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MMP-14 Mediated MMP-9 Expression Is Involved in TGF-Beta1-Induced Keratinocyte Migration

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Abstract The importance of expression of matrix metalloproteinase (MMP) in keratinocyte migration is well established, but its role remains unclear. Here we investigated the function of MMP-14 in TGF-β1-induced keratinocyte migration. TGF-β1 stimulated cell migration and the expression of MMP-2, -9 in HaCaT human keratinocyte cells. When we lowered MMP-14 mRNA with siRNA, cell migration, and MMP-9 expression decreased. Furthermore, the MMP-14 siRNA also reduced activation of JNK in response to TGF-β1, and a JNK-specific inhibitor decreased both cell migration and MMP-9 expression. Taken together, these results suggest that TGF-β1-induced HaCaT cell migration is mediated by MMP-14, which regulates MMP-9 expression via JNK signaling. J. Cell. Biochem. 104: 934–941, 2008.

Key words: cell migration; MMP; TGF-beta1; JNK

Cell migration is a complex process that requires controlled degradation of the extracellular matrix (ECM), which is achieved by extracellular proteases such as serine proteases and matrix metalloproteinases (MMPs) [Okada et al., 1997; Kerkelä and Saarialho-Kere, 2003]. The MMPs are a family of structurally related zinc-dependent endopeptidases that are secreted in inactive form and activated by removal of N-terminal propeptides [Chakraborti et al., 2003]. The MMP family has been classified into gelatinases, stromelysins, collagenases, and membrane type-MMPs (MT-MMPs), depending on their substrate specificity and structural properties. Many physiological processes including wound healing, tissue remodeling, angiogenesis, and embryonic development are regulated by MMPs [Stetler-Stevenson et al., 1993; Sternlicht and Werb, 2001]. However, the abnormal expression of various MMPs is known to contribute to a variety of pathological conditions such as rheumatoid arthritis, atherosclerosis, and tumor invasion [Ravanti and Kähäri, 2000; Chakraborti et al., 2003; Kerkelä and Saarialho-Kere, 2003]. The expression of these MMPs is tightly regulated at the transcriptional and post-transcriptional levels [Stetler-Stevenson et al., 1993].

MT1-MMP (MMP-14), which is constitutively anchored to the cell membrane, activates MMPs such as MMP-2, and cleaves various types of ECM proteins such as collagens, laminins, and its ligands, the integrins [Okada et al., 1997; Chakraborti et al., 2003]. The latent forms of some cytokines are also cleaved and activated by MMP-14 [Sato et al., 2005]. In addition, reduction of MMP-14 mRNA decreases angiogenesis and tumor invasiveness [Robinet et al., 2005; Rutkauskaite et al., 2005]. These data suggest that MMP-14 plays an important role in cell migration not only by regulating the activity of downstream MMPs, but also by processing and activating migration-associated proteins such as integrins and ECMs, and inducing a variety of intracellular signaling events [Baumann et al., 2000; Seiki, 2003]. Although the role of MMP-14 in cell migration has been

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investigated [Endo et al., 2003; Udayakumar et al., 2003], its mechanism of action is poorly understood.

Cell migration requires the coordinated expression of various signaling molecules including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), small GTPases, macrophage stimulating protein (MSP), and TGF-β1 [Santoro and Gaudino, 2005]. TGF-β has many roles in embryogenesis, morphogenesis, differentiation, senescence, tumorigenesis, and apoptosis [O'Kane and Ferguson, 1997; Gold, 1999; Siegel and Massague, 2003], but it is also known to be the most potent cytokine in terms of stimulating cell migration [O'Kane and Ferguson, 1997; Philipp et al., 2004; Hosokawa et al., 2005].

In this study, we tested whether TGF-β1-induced HaCaT human keratinocyte migration is linked to MMP-14 expression. Attenuation of MMP-14 expression led to decreased MMP-9 expression and reduced the stimulation of cell migration via the JNK pathway. These results suggest that, during TGF-β1-induced keratinocyte migration, MMP-14, in addition to its well-known role in MMP-2 activation, may stimulate MMP-9 expression by altering level of JNK signaling.

MATERIALS AND METHODS

Cell Culture and Reagents

Human keratinocyte cell line HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum and Gentamycin (Invitrogen, Carlsbad, CA). TGF-β1 was purchased from PeproTech (London, UK). GM6001, SP600125, and MMP-9 specific inhibitor (#444278) were purchased from Calbiochem (Darmstadt, Germany).

Migration Assay

For the migration assay, confluent HaCaT cells in 6 well plates were wounded by the manual scraping with a yellow pipette tip. Plates were washed two times to remove non-adherent cells and replaced serum free media with or without TGF-β1 (4 ng/ml). Assessment of cell migration was performed under microscopy after 24 h of TGF-β1 treatment followed by methanol fixation, and crystal violet staining. The photograph was taken by an inverted microscope (Axiovert S100, Carl Zeiss Meditec, Germany) with digital camera (Axiocam, Carl Zeiss Meditec). The cell migration was analyzed by measuring wound closure width. In each samples, at least 10 areas of wound closure were measured statistically analyzed in three separated experiments.

Western Blot Analysis

The cells were harvested in RIPA lysis buffer. Protein concentration was determined by BCA protein assay kit (Pierce, Rockford, IL). The lysates containing 10 or 20 μg of proteins were loaded and separated on 8% or 10% SDS–PAGE gel, and transferred to a nitrocellulose membrane (Amersham Life Science, Cleveland, OH). 5% skim milk in TBS-T was used for blocking and antibody dilution buffer. The membrane was developed by enhanced chemiluminescence solution (Santa Cruz, Santa Cruz, CA). Prestained molecular weight standards were purchased from Elpis-Biotech (EBM-1018, DaeJeon, Korea). Antibodies used in this study were obtained as follows: Akt, phospho-Smad2/3, Smad2/3, MMP-2, -9, and -14 were from Millipore (Beverly, MA). JNK was from BD Bioscience (San Jose, CA). Phospho-ERK, phospho-p38, phospho-Akt, and p38 were from Cell Signaling (Denvers, MA). ERK, c-Jun, phospho-c-Jun, and phospho-JNK were from Santa Cruz. Actin was from Sigma (St. Louis, MO).

Zymography

The cell culture supernatants were concentrated using Microcon-30 (Millipore). The concentrates of 400 μl medium were applied to 8% SDS–PAGE gel containing 1 mg/ml of gelatin. After electrophoresis, the gel was washed with renaturation buffer (50 mM Tris–Cl, pH 7.5, 2.5% Triton X-100) to remove SDS and incubated in an activation buffer (50 mM Tris–Cl, pH 7.5, 10 mM $CaCl_2$, 1 μM $ZnCl_2$) for 4 h at 37°C. The gel was stained with Coomassie brilliant blue R-250 for 1 h at room temperature and destained in 30% ethanol, 10% acetic acid.

MMP-14 siRNA Stable Transfection

Predesigned two sets of MMP-14 specific siRNA sequences (M14si-1 and M14si-2) and scrambled controls were purchased from Ambion (Austin, TX). From these sequences, we constructed a MMP-14 specific siRNA expression vector using pSilencer 2.1-U6 neo vector (M14si)

system according to manufacture's manual (Ambion). For stable transfection, HaCaT cells were changed the medium to Opti-MEM (Invitrogen) and the cells were transfected with the 1 μg of M14si vector using SiPORT™ *Lipid* siRNA transfection reagent (Ambion). The M14si and scrambled control expressing cells were selected by 500 μg/ml of Geneticin (G418, Invitrogen). Completely attenuation of MMP-14 mRNA was confirmed by RT-PCR and Western blot analysis.

RT-PCR

Total RNA was isolated from scrambled, M14si-1, and M14si-2 stably expressed HaCaT cells using TRIzol reagent (Invitrogen). Reverse transcription was performed with 1.5 μg of RNA in a 50 μl reaction volume. PCR was carried out 3 μl of cDNA with each MMP-14 and β-actin specific primers.

Over-Expression of MMP-2 and MMP-9

pIRES-neo vector which encoded human full length MMP-2 cDNA [Seomun et al., 2001], and MMP-9 (purchased from Invitrogen, #4054882, a full length human MMP-9 cDNA) inserted in pcDNA3.1 vector (Invitrogen) was prepared for stable transfection, respectively. One microgram of each plasmid were used to transfection using the Wellfect-EX transfection reagent (Welgene, Korea). At 2 days after transfection, medium containing 600 μg/ml of G418 was applied to select the transfectants for 2 weeks with media changed every day. Twelve colonies (for MMP-2) and eight colonies (for MMP-9) were selected. The selected colonies were grown and expanded for further experiments and maintained containing 200 μg/ml of G418.

Statistical Analysis

The wound width of cells in Figures 1A, 2B, 3B,C, and 5B were calculated by using Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

TGF-β1 Induces Cell Migration and MMP Expression

To confirm an effect of TGF-β1 on keratinocyte migration, we added 4 ng/ml of TGF-β1 to wounded HaCaT cells for 24 h. Crystal violet staining showed that TGF-β1 increased the cell migration (Fig. 1A). As it is well-known that cell migration is controlled by MMPs [Okada et al., 1997; Ravanti and Kähäri, 2000; Santoro and Gaudino, 2005], we examined the effect of GM6001, a broad range MMP inhibitor. 20 μM of GM6001 blocked TGF-β1-induced cell migration. Above results of keratinocyte migration was quantified by measuring wound width under the microscope. The wound width of the TGF-β1-treated cells was 0.39 ± 0.1 mm, and that of the control cells was 1.08 ± 0.05 mm. The wound width of the GM6001 pretreated cells with and without TGF-β1 was similar to that of the control cells (1.02 ± 0.12 mm and 1.11 ± 0.09 mm, respectively).

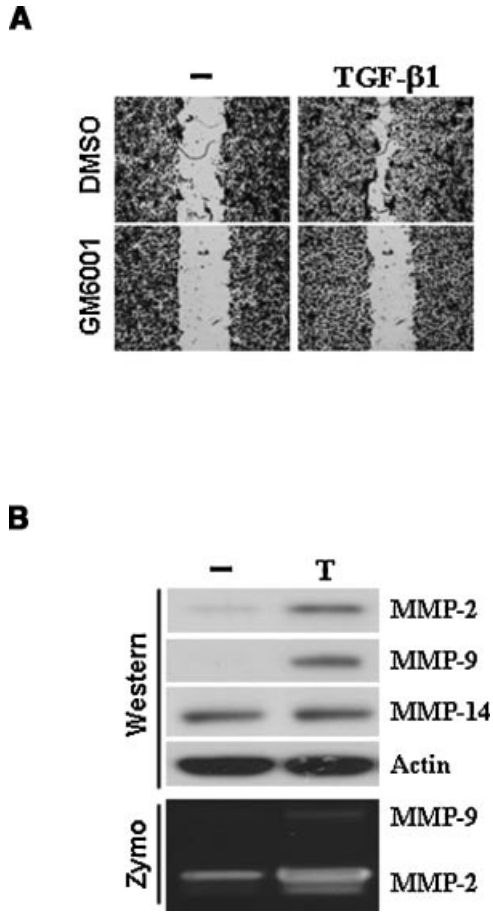

Fig. 1. TGF-β1-induced HaCaT cell migration and MMP expression. **A**: A wound was made with a yellow tip in confluent HaCaT cells, and the cells were pretreated with DMSO or GM6001 (20 μM) before addition of 4 ng/ml TGF-β1. After 24 h, crystal violet staining was used to assess TGF-β1-induced cell migration. **B**: The expressions of MMP-2, -9, and -14 by TGF-β1 were analyzed in total cell lysates (for MMP-14 and Actin) and concentrated cell culture medium (for MMP-2 and -9) by Western blotting and zymography, respectively. The results shown are from one of three separate experiments.

Next, we investigated which MMPs may be involved in the TGF-β1-induced cell migration. The expressions of MMP-1, -3, -7, -10, -11, and -13 mRNA, which were expressed in wounded skin [Ravanti and Kähäri, 2000; Santoro and Gaudino, 2005], were not increased by TGF-β1 (data not shown). Western blotting and zymography revealed increased expression of MMP-2 and MMP-9 (Fig. 1B). MMP-14 did not significantly increased by TGF-β1, but showed steady level of expression both control and TGF-β1-treated cells.

Attenuation of MMP-14 Blocks Cell Migration and MMP-9 Expression

Next, we checked the role of MMP-14, since it is a well-known upstream activator of MMPs such as MMP-2 [Okada et al., 1997; Baumann et al., 2000], and there are evidences that attenuation of MMP-14 with siRNA affects angiogenesis and tumor invasiveness [Robinet et al., 2005; Rutkauskaite et al., 2005]. We made an MMP-14 mRNA specific siRNA expression vector and selected transformants stably over-expressing the siRNA (M14si) using G418.

We isolated individual cell lines (M14si-1 and M14si-2) to determine the effect of different levels of MMP-14 attenuation on TGF-β1-induced cell migration and the expression of MMP-2 and MMP-9. M14si-2 cells were more attenuated in MMP-14 expression (Fig. 2A), migrated less (1.11 ± 0.03 mm, Fig. 2B) than M14si-1 (0.52 ± 0.04 mm) and scrambled control (SC) cells (0.16 ± 0.03 mm). The expression of MMP-9, but not that of MMP-2 in response to TGF-β1, correlated with the extent of reduction of MMP-14 expression (Fig. 2C; compare SC, M14si-1 and -2). In addition, the expression of MMP-2 was not decreased in M14si-2, but active-form of MMP-2 was completely decreased (data not shown). However, attenuation of MMP-14 mRNA in M14si-1 and -2 had no significant affect on expression of MMP-1, -2, -3, -7, -9, -10, -11, and -13 in HaCaT cells (data not shown).

MMP-9-Mediated Cell Migration in HaCaT Cells

Above results indicated that TGF-β1-induced cell migration was inhibited by MMP-14 attenuation followed by MMP-9 reduction. We tested whether MMP-9 is involved in keratinocyte migration. We stably over-expressed MMP-2 and MMP-9 in HaCaT cells. Stable over-

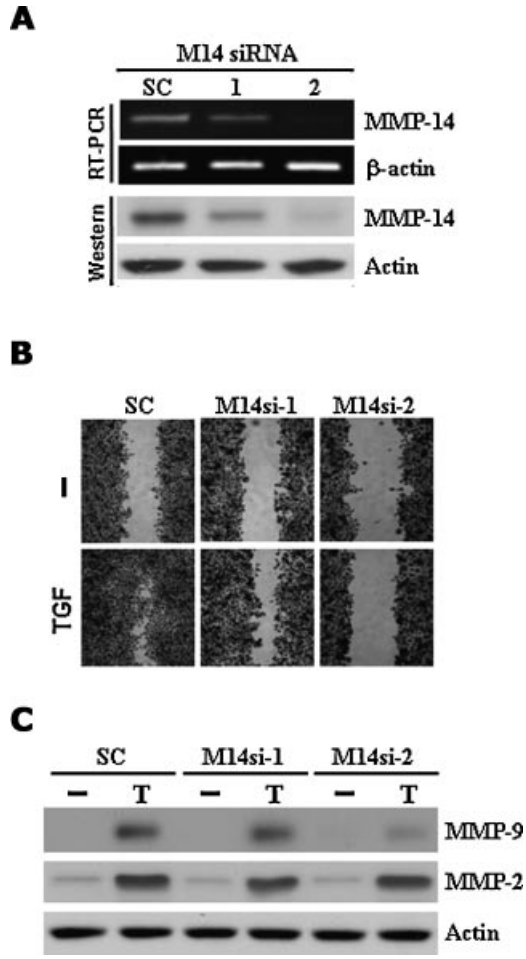

Fig. 2. Attenuation of MMP-14 mRNA by siRNA. **A**: MMP-14 siRNA (M14si-1 and -2) and scramble control (SC) HaCaT cells were analyzed the effect of MMP-14 attenuation by RT-PCR and Western blot. **B**: Cell migration of SC, M14si-1 and -2 cells with or without TGF-β1 for 24 h was measured by microscope following crystal violet staining. **C**: TGF-β1-induced MMP-2 and -9 expressions in SC, M14si-1 and -2 cells were analyzed by Western blot. Results are representative of three independent experiments.

expressions of both genes were confirmed by zymography (Fig. 3A). The MMP-9 over-expressing cells without TGF-β1 stimulation displayed significantly increase of cell migration (0.45 ± 0.06 mm), whereas MMP-2 over-expressing cells (0.63 ± 0.05 mm) did not show this effect like as Mock transfected cells (0.68 ± 0.05 mm, Fig. 3B). MMP-9 mediated cell migration was confirmed using MMP-9 specific inhibitor treatment. MMP-9 inhibitor displayed the inhibition of cell migration (0.96 ± 0.05 mm of MMP-9 inhibitor treated cells and 0.4 ± 0.09 mm of control cells) caused by TGF-β1 (Fig. 3C).

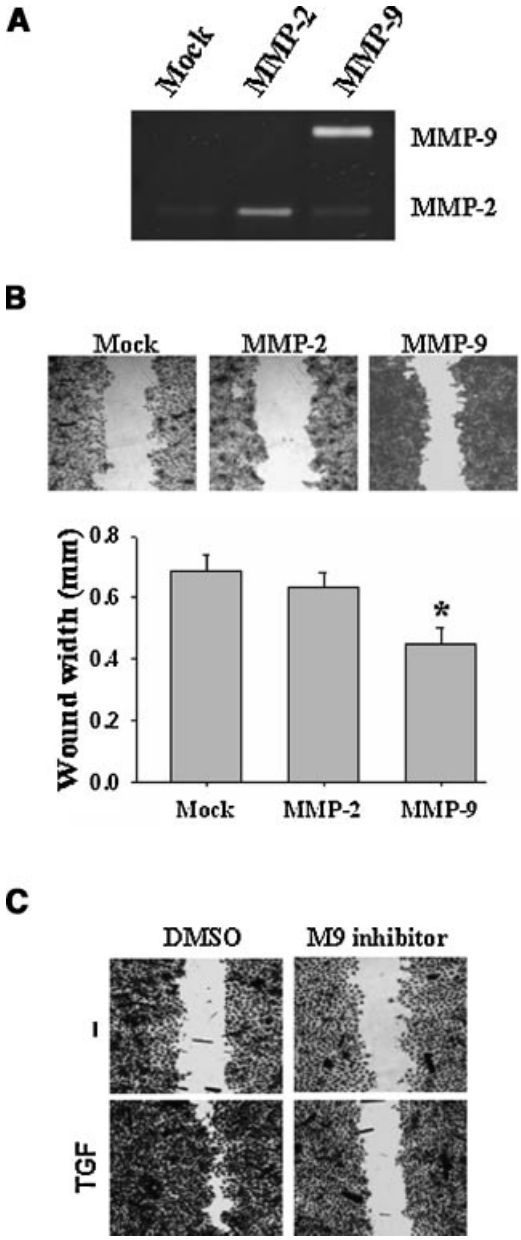

Fig. 3. MMP-9 over-expression induced cell migration. **A**: Over-expression of MMP-2 and MMP-9 protein confirmed by zymography. **B**: Cell migration of Mock, MMP-2, and MMP-9 over-expressing cells was measured after 36 h of wounding. The graph showed the average width of the wound (see Materials and Methods Section). **C**: MMP-9 inhibitor (5μM) was pretreated before TGF-β1 addition, and cell migration was assessed after 24 h. The results are representative of four independent experiments. * Significantly different from Mock and MMP-2 transfected cells at $P < 0.05$.

MMP-14-Mediated JNK Activation

Because it was not clear how the expression of MMP-9 is regulated by MMP-14, we examined various potential downstream mediators of TGF-β1 in SC and M14si-2 cells. ERK, p38 MAPK, JNK, Akt, and Smad2/3 were all activated by TGF-β1, which are known as a regulator of MMPs [O'Kane and Ferguson, 1997; Hintermann et al., 2001; Chakraborti et al., 2003; Philipp et al., 2004; Hosokawa et al., 2005]. Figure 4 shows that TGF-β1 stimulates the phosphorylation each of these signaling molecules. Interestingly, only the activation of JNK was reduced in the M14si-2 cells, indicating that MMP-9 may be activated by MMP-14 via the JNK pathway. To confirm this idea, we treated cells with SP600125, a specific inhibitor of JNK prior to TGF-β1 treatment. As shown in Figure 5A, TGF-β1-induced expression of MMP-9 and the phosphorylation of c-Jun, a downstream kinase of JNK was blocked by SP600125 whereas MMP-2 expression was unaffected. Furthermore, TGF-β1-induced cell migration (0.43 ± 0.09 mm) was also blocked by SP600125 (1.13 ± 0.03 mm, Fig. 5B).

DISCUSSION

The regulation of cell migration by MMP-14 has been well studied. Transfection of MMP-14 into HT1080 cells resulted in increased cell migration [Takino et al., 2004], while attenuation of MMP-14 expression by siRNA decreased fibroblast invasiveness [Rutkauskaite et al., 2005] and reduced angiogenesis in microvascular endothelial cells [Robinet et al., 2005]. Recently, Itoh [2006] proposed that four routes of cell migration are regulated by MMP-14: direct ECM proteolysis, amplification of ECM

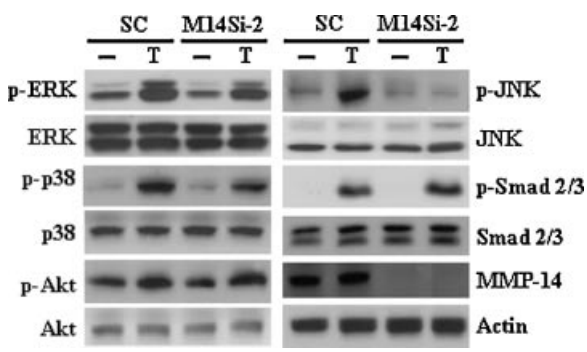

Fig. 4. MMP-14 dependent JNK activation in response to TGF-β1. TGF-β1 treated or untreated SC and M14si-2 cells were collected at indicated time point to examine the phosphorylation of ERK (15 min), Akt (15 min), p38 (30 min), Smad2/3 (5 min), and JNK (60 min), respectively by Western blot. Phosphorylation of ERK, p38 MAPK, Akt, JNK, and Smad2/3 in response to TGF-β1 was analyzed using their phospho-specific antibodies. The results are representative of three independent experiments.

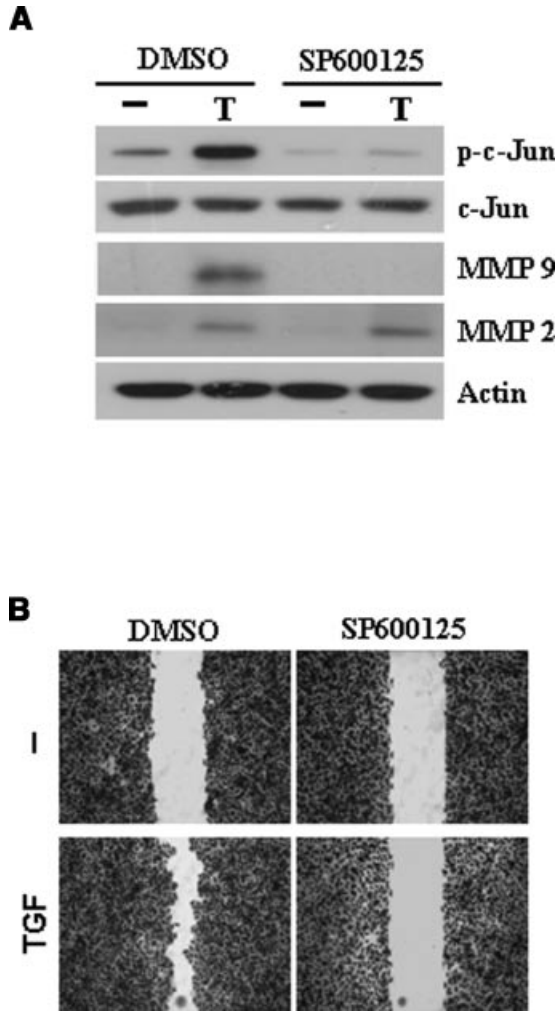

Fig. 5. JNK-mediated MMP-9 induction and cell migration in TGF-β1-treated cells. HaCaT cells were incubated with 10 μM SP600125 (JNK inhibitor) before TGF-β1 treatment. **A**: Cells were harvested 1 h (lysates, for c-Jun and Actin) and 24 h (medium, for MMP-2, -9) after TGF-β1 treatment, and subjected to Western blot analysis. **B**: Cell migration was measured by crystal violet stain after 24 h of TGF-β1 treatment with absence and presence of SP600125. The results are representative of three independent experiments.

proteolysis by activating MMP-2 and MMP-13, processing of cell adhesion molecules, and release of ECM fragments that stimulate cell migration. MMP-14-mediated MMP-2 activation is also well-known during cell migration, tumor invasion, and angiogenesis [Okada et al., 1997; Baumann et al., 2000; Decline et al., 2003; Kerkelä and Saarialho-Kere, 2003]. We found that attenuation of MMP-14 expression in TGF-β1-treated cells reduced cell migration (Fig. 2B) and lowered the level of the active form of MMP-2 (data not shown). This shows that MMP-2 activation by MMP-14 is needed for cell migration, as previously reported [Okada et al., 1997; Baumann et al., 2000]. However, Holmbeck et al. [1999] found severe defects in the development of MMP-14 deficient mice, whereas the development of MMP-2 deficient mice was not as defective. This result suggested that MMP-14 may have functions in addition to activation of MMP-2. Similarly, over-expression of the MMP-2 pro-form alone did not induce cell migration (Fig. 3B). Furthermore, in M14si-2 cells exposed to TGF-β1, MMP-2 expression did not decrease but cell migration was blocked (Fig. 2B,C). These results suggest that MMP-14 performs an additional essential function during cell migration.

Our findings suggest that MMP-9 is another MMP involved in TGF-β1-induced cell migration (Fig. 3B,C), and that its expression is MMP-14 dependent (Fig. 2C). Studies have shown that expression of MMP-9 is critical for keratinocyte tumorigenesis and migration [Cooper et al., 2004; Bigelow et al., 2005], but those studies focused on EGF receptor-dependent processes. On the other hand, TGF-β modulates MMP-9 production through the Ras/MAPK pathway in transformed mouse keratinocytes [Santibanez et al., 2002] and NF-κB induces cell migration by binding to the MMP-9 promoter in human skin primary culture [Han et al., 2001]. We have provided good evidence that MMP-9 over-expression induces keratinocyte migration without the need for stimulation by factors such as TGF-β1 (Fig. 3B).

Despite the importance of MMP-9 in keratinocyte migration, it is not clear how TGF-β regulates the expression of MMP-9. We noted that attenuation of MMP-14 expression decreased TGF-β1-mediated MMP-9 expression (Fig. 2C), and suggested that TGF-β1-induced MMP-9 expression requires MMP-14. There is evidence that MMP-14 activates a number of intracellular signal pathways including the extracellular signal-related kinase (ERK) pathway, the focal adhesion kinase (FAK), Src, Rac, and CD44 during cell migration and tumor invasion [Takino et al., 2004; Sato et al., 2005; Sounni and Noel, 2005]. ERK signaling is known to be activated by MMP-14, and in COS-7 cells, ERK activation is essential for cell migration and is induced by over-expression of MMP-14 [Gingras et al., 2001]. Work with MMP-14-transfected HT1080 cells also revealed that MMP-14 plays an important role in the activation of ERK and of cell migration [Takino

et al., 2004]. However, we have found, in work with an ERK specific inhibitor and the MMP-14 promoter, that ERK signaling regulates MMP-14 and MMP-2 expression in TGF-β1-treated HaCaT cells (unpublished observation). This indicates that TGF-β1-induced ERK activation acts upstream of MMP-14 and MMP-2, but not of MMP-9, in HaCaT cells.

Smad2/3, p38 MAPK, and Akt were all activated by TGF-β1 in both SC and M14si-2 cells (Fig. 4). Interestingly, only activation of JNK was defective in the TGF-β1-treated M14si-2 cells. Furthermore, the JNK specific inhibitor clearly decreased MMP-9 expression and cell migration in response to TGF-β1 (Fig. 5A,B). This is indirect evidence that JNK signaling mediates MMP-9 expression and may be controlled by MMP-14. However, further studies are needed to understand how MMP-14 regulates JNK signaling.

In summary, TGF-β1-induced keratinocyte migration requires upregulation of MMP-9 expression as well as an increase of the active form of MMP-2, and MMP-14 regulates MMP-9 expression via JNK signaling. Our results point to a novel role of MMP-14 in mediating MMP-9 expression via the JNK pathway during TGF-β1-induced keratinocyte migration.

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