promoter and phosphorylates SMAD3.[12] Thus, we considered it important to investigate whether acetaldehyde induces expression of SMADs 3 and 4 and whether this is an early event directly induced by acetaldehyde per se or a late event resulting from up-regulation of TGF-β1. We determined the time course of expression of SMAD3 and SMAD4 mRNA and protein in HHSCs incubated with acetaldehyde. Acetaldehyde up-regulated the mRNA expression of SMADs 3 and 4 approximately threefold ($P < 0.05$ for both) and up-regulated SMAD3 protein expression 4.2-fold ($P < 0.05$) and SMAD4 protein expression 3.5-fold ($P < 0.05$) (Figure 3). This effect was observed by approximately 24 hours after incubation with acetaldehyde and lasted up to 48 hours, a time period that coincided with acetaldehyde-induced up-regulation of TGF-β1 mRNA expression.

Both SMAD3 and SMAD4 Are Limiting Factors in Acetaldehyde-Elicited Up-Regulation of *COL1A2*

To further test the requirement of SMAD3 and SMAD4 in acetaldehyde-elicited up-regulation of *COL1A2*, we cotransfected HHSCs with the −378COL1A2LUC reporter vector and a vector that overexpressed either SMAD3 or SMAD4 and determined the reporter activity in cells treated with or without 200 μmol/L acetaldehyde. Overexpression of either SMAD3 or SMAD4 alone did not significantly enhance reporter activity induced by acetaldehyde. However, when HHSCs were cotransfected with the reporter vector and

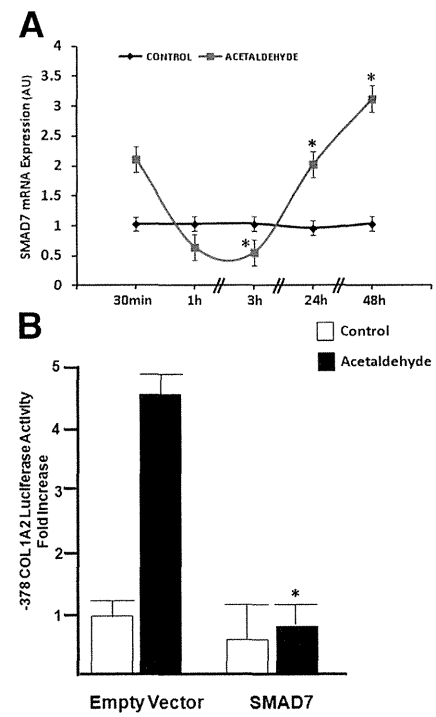

Figure 5 Acetaldehyde modulates SMAD7 expression. **A:** Total RNA was obtained from HHSCs cultured in the absence or presence of 200 μmol/L acetaldehyde for times ranging from 30 minutes to 48 hours. Acetaldehyde down-regulates the expression of SMAD7 mRNA in a time-dependent manner, reaching the maximum down-regulation of 60% after 3 hours, relative to the value at 30 minutes, after which time expression increases steadily and reaches values threefold above basal levels by 48 hours. SMAD7 mRNA expression levels were corrected for differences in loading using *GAPDH* as a housekeeping gene. $*P < 0.05$ versus control. **B:** HHSCs were transiently cotransfected with the −378COL1A2 reporter plasmid and a cytomegalovirus-driven SMAD7 expression vector. At 12 hours after transfection, cells were treated with 200 μmol/L acetaldehyde for 36 hours. Cells were harvested and used to measure luciferase activity. Acetaldehyde failed to up-regulate the expression of *COL1A2* reporter vector in HHSCs cotransfected with a vector overexpressing SMAD7 and the −378COL1A2LUC reporter vector. Data are expressed as means ± SEM. $*P < 0.05$ versus acetaldehyde-treated empty vector. AU, arbitrary units.

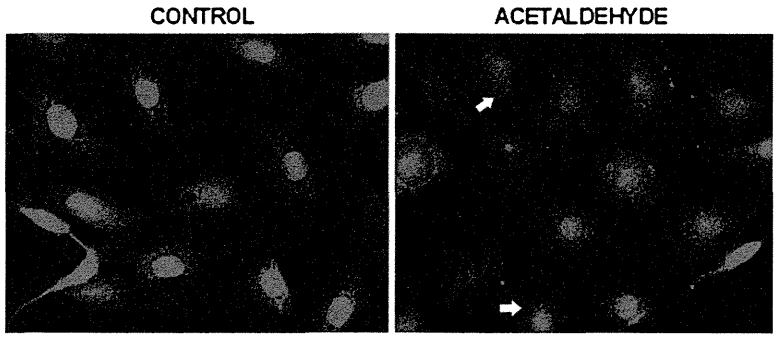

Figure 6 Acetaldehyde modifies cellular distribution of Ski. Immunofluorescence microscopy of mouse HSCs cultured in the absence or presence of 200 μmol/L acetaldehyde for 30 minutes. Cells were fixed and stained with a rabbit anti-Ski antibody followed by a Cy3 (red) labeled anti-rabbit immunoglobulin. Although fluorescence in control untreated cells was concentrated predominantly in the nuclei, in acetaldehyde-treated cells the intensity of the nuclear signal decreased and the cytosol signal increased. In addition, acetaldehyde-treated cells appear more granular and diffused (**arrows**). Original magnification, ×20.

also the two vectors expressing SMAD3 and SMAD4, there was a twofold increase in reporter activity ($P < 0.05$), compared with that induced by acetaldehyde alone or cells expressing either SMAD3 or SMAD4 (Figure 4).

Acetaldehyde Down-Regulates Expression of SMAD7 and Overexpression of SMAD7 Blunts Up-Regulation of *COL1A2*

We and others have shown that SMAD7 is a repressor of collagen gene expression.[24,25] In the present study, therefore, we investigated whether acetaldehyde has any effect on expression of SMAD7. Acetaldehyde down-regulated expression of SMAD7 mRNA in a time-dependent manner, reaching the maximum down-regulation of 60% ($P < 0.05$) after 3 hours, relative to the value at 30 minutes, after which time it increased steadily and reached values threefold ($P < 0.05$) above basal levels by 48 hours (Figure 5A). Interestingly, when HHSCs were cotransfected with both a vector overexpressing SMAD7 and the −378COL1A2LUC reporter vector, acetaldehyde failed to up-regulate expression of *COL1A2* reporter vector (Figure 5B). Overall, these findings suggest that acetaldehyde stimulates *COL1A2* gene expression at least in part by down-regulating expression of SMAD7 mRNA.

Ski, a Known Repressor of TGF-β1–Responsive Genes, Is Colocalized with SMAD4 in the Nucleus

Ski/SnoN is a member of an oncogene family of proteins that are involved in TGF-β1–mediated transcription and repression of some cytokine target genes.[26] In mouse myoblasts, the activity of Ski is mediated primarily through its binding with SMAD4 (but not with SMAD2).[14] Having found that acetaldehyde-mediated up-regulation of *COL1A2* requires SMAD4, we next investigated whether mouse HSCs also express Ski and whether acetaldehyde has any effect on its expression and subcellular localization. Immunocytochemical analysis of control and acetaldehyde-treated mouse HSCs revealed that Ski is localized mainly in the nucleus. With acetaldehyde treatment, however, Ski is translocated to the cytosol, where it has a granular appearance and extends from the perinuclear area to the plasma membrane (Figure 6).

To determine whether Ski cytosolic translocation could be clinically relevant, we analyzed Ski status in response to acetaldehyde and TGF-β1 in HHSCs. Western blot analysis of nuclear Ski in control, acetaldehyde-treated, and TGF-β1–treated HHSCs revealed that nuclear Ski decreased by 40% ($P < 0.05$) within 15 minutes and by 80% ($P < 0.05$) after 30 minutes of TGF-β1 treatment, compared with control. By contrast, acetaldehyde treatment decreased nuclear Ski by 40% ($P < 0.05$) of the control by 15 minutes, and had not decreased further at 30 minutes (Figure 7, A and B). As a consequence of Ski nuclear translocation, cytosolic Ski increased threefold ($P < 0.001$) after 15 minutes of acetaldehyde exposure and fivefold ($P < 0.001$) after 15 minutes of TGF-β1 treatment. Significantly, cytosolic Ski increased by ninefold with acetaldehyde and TGF-β1 treatment within 30 minutes ($P < 0.05$ and $P < 0.05$, respectively) (Figure 7, C and D).

Additional fluorescence microscopy data using antibodies to simultaneously detect both Ski and SMAD4 revealed that

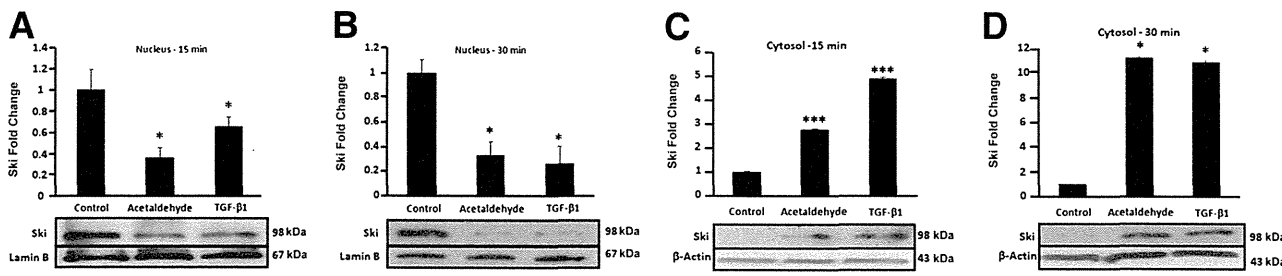

Figure 7 Acetaldehyde and TGF-β1 down-regulate the expression of Ski in HSCs. **A–D:** Western analysis of Ski in nuclear proteins extracted from HHSCs at 15 (**A**) and 30 minutes (**B**) and in cytosolic extracts at 15 (**C**) and 30 minutes (**D**) after treatment with either 200 μmol/L acetaldehyde or 8 ng/mL of TGF-β1. Data are expressed as means ± SEM of triplicate experiments. $*P < 0.05$, $***P < 0.001$ versus control.

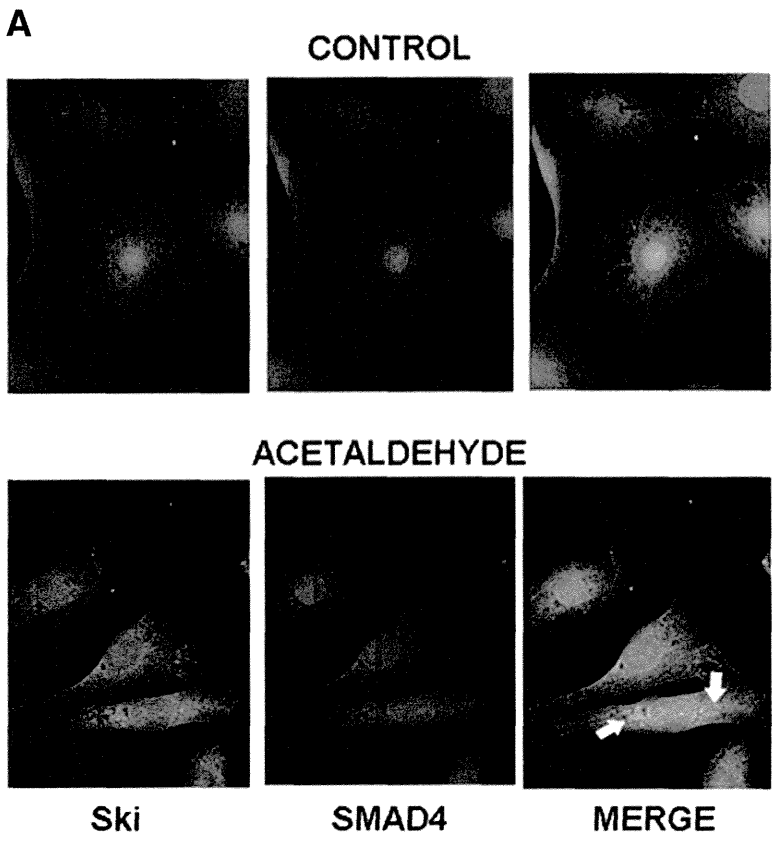

Figure 8 Ski and SMAD4 colocalize in the nuclei of HSCs and translocate to the cytosol after acetaldehyde administration. **A:** Immunofluorescence microscopy of mouse HSCs cultured in the absence or presence of 200 μmol/L acetaldehyde for 30 minutes. Cells were stained with antibodies to SMAD4 and Ski, followed by the corresponding secondary antibodies labeled with fluorescein isothiocyanate (green) for Ski and Cy3 (red) for SMAD4. Both Ski and SMAD4 localized in the nuclei of HSCs; yellow fluorescence indicates colocalization. After administration of acetaldehyde, both proteins are exported to the cytosol (**arrow**). However, a fair amount of SMAD4 is present in the nucleus (red). **B:** Immunoprecipitation (IP) of SMAD4, followed by Western analysis for the detection of Ski in HHSCs cultured in the absence or presence of 200 μmol/L acetaldehyde (**B**). Results are representative of three independent experiments. Original magnification, ×40.

SMAD4 is also localized in the nucleus of untreated HSCs. Moreover, SMAD4 appears to colocalize with Ski, and both appear to be translocated together to the cytosol after acetaldehyde treatment. However, a significant amount of SMAD4 remains in the nucleus, as demonstrated by positive staining with anti-SMAD4 antibodies (Figure 8A). To further test whether SMAD4 and Ski interact with each other, we performed coimmunoprecipitation assays using an antibody against SMAD4 and then verified whether Ski coprecipitated with SMAD4 by Western blot analysis using Ski antibody. SMAD4 and Ski formed complexes in both untreated control and acetaldehyde-treated cells (Figure 8B).

Lactacystin, an Inhibitor of Proteasomal Degradation, Inhibits Acetaldehyde-Mediated *COL1A2* Promoter-Driven Reporter Activity and Gene Transcription

Because Ski is degraded by proteasomes,[27] we investigated whether lactacystin, an inhibitor of proteasomal degradation, has any effect on the levels of Ski and on the level of response of the −378COL1A2LUC reporter vector to acetaldehyde. In HHSCs treated with lactacystin alone, Ski levels were higher than in control cells ($P < 0.05$) (Figure 9A). Furthermore, lactacystin prevented acetaldehyde-mediated decrease in nuclear Ski by approximately 50% ($P < 0.05$). Likewise, lactacystin also prevented acetaldehyde-mediated up-regulation of the −378COL1A2LUC reporter vector activity by 50% ($P < 0.05$) (Figure 9B).

To determine the specificity of this event, we tested the effect of lactacystin on acetaldehyde-induced transcription of *COL1A2* (Figure 10A) and of *FN1*, the nonspecific fibronectin gene (Figure 10B). Lactacystin prevented acetaldehyde-mediated up-regulation of *COL1A2* by 60% ($P < 0.05$), but had no effect on up-regulation of fibronectin transcription (Figure 10). This is in agreement with our previous studies suggesting that acetaldehyde-dependent regulation of collagen and of fibronectin follows different pathways,[9] and that TGF-β1–dependent up-regulation of fibronectin is SMAD independent.[28,29]

Discussion

Acetaldehyde, the first metabolite of ethanol, is fibrogenic and induces expression of both *COL1A1* and *COL1A2* genes by a mechanism dependent on the generation of H_2O_2. These events are partially reproduced by adding this reactive oxygen species to cultured HSCs and can be prevented by the addition of catalase.[2,12] Although the mechanisms whereby acetaldehyde induces generation of H_2O_2 remain to be established, our findings suggest that it may derive from mitochondrial dysfunction and not from the activation of the NADP(H) oxidase.[12] This suggestion is based on the fact

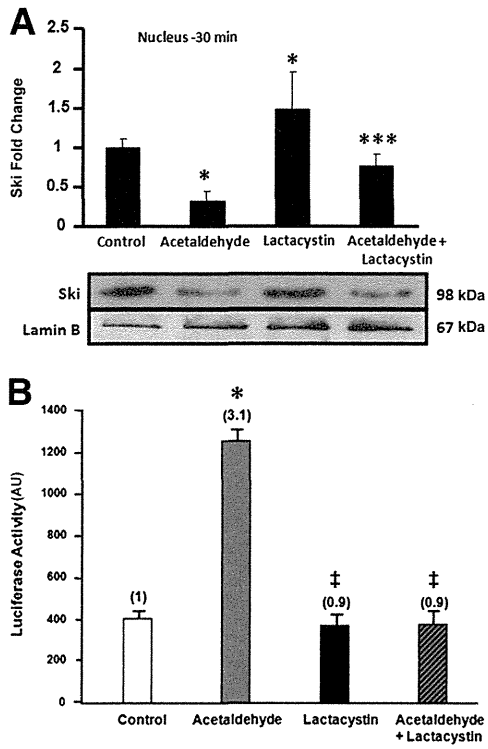

Figure 9 Lactacystin, an inhibitor of proteasomal degradation, inhibits acetaldehyde-mediated expression of *COL1A2* promoter-driven reporter activity. **A:** Western analysis of Ski in nuclear proteins extracted from HHSCs treated with 200 μmol/L acetaldehyde, 30 μmol/L lactacystin, or both. In HHSCs treated with lactacystin alone, Ski levels were higher than in control cells. **B:** HHSCs were transfected with the −378COL1A2LUC reporter vector; 12 hours later, cells were incubated with 30 μmol/L lactacystin. After 30 minutes, acetaldehyde (200 μmol/L final concentration) was added; 36 hours later, cells were harvested and luciferase activity was determined. Controls for these experiments were transfected cells without treatment, cells treated with lactacystin alone, and cells treated with acetaldehyde alone. Lactacystin prevented acetaldehyde-mediated decrease in nuclear Ski by approximately 50% and up-regulation of the −378COL1A2LUC reporter vector activity by 50%. Data are expressed as means ± SEM. $^{*}P < 0.05$ versus control. $^{\dagger}P < 0.05$ versus lactacystin treated cells. $^{\ddagger}P < 0.05$ versus acetaldehyde-treated cells.

that phenyliodonium, an inhibitor of NADP(H) oxidase, had no effect on acetaldehyde-dependent up-regulation of α1(I) collagen mRNA.[30] By contrast, ethanol is well known to cause mitochondrial alterations and redox changes.[9,31] However, these results do not rule out the possibility that other cell types, such as inflammatory and Kupffer cells, known to express NADP(H) oxidase could contribute to the overall pool of H_2O_2 *in vivo*.[8]

Previous work from our laboratory has shown that some fibrogenic actions of acetaldehyde are mediated and/or enhanced by TGF-β1.[1,12] Acetaldehyde induces expression and activation of TGF-β1 and of its type II receptor.[1,12,16] However, the early events triggered by acetaldehyde occurring within the first 6 to 12 hours after acetaldehyde administration are not dependent on TGF-β1 and protein synthesis. Furthermore, both acetaldehyde and TGF-β1 stimulate phosphorylation and binding of SMAD3/4—containing transcriptional complexes to the *COL1A2* promoter region.[12] However, in contrast to the ability of TGF-β1 to up-regulate expression of SMADs 3 and 4 at the mRNA and protein levels and to enhance the phosphorylation of SMAD2,[32] acetaldehyde has no effect on SMAD2 phosphorylation[12] and does not significantly up-regulate expression of SMAD2 protein. In the present study, although neither acetaldehyde nor TGF-β1 alone had significant stimulatory effect, the combination elicited the maximum additive stimulatory activity of *COL1A2* reporter vector (Figure 1).

Overall, these findings strongly suggest that the fibrogenic signaling pathways triggered by acetaldehyde and TGF-β1 are distinctly different. The fact that the stimulatory effect of acetaldehyde after 24 hours on *COL1A2* gene expression was significantly blunted by TGF-β1 neutralizing antibody, but not by a nonspecific antibody (Figure 2), confirms that acetaldehyde-induced expression of TGF-β1 is responsible for the up-regulation of *COL1A2*. That the concomitant expression of both SMAD3 and SMAD4 (Figure 3) is obligatory for this effect of acetaldehyde on TGF-β1 expression is shown by the fact that neither SMAD3 nor SMAD4 vector overexpression alone caused acetaldehyde-induced stimulation of *COL1A2* reporter activity, whereas in combination the two SMADs caused marked stimulation (Figure 4). On the other hand, the ability of acetaldehyde to up-regulate *COL1A2* reporter activity in

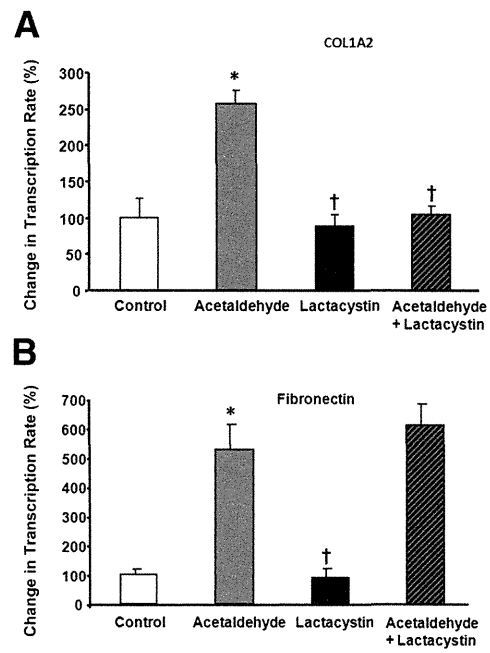

Figure 10 Lactacystin blocks acetaldehyde-mediated *COL1A2* gene transcription, but not acetaldehyde-mediated fibronectin gene transcription. **A** and **B:** Lactacystin prevented acetaldehyde-mediated up-regulation of *COL1A2* by 60% ($P < 0.05$) (**A**), but had no effect on the up-regulation of fibronectin transcription (**B**). Controls are assigned a value of 100%. All values were corrected for loading differences using an S14 ribosomal protein cDNA. Data are expressed as means ± SEM, representative of three independent experiments. $^{*}P < 0.05$ versus control. $^{\dagger}P < 0.05$ versus acetaldehyde-treated cells.

normal HSCs, but not in SMAD7-overexpressing HSCs (Figure 5), indicates that the action of acetaldehyde involves the suppression of SMAD7, a potent repressor of *COL1A2* gene expression.

Our immunocytochemical studies demonstrated that Ski is preferentially localized in the nuclei of activated HSCs (Figures 6 and 7) and that acetaldehyde induces translocation of the repressor protein Ski from the nucleus to the cytoplasm by forming a complex with SMAD4 and thereby leading to the activation of nuclear SMAD4 (Figure 8). The key repressor role of Ski in the regulation of *COL1A2* gene expression was clearly established (Figures 9 and 10). Lactacystin, a potent proteasomal inhibitor, blunted acetaldehyde-dependent up-regulation of *COL1A2* gene transcription and reporter activity of a luciferase vector driven by the acetaldehyde-responsive element of *COL1A2*, but had no effect on the induction of housekeeping fibronectin gene transcription.

Thus, we have shown that, in addition to up-regulating *COL1A2* gene expression by enhancing expression and activity of positive transactivators, acetaldehyde also down-regulates expression of the gene transcription inhibitor SMAD7. More importantly, acetaldehyde effectively promotes the translocation of the other transcription repressor, Ski, from the nucleus to the cytoplasmic compartment to undergo proteasomal degradation via the ubiquitin pathway. Our findings suggest that transcriptional up-regulation of *COL1A2* by acetaldehyde occurs via two distinct mechanisms. The first occurs very rapidly, is transient, and involves the elimination of repressors of *COL1A2* (such as Ski and SMAD7) and the phosphorylation of SMAD3. The second mechanism is more sustained and corresponds to the expression of TGF-β1 and consequent up-regulation of SMAD3 and SMAD4, a process that starts after 6 to 12 hours of exposure to acetaldehyde. Based on these new findings, as well as on earlier work,[16] we speculate that the cytokine TGF-β1 augments acetaldehyde-dependent expression of *COL1A2*. This suggestion is also supported by the fact that the effects of acetaldehyde and TGF-β1 are additive.

We have shown here that acetaldehyde induces a rapid down-regulation of SMAD7. This SMAD is a negative regulator of the *COL1A2* gene,[24,25] so its decrease occurs at the time when collagen mRNA is being up-regulated. Interestingly, the expression of SMAD7 starts to increase at a time when SMAD3 and SMAD4 are being up-regulated and reaches its maximal level when the expression of *COL1A2* has leveled off or even started to decrease. These findings may explain the up-regulation and down-regulation of collagen gene expression after a single acetaldehyde dose of 200 μmol/L. Thus, although collagen mRNA is up-regulated when repressors are down-regulated, collagen gene expression is shut down when the repressors return to normal levels.

The *SMAD7* promoter has binding motifs for SMAD3 and SMAD4.[33,34] Accordingly, it is conceivable that the up-regulation of SMAD7 mRNA by acetaldehyde results from the late up-regulation of SMAD3 and SMAD4 and subsequent binding to the *SMAD7* promoter region. Taken together, our findings suggest that the regulation of the *COL1A2* by acetaldehyde involves the concerted actions of several transactivators and repressors, as observed in the present study. In the basal state, several repressors limit *COL1A2* expression and thus control the amount of type I collagen present in the normal extracellular matrix. However, acetaldehyde induces degradation of these repressors, thus allowing the binding of transcriptional activators involved in *COL1A2* up-regulation. As shown here and previously,[1,11,12] this process occurs via rapid phosphorylation of SMAD3 and formation of SMAD3/4 complexes that interact with Sp1.

In conclusion, based on the present findings we speculate that Ski plays a major role in the fibrogenic action of acetaldehyde by sequestering the SMAD4–Ski complex from the nucleus to the cytoplasm, leading to the proteasomal degradation of Ski via the ubiquitin pathway and consequent activation of SMAD4. Activated SMAD4 and phosphorylated SMAD3, the two key transcription factors, in turn up-regulate *COL1A2* in the nucleus by interacting with its promoter region. Thus, we suggest that the proteasomal degradation of Ski is an important event in acetaldehyde-mediated up-regulation of *COL1A2* in HSCs. The present findings unveil additional critical steps in the acetaldehyde-mediated fibrogenic process and thus suggest possible new targets for antifibrogenic therapy.

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A novel small compound accelerates dermal wound healing by modifying infiltration, proliferation and migration of distinct cellular components in mice

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ABSTRACT

Background: Impaired wound healing in skin ulcer is one of the major medical issues in the aged society. Wound healing is a complex process orchestrated by a number of humoral factors and cellular components. TGF-β is known to stimulate collagen production in dermal fibroblasts while inhibiting proliferation of epidermal keratinocyte. A screening of small compounds that suppress type I collagen production in fibroblasts has identified HSc025 that antagonizes the TGF-β/Smad signal.
Objective: We examined the effects of HSc025 on dermal wound healing and elucidated the underlying mechanisms.
Methods: Effects of HSc025 on the wound closure process were evaluated in a murine full-thickness excisional wound healing model. Cell proliferation and migration were estimated using primary cultures of human keratinocytes and fibroblasts. Comprehensive analyses of gene expression profiles were performed using untreated and HSc025-treated fibroblasts.
Results: Oral HSc025 administration suppressed macrophage infiltration and accelerated wound closure as early as at day 2 after the dermal excision. Treatment of cultured keratinocytes with HSc025 counteracted the inhibitory effects of TGF-β on cell proliferation and migration. On the other hand, HSc025 stimulated migration, but not proliferation, of dermal fibroblasts independently of TGF-β. Experiments using an artificial dermis graft revealed that HSc025 stimulated migration of collagen-producing cells into the graft tissue. A cDNA microarray analysis of untreated and HSc025-treated fibroblasts identified pirin as a critical mediator accelerating fibroblast migration.
Conclusion: HSc025 accelerates wound healing by modifying infiltration, proliferation and migration of distinct cellular components, which provides a novel insight into the therapy for intractable skin ulcer.

1. Introduction

Impaired wound healing in skin ulcer is one of the major medical issues in the recent aged society [1]. The elderly population is growing faster than any other age groups in the developed countries, and the increased age is a major risk factor for insufficient wound healing due to several underlying conditions such as malnutrition, local ischemia, and low daily life activity [2]. In addition, diabetes,

Abbreviations: TGF-β, transforming growth factor-β; IFN-γ, interferon γ; IFN-β, interferon β; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; siRNA, small interfering RNA; EGFP, enhanced green fluorescent protein; H–E, hematoxylin–eosin.

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obesity, and even some medications also cause a delay in wound healing. Some patients cannot be subjected to surgical treatment because of their poor general conditions, thus the opened wounds are susceptible to ischemia, infection, fasciitis and osteomyelitis, possibly leading to life-threatening complications such as disseminated intravascular coagulation. A number of methods have been developed to treat such an intractable disease, which include the usage of anti-bacterial, debridement, irrigation, vacuum-assisted closure, oxygenation, and moist wound healing [3]. However, none of them is enough for dealing with all of the difficult cases, and a novel treatment strategy based on the mechanisms of dermal wound healing is an eager social and medical desire.

Dermal wound healing is a complex process orchestrated by a number of humoral factors and cellular components [4,5]. Wound closure can be achieved by granulation tissue formation in the dermis and re-epithelialization in the epidermis, where fibroblasts and keratinocytes play critical roles, respectively. Fibroblasts are responsible for initiating angiogenesis, epithelialization and collagen formation, and differentiate into myofibroblasts that cause tissue contraction [5].

Among a number of growth factors that orchestrate the complex sequence of cell migration, division, differentiation, and protein expression, transforming growth factor-β (TGF-β) and its intracellular mediators, Smad proteins, have been implicated in both physiological wound healing and pathological fibrosis. In normal wound healing, TGF-β produced by platelets, macrophages and lymphocytes recruits inflammatory cells, stimulates angiogenesis, and up-regulates collagen synthesis [4]. However, despite the initial prediction that the blockade of the TGF-β signal may suppress wound healing by inhibiting collagen production in the granulation tissue, a study using Smad3-null mice has clearly revealed accelerated wound healing compared with wild type animals [6]. These results therefore indicate that the cellular and molecular mechanisms are similar, but not identical, between physiological wound healing and pathological fibrosis. For example, our recent study has shown differential contribution of dermal resident and bone marrow-derived cells to collagen production during wound healing and dermal fibrosis in mice [7].

We have been studying growth factors and cytokines that antagonize the TGF-β/Smad signal as well as their implication in the treatment of organ fibrosis [8]. Among those factors, interferon γ (IFN γ) is well known to suppress progression of organ fibrosis. We have identified YB-1 as a downstream effector of IFN γ to repress transcription of human type I α2 collagen gene (*COL1A2*) [9]. Nuclear translocation of YB-1 by IFN γ antagonized the TGF-β/Smad3 signal in regulating *COL1A2* transcription *in vitro* [10], and adenovirus-mediated overexpression of YB-1 driven by the enhancer/promoter regions of murine counterpart gene (*Col1a2*) suppressed progression of liver fibrosis and enhanced the anti-fibrotic effect of IFN γ *in vivo* [11]. More recently, we have demonstrated that a novel small compound HSc025 stimulates nuclear translocation of YB-1 and ameliorates experimental fibrosis in several organs including skin, lung and liver [12,13].

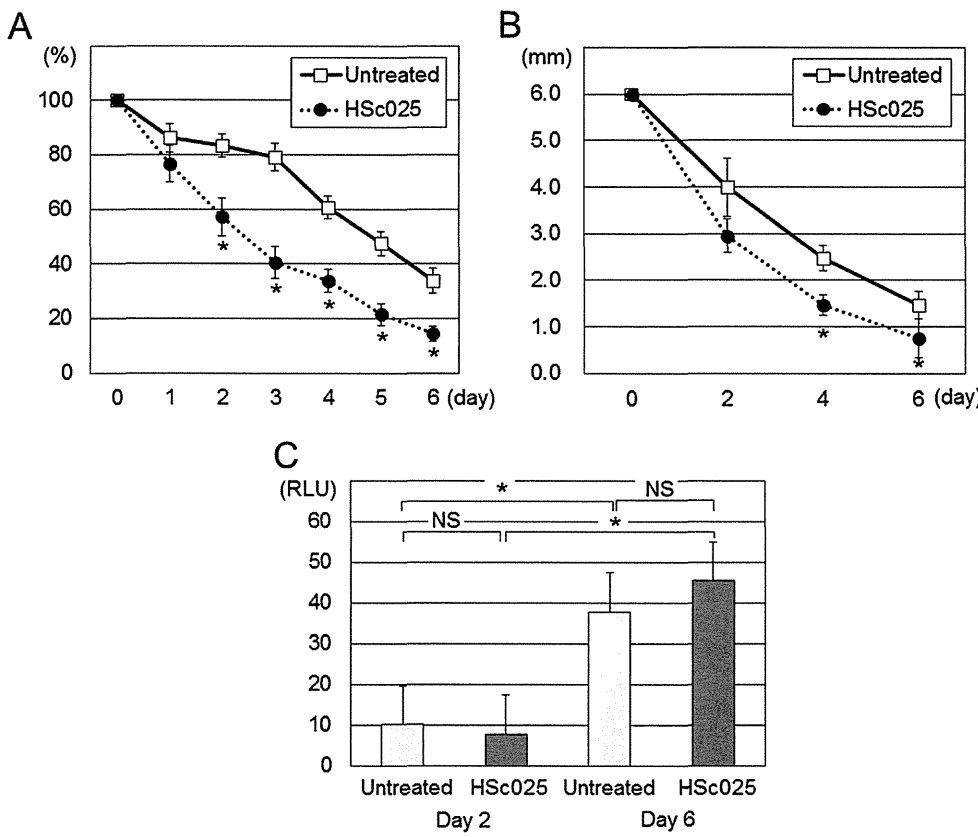

Fig. 1. Wound closure and activation of *Col1a2* promoter after a full-thickness dermal excision. Female COL/LUC mice (20 to 28 weeks of age) underwent a full-thickness 6 mm excisional wounding. They were either untreated or treated with daily oral administration of 75 mg/kg of HSc025. The wound closure was monitored everyday by measuring the area of opened wounds (A). The wound tissues were taken at day 2, 4 and 6, and subjected to H–E staining or luciferase assays to measure the distance between the both sides of wound edges (B) and *Col1a2* promoter activity (C), respectively. Luciferase activity was normalized against the protein concentration of tissue homogenates. The values are expressed as means ± SE from eight wounds in each group. An *asterisk* indicates that the difference between the groups is statistically significant. *RLU*, relative luminescence units; *NS*, not significant.

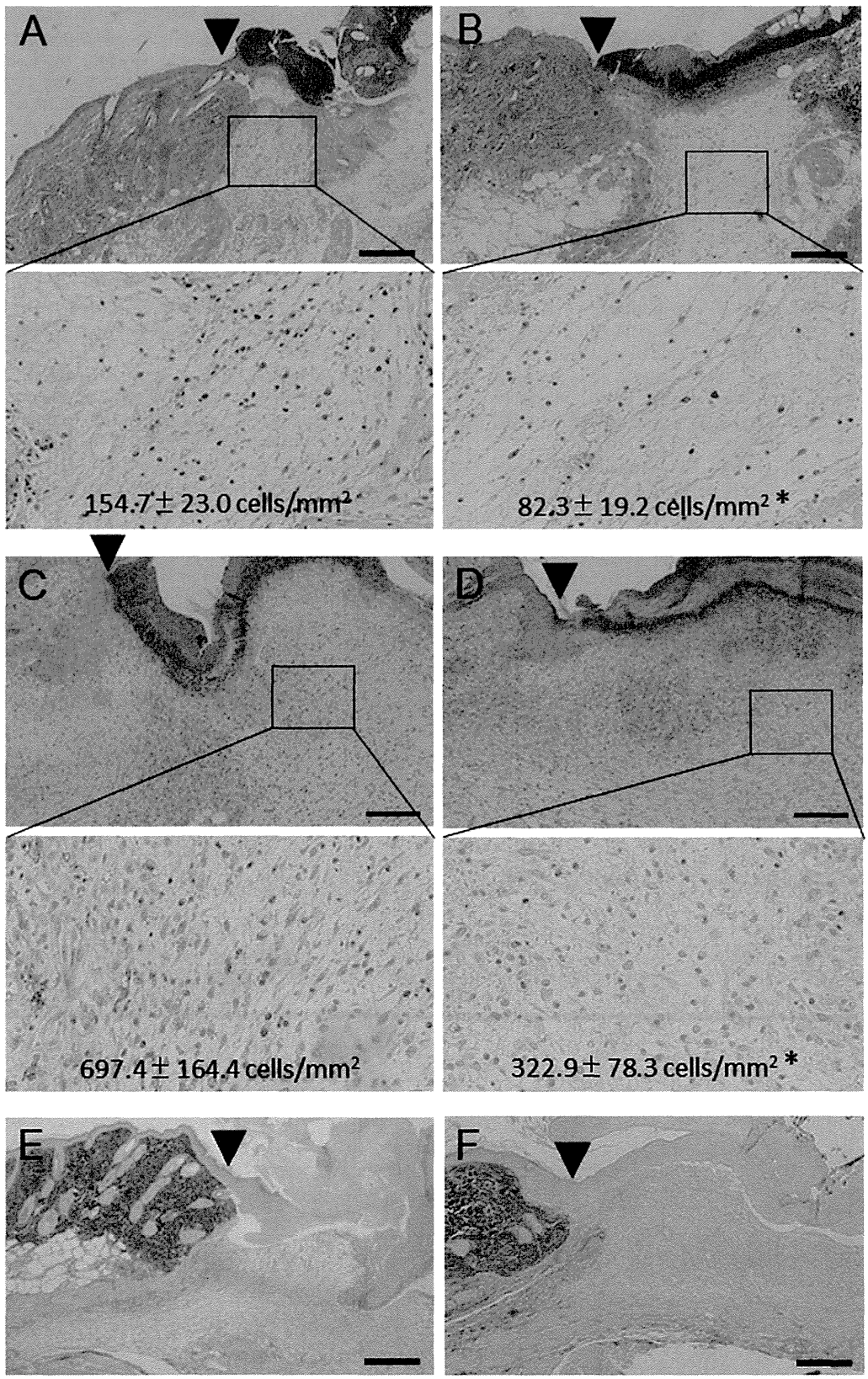

Fig. 2. Histological findings of dermal wound tissues during the healing process. Dermal specimens were obtained from transgenic COL/LUC mice either untreated (A, C and E) or treated with daily oral administration of 75 mg/kg of HSc025 (B, D and F), at day 2 (A and B), day 4 (C and D), or day 6 (E and F) after the wounding. They were subjected to H–E staining (A–D) or Sirius red–Fast green FCF staining (E and F). Representative pictures are shown from eight wounds in each group at day 2 or from six wounds in each group at day 4 and day 6. The wound margins are indicated by *arrowheads*. Pictures taken from the serial sections immunostained with F4/80 antibodies are presented under

However, it is virtually unknown whether HSc025 influences the physiological wound healing process.

The present study was aimed at examining whether HSc025 exerts any effects on the process of wound healing and, if so, what are the underlying mechanisms responsible for the action of HSc025. A combination of a murine excisional wound healing model *in vivo* and experiments using primary cultures of human keratinocytes and fibroblasts *in vitro* indicated that HSc025 accelerated the wound closure process by modifying infiltration, proliferation and migration of distinct cellular components. Moreover, pirin was identified as a critical mediator that stimulated fibroblast migration following HSc025 treatment. The results of the present study lead to better understanding of cutaneous pathophysiology and provide a novel insight into the therapy for intractable skin ulcer.

2. Materials and methods

2.1. Cell culture and reagents

Primary human keratinocytes were purchased from Kurabo, Inc. (Tokyo, Japan) and maintained in Epidercell medium supplemented with 0.1% insulin, 0.5% human epidermal growth factor, 0.1% hydrocortisone, 0.1% gentamycin and amphotericin B, and 0.25% bovine pituitary extract. Primary human fetal fibroblasts (CF37) were described previously [14], and grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. HSc025 was developed and synthesized by Sumitomo Chemical Co. Ltd., and its suppressive effects on skin, lung and liver fibrosis were previously described [12,13]. Stock solution of HSc025 was made by diluting it with dimethyl sulfoxide and stored at 4 °C until use. Recombinant human TGF-β1 was purchased from HumanZyme Inc. (Chicago, IL).

2.2. Cell proliferation and migration assays

Keratinocyte and fibroblasts were grown in 96-well plates until they reached a 30% confluent state, and then treated with different concentrations of HSc025 in the absence or presence of 2.5 ng/ml of TGF-β. After 48 h, cell proliferation was estimated using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). To examine the effects of HSc025 on migration of keratinocytes and fibroblasts, cells were cultured in 6-well plates with culture inserts (ibidi, Tokyo, Japan) set in the bottom of each well. Cells were grown until a confluent state, when the insert was removed to make a strip of area without any cells adhered. After the removal of the inserts, different concentration of HSc025 was added into the culture media with or without 2.5 ng/ml of TGF-β. Cells were further cultured until the cells filled up the vacant areas, while monitoring the cell migration every 6 to 12 h.

2.3. Microarray analysis and real time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from cultured CF37 fibroblasts either untreated or treated with HSc025. After cDNA was synthesized using reverse transcriptase, comprehensive analyses of gene expression profiles were performed using Human Genome U133 Plus 2.0 Array (Affymetrix Inc., Santa Clara, CA). Expression of mouse *pirin* gene was quantified by using MESA Blue qPCR Mastermix (Eurogentec, Seraing, Belgium) as previously described [15]. The PCR primers used were; (forward) 5′-AAAGGGTGGA-CAAGCTTCATT-3′ and (reverse) 5′-CCAAGCACTGCTGTGTGATG-3′. The relative expression levels of *pirin* mRNA were normalized against those of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene in the same RNA preparation.

2.4. Preparation and transfection of small interfering RNA (siRNA)

The sense strand sequence of siRNA used for targeting *pirin* was CCCAUGGCCUACAACUGUGGGUUAA. A non-specific siRNA was used as a control. CF37 fibroblasts were transfected with 2 μg of siRNA using Lipofectamine 2000 (Invitrogen, San Diego, CA) as previously described [10]. Further transfections were repeated every 24 h using the same amount of siRNA for a total of three days before starting the migration assays.

2.5. Western blot analysis

Whole cell lysates were prepared from CF37 fibroblasts and immunoblotted as previously described [9] with rat monoclonal anti-pirin antibody (Cell Signaling, Danvers, MA). Equal loading of protein was confirmed by re-blotting with anti-β-actin antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

2.6. Mice and wound healing model

Transgenic mouse strains that contain the −17,000 to −15,500 tissue-specific enhancer and the −350 to +54 promoter of mouse *Col1a2* upstream sequence linked to either firefly *luciferase* (COL/LUC) or *enhanced green fluorescent protein (EGFP)* reporter gene (COL/EGFP) have previously been described [16,17]. Mice were anesthetized by an intraperitoneal injection of a mixture of tribromo-ethanol (Wako, Tokyo, Japan) and xylazine (Bayer HealthCare, Leverkusen, Germany). After shaving the dorsal hairs, full-thickness excisional wounds were made on the dorsum of mice using a 6 mm biopsy punch (Kai Medical, Tokyo, Japan). Each mouse was housed individually in a sterilized cage and given autoclaved food and redistilled water in order to prevent bacterial infection. HSc025 (75 mg/kg body weight/day) suspended in 1% carboxyl methylcellulose was administered orally every day until the wounds were excised at day 2, 4, 6, or 9 after the wounding. Obtained tissues were subjected to RNA preparation, luciferase assay, and histological examination. All animals received humane care, and the experiments were approved by the Animal Experiment Committee of Tokai University.

2.7. Luciferase assay

Wound specimens obtained from COL/LUC mice were assayed for luciferase enzyme activity to quantify the activation of *Col1a2* promoter. Luciferase assays were performed as previously described [18], and the transcriptional activity was normalized against the protein concentration of the tissue homogenates.

2.8. Histological examination

Excised wound specimens were fixed with 10% buffered formalin, and stained with hematoxylin–eosin (H–E) or Sirius

high magnification below the corresponding H–E pictures together with the mean number of macrophages infiltrating into the wound bed at day 2 (A and B) and day 4 (C and D). The number of F4/80-positive cells was counted in high-power fields throughout the wound bed. An *asterisk* indicates that the cell number in HSc025-treated mice is significantly smaller than that in untreated animals. *Scale bars*, 200 μm.

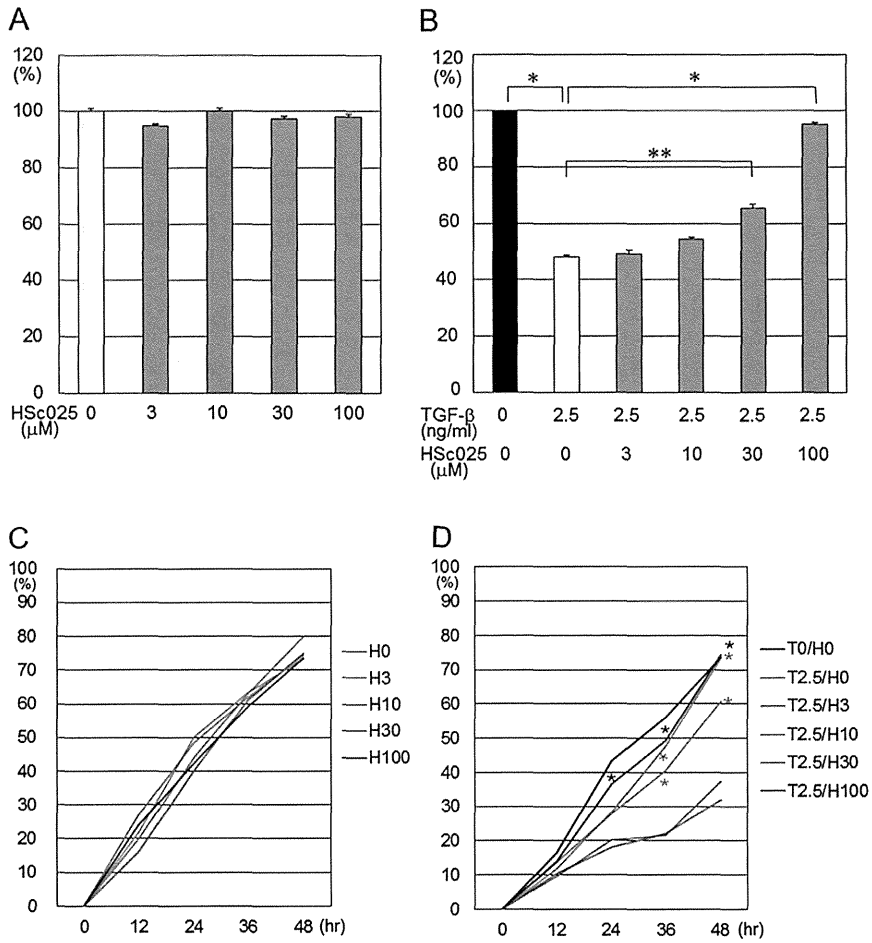

Fig. 3. Effects of HSc025 treatment on proliferation and migration of primary keratinocytes. Primary culture of human keratinocytes were either untreated or treated with the indicated concentrations of HSc025 (*H0* to *H100*) in the absence (*T0*, A and C) or the presence of 2.5 ng/ml of TGF-β (*T2.5*, B and D). They were subjected to cell counting assays after 48 h (A and B) or monitored for cell migration every 12 h (C and D). The values are expressed as means ± SE (A and B) or means (C and D) from five samples in each group. An *asterisk* indicates that the difference between the groups is statistically significant (B; $^{*}<0.01$ and $^{**}<0.05$), or the value is significantly higher than that in TGF-beta-treated control (T2.5/H0) at each time point (D; $^{*}<0.05$).

red–Fast green FCF. Immunohistochemical staining of macrophages, neutrophils or lymphocytes was performed as previously described [20] with antibodies against F4/80 (AbD Serotec, Raleigh, NC), Myeloperoxidase (MPO)-1 (Lab Vision Corp., Fremont, CA), and CD3 (AbD Serotec), respectively.

2.9. Migration of collagen-producing cells into artificial dermis grafts

A full-thickness excisional wound made on the dorsum of transgenic COL/EGFP mice was embedded with an artificial dermis graft, Terudermis (Olympus-Terumo Biomaterials, Tokyo, Japan), which is made of a single layer of low antigenic atelocollagen adhered to a silicone membrane. It reconstructs dermis-like tissue at the site of skin defects, and acts as a scaffold for the infiltrating cells and newly formed capillaries [19]. Mice were then given a daily injection of saline or HSc025 (200 μl of 100 μM solution) into the grafts, and were sacrificed at day 7 after the wounding. Obtained graft tissues were analyzed under a fluorescent microscopy Axio plan II (Carl Zeiss, Jena, Germany) to observe EGFP fluorescence. The specificity of EGFP signal was verified using a confocal laser-scanning microscope, LSM 510 META (Carl Zeiss) as previously described [20].

2.10. Statistical analysis

Values were expressed as the mean ± SE. Man–Whitney's *U* test was used to evaluate the significance of differences between the groups. *P* values less than 0.05 were considered significant.

3. Results

3.1. HSc025 accelerated dermal wound healing

Mice treated with daily oral administration of Hsc025 exhibited a faster wound closure than the untreated animals as early as at day 2 after a dermal excision when estimated by measuring the area of opened wound (Fig. 1A). Tissue samples were taken at day 2, 4 and 6, and the distance between the both sides of wound edges was measured using H–E-stained sections. Consistent with the macroscopic findings described above, the mean distance in HSc025-treated mice already showed a tendency to be shorter than that in untreated animals at day 2, and the difference became statistically significant at day 4 and day 6 (Fig. 1B). Immunohistological staining with anti-F4/80 antibodies indicated that HSc025 significantly suppressed macrophage infiltration into the

wound bed at day 2 (Fig. 2A and B) and day 4 (Fig. 2C and B). On the other hand, there was no significant difference between the two groups in the infiltration of neutrophils or lymphocytes (data not shown). Sirius red staining did not show any difference between the two groups in the amount of collagen accumulated in the granulation tissues at day 6 (Fig. 2E and F).

3.2. Activation of collagen gene promoter was not altered by HSc025 administration

Then we performed experiments to quantify the activation of *Col1a2* promoter in granulation tissues taken at day 2 and day 6 after the wounding. As shown in Fig. 1C, luciferase enzyme activities were significantly higher at day 6 than at day 2 in both untreated and HSc025-treated mice. However, there was no significant difference between the two groups at each time point. These data were consistent with the histological findings of Sirius red staining that showed similar degree of collagen accumulation between the two groups (Fig. 2E and F).

3.3. HSc025 counteracted the TGF-β-suppressed proliferation and migration of keratinocytes

The above *in vivo* experiments revealed that HSc025 significantly suppressed macrophage infiltration and accelerated the wound closure process. Then we conducted a series of *in vitro* experiments to examine whether HSc025 acts on the behaviors of keratinocytes and fibroblasts which also play critical roles in dermal wound healing. When primary cultures of human keratinocytes were treated with different concentrations of HSc025 for 48 h in the absence of TGF-β, HSc025 showed no effect on the growth of keratinocytes (Fig. 3A). On the other hand, treatment with 2.5 ng/ml of TGF-β significantly suppressed proliferation of keratinocytes, and simultaneous administration of 30 μM or higher concentrations of HSc025 counteracted this inhibitory effect of TGF-β in a dose-dependent manner (Fig. 3B). Similarly, although HSc025 did not exhibit any effect on migration of keratinocytes in the absence of TGF-β (Fig. 3C), 10 μM or higher concentrations of HSc025 stimulated cell migration by counteracting the inhibitory effect of TGF-β (Fig. 3D).

3.4. HSc025 stimulated migration, but not proliferation, of fibroblasts independently of TGF-β

The same experiments were carried out to examine the effects of HSc025 on the behaviors of dermal fibroblasts. HSc025 showed no effect on proliferation of primary cultures of fibroblasts either in the absence (Fig. 4A) or presence (Fig. 4B) of 2.5 ng/ml of TGF-β. In contrast, 3 μM or higher concentrations of HSc025 significantly stimulated migration of fibroblasts in the absence of TGF-β

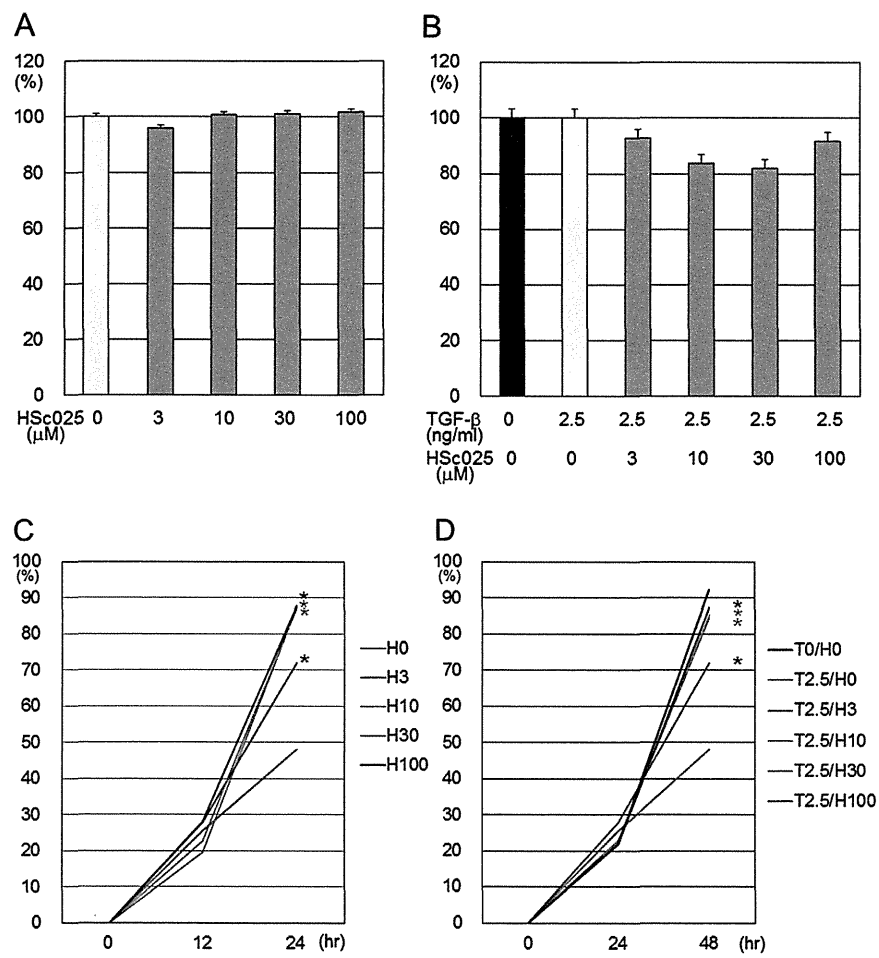

Fig. 4. Effects of HSc025 treatment on proliferation and migration of primary dermal fibroblasts. Primary culture of human dermal fibroblasts were either untreated or treated with the indicated concentrations of HSc025 (*H0* to *H100*) in the absence (*T0*, A and C) or the presence of 2.5 ng/ml of TGF-β (*T2.5*, B and D). They were subjected to cell counting assays after 48 h (A and B) or monitored for cell migration every 12 h (C and D). The values are expressed as means ± SE (A and B) or means (C and D) from five samples in each group. An *asterisk* indicates that the value is significantly higher than that in TGF-β-treated control (*T2.5/H0*) at each time point (D).