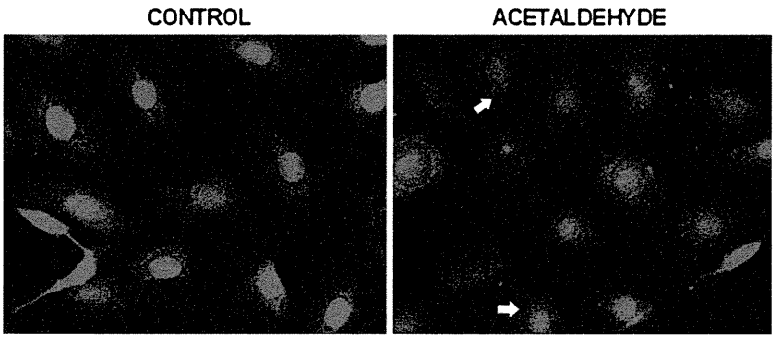

Figure 6 Acetaldehyde modifies cellular distribution of Ski. Immunofluorescence microscopy of mouse HSCs cultured in the absence or presence of 200 μmol/L acetaldehyde for 30 minutes. Cells were fixed and stained with a rabbit anti-Ski antibody followed by a Cy3 (red) labeled anti-rabbit immunoglobulin. 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

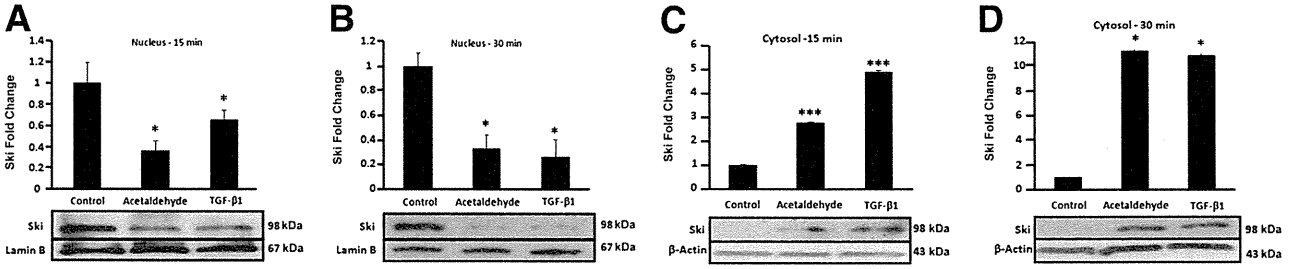

Figure 7 Acetaldehyde and TGF-β1 down-regulate the expression of Ski in HSCs. **A–D:** Western analysis of Ski in nuclear proteins extracted from HHSCs at 15 (**A**) and 30 minutes (**B**) and in cytosolic extracts at 15 (**C**) and 30 minutes (**D**) after treatment with either 200 μmol/L acetaldehyde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

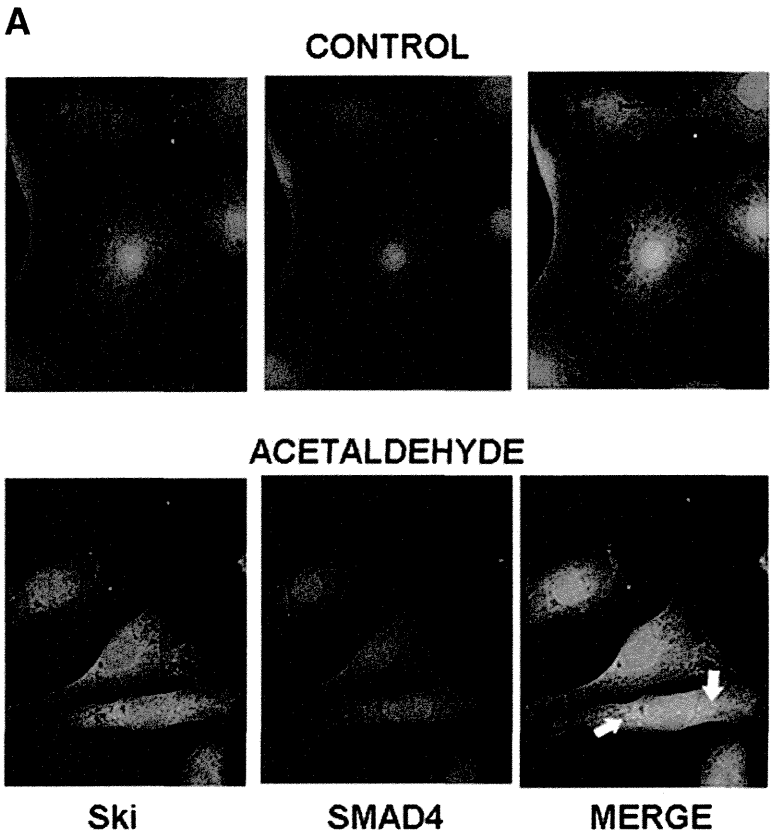

Figure 8 Ski and SMAD4 colocalize in the nuclei of HSCs and translocate to the cytosol after acetaldehyde administration. **A:** Immunofluorescence microscopy of mouse HSCs cultured in the absence or presence of 200 μmol/L acetaldehyde for 30 minutes. Cells were stained with antibodies to SMAD4 and Ski, 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

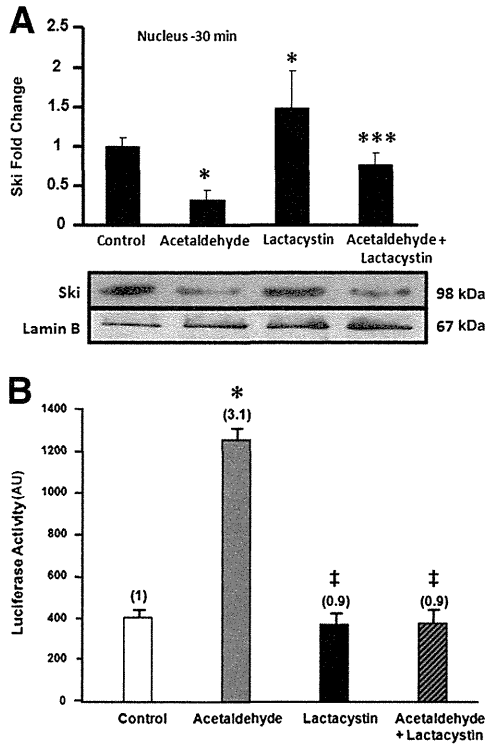

Figure 9 Lactacystin, an inhibitor of proteasomal degradation, inhibits acetaldehyde-mediated expression of *COL1A2* promoter-driven reporter activity. **A:** Western analysis of Ski in nuclear proteins extracted from HHSCs treated with 200 μmol/L acetaldehyde, 30 μmol/L lactacystin, or both. In HHSCs 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

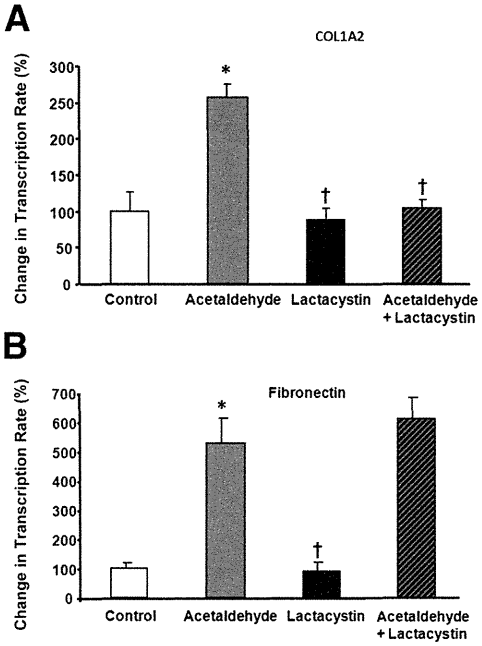

Figure 10 Lactacystin blocks acetaldehyde-mediated *COL1A2* gene transcription, but not acetaldehyde-mediated fibronectin gene transcription. **A** and **B:** Lactacystin prevented acetaldehyde-mediated up-regulation of *COL1A2* by 60% ($P < 0.05$) (**A**), but had no effect on the up-regulation of fibronectin 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].

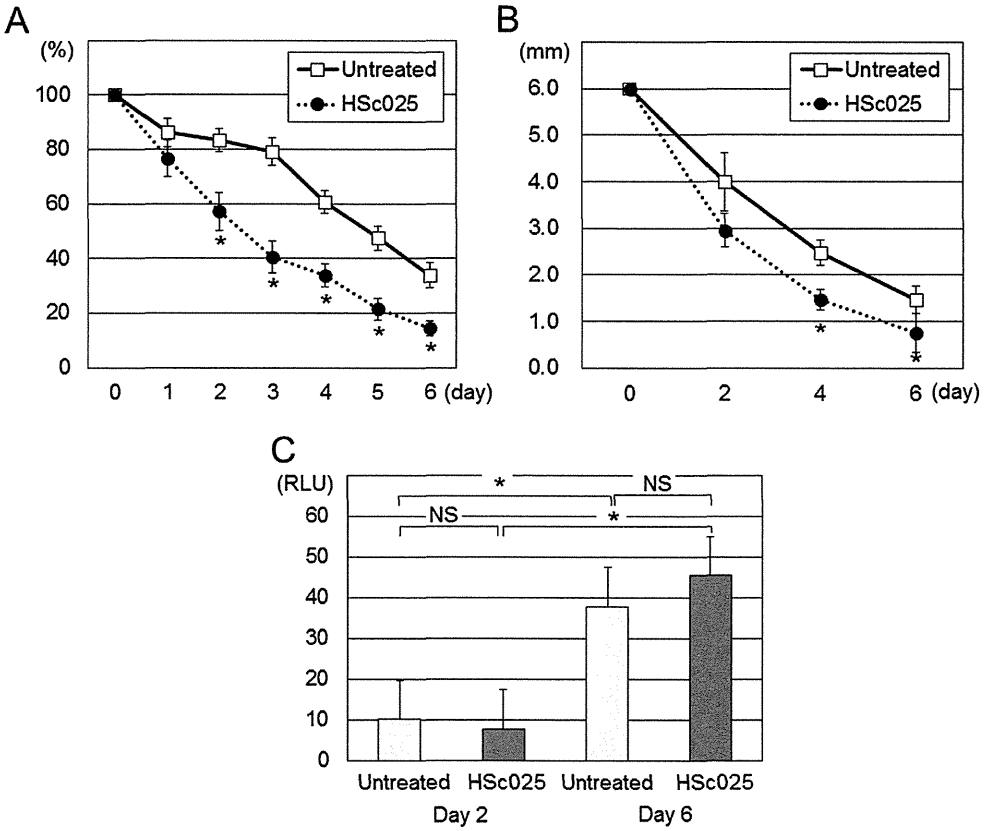

Fig. 1. Wound closure and activation of *Col1a2* promoter after a full-thickness dermal excision. Female COL/LUC mice (20 to 28 weeks of age) underwent a full-thickness 6 mm excisional wounding. They were either untreated or treated with daily oral administration of 75 mg/kg of HSc025. The wound closure 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.

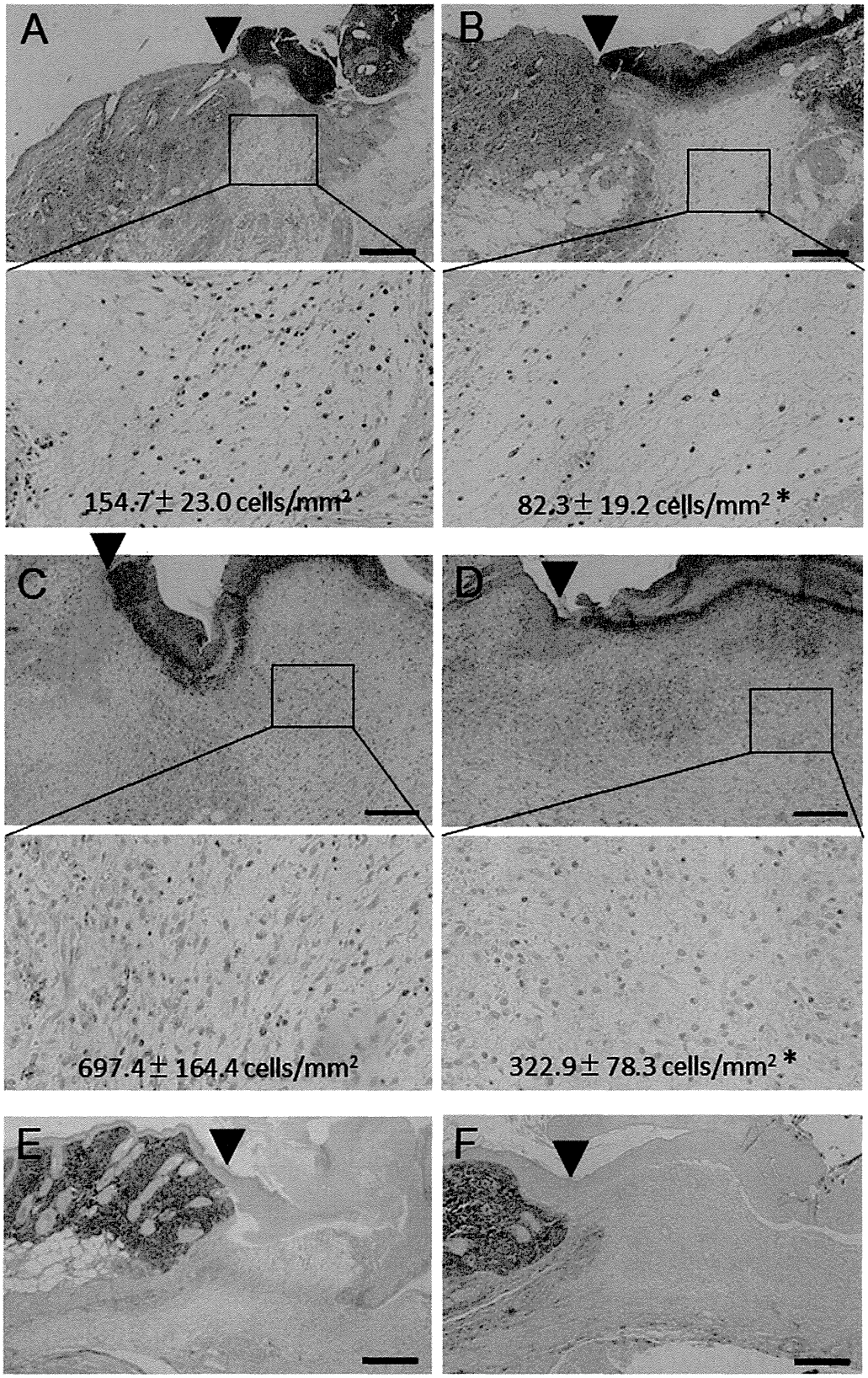

Fig. 2. Histological findings of dermal wound tissues during the healing process. Dermal specimens were obtained from transgenic COL/LUC mice either untreated (A, C and E) or treated with daily oral administration of 75 mg/kg of HSc025 (B, D and F), at day 2 (A and B), day 4 (C and D), or day 6 (E and 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.

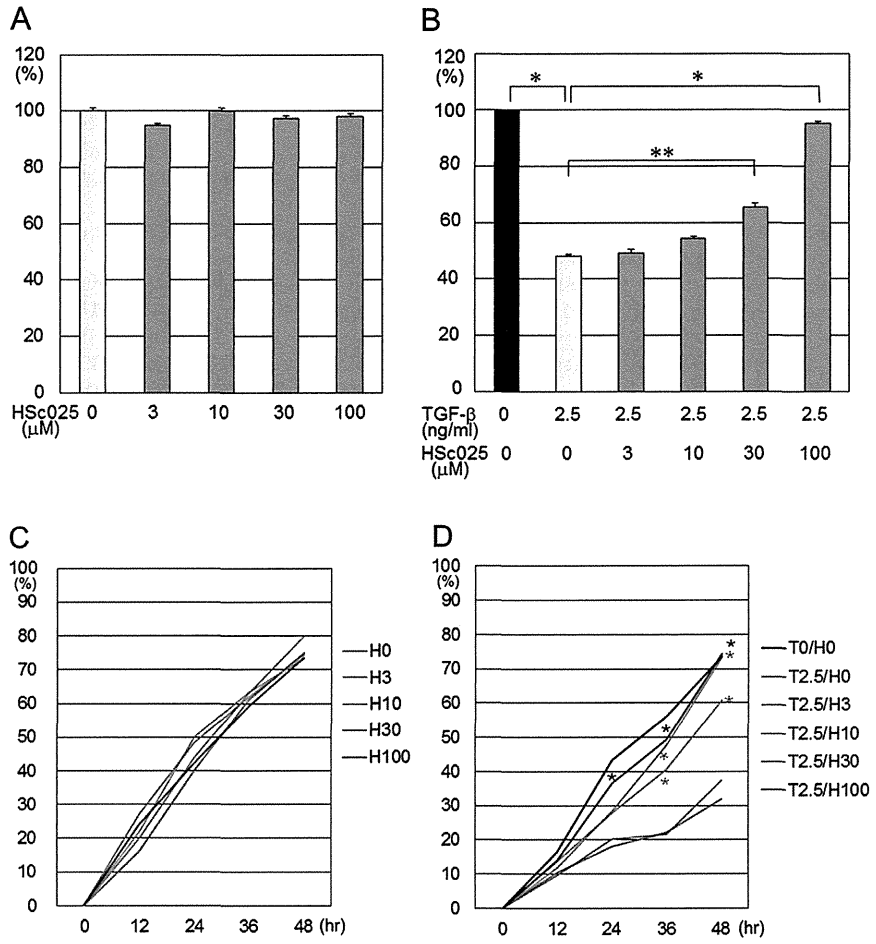

Fig. 3. Effects of HSc025 treatment on proliferation and migration of primary keratinocytes. Primary culture of human keratinocytes were either untreated or treated with the indicated concentrations of HSc025 (*H0* to *H100*) in the absence (*T0*, A and C) or the presence of 2.5 ng/ml of TGF-β (*T2.5*, 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

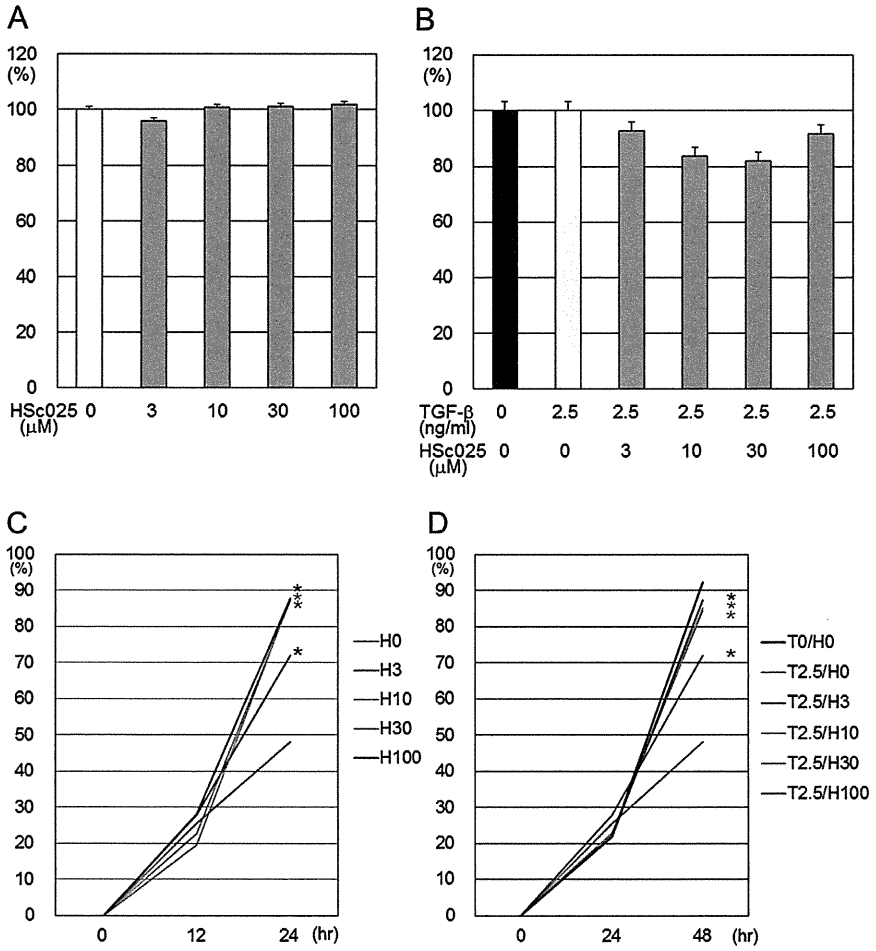

Fig. 4. Effects of HSc025 treatment on proliferation and migration of primary dermal fibroblasts. Primary culture of human dermal fibroblasts were either untreated or treated with the indicated concentrations of HSc025 (*H0* to *H100*) in the absence (*T0*, A and C) or the presence of 2.5 ng/ml of TGF-β (*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).

(Fig. 4C). This stimulatory effect of HSc025 on migration of fibroblasts was also observed in the presence of 2.5 ng/ml of TGF-β (Fig. 4D).

3.5. HSc025 enhanced migration of collagen-producing cells into granulation tissue in vivo

The stimulatory effect of HSc025 on migration of cultured dermal fibroblasts was further verified using an *in vivo* experimental system. For that purpose, an artificial dermis graft was embedded into a fresh wound made on the dorsum of transgenic COL/EGFP mice. The wound tissue including the dermal graft was excised at day 7 and subjected to fluorescent microscopic observation to detect collagen-producing cells migrating into the graft tissue. EGFP-positive cells were observed in both untreated (Fig. 5A) and HSc025-treated mice (Fig. 5B). However, the number of such collagen-producing cells was significantly higher in HSc025-treated mice than in control animals independently of the distance from the wound edge (Fig. 5C). These results confirmed that HSc025 increases the migration speed of dermal fibroblasts *in vivo* as well as *in vitro*.

3.6. Identification of pirin as a mediator of HSc25 in stimulating fibroblast migration

In order to elucidate the mechanisms responsible for the stimulatory effect of HSc025 on fibroblast migration, RNA samples were prepared from cultured dermal fibroblasts untreated or treated with HSc025 and subjected to cDNA microarray analyses (GEO ID: GSE53672). The results indicated an increase in *pirin* gene expression in cells treated with HSc025. This HSc025-induced pirin expression was confirmed at both mRNA and protein levels using real time RT-PCR assays (Fig. 6A and B) and Western blot analysis (Fig. 6C), respectively. HSc025 treatment caused a modest but significant increase in the amounts of *pirin* mRNA and protein in time- and dose-dependent manner. Similarly, real time RT-PCR assays of wound tissue samples showed an increasing tendency of *pirin* gene expression in HSc025-treated mice compared with

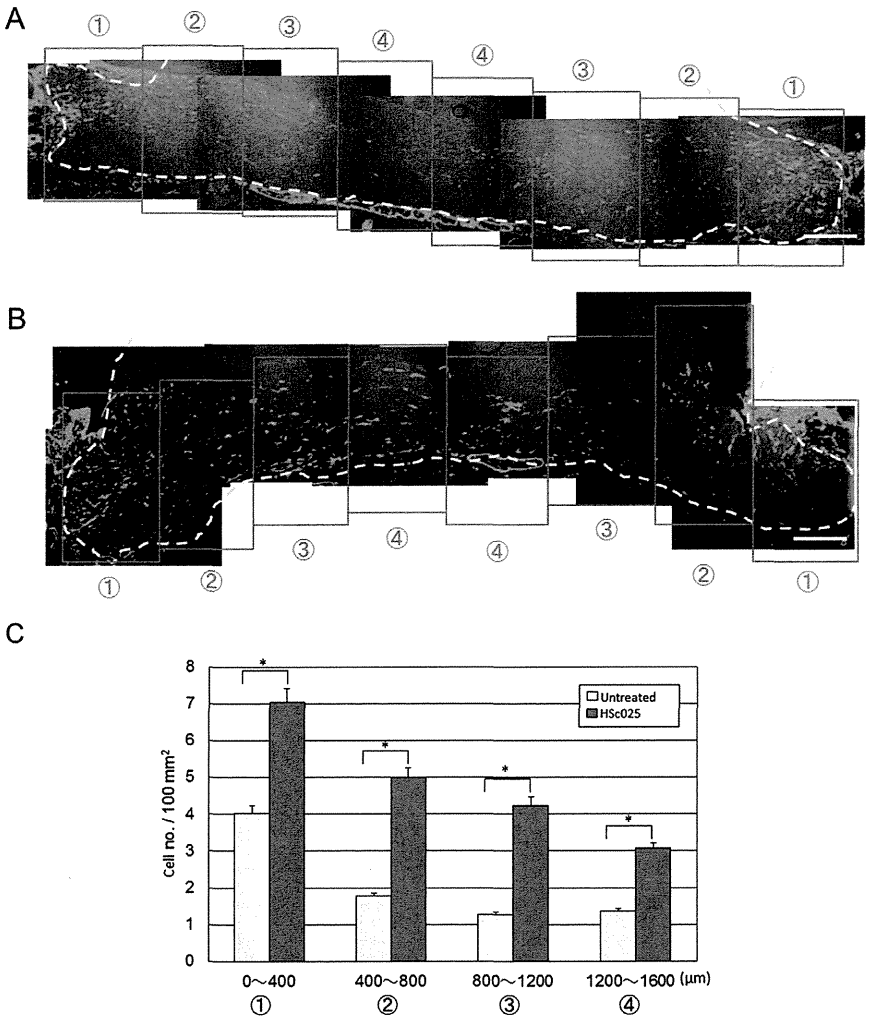

Fig. 5. Migration of collagen-producing cells into artificial dermis grafts. A full-thickness excisional wound made on the dorsum of female COL/EGFP mice (24 to 28 weeks of age) was embedded with an artificial dermis graft. Mice were then treated with daily local injections of 200 μl of either saline (A) or 100 μM of HSc025 (B) into the wounds. Dermal specimens were obtained at day 7 and analyzed under a fluorescent microscope to examine migration of EGFP-positive cells (*green*) into the artificial dermis graft. The *yellow hatched line* indicates the border between the autologous tissue and the embedded artificial dermis graft. The location of migrating EGFP-positive cells was divided into four categories (*red square boxes*) depending on the distance from the wound edge. The cell numbers were counted and compared between untreated and HSc025-treated mice (C). 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. *Scale bars*, 200 μm.

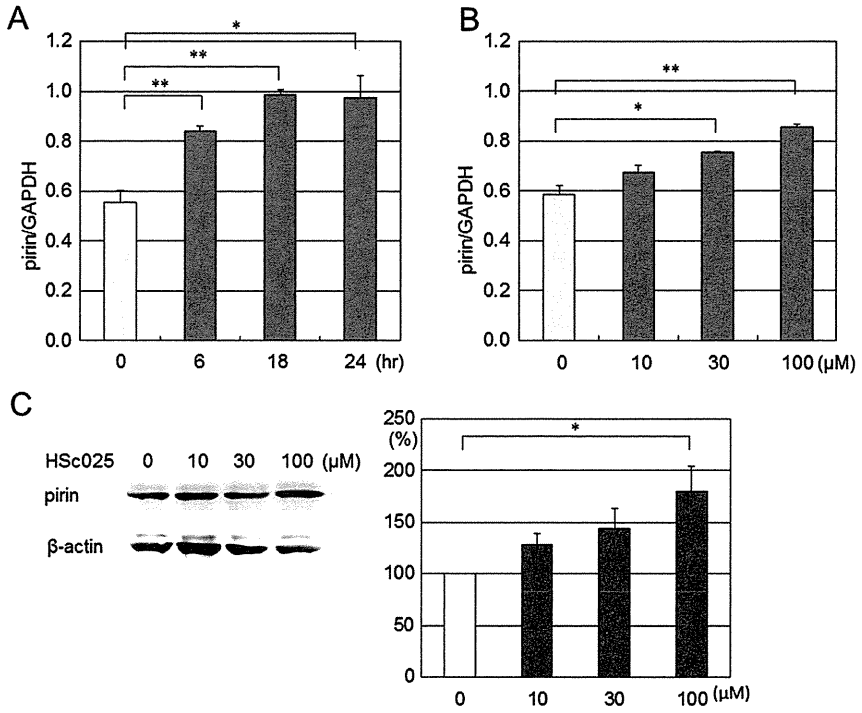

Fig. 6. Induction of pirin expression in HSc025-treated fibroblasts. Primary cultures of dermal fibroblasts were either untreated or treated with 30 μM of HSc025 for the indicated lengths of time (A), or with the indicated concentrations of HSc025 for 18 h (B) or 24 h (C). Total RNA and whole cell lysates were isolated and subjected to real time RT-PCR assays and immunoblot analyses, respectively. The relative expression levels of *pirin* mRNA were normalized against those of *GAPDH* gene in the same RNA preparation, and the values are expressed as means ± SE from one of the repeated experiments with three samples in each group (A and B). A representative picture of immunoblot analyses to detect expression of pirin and β-actin as an internal control is presented with the histograms showing the results of semi-quantitative analyses obtained from three independent experiments (C). An *asterisk* indicates that the value is significantly higher than that in pretreatment (A) or untreated control (B and C); $^{*}<0.05$ and $^{**}<0.01$.

untreated animals at both day 2 (1.3-fold) and day 6 (1.6-fold) after the wounding (data not shown).

3.7. HSc025 enhanced migration of fibroblasts through induction of pirin expression

Since the induction of pirin expression by HSc025 was marginal as described above, we examined the effect of knockdown of pirin expression on migration of cultured fibroblasts. For that purpose, we employed the RNA interference technique using *pirin* siRNA. Transfection of dermal fibroblasts with the specific *pirin* siRNA caused knockdown of the endogenous pirin production nearly 90% at the protein level (Fig. 7A). Under this experimental condition, transfection with *pirin* siRNA, but not control siRNA, completely abolished the stimulatory effect of HSc025 on migration of cultured fibroblasts (Fig. 7B). These results therefore indicate that HSc025 does not simply increase the amount of pirin, but rather modifies its effect on fibroblast migration through unknown mechanisms.

4. Discussion

In this manuscript, we have shown that a novel small compound that was originally identified as an anti-fibrotic reagent suppresses macrophage infiltration and accelerates an early phase of wound healing after a full-thickness dermal excision. Experiments using primary cultures of keratinocytes and fibroblasts indicated that HSc025 acts on the two types of cells through different mechanisms. First, HSc025 stimulated proliferation and migration of primary keratinocytes mostly by antagonizing the TGF-β signal. Consistent with the results of an early study [21], incubation with 2.5 ng/ml of TGF-β suppressed both proliferation and migration of keratinocytes. HSc025 counteracted those inhibitory effects of TGF-β, but it exhibited no effects on the keratinocyte behaviors in the absence of TGF-β. Second, and in contrast to its action on keratinocytes, HSc025 stimulated migration, but not proliferation, of primary cultures of dermal fibroblasts both in the absence and presence of TGF-β. This indicates that the stimulatory effect of HSc025 on fibroblast migration is not a consequence of accelerated cell proliferation, and that HSc025 acts on dermal fibroblasts independently of TGF-β. The stimulatory effect of HSc025 on fibroblast migration was further confirmed by an *in vivo* experiment in which HSc025 treatment certainly increased the number of collagen-producing cells migrating into an artificial dermis graft (Fig. 5).

A comprehensive analysis of the gene expression profiles of untreated and HSc025-treated cultured fibroblasts has identified pirin as a critical mediator of HSc025 that stimulates cell migration. Pirin was originally reported as a highly conserved nuclear protein of unknown functions [22], and it has been reported recently that its interaction with Bcl3 regulates migration of melanoma cells [23]. In that experiment, siRNA knockdown of pirin expression or treatment with a small molecule inhibitor (triphenyl compound A) of the pirin–Bcl3 interaction suppressed migration, but not proliferation, of melanoma cells. Moreover, treatment of melanoma cells with triphenyl compound A, as well as knockdown of the endogenous pirin and Bcl3, down-regulated *SNAI2* expression [23]. Interestingly, SNAI2 (also known as SLUG) has been implicated in mobility of various cell types including keratinocytes [24], and SLUG is considered necessary for proper re-epithelialization during wound healing [25]. The results of the present study suggest that SNAI2/SLUG may also play an important role as a downstream effector of pirin in regulating fibroblast migration. This was supported by the *in vivo* findings that *pirin*

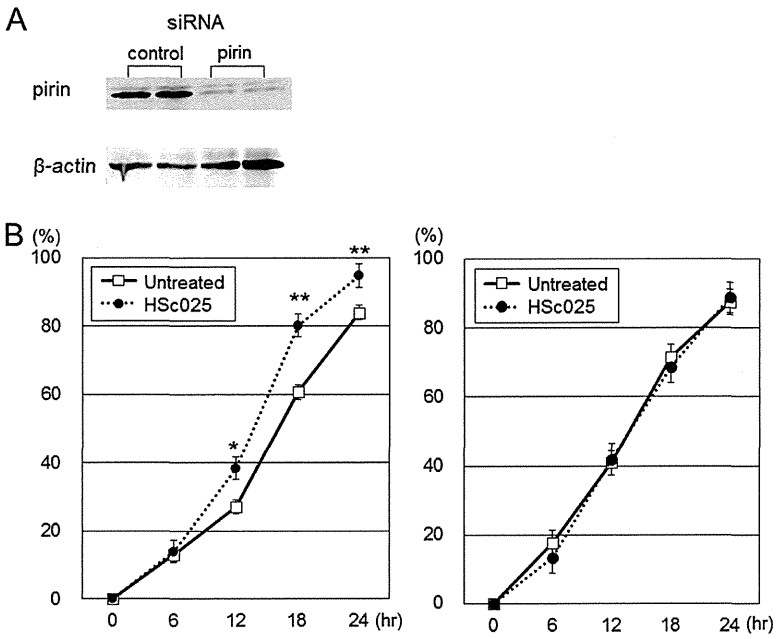

Fig. 7. Effects of knockdown of pirin expression on HSc025-stimulated fibroblast migration. Primary cultures of dermal fibroblasts were subjected to repeated transfection with 2 μg of control or *pirin* siRNA every 24 h for a total of three days. Efficient knockdown of the endogenous pirin expression was confirmed by immunoblot analyses using anti-pirin antibodies (A). Transfected cells with control (*left* in B) or *pirin* siRNA (*right* in B) were monitored for migration every 6 h. The values are expressed as means ± SE from 10 samples in each group. An *asterisk* indicates that the value of HSc025-treated sample is significantly higher than that in untreated cells at each time point; $^{*}<0.05$ and $^{**}<0.01$.

mRNA expression was up-regulated in the HSc025-treated granulation tissues following the excisional wounding.

It should be noted that, although only a marginal increase in pirin expression was induced by HSc025 treatment, knockdown of the endogenous pirin expression by siRNA transfection completely abolished the effects of HSc025 on fibroblast migration (Fig. 7). These findings may suggest that HSc025 does not increase the amount of pirin remarkably, but rather alters the intracellular localization of pirin and/or modifies its interaction with Bcl3 or other nuclear factors. Indeed, we have previously shown that HSc025 interferes with the interaction between YB-1 and its cytoplasmic anchor protein and accelerates nuclear translocation of YB-1, resulting in transcriptional regulation of target genes [13]. Further studies such as experiments using pirin knockout mice are necessary to clarify the detailed molecular mechanisms by which HSc025 enhances the action of pirin and accelerates dermal wound healing.

Early studies using TGF-β neutralizing antibodies [26] and a synthetic TGF-β antagonist [27] have shown that TGF-β is certainly a therapeutic target for accelerating wound healing as well as for reducing excessive scaring. This was confirmed by a study using Smad3-null mice, which clearly demonstrated accelerated wound healing characterized by reduced inflammatory monocyte infiltration and stimulated keratinocyte proliferation [6]. The results of the present study are consistent with those of the Smad3-null mouse experiments: HSc025 suppressed macrophage infiltration (Fig. 2) and accelerated the wound closure process (Fig. 1A and B). However, one of the concerns to use anti-fibrotic reagents for accelerating wound healing is that it may suppress the proper collagen expression and result in the decreased cutaneous tissue integrity. However, there was no significant deference in the overall collagen promoter activities in granulation tissues between untreated and HSc025-treated mice (Fig. 1C). Nor did Sirius red staining show any difference in the amounts of accumulated collagen between the two groups (Fig. 2E and F). This is probably because HSc025 increases the number of collagen-producing fibroblasts migrating into granulation tissue while decreasing the collagen expression levels in an individual cell. Alternatively, different cellular and molecular mechanisms govern the physiological wound healing and pathological dermal fibrosis [7,28], and fibroblasts present in normal and fibrotic dermal tissues respond differently to anti-fibrotic reagents such as HSc025.

It is also important to note that HSc025 accelerated an early phase of physiological healing after a full-thickness dermal excision, but did not shorten the overall period to re-epithelialization dramatically. It is of great worth to examine the effect of HSc025 in pathological conditions where dermal wound healing deteriorates significantly. Work is in progress in our laboratory to explore the detailed signaling pathway and the precise mechanisms responsible for such a beneficial effect of HSc025 on dermal wound healing, which eventually contributes to the development of a novel treatment strategy for intractable skin ulcer.

Acknowledgments

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Drs. K. Higashi, K. Sumida and K. Saito are employees of Sumitomo Chemical Co. Ltd. that has applied for patents to use HSc025 and its analog as anti-fibrotic drugs, but not as agents accelerating wound healing. This collaborative study was performed at Tokai University School of Medicine with a grant support in part from Sumitomo Chemical Co. Ltd.

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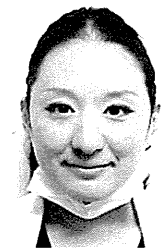

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肝線維化改善の分子・細胞基盤

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索引用語：肝線維症，Matrix metalloproteinase（MMP），Tissue inhibitor of metalloproteinases（TIMP），活性化星細胞，線維化治療

1 はじめに

かつての病理学や肝臓病学の教科書を紐解くと，肝硬変は進行性かつ不可逆的な疾患であると記載されている．ところが，他稿で詳述されるように近年のウイルス性慢性肝炎に対する治療法の進歩は，進行した肝線維症といえども可逆的な病態であることを証明した．これまで，アルコール性肝硬変の禁酒症例など，日常臨床で感じていた肝線維化の可逆性が実証された意義は大きい．組織におけるコラーゲンの含量は，その合成とMatrix metalloproteinase（MMP）による分解とのバランスの上に成り立っており（図1），コラーゲン合成を促進させる原因を取り除くことによって，進行した肝線維症であっても組織学的に改善する．また，原因治療が困難な場合であっても，コラーゲンの合成を抑制する，あるいは分解を適切に誘導することで，肝線維症は治療可能な疾患である．「進行性かつ不可逆的」であったのは，肝線維症に対する効果的な治療法が存在せず，コラーゲン産生をきたす刺激が慢性的に反復していたからに他ならない．

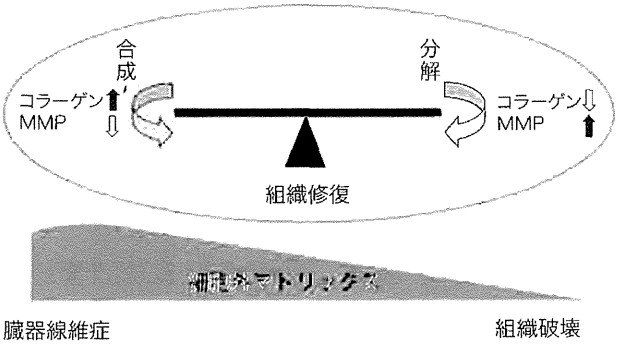

図1 コラーゲンの合成系と分解系
組織におけるコラーゲンの含量は，合成と分解のバランスの上に規定されている．その適切な発現は，組織修復や創傷治癒過程において重要な働きを演じているが，調節機構が破綻をきたすと組織に過剰のコラーゲンが沈着して諸臓器の線維化を引き起こす一方，分解系が優位に傾くと組織破壊や脆弱性が問題となる．

肝線維化の進展機序に関する研究の進歩と

Yutaka INAGAKI et al: Molecular and cellular basis for regression of liver fibrosis
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比較して，改善機序の研究はこれまで立ち遅れている感があったが，最近になって肝線維化の改善過程における活性化星細胞の動態について興味深い知見が得られるようになってきた．

本稿では，肝線維化改善の分子・細胞基盤に関する研究の現状を概説するとともに，硬変肝が正常肝にどこまで近づくかを規定する要因や，肝線維化改善機序の解明に立脚した新規治療法の開発に向けて克服すべき問題点についても言及したい．

2 肝線維化改善機序の研究の難しさはどこにあるのか

肝線維化の進展過程において中心的役割を担う星細胞の活性化機序や，活性化星細胞（筋線維芽細胞）によるⅠ型コラーゲンの産生調節機構など，肝線維化の進展機序の研究は最近の20年余りで大きな進歩を遂げた．それと比較して，肝線維化の改善機序に関する研究，とりわけコラーゲン分解系の研究が立ち遅れているのには，いくつかの理由がある．

第一に，ヒトにおいては線維肝組織に増加したコラーゲン線維を分解する主要な間質性コラゲナーゼであるMMP-1が，ラットやマウスといった齧歯類には存在しないことがあげられる．齧歯類においてはMMP-1と相同性が高いMMP-13がこれに代わる間質性コラゲナーゼとされるが，ヒトにもMMP-13は存在している．ヒトにおけるMMP-1の発現が全身の臓器・組織に広範に認められるのに対して，MMP-13の発現は極めて限局的である．多くの実験的肝線維症モデルには齧歯類が用いられているが，種々の病態において齧歯類のMMP-13がヒトのMMP-1と全く同一の発現動態や働きを示すかは不明であり，齧歯類で得られた結果がそのままヒトの肝線維症に当てはまるかは即断できない．

次に，MMPが肝線維化の進展や改善の各病期において相反する病因的意義を有することを指摘しておきたい．例えば，後述するように肝線維化の改善過程においてはMMPの発現増加や活性化が重要な役割を演じるとされるが，MMP-13遺伝子を欠損させたマウスでは肝線維化の進展はむしろ改善していた[1]．これは，肝線維化の初期段階では既存の小葉構造を破壊してⅠ型コラーゲンの沈着を促すうえでMMP-13が重要な働きを演じることを示唆しており，肝線維化の改善過程においてMMP-13が沈着したⅠ型コラーゲンを分解してむしろ線維化改善に寄与する[2]のとは好対照である．

最後に，これまでにMMPの産生細胞と報告されている星細胞（MMP-2, MMP-13）やマクロファージ（MMP-9, MMP-13）は，まさにⅠ型コラーゲン，あるいはその発現を促進するTransforming growth factor-β（TGF-β）やケモカインの産生細胞である．しかもMMPは単にコラーゲンを分解するのみならず，タンパク質分解酵素としてTGF-βの活性化反応にも深く関わっている．肝線維化の進展や改善過程において，同一細胞もしくは同一細胞集団内のコラーゲン合成と分解のシグナルがどのように相反的あるいは協調的に制御されているのかは興味深い問題である[3]．

3 肝線維化改善機序の研究はどこまで進んだか

1. 肝線維化改善過程におけるMMPとその抑制因子の発現動態

肝線維化の進展と改善におけるMMP研究は，線維肝組織中にコラーゲン分解活性を見いだした報告にその端を発する[4]．四塩化炭素の反復投与に代表される齧歯類を用いた実

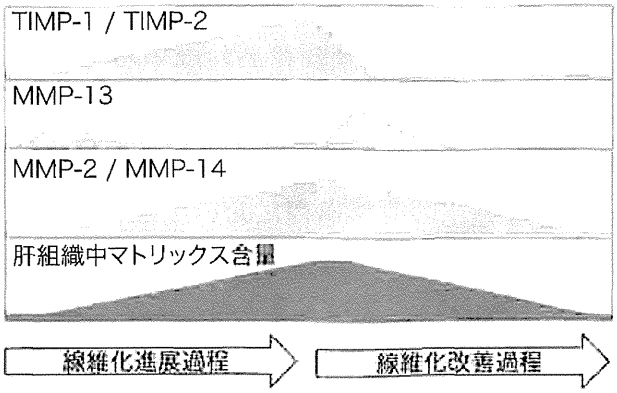

図2　肝線維化の進展ならびに改善過程におけるMMPとTIMPの発現動態

四塩化炭素の反復投与を中止すると，肝線維化の進行に伴って増加していたTIMP-1/TIMP-2の発現は速やかに低下する．この際，MMP-13は一過性に発現が増加し，その抑制因子であるTIMPの発現低下と相まってコラーゲン線維を分解する．また，MMP-2とその活性化を促すMMP-14は，TIMP-1/-2と同様に線維化進展に伴って発現が増加しているが，線維化改善過程においてはTIMPと比較してゆっくりと発現が低下するため，線維分解に寄与すると考えられている（文献2, 5, 10を統合）．

験的肝線維症の特徴は，線維化刺激がなくなると速やかにコラーゲン線維の分解と吸収がみられることである．そこで，このモデルを用いて肝線維化の改善過程におけるMMPやその抑制因子（Tissue inhibitor of metalloproteinases : TIMP）の発現動態や産生細胞が研究されてきた．

MMP-13は，先に述べたように齧歯類における主要な間質性コラゲナーゼである．その発現は正常肝ではほとんど認められず，線維化の進展過程においてもわずかな発現増加にとどまる．しかしながら，四塩化炭素の反復投与を中止するとMMP活性を抑制するTIMP-1およびTIMP-2の発現が急激に低下するため，MMP活性が増加する（図2）．しかも後述するように，この時期に一致して活性化星細胞がアポトーシスに陥るために，新たなコラーゲン産生も抑制されて線維化の改善に至るというモデルが提唱された[5]．TIMP-1の線維化改善における重要性は，TIMP-1トランスジェニックマウスを用いた研究により直接的に証明された．すなわち，TIMP-1を過剰発現すると四塩化炭素投与中止後の活性化星細胞のアポトーシスと肝組織中のMMP-2活性が抑制され，肝線維化の改善が有意に阻害された[6]．

また，同じく四塩化炭素投与モデルを用いた研究により，肝線維化の回復過程に一過性にMMP-13発現が上昇すること，その産生細胞の一部がα-smooth muscle actin（αSMA）陽性の活性化星細胞であることが報告された（図2）[2]．MMP-13の由来としては，線維組織に浸潤したマクロファージとする報告もある[7]．筆者らは，骨髄をEnhanced green fluorescent protein（EGFP）陽性細胞で置換したマウスに四塩化炭素を投与して，骨髄細胞の線維肝組織への浸潤とMMP産生について検討した．その結果，肝線維化の改善過程において骨髄から流入・生着した未分化な幹前駆細胞がMMP-13を発現しており，これらは肝組織中のMMP-13産生細胞の約半数を占めていた[8]．また，MMP-13の下流に位置し，MMP-13によって活性化されるMMP-9についても，既知の好中球やマクロファージに加えて骨髄由来の未分化な前駆細胞による産生が確認され，肝線維化の改善における骨髄と末梢の臓器相関が示された[9]．

MMP-2は，その上流に位置する活性化因子MMP-14とともに，肝線維化の進展に伴って発現が著しく増加する．その主たる基質は基底膜の構成成分であるⅣ型コラーゲンであるが，Ⅰ型コラーゲンや部分的に分解されたゼラチンに対する分解活性も有するため，TIMP発現が低下した状況下ではMMP-13と

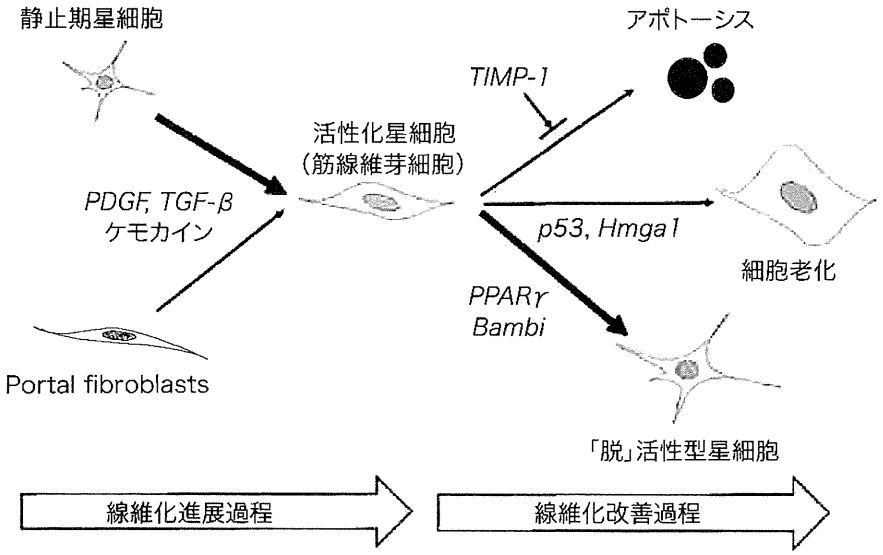

図3　コラーゲン産生細胞の活性化と「脱」活性化

肝実質内の星細胞や門脈域に存在するportal fibroblastsは，肝線維化刺激によって筋線維芽細胞(様細胞)に形質転換して，活発にコラーゲンを産生する．一方，線維化の改善過程においては，活性化した星細胞はアポトーシスや細胞老化に陥ることで排除されると考えられていたが，最近になってその約半数が非活性型に移行(「脱」活性化)することが報告された．

協調して肝線維化の改善に寄与する可能性が考えられる(図2)[10]．

2. 肝線維化改善過程における活性化星細胞の変容

前述したように，四塩化炭素の反復投与を中止して肝線維化所見が改善する際に活性化星細胞数は急激に減少し，アポトーシスに陥ると考えられてきた[5]．しかも，筋線維芽細胞のアポトーシスはMMP-14を介してMMP-2活性を増加させることから，いっそう線維化を改善させる[11]．加えて，肝線維化の改善には活性化星細胞の老化(senescence)が深く関わることが報告された[12]．細胞老化に陥った活性化星細胞はNK細胞の標的となって排除されることで，線維化の改善が促進されるという(図3)．ところが，活性化星細胞のアポトーシスや細胞老化は線維化刺激により増加し，線維化改善過程においてのみ見られる現象ではない．しかも，線維化改善過程においてアポトーシスや細胞老化に陥る細胞数は限られている[5,12]．

最近，実験的肝線維症の改善過程において活性化した星細胞の約半数は非活性型に戻る(「脱」活性化)という報告が相次いでなされた(図3)[13,14]．これまでも，培養星細胞の活性型と非活性型との相互移行については脂肪細胞の分化・脱分化過程と類似してperoxisome proliferator-activated receptor γ (PPARγ)などの転写因子による制御が報告されていたが，この可逆性が*in vivo*においても証明された意義は大きい．しかしながら，線維化改善に伴って脱活性化した星細胞は線維化刺激を全く受けていない静止期の星細胞と同一とはいえず，再度の線維化刺激に対する反応性も高いという．星細胞が有するこの可逆性を肝線維症の治療に応用するには，さらに詳細なメカニズムの解明が必要である．また，活性化星細胞以外にも，門脈域に存在するportal fibroblastsは胆管結紮モデルに代表される胆汁うっ滞に伴う肝線維症の発症と進展におい

て重要な役割を演じている．脱活性化が星細胞のみに認められる現象か，portal fibroblastsから派生した筋線維芽細胞においても共通してみられるかは，肝線維症に対する原因別テーラーメード治療の必要性を考えるうえでも重要な問題である．

4 硬変肝はどの程度まで正常肝に近づくのか

肝線維症の可逆性を論じる場合には，“Reversal”と“Regression”という2つの用語を意識して使い分ける必要がある．Reversalとは進行した肝線維症であっても正常な肝小葉構造に復する意味合いが強く，これに対してRegressionは線維化の程度に一定の改善が認められる場合を指す[15]．完成された硬変肝は，本当に正常肝に戻るのであろうか．細胞外に分泌されて組織に沈着したコラーゲン分子は，その成熟の過程で共有結合を介した架橋形成(cross-link)が完成するとマトリックス構成タンパク質としての安定性が増し，MMPによる分解を受けづらくなる[16]．また，進行した線維肝組織が正常の小葉構造に回復しうるかは，線維化進展に伴う血管走行の不可逆的変化によっても左右されるが，その詳細については他稿に譲る．ヒトの肝硬変を対象とした病理組織学的研究では，micronodular cirrhosisは線維化の改善に伴ってmacronodular cirrhosis，次いで不完全な線維性隔壁へと変化するという[17]．

近年，C型慢性肝炎の治療法がいっそう進歩するに伴って，血中からウイルスが消失してもかつて報告されたような肝線維化の改善が認められない症例が経験されるようになってきた．肥満やアルコール多飲などの他の要因が存在する症例は別としても，抗ウイルス治療法の適応拡大に伴って肝硬変を含むより進行した肝線維化症例を治療対象としていることに留意すべきである．肝線維化の進行症例では，その罹病期間の長さとコラーゲン線維の蓄積量の両面から見てもマトリックスの架橋形成の程度や血管走行の変化が著しく，たとえ原因治療が奏効しても組織に蓄積したコラーゲン線維の分解に長期間を要することは容易に想像できる．

5 コラーゲン分解機序の解明をいかに肝線維化治療につなげるか

肝線維化の進展がコラーゲン合成と分解の不均衡によってもたらされるものならば，その治療は合成系と分解系のバランスの是正に他ならない(図1)．実際，ヒトMMP-1遺伝子[18]もしくはMMP-13遺伝子[19]を，アデノウイルスベクターを用いて線維肝組織に過剰発現させることで実験的肝線維症の改善が報告されている．しかしながら，コラーゲンは組織や臓器の形態保持とともに創傷治癒や組織修復においても重要な役割を担っており，MMPの過剰な発現は組織の脆弱性や関節軟骨の破壊などを引き起こすことが懸念される．アデノウイルスは肝細胞に高い親和性を有することから他臓器への影響は少ないが，これに代わる低分子化合物などを薬剤として投与する際には，線維肝組織に選択的に運搬・作用させる工夫が必要となる．

一方，実際の臨床試験に目を向けると，薬効自体の問題や副作用の懸念のみならず，臨床研究デザインの限界が抗線維化治療薬の開発において大きな障害になっている．すなわち，さまざまな進行速度を有する多くの慢性肝疾患患者の中から比較的均一な対象集団を設定し，通常10年単位の長期経過をたどる肝線維症に対する薬物の投与効果を，1年前後という短期の介入試験で評価することの困