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SOCS3/CIS3 negative regulation of STAT3 in HGF-induced keratinocyte migration

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Abstract

Hepatocyte growth factor (HGF) is a potent mitogen for mature hepatocytes. Because HGF has strong effects on the motility of keratinocytes and is produced by fibroblasts, HGF is thought to regulate keratinocyte migration during wound healing. However, the intracellular signaling mechanism of HGF-induced keratinocyte migration is poorly understood. In this report, we clarify the roles of STAT3 and SOCS/CIS family in HGF-induced keratinocyte migration. HGF activated STAT3 and strongly induced keratinocyte migration. Transfection with the dominant-negative mutant of STAT3 almost completely abolished HGF-induced keratinocyte migration and STAT3 phosphorylation. Next, we studied the mechanisms that regulate STAT3 phosphorylation. HGF enhanced the expression of SOCS3/CIS3 by sixfold within 1 h, but had minimum effect on SOCS1/JAB expression. Transfection with SOCS3/CIS3 almost completely abolished HGF-induced STAT3 phosphorylation and keratinocyte migration, indicating that SOCS3/CIS3 acts as a negative regulator of HGF-induced keratinocyte migration. In conclusion, SOCS3/CIS3 regulates HGF-induced keratinocyte migration by inhibiting STAT3 phosphorylation.

Keywords: Keratinocytes; HGF; Migration; STAT3; STAT1; SOCS3/CIS3; SOCS1/JAB; Wound healing; Adenovirus vector

The migration of epidermal keratinocytes is an important step in skin wound healing. Several growth factors regulate keratinocyte migration [1]. Hepatocyte growth factor (HGF) is a potent mitogen for mature hepatocytes [2,3]. HGF also has mitogenic, motogenic, morphogenic, and tumor-inhibitory activities for a variety of cells, including epithelial, endothelial, and some types of stromal cells [4–6]. Because HGF has potent effects on the motility of keratinocytes and because dermal fibroblasts produce HGF in the skin [7], HGF has been suggested to play a regulatory role in keratinocyte migration during wound healing [8,9]. Recently, an application of HGF has been demonstrated as a potential therapeutic approach for the treatment of cutaneous ulcer [10–13]. However, the intracellular signaling mechanism of HGF-induced migration is poorly understood.

c-Met is an HGF receptor that is autophosphorylated upon binding of HGF. Phosphorylated c-Met recruits a number of substrates with Src homology (SH)2 domains, such as phosphatidylinositol 3-kinase [14], Grb-2 (ASH)/Sos complex [15], Ras GTPase activating protein, $pp60^{src}$, and phospholipase C [15]. Grb-2 has also been implicated in the recruitment of the large adaptor protein Grb-2-associated binding protein-1 (Gab1) to the Met signaling complex [16–18]. These signaling molecules lead to mitogenic activity via the Ras–Raf1–MEK–MAPK pathway. In addition to these

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pathways, two signal transducers and activators of transcription (STAT) proteins, STAT1 and STAT3, are signaling cascade proteins located downstream from c-Met [19].

The STAT signaling pathways are negatively regulated by proteins of the suppressor of cytokine signaling (SOCS)/cytokine-inducible SH2-containing protein (CIS) family to avoid oversignaling [20]. The SOCS/CIS family is induced by cytokine stimulation and binds to tyrosine-phosphorylated sites of the cytokine receptor or to Jak through an SH2 domain, resulting in the inhibition of tyrosine kinase phosphorylation. CIS was the first member of the SOCS/CIS family to be identified; it binds to the tyrosine-phosphorylated site of the erythropoietin receptor and inhibits the STAT5 signal downstream [21]. Additional members of the SOCS/CIS family have been identified independently [22,23]. SOCS1/JAB binds mainly to Jak2 [24] and regulates the IFN-γ/STAT1 [25] and IL-6/STAT3 signaling pathways [23]. SOCS2/CIS2 regulates insulin-like growth factor and the insulin-like growth factor receptor signaling pathways [26]. SOCS3/CIS3 regulates the IL-6/STAT3 and IFN-γ/STAT1 signaling pathways.

Because STATs are involved in the HGF-c-Met signaling pathway, we hypothesized that the SOCS/CIS family regulates HGF-induced keratinocyte migration by inhibiting STAT pathways. To prove this, we first studied whether STAT3 was involved in HGF-induced keratinocyte migration. Next, we tested whether the SOCS/CIS family affects HGF-induced keratinocyte migration through the inhibition of STAT3.

Materials and methods

Reagents and antibodies. Recombinant HGF was kindly provided by Dr. Kunio Matsumoto (Osaka University, Osaka, Japan). Antibodies were purchased as follows: mouse monoclonal STAT3 (Transduction Laboratories) and phospho-STAT3 (New England Biolabs).

Keratinocyte culture. Human skin samples were obtained after plastic surgery under a protocol approved by the Institutional Review Board of Ehime University School of Medicine. Primary normal human keratinocytes were isolated from the normal human skin. Normal human keratinocytes were cultured with MCDB153 medium supplemented with insulin (1 μg/ml), hydrocortisone (0.5 μM), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), bovine hypothalamic extract (BHE; 50 μg/ml), and Ca^{2+}(0.1 mM). This supplement was as described elsewhere [27].

Migration assay. Keratinocytes were cultured at 1×10^5 cells per 35-mm type I-collagen-coated culture plate in culture medium without BHE for 12 h. After stimulation, keratinocyte migration was observed using time-lapse video microscopy (IX-IBC, CK30; Olympus, Tokyo, Japan) in a controlled chamber at 37 °C and 5% CO_2.

Keratinocyte migration was assayed quantitatively with a Boyden chamber, as previously described [28]. Designated amounts of HGF were added to the bottom wells of a 48-well Boyden chamber (Neuro Probe, Cabin John, MD), and an 8-μm pore-size polyvinylpyrrolidone-free polycarbonate membrane (Neuro Probe) was placed on the wells. The membrane was precoated with type I collagen (10 μg/ml in PBS, Nitta Gelatin, Osaka, Japan) at room temperature for 1 h and then extensively washed with PBS. Subconfluent keratinocytes were harvested with trypsin–EDTA (0.05% trypsin and 0.5 mM EDTA) and resuspended in the culture medium without BHE at 1×10^5 cells/ml. Fifty microliters of the keratinocyte suspension (5000 cells/well) was added to the upper wells, and the chamber was incubated overnight at 37 °C in a humidified atmosphere of air with 5% CO_2. Cells that adhered to the upper surface of the filter membrane were removed by scraping with a rubber blade. Cells that moved through the filter and stayed on the lower surface of the membrane were considered to be migrated cells. The membrane was fixed with 10% buffered formalin overnight and then stained with Gill's hematoxylin overnight. The membrane was then mounted between two glass slides with 90% glycerol and the number of migrated cells was determined by counting under a microscope.

Western blotting. Subconfluent keratinocytes were starved for 2 h in BHE-free medium and stimulated with HGF as indicated. Cells were harvested on ice in lysis buffer containing 5 mM EDTA, 100 μM sodium orthovanadate, 100 μM sodium pyrophosphate, 1 mM sodium fluoride, 5 μM 3,4-dichloroisocoumarin, 1 μg/ml aprotinin, and 1% Triton X-100 in PBS. Twenty micrograms of protein was separated on 10% SDS–PAGE and then transferred to a PVDF membrane. The membranes were blocked with 5% skimmed milk in PBS overnight at 4 °C. The blocked membranes were incubated for 6 h with the first antibody as indicated. After three washes with PBS containing 0.05% Tween 20, the membranes were treated with ABC reagents (VECTOR Laboratories, Burlingame, CA) for 20 min at room temperature, washed three times with PBS containing 0.05% Tween 20, treated with ECL detection reagents (Amersham–Pharmacia Biotech, Piscataway, NJ) for 1 min at room temperature, and then exposed to films (Kodak, Rochester, NY).

Quantitative PCR analysis. Total RNA from cultured human keratinocytes was prepared using Isogen (Nippon Gene, Toyama, Japan) and was treated with 50 U/ml DNase I (Clontech) at 37 °C for 30 min to remove any genomic DNA contamination.

To quantify the mRNA expression in vivo, we performed quantitative RT-PCR using the ABI Prism 7700 sequencer detection system (Perkin–Elmer Applied Biosystems, Foster City, CA). RT-PCR mixtures were prepared according to the manufacturer's instructions for the TaqMan One-Step RT-PCR Master Mix Reagent kit (Perkin–Elmer Applied Biosystems). Briefly, 50 ng of total RNA was added to each 50-μl reaction mixture containing 1 μl master mix, 1× multiScribe and RNase inhibitor mix, 200 nM of each primer, and 100 nM hybridization probe for specific detection of target cDNA. For SOCS1/JAB detection, the sense primer 5′-TTTTTCGCCCTTAGC GTGAA-3′, the antisense primer 5′-GCCATCCAGGTGAAAGCG-3′, and the probe 5′-CCTCGGGACCCACGAGCATCC-3′ were added. For SOCS3/CIS3 detection, the sense primer 5′-TTCAGCA TCTCTGTCGGAAGAC-3′, the antisense primer 5′-GCATCGTAC TGGTCCAGGAACT-3′, and the probe 5′-AACGGCCACCTGG ACTCCTATGAGAAA-3′ were added. The probe was labeled with a reporter fluorescent dye, FAM (6-carboxyfluorescein), at the 5′-end. For GAPDH detection, 1 μl of Pre-Developed TaqMan Assay Reagent (Perkin–Elmer Applied Biosystems) was added. The thermal conditions were 48 °C for 30 min for reverse transcription and 95 °C for 10 min, followed by 45 amplification cycles of 95 °C for 15 s for denaturing and 55 °C for 1.5 min for annealing and extension. The PCR products were sequenced to confirm the proper amplification. To compare mRNA expression, the results were estimated as relative values using GAPDH as an internal reference. The relative quantified expression was calculated using the following formula: relative expression $= 2[-(\sum CT_s/N_s - \sum CT_{GAPDH_s}/N_s) - (\sum CT_r/N_r - \sum CT_{GAPDH_r}/N_r)]$, where CT_s denotes the cycle threshold for the candidate gene in the sample, N_s is the number of samples, CT_{GAPDH_s} is the cycle threshold for GAPDH in the same sample, CT_r is the cycle threshold for the candidate gene in the overall reference sample, N_r is the number of reference samples, and CT_{GAPDH_r} is the cycle threshold for GAPDH in the same reference sample. In each group, there were $n = 3$ samples.

Adenovirus vectors (Ax). STAT3 has a phosphorylation site at tyrosine705. In the dominant negative mutants of STAT3 (STAT3F), the phosphorylatable tyrosine residue is substituted with phenylalanine. Axs encoding STAT3F (AxCA STAT3F), SOCS1/JAB (AxCAJAB), and SOCS3/CIS3 (AxCACIS3) were generated as previously described [29] using the COS–TPC method [30]. Ax encoding lacZ (Ax LacZ) was a gift from Dr. Izumi Saito (University of Tokyo). Virus stocks were prepared using a standard procedure [30]. Concentrated, purified virus stocks were prepared using a CsCl gradient, and the virus titer was checked using a plaque formation assay. We infected normal human keratinocytes with Ax at a multiplicity of infection (MOI) of five.

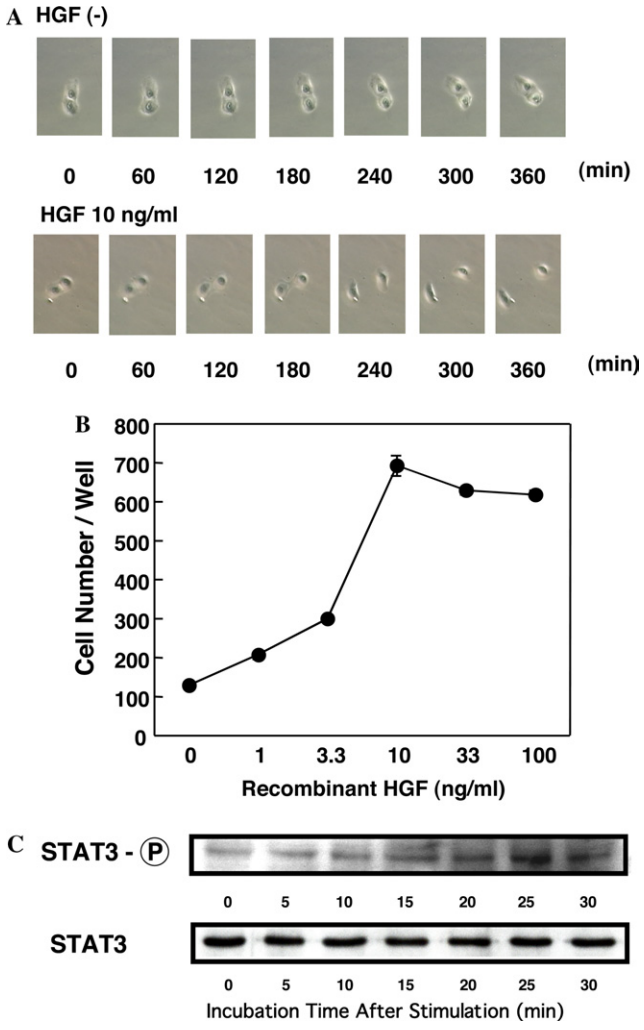

Fig. 1. Keratinocyte migration and phosphorylation of STAT3 by HGF. (A) HGF-induced keratinocyte migration. After adding 10 ng/ml HGF, keratinocyte migration was observed under time-lapse video microscopy every 60 min. (B) Quantification of keratinocyte migration induced by HGF. The indicated amount of HGF was added to the bottom wells of a 48-well Boyden chamber, and then an 8-μm pore-size polyvinylpyrrolidone-free polycarbonate membrane was placed on the wells. Keratinocytes were added to the upper wells at 5000 cells/well. After overnight incubation, the membrane was stained with Gill's hematoxylin. The number of cells that had migrated through the filter was determined by counting under a microscope. Each point shows means ± SD of quadruplicate measurements. (C) Phosphorylation of STAT3 by HGF. Subconfluent keratinocytes were starved for 2 h in BHE-free medium and stimulated with 10 ng/ml HGF. Cells were harvested and the phosphorylation of STAT3 was analyzed by Western blotting.

Results

HGF induces keratinocyte migration and phosphorylates STAT3

We first observed whether HGF induces the migration of normal human keratinocytes by using time-lapse video microscopy (Fig. 1A). Keratinocytes started to migrate within 180 min after HGF stimulation. Without HGF, no migration occurred. Then, the migration was analyzed quantitatively using the Boyden chamber assay (Fig. 1B). HGF induced keratinocyte migration sixfold that of control. The optimum concentration of HGF was 10 ng/ml. The phosphorylation of STAT3 was analyzed by Western blotting (Fig. 1C). HGF phosphorylated STAT3 at 25 min.

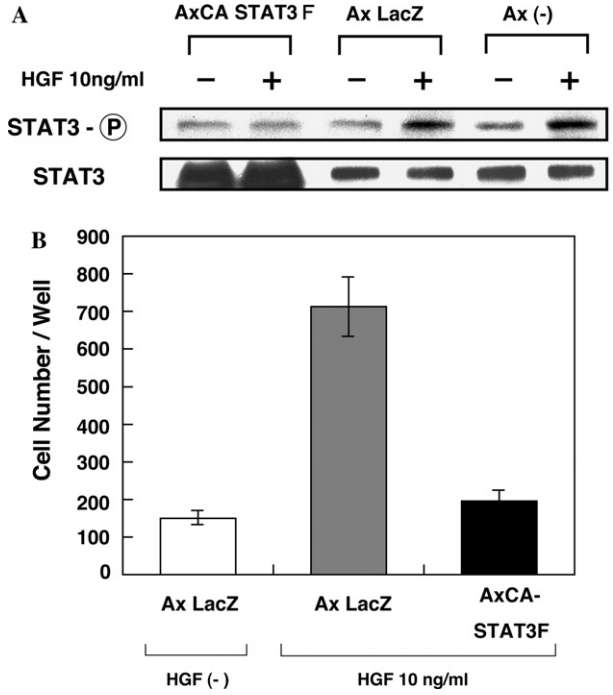

Fig. 2. Inhibition of HGF-induced phosphorylation of STAT3 and keratinocyte migration by STAT3F. (A) Inhibition of STAT3 phosphorylation. Ax LacZ and AxCA STAT3F were transfected into normal human keratinocytes at an MOI of 5. After 24 h, the keratinocytes were stimulated with 10 ng/ml HGF or vehicle alone for 25 min. Then the cells were harvested and analyzed by Western blotting. (B) Inhibition of keratinocyte migration. Ax LacZ and AxCA STAT3F were transfected into normal human keratinocytes at an MOI of five. After 24 h, the keratinocytes were harvested and transferred to a Boyden chamber and HGF (10 ng/ml) was added to the lower chamber. The migration was analyzed as in Fig. 1B. Each point shows the mean ± SD of quadruplicate measurements.

Phosphorylation of STAT3 is essential for HGF-induced keratinocyte migration

We next constructed dominant negative mutants of STAT3 (STAT3F) to study the role of STAT3 in HGF-induced keratinocyte migration. The expression of STAT3F using Ax (AxCA STAT3F) almost completely blocked HGF-induced STAT3 phosphorylation, while the transfection of LacZ had no effect on the STAT3 phosphorylation (Fig. 2A).

Using AxCA STAT3F, we analyzed the functions of STAT3 in HGF-induced keratinocyte migration. After the transfection of keratinocytes with AxCA STAT3F, the HGF-induced migration of the keratinocytes was quantitatively analyzed with the Boyden chamber assay (Fig. 2B). The expression of STAT3F almost completely blocked HGF-induced keratinocyte migration, while the expression of LacZ had no effect on migration. Because STAT3 was phosphorylated by HGF, and because HGF-induced migration was blocked by STAT3F, we concluded that the phosphorylation of STAT3 is essential for HGF-induced keratinocyte migration.

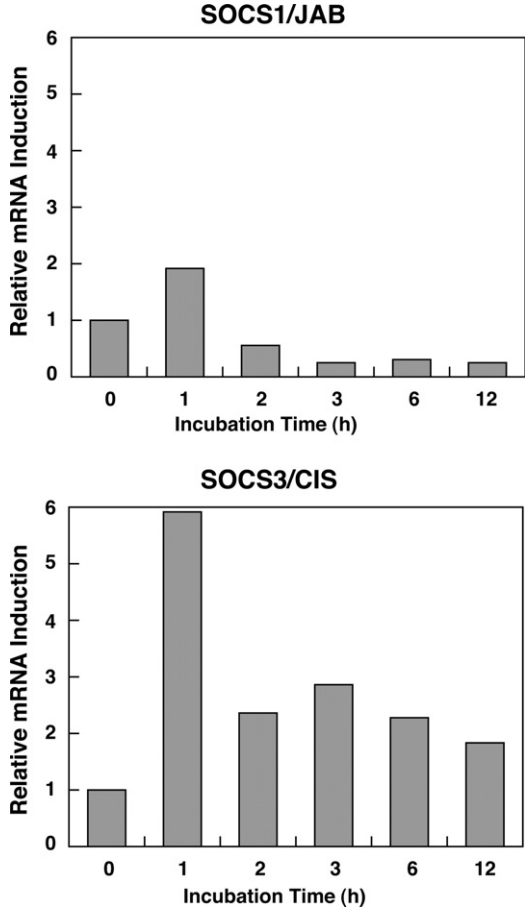

Fig. 3. Induction of SOCS3/CIS by HGF. Subconfluent keratinocytes were stimulated with 10 ng/ml HGF. Cells were harvested at the indicated time. The mRNA expression of SOCS1/JAB and SOCS3/CIS was analyzed using real-time PCR. The results were adjusted to relative values using GAPDH as an internal reference.

HGF induces SOCS3/CIS3

The SOCS/CIS family is inducible de novo by stimulation and negatively regulates the STAT family. Therefore, it is possible that the SOCS/CIS family regulates HGF-induced keratinocyte migration. To prove this, we first determined whether HGF induces the SOCS/CIS family in keratinocytes. As shown in Fig. 3, HGF enhanced the SOCS3/CIS3 mRNA expression sixfold at 1 h after stimulation, while the induction of SOCS1/JAB by HGF was not as significant as that of SOCS3/CIS3.

SOCS1/JAB and SOCS3/CIS3 inhibit HGF-induced STAT3 phosphorylation and keratinocyte migration

First, we determined the effects of SOCS1/JAB and SOCS3/CIS3 on HGF-induced STAT3 phosphorylation. AxCAJAB and AxCACIS3 were transfected into keratinocytes; the expression of either SOCS1/JAB or SOCS3 / CIS3 almost completely blocked HGF-induced STAT3 phosphorylation (Fig. 4A).

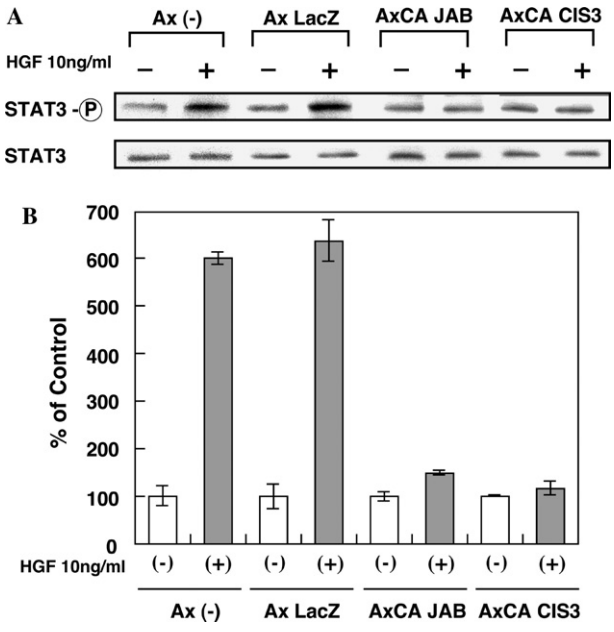

Fig. 4. Inhibition of HGF-induced STAT3 phosphorylation and keratinocyte migration by SOCS1/JAB and SOCS3/CIS3. (A) Inhibition of STAT3 phosphorylation. Ax LacZ, AxCAJAB, and AxCACIS3 were transfected into normal human keratinocytes at an MOI of 5. After 24 h, the keratinocytes were stimulated with 10 ng/ml HGF or vehicle alone for 25 min. Then, the cells were harvested and analyzed by Western blotting. (B) Inhibition of keratinocyte migration. Ax LacZ, AxCAJAB, and AxCACIS3 were transfected into normal human keratinocytes at an MOI of 5. After 24 h, the keratinocytes were harvested and transferred to a Boyden chamber for the analysis of migration, as described in Fig. 1B. HGF (10 ng/ml) was added to the lower chamber, and the set-up was incubated overnight. Each point shows the mean ± SD of quadruplicate measurements.

Using AxCAJAB and AxCACIS3, we analyzed the regulatory mechanism of STAT3 in HGF-induced keratinocyte migration. After the transfection of keratinocytes with AxCAJAB or AxCACIS3, the HGF-induced migration of the keratinocytes was analyzed quantitatively with the Boyden chamber assay (Fig. 4B). The expression of either SOCS1/JAB or SOCS3/CIS3 almost completely blocked HGF-induced keratinocyte migration. Because SOCS3/CIS3 was induced by HGF and SOCS3/CIS3 blocked HGF-induced phosphorylation of STAT3 and migration, we concluded that SOCS3/CIS3 regulates HGF-induced keratinocyte migration.

Discussion

The intracellular signaling mechanisms involving c-Met have been studied in hepatocytes primarily. Although it was demonstrated that HGF induces keratinocyte migration [8], the intracellular signaling mechanisms remained unclear. It was suggested that the major signaling pathway downstream from HGF/c-Met is the MAPK cascade [31]. However, the involvement of STAT signaling cascades in HGF-induced signal transduction was also reported [19]. Because EGF-induced keratinocyte migration was abolished in STAT3-disrupted keratinocytes [32], STAT pathways were thought to be involved in HGF-induced keratinocyte migration. Therefore, we studied whether SOCS/CIS regulates HGF-induced keratinocyte migration.

The SOCS/CIS family negatively regulates STAT pathways. However, the inhibitory functions of the SOCS/CIS family differ among cell types and cell conditions. In this study, we showed that SOCS3/CIS3 was induced and inhibited STAT3 phosphorylation and migration, indicating that SOCS3/CIS3 acts as a self-limiting factor to avoid overstimulation. Although HGF induces SOCS3/CIS3, EGF, a growth factor that strongly induces keratinocyte migration [33], does not induce SOCS1/JAB or SOCS3/CIS3 [29]. Therefore, EGF-induced keratinocyte migration does not involve the SOCS1/JAB- or SOCS3/CIS3-mediated self-regulatory mechanism of STAT3 activation in keratinocytes. This indicates that the intracellular regulatory mechanism of keratinocyte migration differs among growth factors.

In this study, we showed that SOCS1/JAB inhibited HGF-induced keratinocyte migration. However, SOCS1/JAB was not induced by HGF, suggesting that SOCS1/JAB has another role other than acting as a self-limiting factor. It is possible that cytokine-induced SOCS1/JAB or SOCS3/CIS3 affects HGF-induced keratinocyte migration. Although the inhibitory and negative regulatory mechanisms of cytokine signals in epidermal keratinocytes have not been assessed fully, cytokines such as IFN-γ, IL-4, and IL-6 are implicated in a variety of physiological and pathological conditions of the skin. IFN-γ enhances SOCS1/JAB and SOCS3/CIS3 expression [29]. In addition, IL-4 and IL-6 enhance the expression of SOCS1/JAB and SOCS3/CIS3, respectively [29]. Therefore, cytokines in various inflammatory skin conditions might affect wound healing by regulating keratinocyte migration via the induction of SOCS1/JAB or SOCS3/CIS3.

In conclusion, the SOCS3/CIS3 negative feedback mechanism of STAT3 activation is a key pathway of HGF-induced keratinocyte migration.

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