Proteolytic and non-proteolytic activation of keratinocyte-derived latent TGF-β1 induces fibroblast differentiation in a wound-healing model using rat skin

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Abstract

Transforming growth factor-β1 (TGF-β1) reportedly causes the differentiation of fibroblasts to myofibroblasts during wound healing. We investigated the mechanism underlying the activation of latent TGF-β1 released by keratinocytes in efforts to identify promising pharmacological approaches for the prevention of hypertrophic scar formation. A three-dimensional collagen gel matrix culture was prepared using rat keratinocytes and dermal fibroblasts. Stratified keratinocytes promoted the TGF receptor-dependent increase in α-smooth muscle actin (α-SMA) immunostaining and mRNA levels in fibroblasts. Latent TGF-β1 was found to be localized suprabasally and secreted. α-SMA expression was inhibited by an anti-α_v-integrin antibody and a matrix metalloproteinase (MMP) inhibitor, GM6001. In a two-dimensional fibroblast culture, α-SMA expression depended on the production of endogenous TGF-β1 and required α_v-integrin or MMP for the response to recombinant latent TGF-β1. In keratinocyte-conditioned medium, MMP-dependent latent TGF-β1 secretion was detected. Applying this medium to the fibroblast culture enhanced α-SMA production. This effect was decreased by GM6001, the anti-α_v-integrin antibody, or the preabsorption of latent TGF-β1. These results indicate that keratinocytes secrete latent TGF-β1, which is liberated to fibroblasts over distance and is activated to produce α-SMA with the aid of a positive-feedback loop. MMP inhibition was effective for targeting both keratinocytes and fibroblasts in this model.

Keywords: keratinocyte, fibroblast, transforming growth factor-β1, α_v-integrin, matrix metalloproteinase

Introduction

The wound healing process involves sequential interactions of many different cell types. This process is divided into three phases: inflammation, proliferation, and scar maturation (1). Following the inflammation phase, repair of the epidermal and dermal layers is initiated in which the invasion of fibroblasts, macrophages, and vascular cells into the wound area produces granulation tissue. In that tissue, fibroblasts are known to start acquiring a contractile phenotype. These predominant cells in a contracting wound were initially called myofibroblasts (2), and reportedly contribute to wound contraction (1,3). Myofibroblasts are characterized by the *de novo* expression of α-smooth muscle actin (α-SMA), which generates a contractile force in granulation tissue (4,5). This differentiation of fibroblasts toward myofibroblasts is believed to be of major importance in pathogenic scarring and fibrosis (6). The finely tuned balance between the activated and quiescent fibroblast subpopulations seems to determine scar quality (7). Therefore, pharmacological approaches to alter this balance may be beneficial for the prevention of hypertrophic scar formation and contractures.

Fibroblasts/myofibroblasts are subjected to paracrine signals from many types of surrounding cells; especially, the epithelial-mesenchymal interaction is likely to be prominent after the initial inflammatory phase (8). Epithelial cells that are located at the wound edge migrate rapidly over the granulation tissue to produce a new outer layer of skin (re-epithelialization), probably contributing to the paracrine production of growth factors and cytokines. Of particular interest is the potential of fibroblasts to undergo phenotypic changes as a result of transforming growth factor-β1 (TGF-β1) signaling. TGF-βs are known to be secreted by platelets, fibroblasts, macrophages, and keratinocytes within an injury and to act as stimulators of granulation tissue formation

and the production of myofibroblasts (3,9-11). It should be noted, however, that TGF-βs are commonly secreted as biologically inactive complexes (12,13). The small latent TGF-β complex (SLC) consists of two components, the active TGF-β homodimer and the N-terminal propeptide homodimer. The former is non-covalently associated with the latter, which is known as the latency-associated peptide (LAP). LAP binds to the latent TGF-β binding protein (LTBP) using disulfide bonds to produce the large latent TGF-β complex (LLC), which is incorporated into the extracellular matrix (ECM) (14). The ECM acts as a reservoir from which growth factors and cytokines can be released as required and modifies their availability and activity (7,13).

A previous report using a keratinocyte-fibroblast monolayer co-culture system showed that cell-cell contact between two types of cells enhanced myofibroblast formation through the activation of TGF-β (8). However, there must be some other mechanism to explain the differentiation of fibroblasts that were located far from the keratinocyte layers. Such an inconsistency may be solved by considering the proteolytic degradation of the LLC, which reportedly releases the latent form of TGF-β from the ECM (15,16). Serine proteases, such as plasmin, and matrix metalloproteinases (MMPs), such as MMP-2 and -9, can release latent TGF-β complexes from the ECM (13). Thereafter, the released latent TGF-β complexes need to be processed further for the activation of TGF-β at the site of action (13). For example, binding of the LAP to $\alpha_v\beta_3$-, $\alpha_v\beta_5$-, and $\alpha_v\beta_6$-integrin induces a conformational change in the complex, resulting in the unmasking of the mature TGF-β moiety (7,17,18). An alternative mechanism for the activation of TGF-β is induced by the increased concentration of the SLC on the cell surface through binding to $\alpha_v\beta_8$-integrin, which is followed by the activation of membrane type-1 MMP (MT1-MMP), leading to the release of the mature TGF-β1

moiety from the LAP (19).

In the pathophysiological setting, although many studies have reported several mechanisms underlying the activation of latent TGF-β1, it has not yet been demonstrated fully what type of TGF-β1 complex (latent or active) is secreted from keratinocyte layers and how such a form is activated and stimulates the differentiation of fibroblasts sufficiently over distance. In addition, the presence of bi-directional regulation between epithelial cells and fibroblasts needs to be considered; in human skin equivalents, myofibroblasts contribute to a hyperproliferative epidermis (20). Conversely, in the therapeutic setting, one of the potential pharmacological approaches to prevent hypertrophic scar formation should be inhibition of the TGF-β1 receptor. However, although this appears to be a rational strategy, it may induce severe adverse effects, namely, inhibition of immune regulation and many physiological functions (21,22). Instead, inhibition of the activation of the latent form is likely to be a suitable pharmacological approach to ameliorate hypertrophic scar formation.

Using a reconstruction model derived from epithelial cells and fibroblasts isolated from neonatal rat skin, we investigated the mechanism underlying the activation of the TGF-β1 complex during the epithelial regulation of α-SMA production in myofibroblasts and tested possible promising pharmacological approaches. Briefly, freshly isolated rat keratinocytes were overlaid on a collagen gel in which dermal fibroblasts had been embedded [three-dimensional (3D) culture]. Furthermore, to clarify the site of pharmacological action, any possible feedback regulation from myofibroblasts to keratinocytes should be excluded. Thus, we also prepared a bioassay system using a fibroblast culture [two-dimensional (2D) culture] to assess α-SMA production induced by keratinocyte-conditioned medium. Then, we examined the types of TGF-β1 complex

present and the sites of pharmacological action of latent TGF-β1.

Materials and Methods

Antibodies and reagents

The following primary antibodies were used: mouse monoclonal anti-α-SMA (clone 1A4; Dako, Glostrup, Denmark), rabbit polyclonal anti-TGF-β1 (G122A; Promega, Madison, WI, USA), mouse monoclonal anti-TGF-β1 (clone 9016; R&D Systems, Minneapolis, MN, USA), goat polyclonal anti-LAP (T-17; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-α_v-integrin (clone 21/CD51; BD Biosciences, San Jose, CA, USA or clone 10D5; Abcam, Cambridge, UK), and mouse monoclonal anti-cytokeratin 10 (CK10; Dako). Recombinant human TGF-β1 (HumanZyme, Chicago, IL, USA) and recombinant human SLC (R&D Systems) were used. LY364947 (Cayman Chemical, Ann Arbor, MI, USA) and GM6001 (Enzo Life Sciences, Farmingdale, NY, USA) were dissolved in dimethyl sulfoxide to make stock solutions that were diluted more than 1,000 fold before use. The other compounds were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

Dissociation of keratinocytes and dermal fibroblasts

Two-day-old Wistar rats were killed by inhalation of anesthetic isoflurane. The dorsal skin was removed after disinfecting it with ethanol. Permission for the procedures used was granted by the Animal Research Committee of Fukuoka Dental College. The skin was incubated overnight at 4°C in modified Eagle's medium containing dispase (750 protease units/mL, Dispase II; Godo Shusei, Tokyo). To obtain keratinocytes, epidermal tissue was digested at 37°C for 1 min in phosphate-buffered saline containing 0.1% trypsin and 0.65 mM EDTA (Gibco-Life Technologies, Carlsbad, CA, USA). Enzyme activity was stopped by adding fetal bovine serum (FBS). The

suspension was filtered (Cell Strainer; Becton Dickinson, Franklin Lakes, NJ, USA), and then resuspended in Ham's F-12 medium (Gibco) immediately before use of the reconstruction culture.

Dermal tissue was minced into rectangular pieces and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 15% FBS. To collect fibroblasts that sprouted from the dissected skin, the fibroblasts surrounding the tissue were digested at 37°C for 5 min in phosphate-buffered saline containing 0.1% trypsin and 0.65 mM EDTA. Enzyme activity was stopped by adding FBS. After centrifugation, the fibroblasts were resuspended in fresh medium containing 10% FBS for 5–6 days, followed by subculturing once or twice.

Collagen gels were prepared on ice by mixing type-I collagen (0.725 volume; Nitta Gelatin, Osaka) with a reconstitution buffer (0.1 volume; 2.2 g $NaHCO_3$ and 4.77 g HEPES in 100 mL of 50 mM NaOH solution, pH 7.0), 5-times concentrated DMEM (without $NaHCO_3$, 0.15 volume), and 10-times concentrated Ham's F-12 medium (without $NaHCO_3$, 0.025 volume) (Fig. 1A). The final concentrations of $NaHCO_3$ and HEPES were 26 and 20 mM, respectively. We mixed 2.5 mL of the gel with 1.25×10^5 dermal fibroblasts and poured this into a cell culture insert (2.5-cm diameter, Millicell CM; Millipore, Temecula, CA, USA). The gel was solidified by warming at 37°C for 30 min. A culture insert without fibroblasts was also prepared.

Construction of the rat skin model using collagen gel matrix culture

Epithelial cells were overlaid on the collagen gel, which had been prepared 2 days before, in the presence or absence of fibroblasts. In our experiments, 4–5 culture inserts could be generated from the back skin of 4 newborn rats (Fig. 1A). The culture insert

was placed in an outer dish (10-cm diameter) containing DMEM and Ham's F-12 medium (3:1 volume) supplemented with 10% FBS and a growth factor cocktail (0.4% v/v bovine pituitary extract, 0.2 ng/mL human epidermal growth factor [EGF], 5 μg/mL transferrin, 5 μg/mL insulin, and 0.18 μg/mL hydrocortisone; HKGS Kit; Gibco), 250 μM ascorbic acid (Sigma-Aldrich Co.), and 20 μM phosphorylethanolamine (Sigma-Aldrich Co.), and incubated at 37°C in 5% CO_2.

After the epithelial cells grew to confluency, the gel surface was transferred to the air-liquid interface by removing the inner medium and reducing the amount of the outer medium to a level that would avoid immersing or drying the surface of the gel (air-lift, Fig. 1A). New culture medium was added every other day to make a heterotypic model after 9–10 days.

Histological and immunohistochemical examination of the reconstructed model

The reconstructed model was fixed with a 10% formalin solution, routinely processed, and embedded in paraffin as reported previously (23). Three-micrometer-thick sections were used for hematoxylin and eosin (H-E) staining and 3,3′-diaminobenzidine visualization of immunostaining. The sections were counterstained with hematoxylin.

Real-time PCR measurement of mRNA levels

Total RNA was isolated from the fibroblasts/myofibroblasts in the 3D-reconstruction model or those cultured on plastic plates, and the mRNA was reverse transcribed into cDNA. Quantitative PCR with the comparative $\Delta\Delta C_T$ method was performed on the samples in duplicate using an Applied Biosystems 7500 Real-time

PCR System (Grand Island, NY) with SYBR^{R} Premix Ex Taq II (Takara Bio, Shiga) and a pair of specific primers to target rat α-SMA (forward: 5′-ACTGGGACGACATGGAAAAG-3′, reverse: 5′-CATACATGGCAGGGACATTG-3′, GenBank accession number: NM_031004), rat TGF-β1 (forward: 5′-TATCCCGGTGGCATACTGAG-3′, reverse: 5′-CCCAAGGAAAGGTAGGTGATAGTC-3′, GenBank accession number: X52498), and an endogenous control, GAPDH (forward: 5′-GTGCCAGCCTCGTCTCATAG-3′, reverse: 5′-GACTGTGCCGTTGAACTTGC-3′, GenBank accession number: NM_017008) under the thermal cycling conditions recommended by the manufacturer. The mean ΔC_T value in the control group was used as an internal calibrator, and the relative quantity of the target gene in the test groups was calculated as $2^{-\Delta\Delta C_T}$ (range = $2^{-(\Delta\Delta C_T - S)}$ to $2^{-(\Delta\Delta C_T + S)}$), where S is the standard error of the mean (S.E.M.) of the $\Delta\Delta C_T$ value (24). PCR products were also analyzed on an ethidium bromide-stained agarose (2%) gel.

Immunoassay for the quantification of TGF-β1 concentration

For quantitative determination of the concentration of active TGF-β1 in the cell culture supernatant, an enzyme-linked immunosorbent assay (ELISA) was performed. Seven or nine days after air-lift, the culture inserts were placed in an outer dish (60-mm diameter) containing a 3 mL mixture of serum-free DMEM and Ham's F-12 medium (3:1 volume) supplemented with the growth factor cocktail. The culture supernatant was collected the next day and mixed with a known concentration of control TGF-β1 to provide a sample solution for ELISA (Quantikine; R&D Systems). The optical density of the TGF-β1 standards (31.2 to 1,000 pg/mL), samples, control (approximately 100 pg/mL), and blank (medium only) in duplicate was measured using a microplate reader

(1420 ARVO MX; PerkinElmer, Inc., Waltham, MA, USA). For quantitative determination of total TGF-β1 (active and latent), the culture supernatant was treated with 1 M HCl to change the latent form into an immunoreactive form; thereafter, pH was neutralized with NaOH. On the basis of the regression curve constructed in each experiment using the standards, the concentration of TGF-β1 (active and total) in the culture supernatant was estimated.

Immunoblotting analysis

Protein samples were treated with 2% sodium dodecyl sulfate (SDS) under non-reducing conditions at room temperature, then subjected to 4–15% gradient SDS-polyacrylamide gel electrophoresis (PAGE), and finally blotted to a PVDF membrane (Hybond-P; GE Healthcare, Wauwatosa, WI, USA). Immunoblots were obtained using the anti-TGF-β1 (G122A) and LAP antibodies followed by horseradish peroxidase-conjugated anti-rabbit and anti-goat immunoglobulin G (IgG) secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA), respectively, and detected by means of enhanced chemiluminescence (ImmunoStar LD; Wako Pure Chemical, Osaka).

Two-dimensional culture of rat dermal fibroblasts

The rat 2D fibroblast culture was maintained initially in DMEM containing 10% FBS for 5–6 days, followed by subculture in 2 mL of serum-free DMEM + Ham's F-12 medium (3:1 volume) containing EGF, transferrin, insulin, and 5 mg/mL bovine serum albumin (type IV; Invitrogen-Life Technologies, Carlsbad, CA, USA) on 6-well plastic culture plates (10 cm^2 for each well). Recombinant TGF-β1 and SLC were added for 24 h

in the presence or absence of the anti-α_v-integrin antibody or its control IgG before extraction of total RNA. In the RNA interference study, the fibroblasts were transfected with a mixture of two types of TGF-β1 small interfering RNA (siRNA; forward strands, 5′-UAUUCCGUCUCCUUGGUUCdTdT-3′ and 5′-AAAGUCAAUGUACAGCUGCdTdT-3′; total amount, 150 pmole) (25) or their control RNA, which had been added to 7.5 μL Lipofectamine RNAiMAX reagent (Invitrogen), and were cultured for 48 h before use.

To detect keratinocyte-derived α-SMA-increased substances using the 2D culture, 6 mL of supernatant were collected from stratified keratinocytes that had been cultured in serum-free DMEM and Ham's F-12 medium (3:1 volume) supplemented with the growth factor cocktail for 5 days after air-lift and seeded on collagen gels containing no fibroblasts. We added 1 mL of the conditioned medium to the fibroblast culture (total, 2 mL medium). To remove TGF-β1 and/or LAP from the conditioned medium (preabsorption experiments), the medium was incubated at 4°C with the anti-TGF-β1 antibody (clone 9016; 1 μg/mL) or anti-LAP antibody (1 μg/mL) for 1 h followed by a further incubation for 1 h with protein A/G agarose (Santa Cruz). The supernatant obtained following centrifugation was used in the bioassay with the 2D culture.

Statistical analysis

All values are presented as the mean ± S.E.M. (N, number of observations). Statistical analysis for the comparison of 2 groups was performed using Student's *t*-test. For more than 3 groups, the analysis was performed using one-way analysis of variance (ANOVA) for a randomized block experiment followed by a *post-hoc* test. A *P* value less than 0.05 was considered statistically significant.

Results

Effects of keratinocyte layers on the differentiation of fibroblasts to the contractile cell type in the collagen matrix

Firstly, we conducted histological and immunohistochemical assessments of the 3D reconstruction model (10 days). The culture model retained a multilayered epithelium-like structure (Fig. 1B) as reported previously (26-28). H-E staining revealed that an intense eosinophilic pattern was present in the outermost layer, which was the equivalent of the cornified layer of the skin. Under this layer, dense dot-like staining of keratohyalin granules was observed in flattened keratinocytes (granular layer). CK10 is known to be located in the suprabasal layer and is accompanied with orthokeratinization. The anti-CK10 antibody exhibited intense immunostaining of the layers that correspond to the granular and spinous layers of the skin, but not the cells located just above the collagen gel (basal layer) (Fig. 1B). These results suggest that the keratinocytes were stratifying and differentiating at 7–10 days after air-lift in the 3D reconstruction model.

We investigated the expression of α-SMA in different types of reconstruction models derived from fibroblasts, in the presence or absence of stratified keratinocytes, isolated from neonatal rat skin (Fig. 1C). Immunohistochemistry using the anti-α-SMA antibody (clone 1A4, 1:100) revealed the more intense expression of α-SMA in the fibroblasts embedded in the collagen gel covered with keratinocyte layers at 10 days after air-lift (10-day heterotypic model) (Fig. 1C). Immunopositive staining was remarkable in the fibroblasts located approximately in the middle of the gel as well as in those underneath the keratinocyte layers. However, α-SMA expression was weaker in the fibroblasts of the 7-day heterotypic model. In the model without keratinocytes, α-SMA expression was

almost absent at day 7, but a few fibroblasts possessed α-SMA at day 10 (Fig. 1C). These results suggest a stimulatory role for stratified keratinocytes in the differentiation of fibroblasts to myofibroblasts within the gel.

Endogenous latent TGF-β1 is involved in the differentiation of fibroblasts in the 3D reconstruction model

We examined whether α-SMA production is mediated by the TGF-β signaling pathway by using LY364947, a TGF-β type I receptor inhibitor. This inhibitor strongly attenuated the α-SMA-positive immunostaining and the α-SMA mRNA levels in fibroblasts (Fig. 2A and 2B), suggesting that endogenous α-SMA production is mediated by the TGF-β type I receptor.

Next, we tested whether TGF-β1 is present within the epithelium and whether it is released from the epithelium to the underlying collagen gel. We validated the anti-TGF-β1 antibody (G122A) in SDS-PAGE of recombinant TGF-β1 and SLC under non-reducing conditions followed by immunoblotting (Fig. 2C). The anti-TGF-β1 antibody recognized the recombinant active TGF-β1 dimer (approximately 22 kDa) and the latent form (approximately 105 kDa, including TGF-β1), but did not recognize the LAP dimer (approximately 85 kDa), which was partly dissociated from SLC by SDS treatment and was recognized by the anti-LAP antibody, suggesting that the TGF-β1 antibody (G122A) can recognize the active and latent forms of TGF-β1. Using this antibody (1:200), we found that the active and latent forms of TGF-β1 were present in the keratinocytes, especially in the suprabasal layers, as well as in myofibroblasts of the 10-day model (Fig. 2D).

An ELISA that can selectively detect the active form of TGF-β1 revealed the basal

level of active TGF-β1 in the culture supernatant from the 9-day model, and failed to show a clear difference in the production of TGF-β1 in the presence [KC(+)] or absence [KC(-)] of keratinocytes, suggesting that the active form of TGF-β1 was, in part, secreted from myofibroblasts (Fig. 2E). Some physical conditions (e.g., heat and pH extremes) have been shown to denature LAP and release active TGF-β1 (29). Interestingly, when the culture supernatant was acid-treated for 10 min and then the pH neutralized, the model produced significantly more total TGF-β1 (i.e., both active and latent forms) in KC(+) cultures than in KC(-) cultures (Fig. 2E). The value of the latent form in either culture was estimated by subtracting the value of active TGF-β1 from that of total TGF-β1. These results indicate that latent TGF-β1 is the main component released endogenously from keratinocytes.

Role of α_v-integrin in the differentiation of fibroblasts in the 3D reconstruction model

To investigate the role of integrins in fibroblast differentiation in the 3D model, which is possibly mediated by keratinocyte-derived latent TGF-β1, we focused on α_v-integrin, which is known to cause a conformational change of latent TGF-β1 (7,17). The antibody for α_v-integrin (clone 21/CD51, 1:50) revealed the presence of this integrin in keratinocytes and myofibroblasts (Fig. 3A). The application of a neutralizing antibody to α_v-integrin (clone 10D, 1 μg/mL) significantly attenuated α-SMA immunoreactivity and α-SMA mRNA levels, suggesting the involvement of α_v-integrin in the differentiation of myofibroblasts (Fig. 3B and C). We also observed the relatively weak action of the anti-TGF-β1 antibody (clone 9016, 1 μg/mL), likely to be due to its weaker association with the latent form of TGF-β1.

Requirement of protease activity for the differentiation of fibroblasts

Identification of the mechanism underlying the release of the latent TGF-β1 that is covalently linked to the ECM is required to clarify the paracrine interaction of keratinocytes with fibroblasts/myofibroblasts over distance. We tested whether protease inhibitors affect the production of α-SMA. GM6001 (MMP inhibitor) markedly attenuated α-SMA immunoreactivity, while aprotinin (serine protease inhibitor) only weakly affected it (Fig. 4A). Leupeptin (a cystein and serine protease inhibitor), but not E64 (a cysteine protease inhibitor) or aprotinin, significantly attenuated α-SMA mRNA levels (Fig. 4B). Among the protease inhibitors, GM6001 (1 and 5 μM) diminished α-SMA mRNA levels most intensely, suggesting that MMPs are most likely involved in the proteolytic cleavage of latent TGF-β1 to induce myofibroblast differentiation (Fig. 4B).

Effects of exogenous TGF-β1 and SLC on dermal fibroblasts in the 2D culture

Although we demonstrated that MMP and α_v-integrin play important roles in the differentiation of fibroblasts, their site of action within the 3D model—keratinocytes, fibroblasts/myofibroblasts, or both—has not yet been demonstrated fully. Thus, we cultured the two types of cells separately in the following experiments.

First, we checked the recombinant TGF-β1 and SLC proteins using SDS-PAGE under non-reducing conditions followed by immunoblotting with antibodies against active TGF-β1 (G122A) and LAP (Fig. 2C). Recombinant SLC (approximately 105 kDa, including TGF-β1 and LAP dimers) was recognized by the anti-TGF-β1 and anti-LAP antibodies. The recombinant active TGF-β1 dimer (approximately 22 kDa) and the 22 kDa form, which dissociated partially from SLC due to SDS treatment, were recognized

by the anti-TGF-β1 antibody, but not by the anti-LAP antibody. The approximately 85-kDa form, which dissociated partially from SLC (LAP dimer), were recognized by the anti-LAP antibody, but not by the anti-TGF-β1 antibody. These results confirmed the molecular composition of the recombinant proteins and validity of the antibodies used in this study.

Rat dermal fibroblasts were cultured on plastic plates to quantify the effects of exogenous TGF-β1 and its latent form, SLC (Fig. 5A). When either recombinant TGF-β1 (2 ng/mL) or SLC (10 ng/mL) was applied to the dermal fibroblasts, α-SMA production was increased by 2- to 3-fold (Fig. 5B). Notably, the anti-α_v-integrin antibody inhibited the SLC-induced increase of α-SMA production, but not the TGF-β1-induced increase. Similarly, GM6001 significantly inhibited the SLC-induced increase of α-SMA production, but not the TGF-β1-induced increase. These results indicate that the dermal fibroblasts were capable of responding to the active and latent forms of TGF-β1. In addition, α_v-integrin or MMPs expressed on the fibroblasts are likely to be required for the activation of latent TGF-β1.

Keratinocyte-derived latent TGF-β1 increases α-SMA production in fibroblasts

In order to characterize the form of TGF-β1 (active or latent) released from the stratified keratinocytes, we collected conditioned medium in which only stratified keratinocytes were maintained on the collagen gel (KC medium) and then applied this medium to the 2D fibroblast culture (Fig. 5A). The conditioned medium significantly increased α-SMA mRNA levels in the fibroblasts (Fig. 5C). This action was significantly inhibited by GM6001 (5 μM) and by the anti-α_v integrin antibody (1 μg/mL).

Then, the efficiency of preabsorption with the anti-LAP or anti-TGF-β1 (clone 9016)

antibody combined with protein A/G agarose was examined using medium containing either recombinant SLC (160 ng/mL) or TGF-β1 (40 ng/mL) (Suppl. Fig. 1). In the SLC-containing medium, the TGF-β1 content of the supernatant fraction was attenuated by treatment with the anti-LAP antibody (1 μg/mL) and, to a lesser degree, with the anti-TGF-β1 antibody (1 μg/mL). In contrast, the TGF-β1 content was inhibited by only the anti-TGF-β1 antibody in the active TGF-β1-containing medium (Suppl. Fig. 1). These findings suggest the efficiency of preabsorption using the anti-LAP or anti-TGF-β1 antibody.

After endogenous latent TGF-β1 was preabsorbed using the anti-LAP antibody (1 μg/mL), the culture medium was applied to the 2D culture. Preabsorption using the anti-LAP antibody plus protein A/G beads caused a significant decrease in the KC medium-induced increase of α-SMA mRNA levels down to the control level (Fig. 5C). To confirm that TGF-β1 complexes had been preabsorbed, the protein A/G beads were treated with SDS under non-reducing conditions. Immunoblotting clearly showed the presence of TGF-β1, suggesting the release of TGF-β1 tightly associated with LAP in the KC medium (Fig. 5D). Preabsorption using the anti-TGF-β1 antibody (clone 9016, 1 μg/mL) plus protein A/G beads also produced a modest but significant inhibitory effect (Fig. 5C). Immunoblotting indicated the partial removal of TGF-β1 from the KC medium by the anti-TGF-β1 antibody (Fig. 5D).

To examine the involvement of MMPs in the release of TGF-β1 from keratinocytes, we collected the conditioned medium from keratinocytes that had been incubated in the presence or absence of GM6001 (5 μM) for 5 days, and total TGF-β1 levels were detected by ELISA. As a result, TGF-β1 levels were significantly attenuated by pretreatment with GM6001 (Fig. 5E). These results suggest that: 1) keratinocytes released the

α-SMA-increasing latent TGF-β1, at least in part, with the aid of MMPs; 2) the substances were mainly associated with LAP; and 3) the activation of TGF-β1 was dependent on α_v-integrin and MMPs located in the dermal fibroblasts.

Stimulation of α-SMA production in dermal fibroblasts by TGF-β1 secreted in an autocrine or paracrine manner

As described earlier (Fig. 2C), immunocytochemistry showed the expression of TGF-β1 in the myofibroblasts of the 3D reconstruction model. Therefore, using the 2D fibroblast culture, we investigated whether TGF-β1 was produced by the fibroblasts and whether the exogenous application of TGF-β1 and SLC augmented the production of α-SMA partly through TGF-β1 secretion in an autocrine or paracrine manner. In the control, in which TGF-β1 siRNA was used to knock down TGF-β1 mRNA levels (Fig. 6B), α-SMA production was inhibited to approximately 50% of the level observed in the control RNA-treated cells (Fig. 6A). Exogenous stimulation with TGF-β1 or SLC increased the α-SMA mRNA levels, an effect that was substantially suppressed by TGF-β1 siRNA. These results suggest that exogenous stimulation of the TGF-β1 pathway failed to cause α-SMA production when the autocrine or paracrine action of TGF-β1 was suppressed in fibroblasts. With the aid of the endogenous production of TGF-β1, exogenous TGF-β1 stimulation is likely to induce the production of α-SMA effectively in a positive-feedback fashion.

Discussion

During wound healing, fibroblasts in granular tissue are known to differentiate into myofibroblasts with a contractile phenotype via an extremely complicated process (1,3). The specific expression of α-SMA was shown to be mainly associated with the presence of active TGF-β1 and mechanical tension (3,30). Bell *et al.* (31) reported the potential use of an *in vitro* model to study connective tissue reorganization during wound contraction. Their model consisted of a tissue-like structure made of fibroblasts incorporated in hydrated collagen lattices (32). The present 3D reconstruction model, consisting of keratinocytes and fibroblasts/myofibroblasts derived from rat skin, represents a good wound healing model and directly shows the importance of the process termed re-epithelialization, where keratinocytes migrate over the damaged area of skin. This model clarifies the interaction between keratinocytes and fibroblasts/myofibroblasts, and removes the influence of complicated inflammatory processes (i.e., neutrophils, monocytes, and macrophage progenitors) and the substances released from these inflammatory cells. Previously, Rompré *et al.* (33) reported that keratinocytes produced a severe contraction of human skin that occurred in a seeding density-dependent fashion. A previous report using a keratinocyte-fibroblast monolayer co-culture system showed that cell-cell contact between the two types of cells enhanced myofibroblast formation through the activation of latent TGF-β (8). In the present heterotypic 3D model, in combination with the 2D fibroblast culture, we clarified the mechanism underlying the effect of keratinocyte-derived TGF-β1 on myofibroblast differentiation over distance and revealed several targets for pharmacological approaches: 1) the existence of TGF-β1 complexes in the keratinocyte layers (Fig. 7A); 2) the release of latent TGF-β1 (SLC or

LLC) into the culture medium, probably through the activity of MMPs (Fig. 7A); 3) the activation of latent TGF-β1 on the surface of fibroblasts by α_v-integrin and/or proteases (Fig. 7B and 7C); 4) the TGF-β1-dependent production of α-SMA through the activation of TGF-β type I receptors (Fig. 7B and 7C); and 5) the presence of a positive-feedback loop in the TGF-β1-induced production of α-SMA in fibroblasts (Fig. 7C).

The present study demonstrated that the expression of α-SMA in fibroblasts is at least, in part, stratified keratinocyte- and TGF-β1-dependent (especially the latent form) (Figs. 1, 2, and 7A). Immunohistochemical data suggest the dense localization of TGF-β1 in suprabasal keratinocytes (Fig. 2D). This was supported by previous studies demonstrating that TGF-β1 was expressed in the suprabasal epithelial cells of human skin equivalents (20). The expression of latent TGF-β1 was shown to increase markedly in suprabasal keratinocytes after injury, although its localization was faint in normal mouse epidermis (11). These findings indicate that dividing keratinocytes are one of the major cellular sources of TGF-β1 during wound healing. It was noteworthy, however, that in the ELISA study, we failed to obtain clear evidence of the keratinocyte-dependent production of active TGF-β1 in the culture medium, but we were able to evaluate the epithelium-dependent production of total TGF-β1 (activated and latent) following acid treatment for the activation of latent TGF-β1 (Fig. 2E). Furthermore, the conditioned medium in which SLC had been preabsorbed using the anti-LAP antibody was unable to increase α-SMA production in the 2D fibroblast culture (Fig. 5C). Similarly, the presence of latent TGF-β1 was reported previously in fibroblast-conditioned medium monitored using a radioreceptor assay and soft agar growth assay (29,34).

Identification of the mechanism underlying the release of latent TGF-β1 that is

covalently linked to ECM is required to clarify a paracrine interaction between two types of cells located over distance and constrained by diffusion. MMPs are structurally related enzymes that can cleave ECM molecules, growth factors, and cytokines (13,35). In general, MMPs are not or are only weakly expressed in healthy skin; however, the chemical and physical changes in the cellular environment after skin injury induce the expression of various types of MMPs such as MMP-2, -3, and -9 (35-37). MMP-2 is expressed exclusively in granulation tissue (37), and is suggested to activate TGF-β1 by cleaving LAP and LTBP and releasing TGF-β1 from osteoclasts (38). MT1-MMP reportedly releases TGF-β1 from the ECM surrounding endothelial cells via the proteolytic processing of LTBP-1 (16). In the present study, among several protease inhibitors tested, the most potent inhibitor of α-SMA expression was GM6001, suggesting that MMPs mainly facilitate the action of TGF-β1, probably by the cleavage of LTBP sequestered in the ECM and the release of SLC or LLC (Figs. 4 and 7A). In fact, incubation of stratified keratinocytes with GM6001 reduced the total amount of TGF-β1 secreted into the medium (Fig. 5E). Previous studies have also raised the possibility of the same mechanism of TGF-β1 activation in a collagen-contraction experiment (39) and in a mouse skin wound experiment (40). We cannot rule out the involvement of other proteases during wound healing, as shown in a previous report on the fibrinolytic enzyme plasmin (15), since the other proteases assessed in the present study partially decreased α-SMA expression.

The direct contact of SLC with thrombospondin-1 or a subset of integrins, such as $\alpha_v\beta_3$-, $\alpha_v\beta_5$-, and $\alpha_v\beta_6$-integrin, causes the non-proteolytic, conformational modification of SLC, leading to the presentation of active TGF-β1 to its receptors (18,19,41). *Itgb6*$^{-/-}$ mice were previously demonstrated to exhibit a phenotype similar to *Tgfb1*$^{-/-}$ mice (41).

$\alpha_v\beta_6$-Integrin expression is reportedly upregulated in epithelial cells during wound healing and colocalized with TGF-β1 in humans and pigs, although it is not expressed under normal conditions (42,43). In the present study, we showed that α_v-integrins were expressed in myofibroblasts and that a neutralizing antibody against α_v-integrin strongly inhibited myofibroblast differentiation (Fig. 3), suggesting that α_v-integrin is involved in the non-proteolytic, conformational modification of SLC to present TGF-β1 to its receptors on myofibroblasts in our heterotypic reconstruction model (Fig. 7B). Furthermore, an anti-α_v-integrin antibody attenuated the expression of α-SMA in our 2D fibroblast culture, which was induced by SLC applied exogenously and by KC medium, but not by active TGF-β1 (Fig. 5B and 5C). Our results are supported by previous studies in that the TGF-β1-induced expression of α-SMA in human dermal myofibroblasts was inhibited by an anti-α_v-integrin antibody (44) and that fibrosis was attenuated by an anti-α_v-integrin antibody (45-48). In addition, the second MMP-induced proteolysis is presumably involved in the cleavage of the latent form to produce active TGF-β1 near fibroblasts since GM6001 inhibited myofibroblast differentiation in the 2D fibroblast culture induced by SLC and KC medium (Figs. 5B, 5C, and 7C). In either situation, since the two types of cells are not in direct contact in our heterotypic model, which is similar to the *in vivo* situation, or in the 2D fibroblast culture stimulated by KC medium, the liberation of a soluble and still latent form of TGF-β1 should be a prerequisite for its subsequent non-proteolytic and/or proteolytic activation.

The present 2D culture experiment demonstrated that TGF-β1 siRNA attenuated the expression of α-SMA mRNA in the presence or absence of exogenous stimulation with TGF-β1 or SLC (Fig. 6A). These results suggest the possible presence of a

positive-feedback loop in the TGF-β1-induced production of α-SMA in fibroblasts (Fig. 7C). In addition to the basal production of α-SMA in fibroblasts/myofibroblasts, keratinocyte-derived TGF-β1 complexes are likely to require the production of active TGF-β1 in the fibroblast culture (Fig. 2E), which effectively increased α-SMA production in an autocrine or paracrine fashion. Although it is well known that TGF-β1 governs the fibroblast phenotype, other cell-cell interactions should also be taken into consideration for the keratinocyte-induced differentiation of fibroblasts, such as platelet-derived growth factor and activin, reportedly detected in keratinocytes and involved in the promotion of fibroblast differentiation (30). In contrast, keratinocyte-derived interleukin-1 following NF-κB activation in fibroblasts is reportedly an inhibitory factor of the TGF-β1 signaling pathway (8).

It is important to consider the possibility that an alternative source of fibroblast cells is involved in the production of myofibroblasts. The epithelial-mesenchymal transition (EMT) has been postulated as a major mechanism by which new fibroblasts can arise during tissue fibrosis (6,49). TGF-βs were shown to promote type 2 EMT in renal and pulmonary epithelial cells (50,51). However, it has not yet been clarified whether keratinocytes undergo EMT and then gain the contractile phenotype during wound healing, pathogenic scarring, and fibrosis, unlike the well-known reproducible phenomena elicited by exogenous TGF-βs in *in vitro* studies (52,53).

In conclusion, in the culture models made from rat keratinocytes and dermal fibroblasts, stratified keratinocytes afford a contractile phenotype (α-SMA production) to distant fibroblasts through: 1) the release of latent TGF-β1 due to the proteolysis of LLC by MMPs from the ECM; and 2) the subsequent activation of latent TGF-β1 due to conformational and proteolytic changes induced by α_v-integrin and MMPs in the

fibroblast culture in a positive feedback fashion. We found that an MMP inhibitor is effective at inhibiting the differentiation of dermal fibroblasts by targeting different sites during the activation of latent TGF-β1, suggesting possible promising therapeutic approaches for the prevention of hypertrophic scar formation and contractures. Due to the lack of *in vivo* data to corroborate the present results, further studies will be required using preclinical models to strengthen the potential of the present findings in translational research.

Acknowledgments

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Figure legends

Fig. 1. Dependency of keratinocytes on the differentiation of fibroblasts to the contractile phenotype in the 3D reconstruction model. A, Schematic diagram of the rat skin model. The collagen gel surface was transferred to the air-liquid interface (air-lift) to induce stratification of the keratinocytes. B, Histological and immunohistochemical assessments of the 3D reconstruction model. H-E; hematoxylin-eosin staining. An anti-CK10 antibody was used. Ten-day models with stratified keratinocytes [KC(+)]. Dense dot-like staining of keratohyalin granules was observed (arrowhead). The basal layer is indicated by the arrow. C, α-SMA immunostaining in the 7-day and 10-day models without keratinocytes [KC(-)], and in the 7-day and 10-day KC(+) models. The immunopositive cells are indicated by arrowheads and a section of the image was enlarged (insets).

Fig. 2. Keratinocyte-induced release of TGF-β1 and the involvement of the TGF-β type I receptor in α-SMA expression. A, TGF-β type I receptor inhibitor attenuated the α-SMA immunostaining of the 7-day 3D models. We added 3 μM LY364947 (LY) to the culture medium just after air-lift. B, LY reduced the α-SMA mRNA levels. (Upper) A typical RT-PCR result. (Lower) Quantitative comparison of α-SMA mRNA levels in fibroblasts/myofibroblasts embedded in the collagen gel in the presence or absence of 3 μM LY (real-time PCR). The mRNA levels are expressed as $2^{-\Delta\Delta C_T}$ (range = $2^{-(\Delta\Delta C_T - S)}$ to $2^{-(\Delta\Delta C_T + S)}$) (see Materials and Methods) and normalized to the values obtained without LY. *$p < 0.05$; paired *t*-test ($n = 4$). C, Immunoblots for recombinant active TGF-β1 (5 ng/lane) and the latent form (20 ng/lane, SLC) by using anti-TGF-β1 (G122A) and anti-LAP antibodies. D, Immunohistochemistry revealed the existence of TGF-β1

complexes in the keratinocyte layers of the 10-day model. TGF-β1 complexes were present in the suprabasal keratinocytes and fibroblasts (arrowheads). E, Release of activated TGF-β1 into the culture supernatant was evaluated using ELISA. The culture medium was free of serum. The total amount of TGF-β1 (active + latent) was estimated in culture supernatant that had been acid treated for 10 min followed by neutralization of pH. *$p < 0.05$; paired *t*-test, mean ± S.E.M. (n = 6). The value of the latent form was estimated by subtracting the value of active TGF-β1 from that of total TGF-β1.

Fig. 3. Roles of TGF-β1 and integrins on the differentiation of fibroblasts. A, Immunostaining of α_v-integrin in keratinocytes and myofibroblasts of the 10-day 3D models. The locations of the integrins in keratinocytes and myofibroblasts are indicated by arrowheads. B and C, Neutralizing antibodies against α_v-integrin and TGF-β1 decreased α-SMA immunostaining and α-SMA mRNA levels in the 10-day 3D models. The mRNA levels are expressed as $2^{-\Delta\Delta C_T}$ (range = $2^{-(\Delta\Delta C_T - S)}$ to $2^{-(\Delta\Delta C_T + S)}$) (see Materials and Methods) and normalized to the values for the control mouse IgG. *$p < 0.01$; one-way ANOVA for a randomized block experiment using the $2^{-\Delta\Delta C_T}$ values followed by the *post-hoc* Fisher's PLSD test (n = 4).

Fig. 4. Involvement of protease activity in the differentiation of fibroblasts. A, Effects of GM6001 (1 μM) and aprotinin (2 μM) on α-SMA immunostaining in the 10-day 3D models. The immunopositive cells are indicated by arrowheads. B, Effects of GM6001, E64, aprotinin, and leupeptin on α-SMA mRNA levels. The mRNA levels are expressed as $2^{-\Delta\Delta C_T}$ (range = $2^{-(\Delta\Delta C_T - S)}$ to $2^{-(\Delta\Delta C_T + S)}$) (see Materials and Methods). *$p < 0.05$, **$p < 0.01$; one-way ANOVA for a randomized block experiment using the $2^{-\Delta\Delta C_T}$ values

followed by the *post-hoc* Fisher's PLSD test (n = 4).

Fig. 5. Increase in α-SMA production in dermal fibroblasts (2D culture) by keratinocyte-derived SLC. A, Schematic diagram of the experimental procedures. Letters B–E in parentheses indicate the results shown in the corresponding panels. B, Effects of exogenously applied TGF-β1 and its latent form on dermal fibroblasts in the presence or absence of GM6001 or neutralizing antibodies to α_v-integrin. C, Keratinocyte-conditioned medium (KC medium) increased the production of α-SMA in dermal fibroblasts. The anti-LAP antibody potently preabsorbed SLC from KC medium and reduced the production of α-SMA. The mRNA levels are expressed as $2^{-\Delta\Delta C_T}$ (range = $2^{-(\Delta\Delta C_T - S)}$ to $2^{-(\Delta\Delta C_T + S)}$) (see Materials and Methods). *p < 0.05, **p < 0.01, ***p < 0.001 compared with control, #p < 0.05, ##p < 0.01, ###p < 0.001 compared with the corresponding data in the absence of the drug or antibody; one-way ANOVA for a randomized block experiment using the $2^{-\Delta\Delta C_T}$ values followed by the *post-hoc* Fisher's PLSD test (n = 4–5). D, Immunoprecipitation was performed on the KC medium using either the anti-LAP, anti-TGF-β1 (clone 9016), or control IgG antibodies and protein A/G beads. The protein beads were treated with SDS under non-reducing conditions, and the anti-TGF-β1 antibody (G122A) was used for the detection of active TGF-β1 dimers on the immunoblots. E, Pretreatment of stratified keratinocytes with GM6001 reduced the release of total TGF-β1 into the culture supernatant evaluated using ELISA. The culture media were free of serum. **p < 0.01; paired *t*-test, mean ± S.E.M. (n = 6).

Fig. 6 Endogenous stimulation of α-SMA production by TGF-β1 in dermal fibroblasts. Treatment with TGF-β1 siRNA decreased the levels of α-SMA (A) and TGF-β1 (B)

mRNA in the presence or absence of TGF-β1 and SLC. The mRNA levels are expressed as $2^{-\Delta\Delta C_T}$ (range = $2^{-(\Delta\Delta C_T - S)}$ to $2^{-(\Delta\Delta C_T + S)}$) (see Materials and Methods). *$p < 0.05$ compared with control, #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ compared with the corresponding data in the absence of siRNA,; one-way ANOVA for a randomized block experiment using the $2^{-\Delta\Delta C_T}$ values followed by the *post-hoc* Fisher's PLSD test (n = 4).

Fig. 7 Schematic diagram of dermal fibroblast differentiation induced by keratinocyte-derived latent TGF-β1 in the rat 3D culture model. A, Latent TGF-β1 (SLC or LLC) is released by matrix metalloproteinases (MMPs) from the extracellular matrix surrounding the stratified keratinocytes. B, α_v-Integrin causes a conformational change of the latent TGF-β1 complexes to bind to its receptors (TGF-β RI/RII), resulting in fibroblast differentiation. MMPs are also involved in the activation of the latent TGF-β1 complexes. C, An autocrine or paracrine mechanism is involved in the facilitation of fibroblast differentiation (production of latent TGF-β1 and subsequently α-SMA). Active TGF-β1 may also be included after proteolysis of LAP (see Fig. 2E). Upper right panel denotes symbols representing active TGF-β1 and its latent complexes.

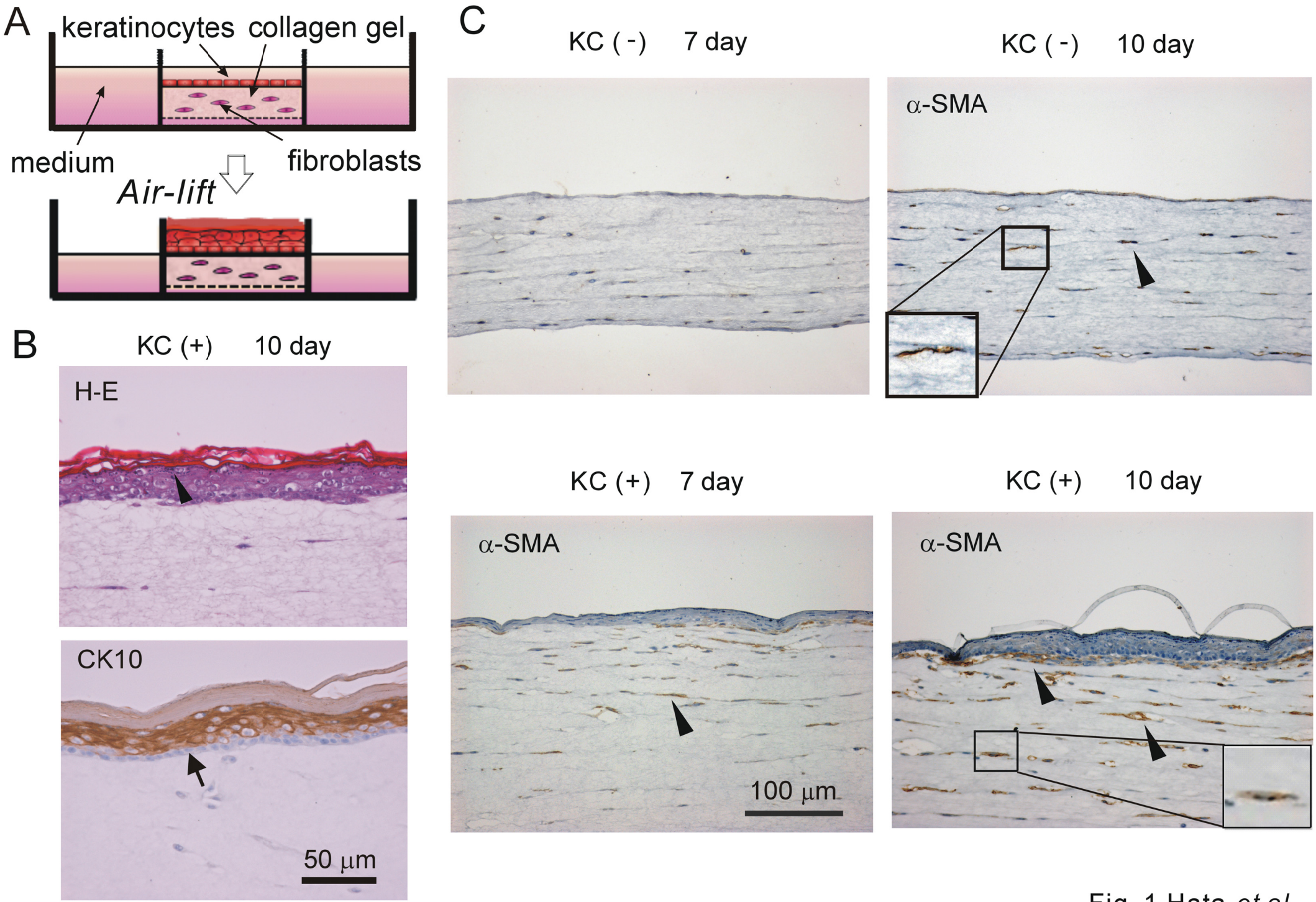


Fig. 1 Hata *et al.*

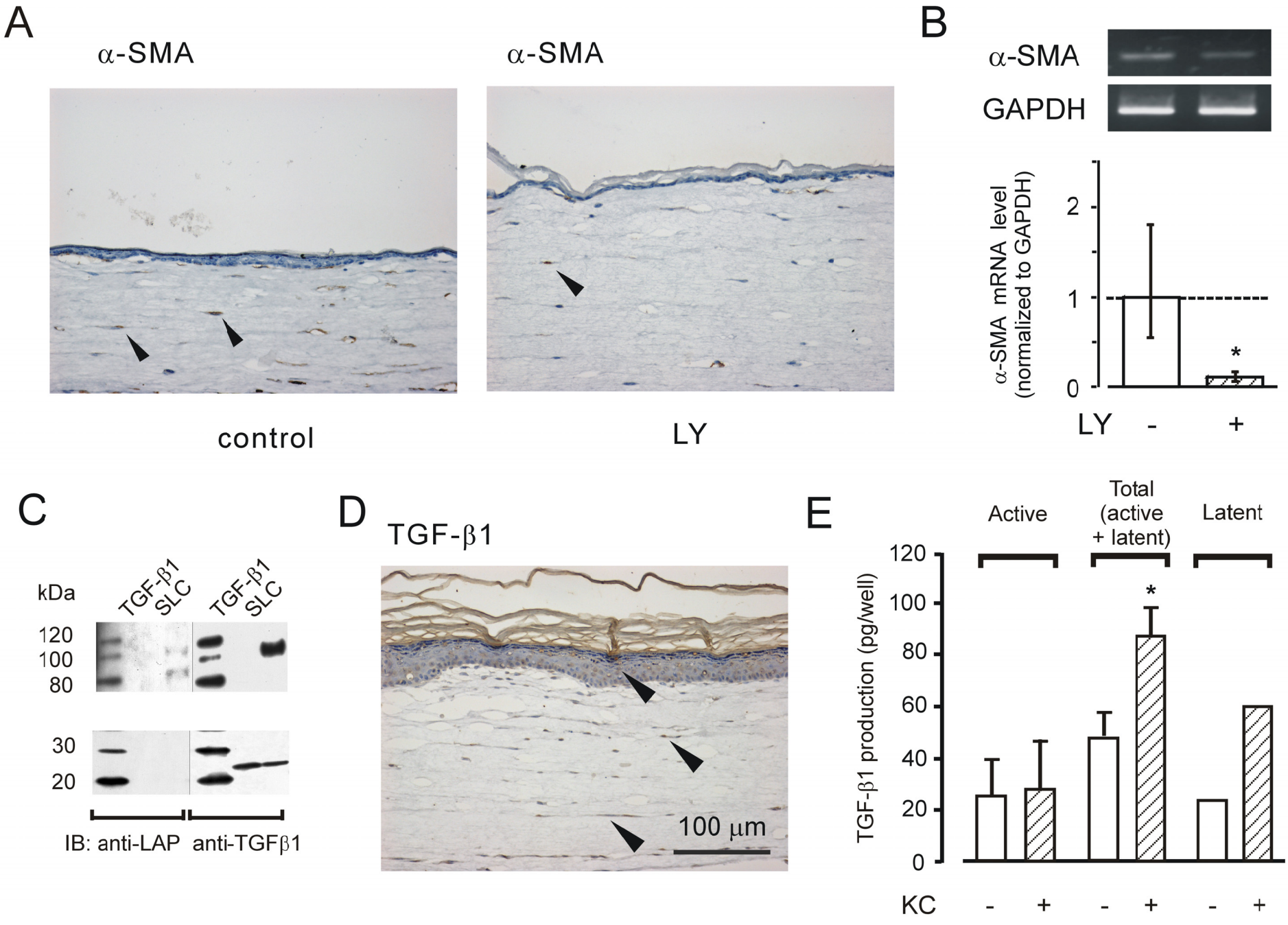
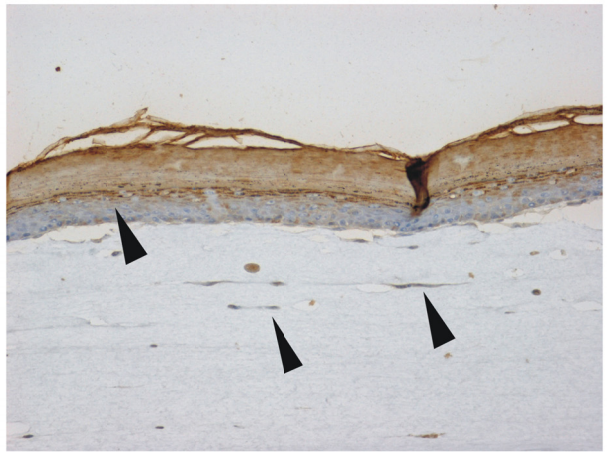


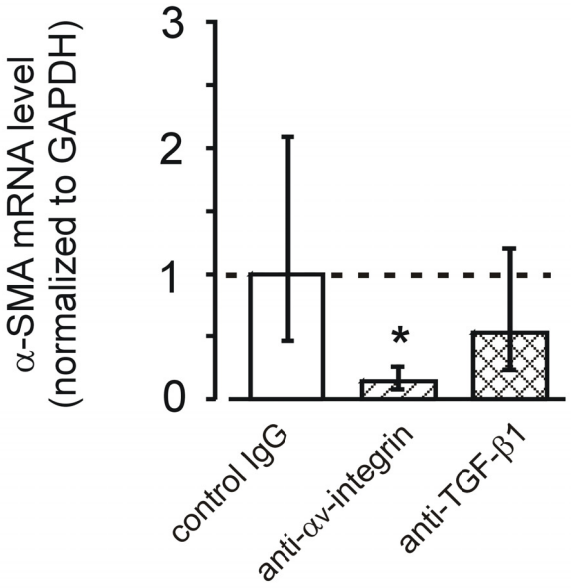
Fig. 2 Hata *et al.*

A

αv-integrin



B

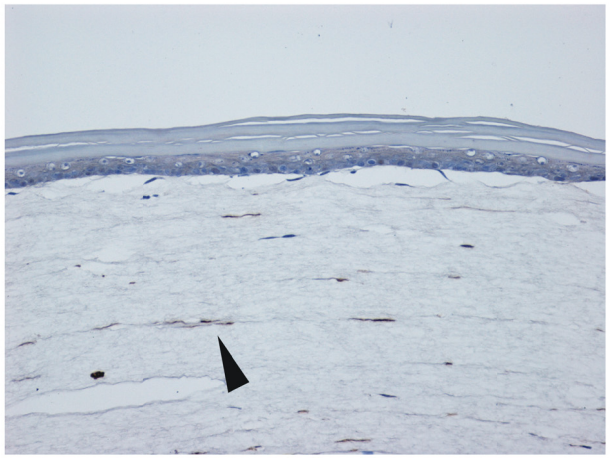


C

α-SMA

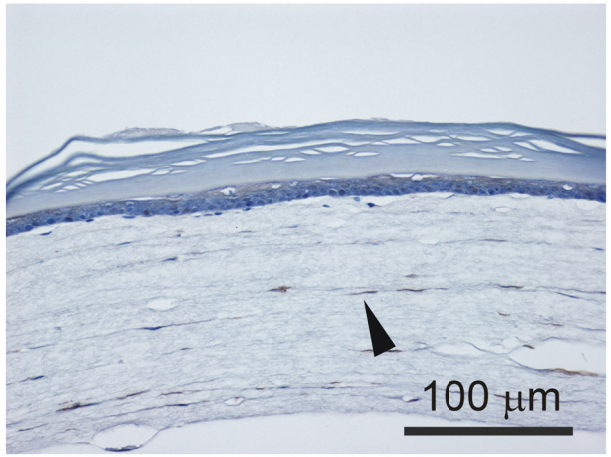
control IgG

α-SMA



Anti-αv-integrin

α-SMA



Anti-TGF-β1

Fig. 3 Hata *et al.*

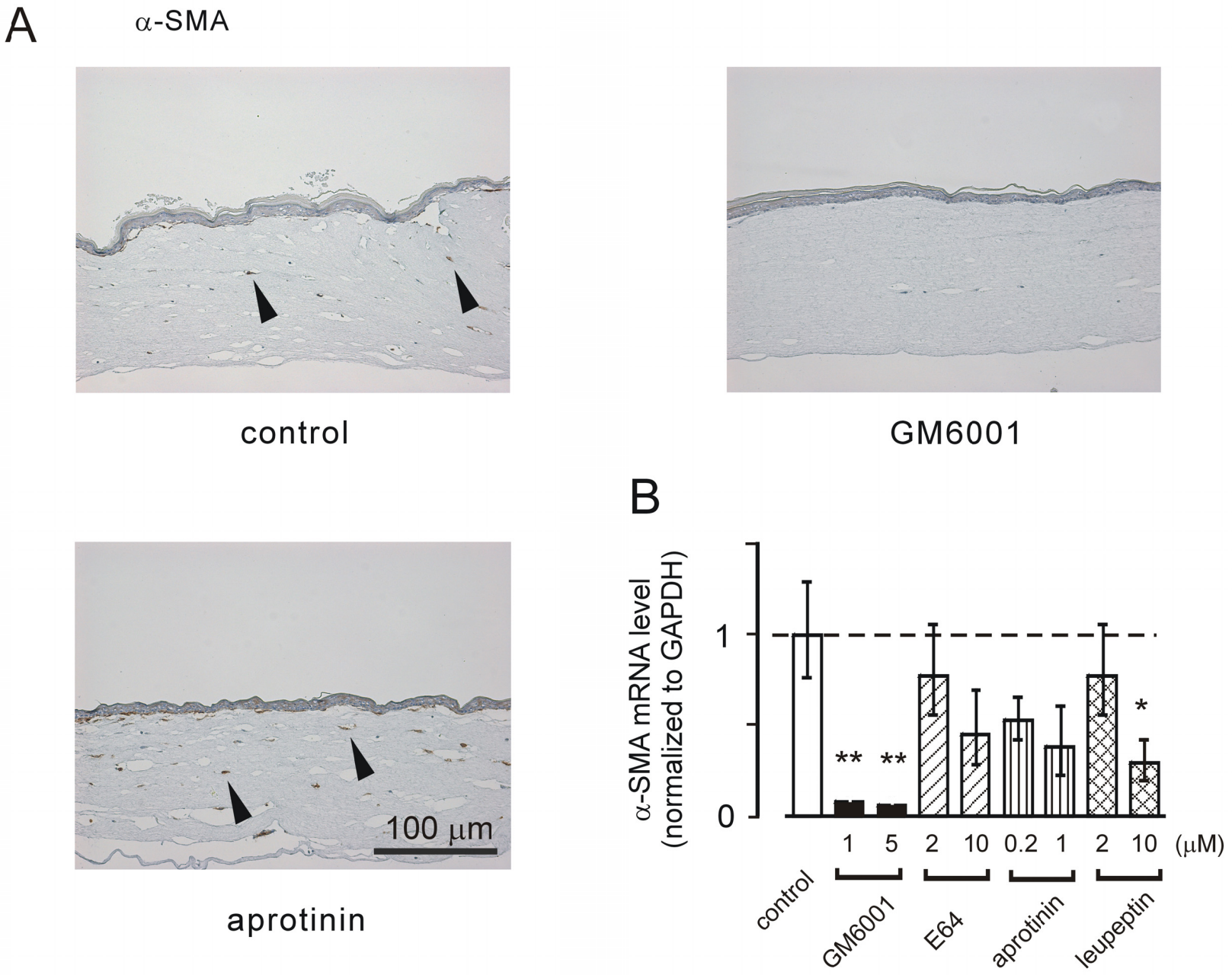


Fig. 4 Hata *et al.*

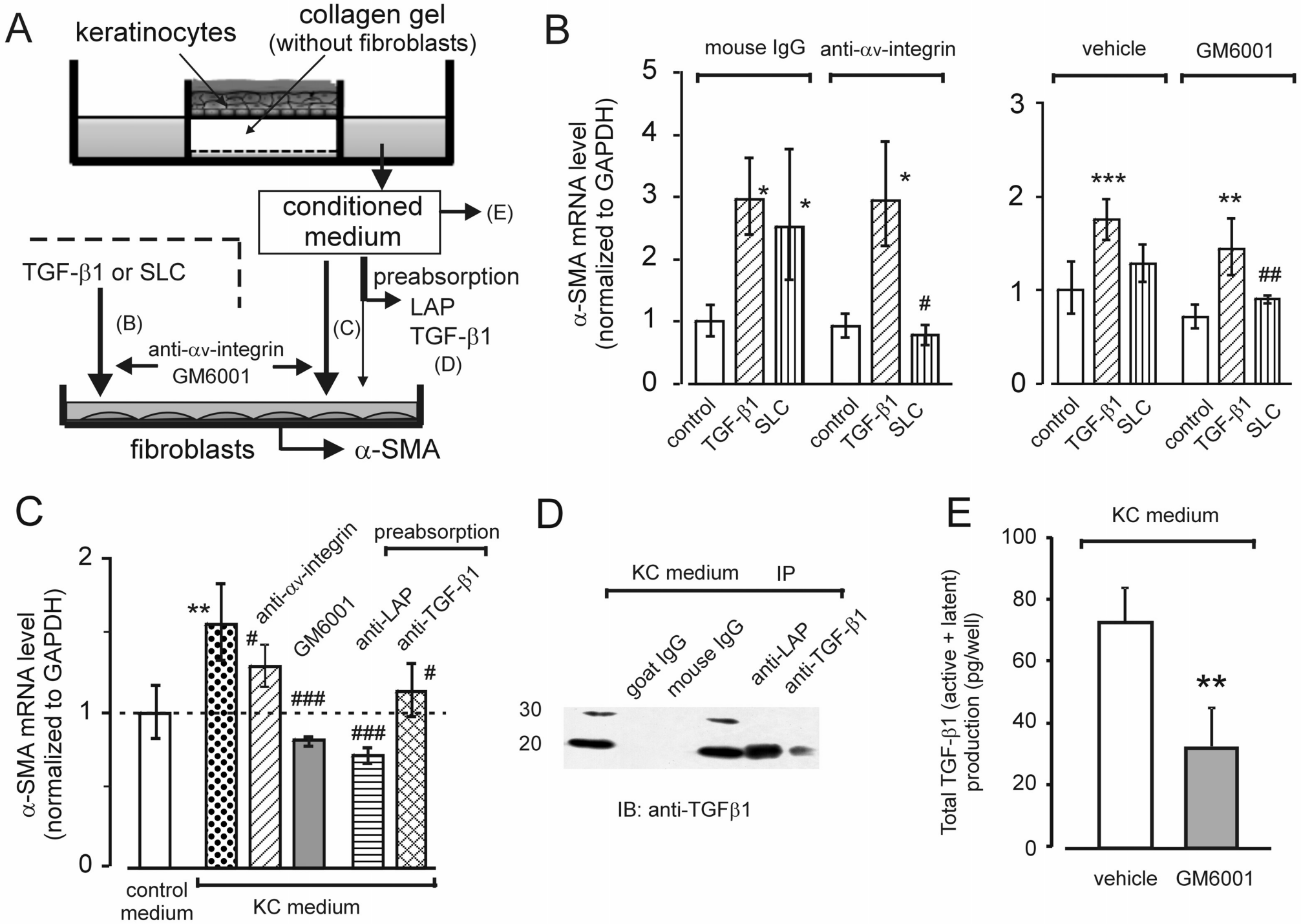


Fig. 5 Hata *et al.*

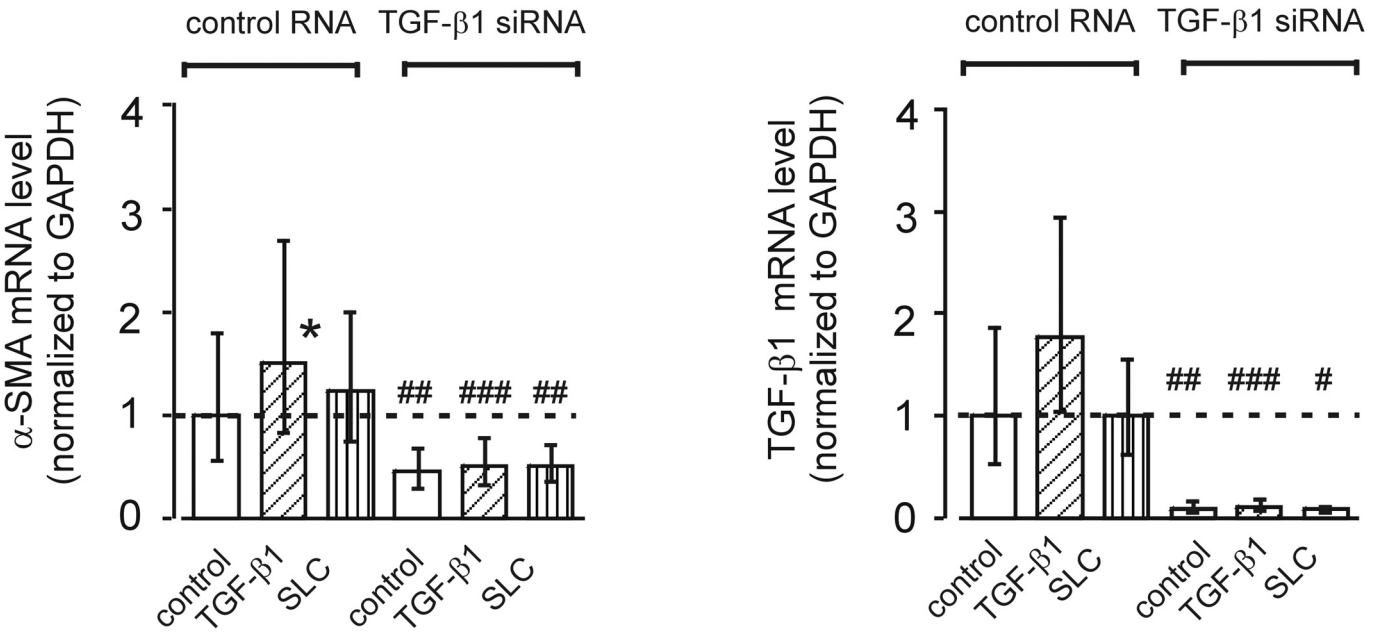


Fig. 6 Hata *et al.*

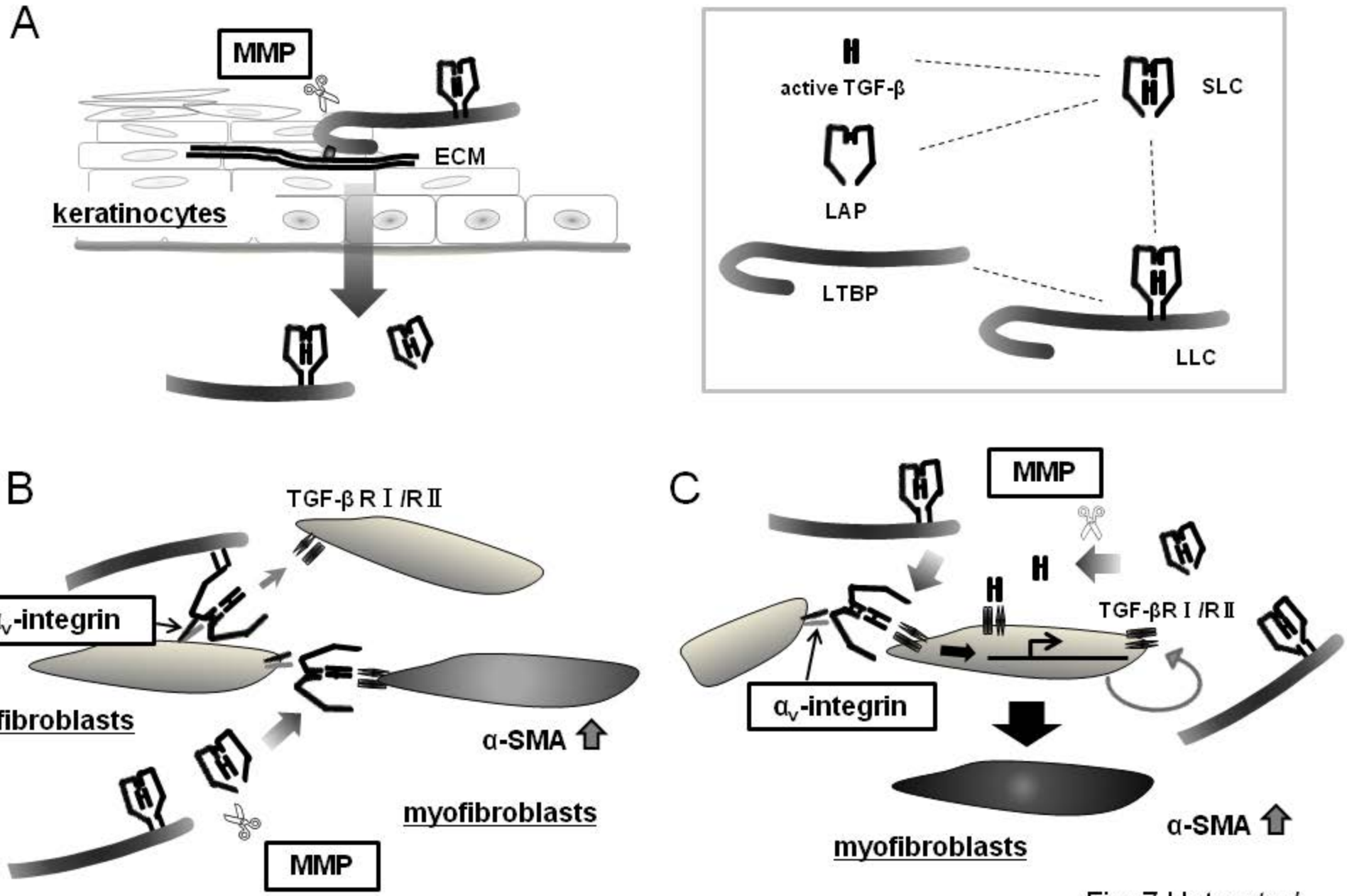
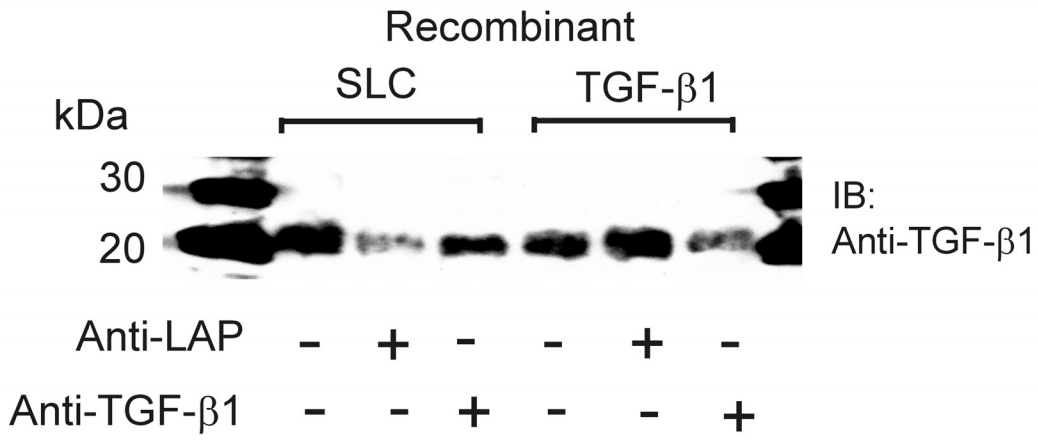


Fig. 7 Hata *et al.*



Suppl. Fig.1 Hata *et al.*

Proteolytic and non-proteolytic activation of keratinocyte-derived latent TGF-β1 induces fibroblast differentiation in a wound-healing model using rat skin

Shozaburo Hata[1)], Kazuhiko Okamura[2)], Mitsutoki Hatta[3)], Hiroyuki Ishikawa[1)], Jun Yamazaki[3)]

Supplemental Material

Figure legend

Suppl. Fig. 1. Efficiency of preabsorption using anti-latency-associated peptide (LAP) and anti-transforming growth factor-β1 (TGF-β1) (clone 9016) antibodies combined with protein A/G agarose in the presence of recombinant small latent complex (SLC) (160 ng/mL) and active TGF-β1 (40 ng/mL). After centrifugation of the suspension that had been mixed using the same time course as in the preabsorption experiments for collagen gel medium, the supernatant fraction was concentrated 10-fold using ultrafiltration membrane units (Amicon Ultracel-3K; Merck, Darmstadt, Germany) and incubated with sodium dodecyl sulfate in non-reducing conditions. An anti-TGF-β1 antibody (G122A) was used to detect active TGF-β1 dimers on the immunoblots.