Article

Rap1 regulates hepatic stellate cell migration through the modulation of RhoA activity in response to transforming growth factor-β1

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Abstract: Although the migration of hepatic stellate cells (HSCs) is important for hepatic fibrosis, the regulation of HSC migration is poorly understood. Interestingly, transforming growth factor (TGF)-β1 induces monocyte migration to sites of injury or inflammation in the early phase but inhibits cell migration in the late phase. In this study, we investigated the role of RhoA signaling in TGF-β1-induced HSC migration. We found that TGF-β1 increased the protein and mRNA levels of α-SMA and collagen type I in HSC-T6 cells. The level of RhoA-GTP in TGF-β1-stimulated cells was significantly higher than that in control cells. Moreover, cofilin phosphorylation and F-actin formation was more strongly detected in TGF-β1-stimulated cells than in control cells. Additionally, TGF-β1 induced the activation of NF-κB and the expression of extracellular matrix proteins and several cytokines in HSC-T6 cells. The active form of Rap1 (Rap1 V12) suppressed RhoA-GTP levels, whereas the dominant negative form of Rap1 (Rap1 N17) augmented RhoA-GTP levels. Therefore, we confirmed that Rap1 regulates RhoA activation in TGF-β1-stimulated HSC-T6 cells. These findings suggest that TGF-β1 regulates Rap1, resulting in RhoA suppression, NF-κB activation and F-actin formation during the migration of HSCs.

Keywords: cell migration 1; hepatic stellate cell 2; TGF-β1 3; Rap1 4; RhoA 5; NF-κB 6

1. Introduction

Hepatic fibrosis is characterized by excessive deposition of extracellular matrix (ECM) mediated by activated hepatic stellate cells (HSCs) [1]. Activated HSCs are believed to play a major role via proliferation and production of ECM and even migrate to fibrotic areas by transforming into myofibroblasts [2]. Although most studies in hepatic fibrosis have focused on proliferating and producing ECM by activated HSCs, recent studies have demonstrated that the migration of HSCs could have an important role in hepatic fibrosis, including ECM and growth factor production [3,4]. Cell migration plays an essential role in a variety of biological events. Cell migration is a dynamic

 doi:10.20944/preprints201809.0133.v1

and cyclic process that is regulated by small GTPase proteins [5]. Cdc42 regulates the direction of movement, and Rac induces membrane protrusion at the leading edge of cells through actin polymerization and stimulation of integrin adhesion complexes [6]. Rho regulates actin-myosin contraction and retraction in the cell body and lagging edge [7,8]. Among the cytoskeleton components, actin filaments have a major role in the formation of stress fibers during cell adhesion and migration. Rho GTPase is known to regulate the actin cytoskeleton, cell polarity, gene expression, microtubule dynamics, and vesicular trafficking [9].

Transforming growth factor (TGF)-β1 is known as a multifunctional cytokine that regulates cell proliferation, differentiation, migration, survival, carcinogenesis, wound healing, immune reaction, and fibrosis [10]. In particular, TGF-β1 is the most important profibrotic cytokine in hepatic fibrosis. Interestingly, TGF-β1 activates Rho subfamily GTPases, including RhoA, Rac1, and Cdc42, in various cell lines, including the HSC-T6 cell line [11-14]. Li et al. demonstrated that TGF-β1-induced RhoA activation in activated HSCs mediates the migration of HSCs via the Smad/JNK/p38 signaling pathway [15].

In addition to the Smad-dependent TGF-β1 signaling pathway, TGF-β1 can induce the activation of the nuclear factor-κB (NF-κB) signaling pathway, leading to the migration of cells [11]. NF-κB is a well-known transcription factor that regulates the expression of cytokines, chemokines, cell adhesion molecules, and inducible proteins to control inflammation, apoptosis, and malignancy [16]. Kim et al. demonstrated that IκB kinase (IKK)-γ stimulates RhoA activation, which leads to the direct phosphorylation of IKKβ and subsequent NF-κB activation that induces chemokine expression and cell migration in response to TGF-β1 [17]. As a member of the Ras superfamily of small GTPases, Rap1 is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Rap1 is associated with the regulation of integrin- and cadherin-mediated cell adhesion and of recycling, avidity, and affinity of integrins via the inside-out activation process [18]. Rap1 signaling can both positively and negatively modulate the activity of Rho family proteins such as Cdc42, Rac1, and RhoA. Moon et al. demonstrated that Rap1 inhibits cell migration by regulating RhoA activity in response to TGF-β1 [19]. Interestingly, Rap1 is an important modulator of the NF-κB signaling pathway [20,21]. Although the influence of Rho GTPase signaling on HSC migration during hepatic fibrosis has been reported [15], the role of NF-κB signaling in response to TGF-β1 via RhoA GTPase activation has not been investigated in activated HSCs. Therefore, in this study, we investigated the mechanism by which Rap1 regulates RhoA activity by NF-κB signaling during TGF-β1-induced HSC migration.

2. Results

2.1. TGF-β1 induces the activation of HSCs

TGF-β1 is known to induce the activation of HSCs. To confirm the activation of HSCs after TGF-β1 treatment (Figure 1A-1D), we investigated the major markers of activated HSCs, namely, α-SMA and collagen type I. Indeed, compared with that in untreated HSC-T6 cells the mRNA expression of α-SMA and collagen type 1 was significantly increased in TGF-β1-treated HSC-T6 cells (Figure 1E and 1F), indicating that TGF-β1 induces the activation of HSCs.

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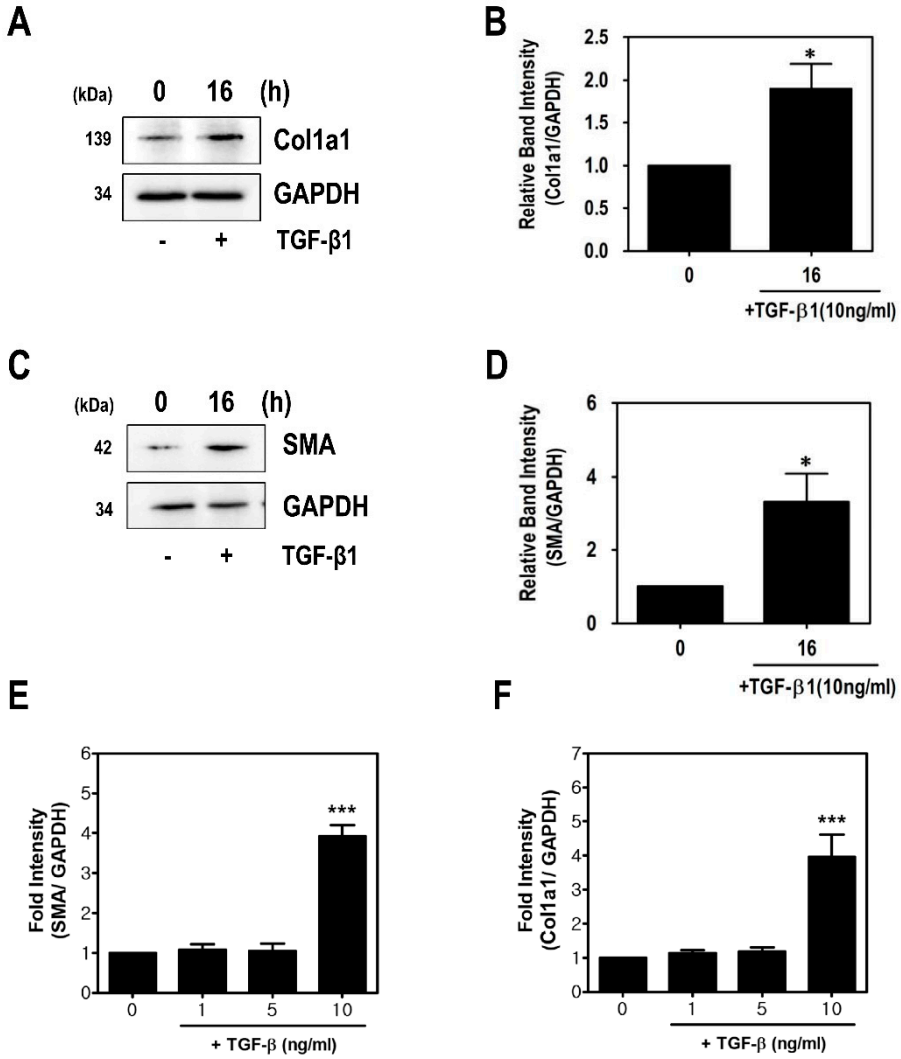

Figure 1. TGF-β1 induces the expression of α-SMA and collagen type 1. (A-D) HSC-T6 cells were treated with or without 10 ng/ml TGF-β1 for 16 h. Western blot analysis of the expression levels of α-SMA and collagen type 1 (mean ± SEM of n = 3, **p < 0.05, Student's t-test). (E and F) Real-time PCR analysis of α-SMA and collagen type 1 mRNA expression. (mean ± SEM of n = 3, ***p < 0.001, one-way ANOVA, Tukey's post hoc test).

2.2. TGF-β1 regulates F-actin formation through RhoA signaling

To examine whether TGF-β1 activates RhoA in HSC-T6 cells, we performed a pull-down assay with the GST-Rhotekin-RBD in HSC-T6 cells with/without TGF-β1 treatment, as previously described [19]. Compared with that in untreated HSCs, the expression level of RhoA-GTP was significantly increased in TGF-β1-treated cells (Figure 2A and 2B).

To further investigate the mechanism of TGF-β1-induced RhoA signaling, we assessed whether TGF-β1 modulates the RhoA-ROCK-LIMK-cofilin pathway. In previous studies, RhoA phosphorylated at Ser188 (p-RhoA) was shown to negatively regulate RhoA activity by promoting interactions with RhoGDI and translocate RhoA from the membrane to the cytosol [22] to enhance phospho-LIMK1/2 (p-LIMK1/2) and phospho-cofilin (p-cofilin) levels (Figure 2A and 2B). Similar to the above results, TGF-β1 induces an increase in the expression of p-cofilin in activated HSCs (Figure 2C and 2D).

Previous studies have shown that RhoA activation plays a role in the regulation of cytoskeleton reorganization through actin stress fiber formation and cell adhesion induction [23,24]. Thus, we

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investigated the effect of TGF-β1 on the formation of actin stress fibers in HSC-T6 cells. Stress fibers were observed to form filamentous actin (F-actin), which was detected with FITC-conjugated phalloidin. As shown in Figure 2E, F-actin formation was more strongly observed in TGF-β1-treated HSC-T6 cells than in untreated HSC-T6 cells (Figure 2E).

To investigate whether RhoA signaling regulates the activation of HSCs, we examined changes in α-SMA and collagen type I expression levels after the inhibition of RhoA by Y27632 (a ROCK inhibitor) or Tat-C3 (a Rho-specific inhibitor). Indeed, treatment with Y27632 or Tat-C3 inhibited the expression of α-SMA and collagen type I in TGF-β1-treated HSC-T6 cells. In addition, the inhibition of RhoA with Y27632 and Tat-C3 significantly decreased p-cofilin levels (Figure 3A and 3B).

To assess the level of inhibition of F-actin formation by Y27632 or Tat-C3, we examined the level of F-actin in TGF-β1-treated HSC-T6 cells. Interestingly, RhoA inhibitors also reduced F-actin formation in response to TGF-β1 (Figure 3C), indicating that RhoA signaling regulates cytoskeletal reorganization, including F-actin formation, during the process of HSC activation.

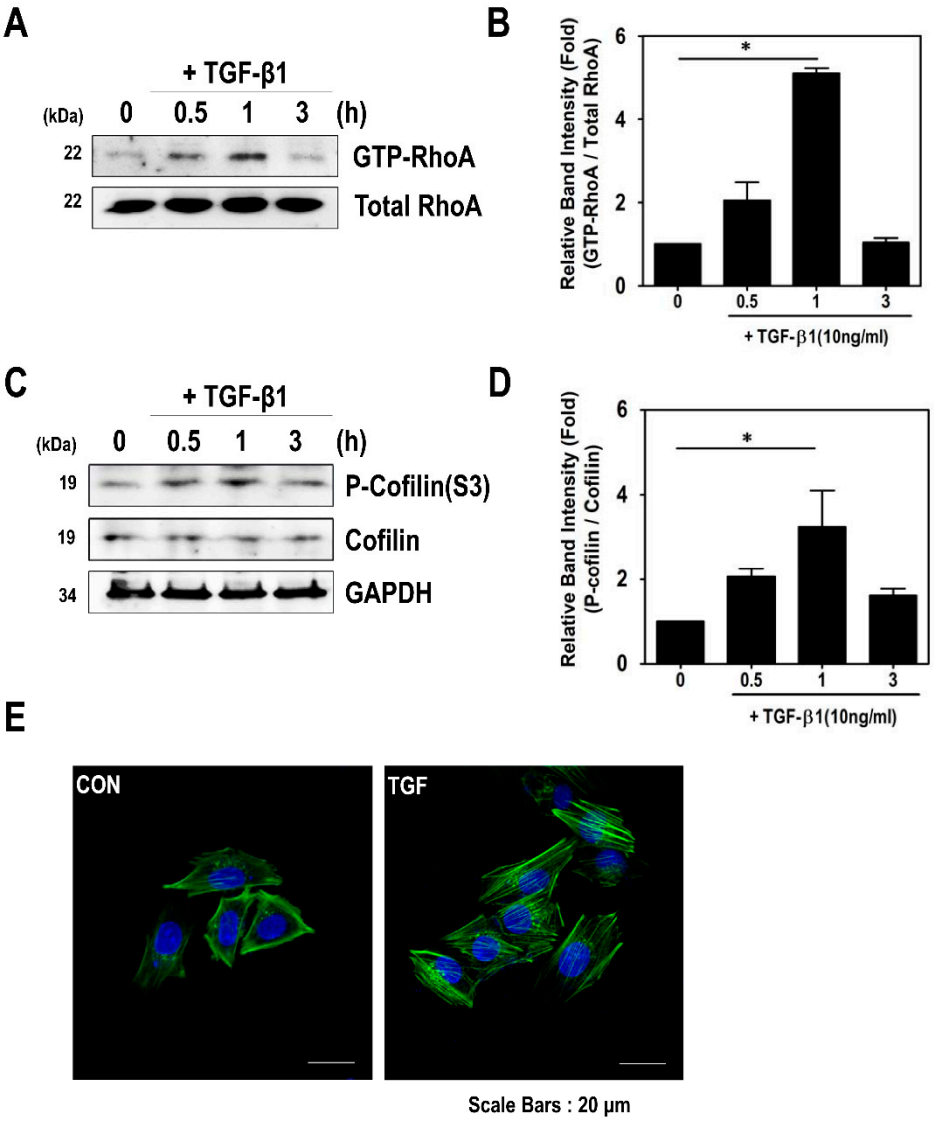

Figure 2. TGF-β1 regulates the RhoA-cofilin pathway. (A and B) Cells were incubated with TGF-β1 for various durations (0 h, 0.5 h, 1 h and 3 h). Detection of GTP-RhoA was performed by GST pull-down assays. Total protein levels of RhoA were detected by Western blotting (mean ± SEM of n = 3, *p < 0.05, one-way ANOVA, Tukey's post hoc test). (C and D) Phosphorylation of cofilin was

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assessed in TGF-β1-stimulated HSC-T6 cells (mean ± SEM of n = 3, *p < 0.05, one-way ANOVA, Tukey's post hoc test). (E) Immunocytochemical staining for F-actin in HSC-T6 cells using Alexa Fluor 488-phalloidin (green). DAPI (blue) was used to counterstain the nuclei. All images are representative of multiple images from three (n = 3) independent experiments (scale bars, 20 μm).

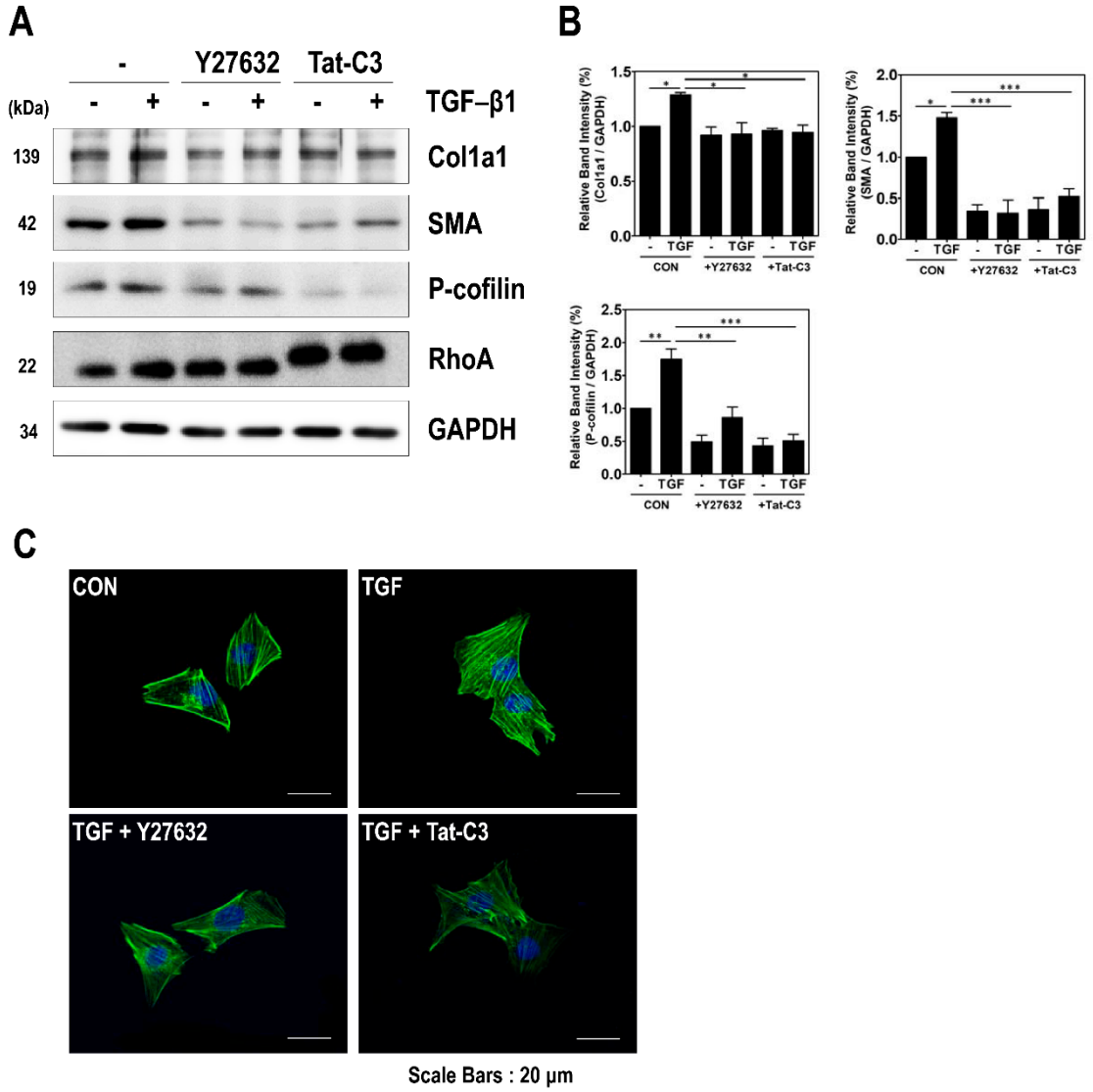

Figure 3. Inhibition of RhoA blocks F-actin formation. (A and B) HSC-T6 cells were incubated with 10 μM Y27632 and 1 μg/ml Tat-C3 in the presence of TGF-β1. Levels of α-SMA, p-cofilin and collagen type 1 were assessed by Western blotting (mean ± SEM of n = 3, *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA, Tukey's post hoc test). (C) Immunocytochemical staining for F-actin in HSC-T6 cells using Alexa Fluor 488-phalloidin (green). DAPI (blue) was used to counterstain the nuclei. All images are representative of multiple images from three (n = 3) independent experiments (scale bars, 20 μm).

2.3. TGF-β1 regulates RhoA via Rap1

Previous studies have reported that Rap1 inhibits cell migration by suppressing RhoA activity in response to TGF-β1 in macrophages [19]. We thus investigated whether TGF-β1 regulates the activity of Rap1 in HSC-T6 cells using a pull-down assay with the His-RalGDS-Rap-binding domain (RBD). Rap1 activity is inhibited by TGF-β1 at 30 min but continues to increase thereafter (Figure 4A and 4B). In contrast, the levels of GTP-RhoA and GTP-Rap1 showed opposite patterns in a time-dependent manner after TGF-β1 stimulation (Figure 2A and 4A). In addition, inhibition of

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Rap1 (using Rap1 N17: the dominant negative form of Rap1) induced RhoA activation, and activation of Rap1 (using Rap1 V12: the constitutively active form of Rap1) blocked RhoA activation. These results indicated that Rap1 regulates the activity of RhoA in TGF-β1-treated HSC-T6 cells (Figure 4C and 4D).

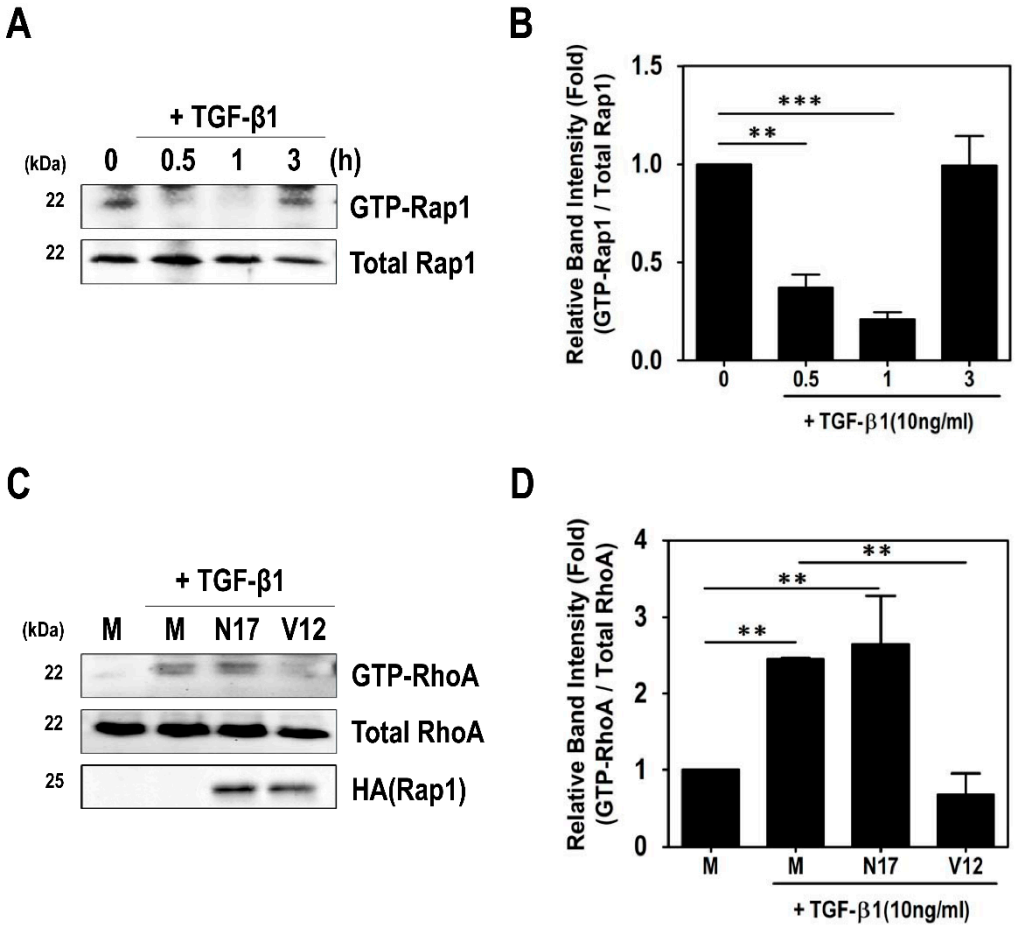

Figure 4. Rap1 downregulates TGF-β1-induced GTP-RhoA. (A and B) Cells were incubated with 10 ng/ml TGF-β1 for various durations (0 h, 0.5 h, 1 h and 3 h). The GTP-bound form of Rap1 was selectively precipitated from cell lysates using the His-RalGDS-RBD fusion protein and Ni-NTA His-Bind resin and detected by immunoblotting with an anti-Rap1 antibody (mean ± SEM of n = 3, **p < 0.01, ***p < 0.001, one-way ANOVA, Tukey's post hoc test). (C and D) MOCK vector, pcDNA-Rap1 N17 (encoding the dominant negative form of Rap1) and pcDNA-Rap1 V12 (encoding the constitutively active form of Rap1) were transfected into HSC-T6 cells, and the cells were stimulated with 10 ng/ml TGF-β1 for 1 h. GTP-RhoA levels were determined using a pull-down assay with GST-Rhotekin-RBD fusion protein and Glutathione-Sepharose beads. RhoA expression was determined by Western blotting using an anti-RhoA antibody (mean ± SEM of n = 3, **p < 0.01, one-way ANOVA, Tukey's post hoc test).

2.4. TGF-β1 regulates HSC-T6 cell migration via TGF-β1-activated kinase 1 (TAK1) and NF-κB signaling

To confirm that the molecular mechanism by which TGF-β1 induces NF-κB activation, we measured the phosphorylation of NF-κB signaling components. Compared with those in untreated cells, the expression levels of p-IKKα/β, p-IκBα, and p-p65 in TGF-β1-treated HSC-T6 cells were significantly increased (Figure 5A and 5B). We measured the expression of ECM proteins and several cytokines in TGF-β1-treated HSC-T6 cells. Treatment with TGF-β1 induced an increase in the expression of fibronectin and TGF-β1, but IL-1β expression was not different between the untreated cells and TGF-treated cells (Figure 5C and 5D).

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Activated HSCs migrate and accumulate at tissue repair sites, regulating ECM production and secretion [25,26]. To confirm this, we measured the migration of activated HSCs using transwell and wound healing assays. Indeed, compared with that of untreated cells, the migration of TGF-β1-treated HSC-T6 cells was significantly increased (Figure 6A-6C). Interestingly, RhoA inhibitors reduced cell migration in TGF-β1-treated HSC-T6 cells (Figure 6E). Additionally, Rap1 activation blocked the migration of HSCs (Figure 6D). Taken together, these findings suggest that TGF-β1 modulates cell migration by regulating ECM protein and chemokine expression through Rap1, RhoA, and NF-κB signaling.

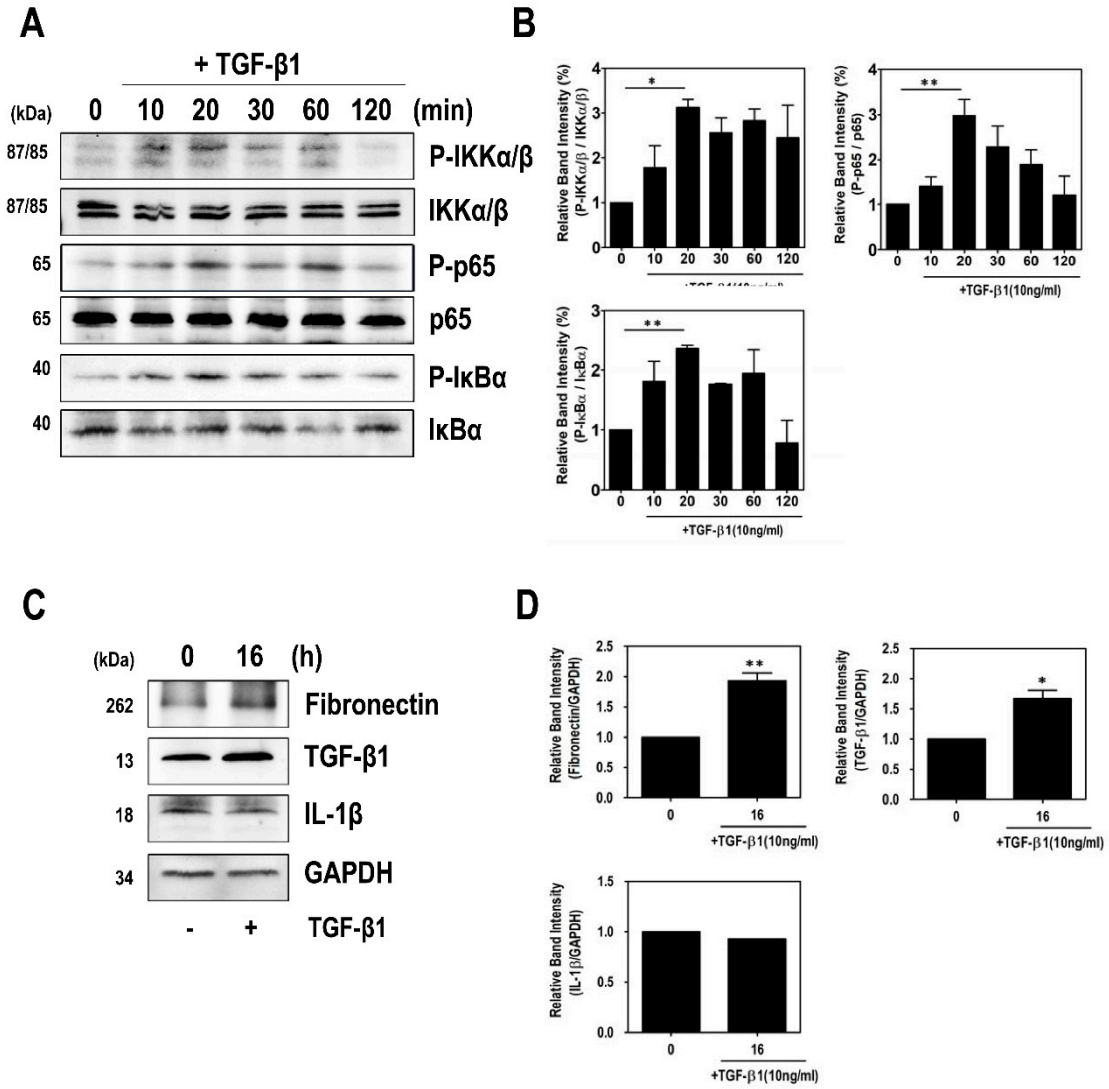

Figure 5. TGF-β1 regulates NF-κB signaling. (A and B) HSC-T6 cells were treated with 10 ng/ml TGF-β1 for various durations (0 min, 10 min, 20 min, 30 min, 60 min and 120 min). The phosphorylation of IκB, IKKα/β, and p65 was analyzed by Western blotting (mean ± SEM of n = 3, *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA, Tukey's post hoc test). (C and D) Cells were treated with or without 10 ng/ml TGF-β1 for 16 h. Western blot analysis of the expression levels of fibronectin, TGF-β1 and IL-β1 (mean ± SEM of n = 3, *p < 0.05, **p < 0.01, Student's t-test).

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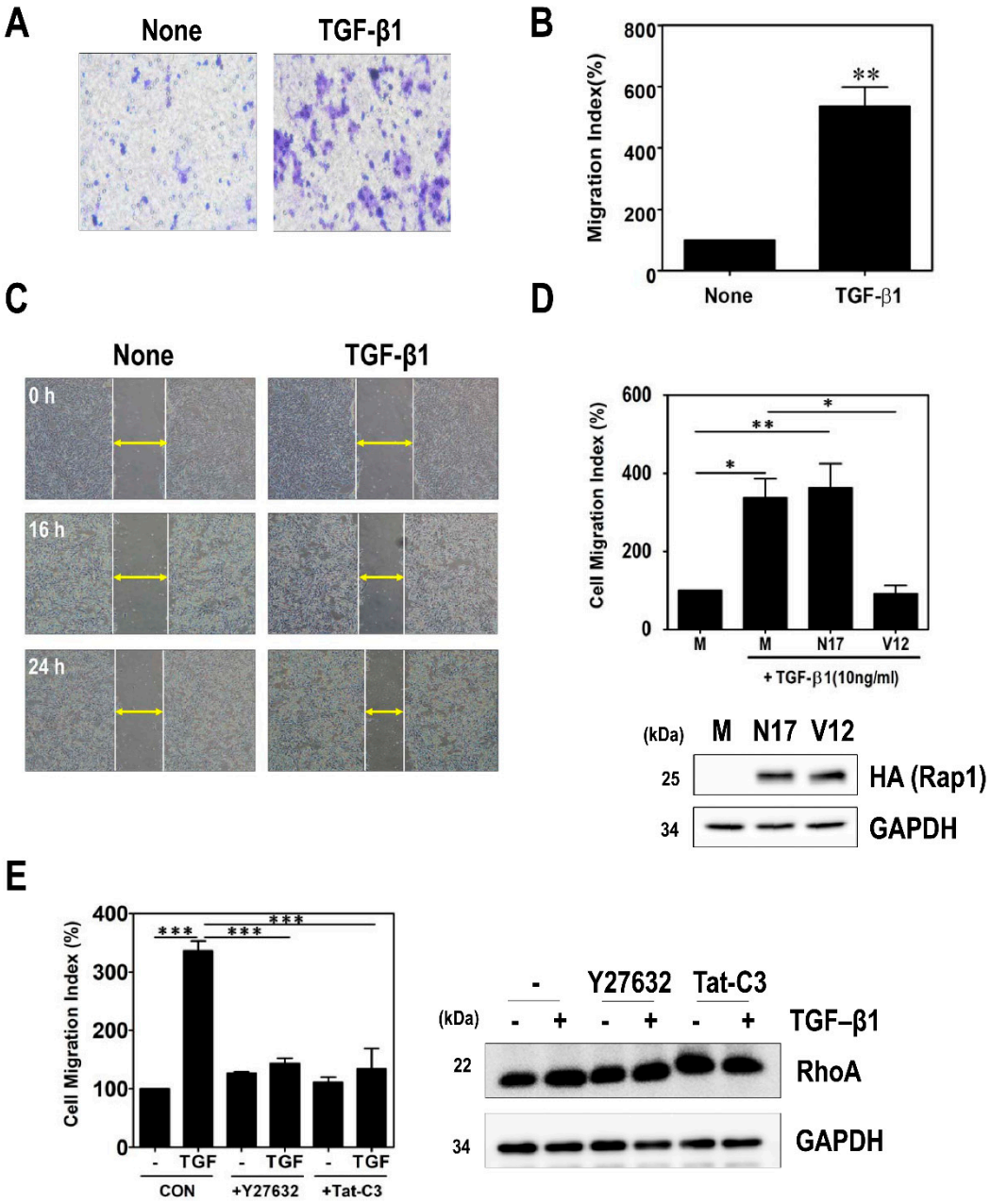

Figure 6. TGF-β1 regulates HSC-T6 cell migration via TAK1 and NF-κB signaling. (A and B) Serum-starved HSC-T6 cells were added to the upper chamber, and 10 ng/ml TGF-β1 was added to the lower chamber. Then, the cells in the upper chamber were allowed to migrate for 16 h (mean ± SEM of n = 3, **p < 0.01, Student's t-test). (C) TGF-β1 induced wound healing in cultured HSC-T6 cells. All images are representative of multiple images from three (n = 3) independent experiments. (D) MOCK vector, pcDNA-Rap1 N17 (encoding the dominant negative form of Rap1) and pcDNA-Rap1 V12 (encoding the constitutively active form of Rap1) were transfected into HSC-T6 cells, and the cells were allowed to migrate for 16 h (mean ± SEM of n = 3, *p < 0.05, **p < 0.01, one-way ANOVA, Tukey's post hoc test). (E) HSC-T6 cells were incubated with 10 μM Y27632 and 1 μg/ml Tat-C3 and allowed to migrate for 16 h (mean ± SEM of n = 3, ***p < 0.001, one-way ANOVA, Tukey's post hoc test).

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3. Discussion

NF-κB modulates various essential functions of hepatocytes, HSCs, and Kupffer cells [27-29]. The crucial role of NF-κB in the liver is emphasized by the fact that deletion of NF-κB signaling components in mouse models leads to spontaneous liver damage, fibrosis, and hepatocellular carcinoma. TGF-β1-induced polyubiquitination of TAK1 lysine 158 is required for TAK1-mediated Smad-independent IKK, JNK, and p38 activation [30,31] Hepatic fibrosis is a reversible wound healing response characterized by the accumulation of fibrillar matrix in the injured liver. Activation of HSCs during hepatic fibrosis is considered a major factor in the progression of chronic liver disease [3,32,33]. HSCs are an important source of growth factors in the liver, and HSCs not only increase in numbers but also increase ECM production per cell, leading to fibrosis [34]. Activated HSCs themselves are a key source of cytokines and establish an autocrine stimulation loop. HSCs are activated by TGF-β1 [35], and the activation of HSCs increases expression of TGF-β1. Our study also demonstrated that TGF-β1 increases the expression of protein and mRNA levels of α-SMA and collagen type I, consistent with previous studies (Figure 1) [36,37].

Cell migration plays a pivotal role in many biological processes, including differentiation and immune responses. Cell migration is a multistep process involving changes in the cytoskeleton and can be divided into the following four steps: 1) lamellipodium extension, 2) new adhesion, 3) cell body contraction, and 4) tail detachment [38]. Rho GTPases regulate many aspects of cell migration and affect other components of the cytoskeleton as well as cell-substrate adhesion and matrix reconstitution. Actin filament reorganization through cofilin activity is observed at the leading edge of migrating cells [39]. RhoA-ROCK-LIMK signaling induces the phosphorylation of cofilin, thereby inactivating it and promoting actin polymerization [40]. We found that TGF-β1 significantly increased the level of p-cofilin that induced cofilin activation, thereby facilitating F-actin formation. In addition, the inhibition of RhoA signaling by Y27632 and Tat-C3 significantly decreased F-actin formation (Figure 3). Iwamoto et al. [41] reported that Y27632 consistently suppressed cell spreading and proliferation and that Y27632 also decreased the gene expression and protein accumulation of collagen type I in primary cultured HSCs. Kato et al. also demonstrated that Tat-C3 treatment in activated HSCs distorted the cell shape and decreased stress fiber formation and the amount of collagen type I [42]. These results were consistent with findings in a rat model of dimethyl nitrosamine-induced hepatic fibrosis [43]. Our results also demonstrated that inhibition of RhoA signaling suppressed the phosphorylation of cofilin and subsequently decreased F-actin formation in activated HSCs, strongly suggesting that RhoA signaling is an important mechanism underlying phenotypic changes in HSCs during the initiation of hepatic fibrosis.

Rap1 is a member of the Ras GTPase family, and it is inactive in its GDP-bound form and becomes active after binding to GTP. GAPs and GEFs regulate Rap1, with GAPs promoting the GDP-bound (inactive) form and GEFs promoting the GTP-bound (active) form. GTP-binding Rap1 binds to effector molecules, including Raf-1, B-Raf, RalGDS and AF-6 [44,45]. Rap1 activation is associated with CD31-dependent adhesion to ICAM and VCAM, thereby mediating the interaction of platelets and leukocytes with vascular endothelial cells and facilitating the transmigration and extravasation of leukocytes [46]. In addition, LFA-1 binds to ICAM-1 after the introduction of constitutively active Rap1 and increases Rap1-dependent adhesion and migration in pro-B cells [47,48]. Regarding the stimulation of cell migration by Rap1, we have already shown that Rap1 inhibits the activity of RhoA and inhibits cell migration in mouse macrophages [19]. Although the mechanism by which Rap1 signaling modulates HSC migration is unknown, our data suggest that Rap1 can regulate the ability of cells to produce F-actin and express cytokines by inhibiting the activity of RhoA.

In this study, we found that TGF-β1 regulated Rap1 activity and thus the RhoA-mediated signal transduction pathway. TGF-β1 increased the activity of RhoA after short-term treatment but reduced the activity of Rap1 (Figures 2 and 3). In a previous report, a Rap1-dependent RhoGAP (ARAP3) was activated by Rap1 and induced RhoA inactivation in PC12 cells with neurite outgrowth in response to nerve growth factor (NGF) [49]. ARAP3 is a potent GAP for RhoA, and Rap1, and PtdIns(3,4,5)P3 activate the Rho GAP activity of ARAP in vitro and in vivo [50]. Similarly,

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dominant negative Rap1 (Rap1 N17) increased the activity of RhoA, whereas constitutively active Rap1 (Rap1 V12) decreased the activity of RhoA (Figure 3).

TGF-β is one of the multifunctional cytokines in most cells. Several mitogen-activated protein kinase kinase kinases (MAPKKKs), including TAK1, play a pivotal role in NF-κB activation. Overexpression of TAK1 and TAK1-binding protein 1 (TAB1) induces the nuclear translocation of the NF-κB p50/p65 heterodimer with the degradation of IκBα and IκBβ [51]. Activated TAK1 via Ubc13-Uev1A-mediated K63-linked polyubiquitin was able to specifically phosphorylate IKKβ at S177 and S181 [52]. IKKγ binds to the RhoA-RhoGDI complex and promotes RhoA activation by destroying the RhoA-RhoGDI complex in response to TGF. Active RhoA-GTP and ROCK phosphorylate IKKβ, followed by the phosphorylation of IκB and p65 to induce NF-κB activation [17]. We also found that TGF-β1 increased the phosphorylation of TAK1, IKKα/β, IκBα and p65 (Figure 5) in activated HSCs. The decreased expression of TGF-β1 reduced the DNA-binding activity of NF-κB and decreased the production of fibronectin and chemokines such as IL-8 and MCP-1 in the kidney [53]. We also demonstrated that TGF-β1 increased the expression of fibronectin and TGF-β1 and the formation of F-actin, ultimately enhancing the migration of cells.

Taken together, our results demonstrated that Rap1 contributes to RhoA inactivation, thereby inhibiting F-actin formation and NF-kB activity, eventually regulating HSC migration. These results are important for understanding the activity of TGF-mediated HSCs, the accumulation of ECM, and mechanisms associated with cell migration.

4. Materials and Methods

4.1. Materials

Bovine serum albumin (BSA), Y27632, and recombinant TGF-β1 protein were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Anti-RhoA, anti-Rap1, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and anti-cofilin antibodies were obtained from Santa Cruz Biotechnology (SantaCruz, Dallas, TX, USA). Anti-α-SMA, anti-collagen type 1, anti-p-cofilin, anti-p-p65, anti-p65, anti-p-IκBα, anti-IκBα, anti-p-IKKβ and anti-IKKα/β antibodies were obtained from Cell Signaling Technology (Cell Signaling Technology, Danvers, MA, USA).

4.2. Cell culture and transfection

HSC-T6 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (HyClone, South Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone), 100 units/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific, Rockford, IL, USA) at 37℃ with 5% CO2. Transient transfections were carried out using the Lipofectamine 2000 reagent (Thermo Fisher Scientific) according to the manufacturer's instructions.

4.3. Western blot analysis

Cells were collected and washed once with ice-cold phosphate-buffered saline (PBS) and lysed with RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 10 mM NaF, 1 mM Na3VO4, 2 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin). The cell lysates were centrifuged at 13,000x g for 20 min, and protein concentrations in the supernatants were analyzed using a Bradford assay (Bio-Rad Laboratories, Inc.). Equal amounts of proteins were separated using SDS-PAGE, transferred to PVDF membranes, and probed with the appropriate antibodies. Immunoreactive bands were visualized on digital images captured with FUSION FX7 SPECTRA (Vilber Lourmat, Eberhardzell, Germany) using EzwestLumi plus Western blot detection reagent (ATTO corporation, Tokyo, Japan), and the band intensities were quantified using ImageJ (NIH) software. Statistical analyses were performed using GraphPad Prism 4 (San Diego, CA, USA).

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4.4. Immunocytochemistry

HSC-T6 cells were treated with 10 ng/ml TGF-β1 in DMEM for the indicated times at 37°C under 5% CO2. The cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. The cells were permeabilized with 0.2% Triton X-100 for 10 min, and then the samples were blocked with 2% BSA in PBS for 15 min at room temperature. For fluorescence labeling, the cells were sequentially incubated with 5% BSA/PBS for 1 h and rabbit polyclonal anti-RhoA (1:100; Santa Cruz Biotechnology) and goat polyclonal anti-PrP (1:200; Santa Cruz Biotechnology) antibodies overnight at 4°C. The cells were washed and incubated with fluorescein isothiocyanate (FITC)- or rhodamine-conjugated anti-mouse or anti-rabbit IgG (1:500) for 1 h at room temperature. The immunolabeled cells were examined using an LSM 700 laser confocal microscope (Zeiss, Oberkochen, Germany).

4.5. Glutathione S-transferase (GST)-Rhotekin-Rho-binding domain (RBD) pull-down assays for activated RhoA

Cells were collected and washed with PBS and then lysed in binding/washing/lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, 1 mM DTT, 5% glycerol, 10 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, and 1 mM EGTA) with a protease inhibitor cocktail tablet. The lysates were centrifuged at 13,000x g for 10 min at 4°C. The supernatant was incubated with GST-Rhotekin-RBD to detect GTP-RhoA. The beads were washed 3 times with binding/washing/lysis buffer. The bound proteins were eluted with 2x Laemmli sample buffer by boiling. The samples were electrophoresed and analyzed by Western blotting with an anti-RhoA antibody.

4.6. Real-time quantitative reverse transcription (RT)-polymerase chain reaction (PCR)

Total RNA was extracted with Isol-RNA lysis reagent (5 Prime Inc., MD, USA). RT of the mRNA and PCR were performed using the Superscript II system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The primers were as follows: α-SMA, forward, 5'-AAGCCCAGCCAGTCGCTGTCA-3' and reverse, 5'-GAAGCCGGCCTTACAGAGCCC-3'; collagen type I, forward, 5'-GCCTCTTAGGGGCCACT-3' and reverse, 5'-ATTGGGGACCCTTAGGCCAT-3' and GAPDH, which was used as an internal control, forward, 5'-TGGCCTTCCGTGTTCCTA-3' and reverse, 5'-GAGTTGCTGTTGAAGTCGCA-3'. Real-time quantitative RT-PCR was performed using a SYBR Green PCR mixture (Enzynomics, Deajeon, Korea).

4.7. Wound healing assay

Cells (5×10^5 per well) were seeded in six-well plates coated with gelatin and grown to confluence in DMEM containing 10% FBS. Confluent cells were incubated in serum-free medium for 24 h. The cells were treated with TGF-β1 for 16 h and washed with PBS, and then the cells layer wounded with a pipette tip. Fresh, complete medium was added, and the cells were allowed to close the wound for 24 h. Photographs were captured at the same position of the wound.

4.8. Cell migration assay

The migration of HSC-T6 cells was examined using a transwell assay (Corning Inc., New York, NY, USA). Approximately 1×10^5 cells were resuspended in 200 μl DMEM and seeded on cell culture inserts with 8.0 μm pores, which were precoated with gelatin. Medium (700 μl) with 10 ng/ml TGF-β1 was added to the bottom chamber. The cells were allowed to migrate for 16 h. The cells were fixed and stained with crystal violet, and the number of cells on the lower surface of the filters was counted under the microscope. A total of 4 fields were counted for each transwell filter.

4.9. Statistical analysis

Data were analyzed using Student's t-test, one-way ANOVA followed by Tukey's post hoc test and multiple comparison tests using the GraphPad Prism 4 software.

 doi:10.20944/preprints201809.0133.v1

Author Contributions: Planning and conceptualizing the study: Sung-Eun Kim; study design: Mi-Young Moon, Hee-Jun Kim, Sung-Eun Kim; drafting the article: Mi-Young Moon, Sung-Eun Kim; performed experiments: Mi-Young Moon, Hee-Jun Kim, Mo- Jong Kim; data analysis and interpretation: Mi-Young Moon, Hee-Jun Kim, Mo- Jong Kim, Ji-Won Park, Ki Tae Suk, Jae-Bong Park, Sung-Eun Kim; All the authors reviewed the manuscript and approved the final version.

Acknowledgments: This research was supported by a grant of the basic science research program through the National Research Foundation (NRF) of Korea funded by the Ministry of Science and ICT (grant number: 2017R1C1B1005996) National Research Foundation of Korea (NRF) grant funded by the Korean Government (NRF-2014R1A1A3052139) and a Hallym University Research Fund (HURF-2015-56).

Conflicts of Interest: The authors declare that they have no conflict of interest.

Abbreviations

HSC	Hepatic stellate cells
TGF	Transforming growth factor
SMA	smooth muscle actin
GAPDH	glyceraldehyde-3 phosphate dehydrogenase
Col1a1	collagen type I
GST-Rhotekin-RBD	glutathione S-transferase-Rhotekin Rho-binding domain
HIS-RalGDS-RBD	His-Ral guanine nucleotide dissociation stimulator Rap binding domain

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