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CCL20 derived from PMA-differentiated macrophages abrogates TGF-β1-induced expression of cancer progression suppressor CXCL14 in HSC-4 cells in PI3K-, MEK1/2-, and NF-κB-dependent manners

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Abstract：Squamous cell carcinoma (SCC) is the most common cancer that develops in the oral cavity. Epithelial-mesenchymal transition (EMT) is known to play an important role in the process of metastasis of SCC cells. In our previous study, we demonstrated that transforming growth factor-β1 (TGF-β1) induces EMT in the human oral SCC (hOSCC) cell line HSC-4. However, the molecular mechanisms of the metastasis after an EMT-induced cancer are poorly understood. Moreover, tumor-associated macrophages (TAMs), which coexist with the cancer tissues, are known to participate in permeation metastasis. However, it still remains uncertain whether TAMs affect the metastatic activity of hOSCC cells. We found that the expression of CXCL14, which is known to suppress the progression of colon, and breast cancers, was upregulated in HSC-4 cells by TGF-β1 stimulation. The Smad3 inhibitor, SIS3, suppressed the TGF-β1-induced expression of CXCL14. Interestingly, the CXCL14 suppressed the migratory and proliferative activities in HSC-4 cells. Moreover, the expression of CXCL14 in HSC-4 cells was downregulated by cocultivation with differentiated macrophages derived from monocytic THP-1 cells, but not by cocultivation with non-differentiated monocytic THP-1 cells. On the other hand, we found that expression levels of CCL20 in the differentiated macrophages derived from THP-1 cells was higher than that in non-differentiated monocytic THP-1 cells. In addition, CCL20 suppressed TGF-β1-induced expression of CXCL14. Intriguingly, the MAPK/ERK kinase (MEK) inhibitor U0126, PI3K inhibitor LY294002, or NF-κB kinase-2 (IKK-2) inhibitor TPCA-1

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abrogated the CCL20-promoted inhibition of TGF-β1-induced expression of CXCL14. Moreover, CCL20 decreased the migratory activity of HSC-4 cells. Taken together, these results suggest that CCL20 derived from TAMs abrogated the TGF-β1-induced expression of the cancer progression suppressor CXCL14 in HSC-4 cells in PI3K-, MEK1/2-, and NF- κB-dependent manners, resulting in the activation of metastatic activity in HSC-4 cells..

Keywords: CCL20, CXCL14, epithelial-mesenchymal transition, squamous cell carcinoma, transforming growth factor-β

Introduction

Squamous cell carcinoma (SCC) is the most frequent cancer in the oral cavity[1]. Recently, comprehensive characterization of head and neck SCC has led to the identification of distinctive gene mutations[2]. Epithelial-mesenchymal transition (EMT) is known to play an important role in cancer metastasis[3]; it induces the loss of characteristics of the epithelia and the gain of characteristics of the mesenchyme in differentiated epithelial cells, which leads to increased cell migration and invasion[4]. EMT is not only an important process in development, adult tissue maintenance and reproduction[5,6], but also in cancer and disease-induced desmoplasia[5,7]. A change in expression from E-cadherin to N-cadherin (cadherin switch) is known to play an important role in the malignant transformation of cancer cells during the EMT process[8,9]. The mechanism underlying regulation of the cadherin switch in human oral squamous cell carcinoma (hOSCC) cells remains to be elucidated. Interestingly, previous studies have reported changes in the expression of various genes related to the cadherin switch in many types of squamous cell carcinoma cells other than hOSCC[10,11]. Transforming growth factor-β (TGF-β) is a crucial inducer of EMT[12,13,14] that has a contradictory dual-faceted nature; it acts both as a tumor suppressor during the initial stages of tumorigenesis as well as an activator in tumor progression. Thus, TGF-β inhibits tumor cell proliferation and promotes apoptosis in early-stage tumor cells. In addition, TGF-β induces invasion and metastasis of cancer through EMT, allows for escape from the immune system, and facilitates angiogenesis in late-stage tumor cells[15]. TGF-β binds to TGF-β receptor type I and type II, which are transmembrane serine/threonine kinases. Smad2 and Smad3, upon being phosphorylated by TGF-β receptor, bind to Smad4 and then translocate to the nucleus. The transcription of several target genes is regulated by the Smad2/3/4 complex in cooperation with other cofactors[16,17].

Tumor-associated macrophages (TAMs) are an important cell type in the cancer microenvironment[18]. TAMs, as well as M2 macrophages, generally progressed the invasion and metastasis of cancer, and offered protection against the immune system. TAMs induce tumor progression and suppress immune dysfunction through the secretion of IL-10, CCL22, and TGF-β, to allow for the survival of cancer cells[18]. TAMs allow for metastasis of non-small cell lung cancer by promoting TGF-β secretion-induced EMT[19]. However, it remains to be clarified that how TAMs affect the metastatic activity in hOSCC cells. TAMs produced many kinds of cytokines

and chemokines depending on their phenotypes[18]. C-C motif chemokine 20 (CCL20), also known as macrophage inflammatory protein-3α (MIP-3α), belongs to the CC chemokine family and binds C-C motif chemokine receptor 6 (CCR6), which is a G protein-coupled receptor (GPCR) on the cell surface[20]. CCL20-induced activation of CCR6 further leads to the activation of the mitogen-activated protein kinase (MAPK) / extracellular signal-regulated kinase (ERK) kinase (MEK) /ERK signaling pathway in lung cancers[21]. Muscella *et al.* demonstrated that CCL20 induces the expression of matrix metalloproteinase (MMP)-2 and MMP-9 through the JAK2/STAT3 and PKC/Akt/NF-κB signaling pathways, respectively, resulting in upregulation of the metastatic activity in tumor cells[22].

CXC motif chemokine 14 (CXCL14), also known as BRAK, is a member of CXC motif chemokine. The binding receptor for CXCL14 has not been identified till date [23]. It is generally known that CXCL14 displays anti-tumor activities against tumor formation and metastasis[24]. CXCL14 suppresses the growth of hOSCC cells, but enhances the invasiveness of breast and prostate cancers, suggesting that it switches between the roles of an enemy or ally to tumor cells in a tumor cell type-specific manner[25]. It has also been reported that epidermal growth factor (EGF) downregulates the expression of CXCL14 in hOSCC cells through the ERK signaling pathway[26]. However, the molecular mechanisms underlying CXCL14 expression in hOSCC cells are not completely understood yet.

In our previous study, we demonstrated that TGF-β1 induced EMT in hOSCC cell line HSC-4. We also reported that the migratory activity of HSC-4 cells is promoted through TGF-β1-induced integrin α3β1/FAK activation[11]. In addition, we found that TGF-β1-induced upregulation of Slug expression positively regulates the migratory activity of HSC-4 cells[11,27]. TGF-β1 also stimulates the invasion ability of HSC-4 cells through the Slug/Wnt-5b/MMP-10 signaling axis[28]. These results suggest that Slug might be an important EMT-related transcription factor which promotes metastasis of hOSCC cells. In addition, we have also demonstrated that Slug dose not participate in the upregulation of N-cadherin expression[9], suggesting that EMT-related transcription factors other than Slug play an important role in this process. It still remains to be clarified whether TGF-β promotes chemokine secretion from hOSCC cells, resulting in the upregulation of the metastatic activity in hOSCC cells in a paracrine-, or autocrine-manners.

In this study, we evaluated the effect of TGF-β1 on CXCL14 expression in hOSCC cells. In addition, we also investigated how the differentiated macrophages regulated CXCL14 expression in HSC-4 cells through the CCL20-mediated cell-to-cell interaction.

Materials and Methods

Materials

Cultured cell lines were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Recombinant human (rh) TGF-β1, CXCL14 and CCL20 were purchased from PeproTech (Rocky Hill, NJ, USA). SB4131542, U0126 and LY294002 inhibitors were obtained from Merck-Millipore (Frankfurt, Germany). SIS3 inhibitor was provided by Selleck Chemicals (Houston, TX, USA). CCR6 inhibitor 1 was purchased from MedChemExpress (MCE) (Monmouth Junction, NJ, USA). TPCA-1 was purchased

from R&D Systems Inc. (Minneapolis, MN, USA). Protease inhibitor cocktail, for use with mammalian cell and tissue extracts, and phosphatase inhibitor cocktails 1 and 2 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma-Aldrich. All other purchased reagents were of analytical grade.

Cell culture

All cell lines were grown at 37° C with 5% CO_2. Human HSC-4 squamous cell carcinoma cells (JCRB0624) were cultured in Eagle's Minimum Essential Medium (MEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Rockville, MD, USA). SAS cells (JCRB0260) were cultured in RPMI 1640 medium (Gibco BRL) supplemented with 10% FBS. HSC-2 (JCRB0622) and HSC-3 cells (JCRB0623) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) with 10% FBS. The culture medium was removed and replaced with serum-free medium 24 h prior to TGF-β1-stimulation experiments. For time-course experiments, 2.0×10^5 hOSCC cells were cultured in 500 μl of serum-free medium containing TGF-β1 (10 ng/ml), for 1 to 48 h in 12 or 24-well tissue culture plates.

Human acute monocytic leukemia THP-1 cells (JCRB112.1) were cultured in RPMI 1640 medium supplemented with 10% FBS. For the induction of macrophage differentiation, 3.0×10^5 THP-1 cells were seeded on each well of 12 well-tissue culture plates. Then, the THP-1 cells were treated with PMA (50 ng/ml) for 48 h. After rinsing with phosphate-buffered saline (PBS) buffer to remove non-adherent cells (non-differentiated monocytic THP-1 cells), adherent cells (PMA-differentiated macrophages) were further treated with PMA (50 ng/ml) for 48 h to complete the macrophage differentiation of the THP-1 cells.

Quantitative real time RT-PCR

For total RNA preparation, 2.0×10^5 cells were cultured in 24-well tissue culture plates. Total RNA was isolated using ISOGEN reagent (Nippon Gene, Toyama, Japan), according to the manufacturer's instructions. RNA was reverse transcribed into first-strand cDNA using an RT-PCR System Kit (Takara Bio, Shiga, Japan). Quantitative real time PCR (qPCR) was performed on a Thermal Cycler Dice Real Time System (Takara Bio) using SYBR® Premix Ex Taq II (Takara Bio) with human gene-specific primers [CXCL14, 5′-GATCCGCTACAGCGACGTGA-3′ (forward) and 5′-GGACACGCTCTTGGTGGTGA-3′ (reverse); CCL20, 5′-TTGATGTCAGTGCTGCTACTCCA-3′ (forward) and5′-TGTGTATCCAAGACAGCAGTCAAAG-3′ (reverse); CD163, 5′-GGCTCAATGAAGTGAAGTGCAAAG-3′ (forward) and 5′-CCAAGGATCCCGACTGCAA-3′ (reverse); CD206 (MRC1), 5′-GCCCGGAGTCAGATCACACA-3′ (forward) and 5′-AGTGGCTCAACCCGATATGACAG-3′ (reverse); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-GCACCGTCAAGGCTGAGAAC-3′ (forward) and 5′-TGGTGAAGACGCCAGTGGA-3′ (reverse)]. Target gene expression was normalized to an internal GAPDH reference and expressed in terms of fold change relative to the control sample using the 2-ΔΔ Ct method[29].

Western blot analysis

For western blot experiments, 3.0×10^6 cells were lysed in RIPA buffer (Sigma-Aldrich) containing a protease and phosphatase inhibitor cocktail (Sigma-Aldrich). The protein

content of the samples was measured using BCA reagent (Thermo Fisher Scientific, Waltham, MA USA). For the preparation of cell lysates to examine marker proteins, 1.0×10^6 cells were cultured in a 6-well plate in serum-free MEM with or without 10 ng/ml TGF-β1 for the indicated times. Cells were dissolved in SDS sample buffer containing a protease and phosphatase inhibitor cocktail (Sigma-Aldrich). The protein samples were separated using SDS-PAGE with 12.5% acrylamide gels (ATTO Co., Tokyo, Japan) and subsequently transferred onto PVDF membranes (Merck-Millipore). The membranes were probed with a rabbit anti-CXCL14 primary antibody (at a 1:1000 dilution, N3C3; GeneTex, Irvine, CA, USA), while a mouse anti-β-actin antibody (at a 1:1000 dilution, clone C4; Santa Cruz Biotechnology) was used as the loading control. The blots were then incubated with alkaline phosphatase-conjugated secondary antibody, following which the signals were detected using an alkaline phosphatase substrate kit (BCIP/NBT Substrate Kit; Vector Laboratories Inc., Burlingame, CA, USA).

Proliferation assay

HSC-4 cell proliferation was evaluated using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, 5.0×10^3 cells/well were subcultured for 24 h in 96-well plates containing MEM supplemented with 10% FBS. The culture medium was then replaced with MEM without FBS with or without rhCXCL14 (100 ng/ml) for 48 h according to the method of Wnag *et al*[30]. The cells were incubated with 10 μl of CCK-8 solution at 37℃ in 5% CO_2 for 2 h. The absorbances at 450 nm were used to measure the reduced forms of reagent using a microplate reader. The assays were performed independently at least 3 times.

Cell migration assay with a Boyden chamber

The Boyden chamber-based cell migration assays were performed as follows: HSC-4 cells were treated with TGF-β1 (10 ng/ml) under serum-free conditions for 48 h. Then, the TGF-β1 -treated cells were further treated; 1) with/without CXCL14 (100 ng/ml), or 2) with/without CCL20 (10 ng/ml) for 48 h. Subsequently, the cells treated in 1), or 2) were plated at a density of 1.0×10^5 cells into the upper chamber of a Boyden chamber apparatus in serum-free media and were allowed to migrate into a medium containing 10% FBS in the lower chamber for 24 h at 37° C, respectively. Following the 24 h incubation period, the filter was fixed in 4% paraformaldehyde and stained with DAPI for 10 min. The cells that migrated to the underside of the membrane were counted in nine random fields under an IX70 fluorescence microscope (Olympus Co., Tokyo, Japan). The data presented here represent the average of triplicate experiments. The values indicate the mean number of migrating cells compared to the control. The level of significance was determined using an unpaired two-tailed Student's *t*-test.

Coculture HSC-4 cells and THP-1 cells treated/non-treated with PMA

HSC-4 (3.0×10^5 cells) cells were seeded into the upper chambers of 12 well-transwell plates (Costar®, Corning Incorporated, Glendale, AZ, USA) and treated with 10 ng/ml TGF-β1. After 48 h of cultivation, the TGF-β1-treated HSC-4 cells were rinsed and washed with PBS buffer and then added with RPMI 1640 medium supplemented with 10% FBS. On the

other hand, PMA-treated/non-treated THP-1 cells (3.0×10^5 cells) were cultured for 48 h in the lower chambers of 12 well-transwell with RPMI 1640 medium supplemented with 10% FBS.

Statistical analysis

All experiments were performed at least in triplicate. Results are expressed as mean ± standard deviation. Differences between the two groups (control and TGF-β1-treated cells) in the time course experiment of CXCL14 expression as well as CXCL14 and CCL20 expression in hOSCC cells were analyzed using an unpaired two-tailed Student's *t*-test. On the other hand, statistical comparison between every pair of two samples among the multiple samples in the inhibitor-treatment experiments were performed using Tukey's multiple comparison test with SPSS Statistics 24 software (IBM, Armonk, NY, USA). Differences were considered statistically significant at $^*P<0.05$ and $^{**}P<0.01$.

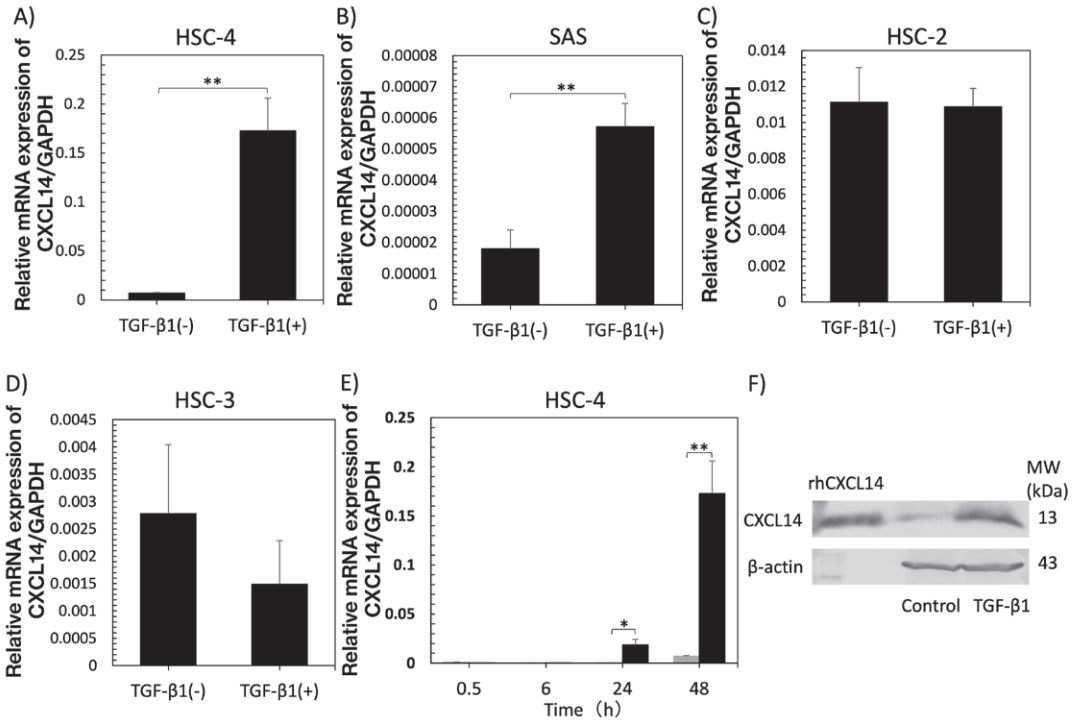

Figure. 1 : *TGF- β 1 induced the expression of CXCL14 in hOSCC cell lines*
hOSCC cells were stimulated by 10 ng/ml TGF-β1 for 48 h and then the expression of CXCL14 was examined using RT-qPCR. (A) HSC-4, (B) SAS, (C) HSC-2 and (D) HSC-3 cell lines were used. (E) Time course study of CXCL14 mRNA expression after TGF-β1 stimulation was performed in HSC-4 cells (dark gray bar, control; black bar, TGF-β1 stimulation). Values have been normalized to the GAPDH mRNA levels. Data are presented as the mean ± SD of quadruplicate experiments. Differences in values between control and TGF-β1-treated cells at each time points were statistically analyzed using Student's t-test ($^{**}P<0.01$ and $^*P<0.05$). (F) The protein levels of CXCL14 were analyzed at 48 h stimulation after with or without 10 ng/ml TGF-β1 stimulation using western blot analysis with anti-CXCL14 antibody. β-actin in the cell lysate was used as a loading control.

Results

TGF-β1 induced the expression of CXCL14 in hOSCC cell lines HSC-4 and SAS

Four kinds of hOSCC cell lines were treated with TGF-β1 (10 ng/ml) for 48 h, and then the mRNA expression level of CXCL14 was examined by RT-qPCR. TGF-β1 upregulated the CXCL14 mRNA expression levels in HSC-4 cells and SAS cells, which are known to be TGF-β1-responsive cells[11] (Fig. 1A, and B). However, the expression level of CXCL14 was not affected by treatment with TGF-β1 (10 ng/ml) in HSC-2 cells and HSC-3 cells, which are known to be TGF-β1-non-responsive cells[11] (Fig. 1C and D). The expression level of CXCL14 mRNA in HSC-4 cells was significantly upregulated at 24 to 48 h after TGF-β1 stimulation (Fig. 1E). The CXCL14 protein expression in HSC-4 cells was increased at 48 h after TGF-β1 stimulation (Fig. 1F).

TGF-β1 upregulated the expression level of CXCL14 through activation of the Smad signaling pathway in HSC-4 cells

TGF-β1 (10 ng/ml) -induced CXCL14 mRNA expression was clearly suppressed by the type I TGF-β receptor ALK5 inhibitor SB431542 (Fig. 2A). In addition, SIS3, a specific

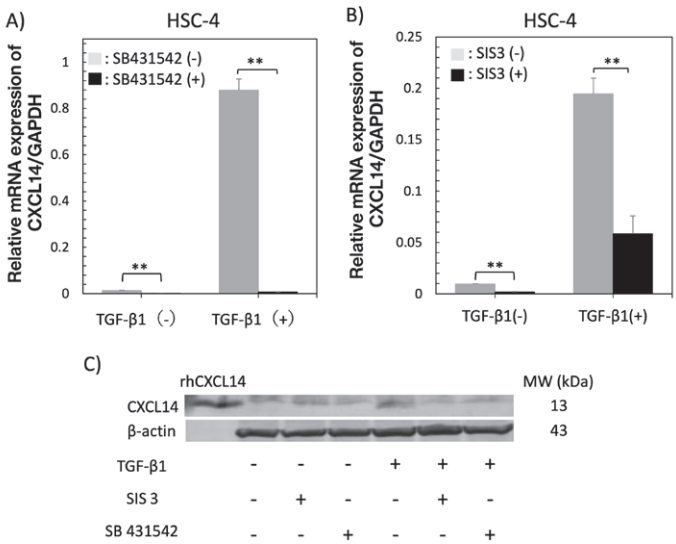

Figure. 2 : *TGF-β1 upregulated the expression level of CXCL14 through activation of Smad signaling pathway in HSC-4 cells*

(A and B) The mRNA expression of CXCL14 was evaluated in HSC-4 cells treated with or without 10 ng/ml TGF-β1 for 48 h. (A) The cells were treated with (black bars) or without (gray bars) 10 μM SB431542 for 30 min before TGF-β1 treatment. (B) The cells were treated with (black bars) or without (gray bars) 0.2 μM SIS3 (black bar) for 30 min before treatment with 10 ng/ml TGF-β1. (A and B) Values have been normalized to GAPDH mRNA levels. Data are presented as the mean ± SD of quadruplicate experiments. Differences in values between two groups were statistically compared using unpaired two-tailed Student's *t*-test (***P*<0.01). (C) Protein expression levels of CXCL14 by TGF-β1 treatment in HSC-4 cells were assayed using western blotting with an anti-CXCL14 antibody. The cells were then treated with 10 ng/ml TGF-β1 for 48 h. Some cells were treated with 10 μM SB431542 or 0.2 μM SIS3 at 30 min before TGF-β1 treatment. 0.3 μg rhCXCL14 was loaded as a positive control. β-actin in the cell lysate was used as a loading control.

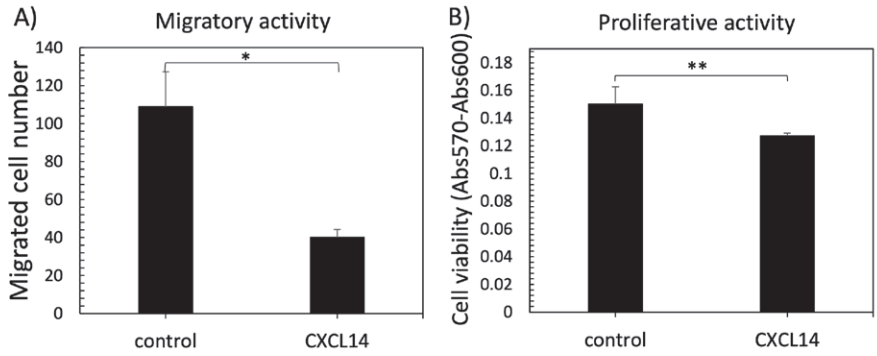

Figure. 3 : *CXCL14 suppressed the migratory-, and proliferative-activities of HSC-4 cells*
HSC-4 cells were stimulated with 10 ng/ml TGF-β1 for 48 h and then used in subsequent experiments. (A) Cell migration ability was evaluated in cells stimulated with 100 ng/ml CXCL14 using a Boyden chamber assay. After 24 h, the migrated cells were counted using with DAP1 staining. Data represent the mean ± SD of triplicate experiments. Differences in values between control and CXCL14-treated cells were statistically analyzed using Student's t-test (*P<0.05). (B) Proliferation ability was monitored in cells stimulated with or without 100 ng/ml CXCL14 using the CCK-8 assay. Data are presented as the mean ± SD of quadruplicate experiments. Differences in values between control and CXCL14-treated cells were statistically analyzed using Student's t-test (**P<0.01).

Smad3 inhibitor, suppressed TGF-β1-induced expression of CXCL14 mRNA (Fig. 2B). Both SB431542, and SIS3 suppressed TGF-β1-induced expression of CXCL14 protein (Fig. 2C). These data indicated that TGF-β1 increased the expression of CXCL14 through the ALK5/Smad3 signaling pathway in HSC-4 cells.

CXCL14 suppressed the migratory and proliferative activities of HSC-4 cells

We examined whether CXCL14 affected statuses of the migration and proliferation of HSC-4 cells. CXCL14 (100 ng/ml) clearly suppressed the migratory and proliferative-activities of HSC-4 cells (Fig 3A and B, respectively). These results indicated that CXCL14 retained a suppressive effect on the metastatic activity of HSC-4 cells. On the other hand, CXCL14 (100 ng/ml) induced cell migration of monocytic THP-1 cells, suggesting that hOSCC recruited immune cells such as monocytes through secretion of CXCL14 (data not shown).

TGF- β1-promoted expression of CXCL14 in HSC-4 cells was suppressed by coculture with PMA-differentiated macrophages derived from THP-1 cells

We confirmed whether monocytic THP-1 cells adequately differentiated into macrophages after treatment with PMA (50 ng/ml) for 48 h. PMA (50 ng/ml) clearly induced macrophage markers CD163 and CD206, indicating that the monocytic THP-1 cells differentiated into macrophages with the PMA stimulation (Fig. 4A and B). TGF-β1 (10 ng/ml) -promoted expression of CXCL14 mRNA in HSC-4 cells was downregulated by coculture with the PMA-differentiated macrophages derived from monocytic THP-1

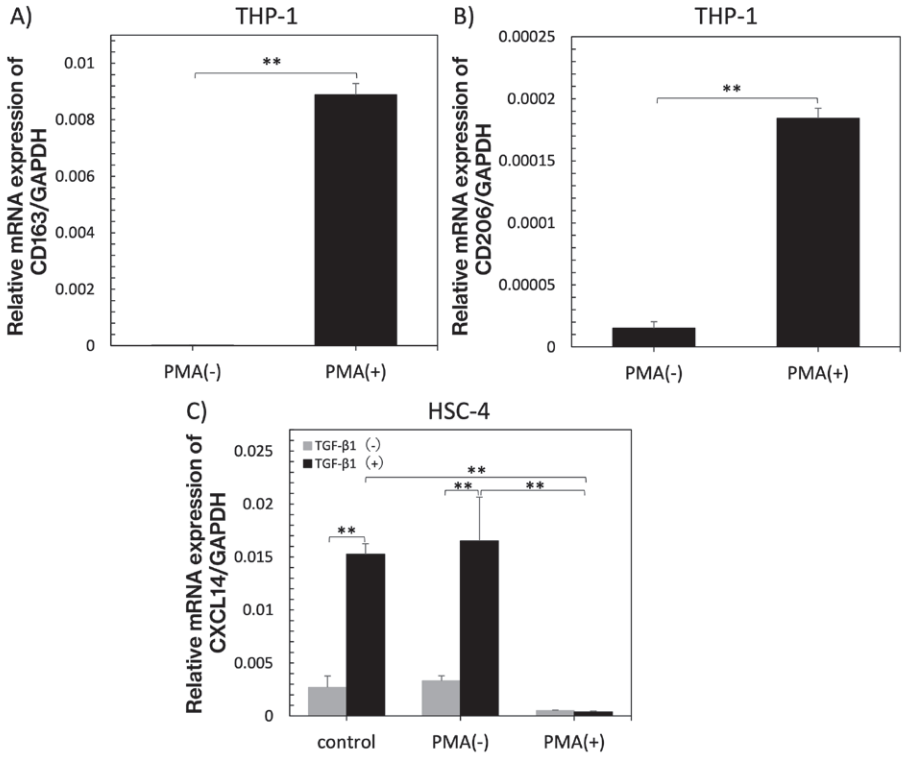

Figure. 4 : *The TGF- β 1-promoted expression of CXCL14 in HSC-4 cells was suppressed by coculturing with PMA-differentiated macrophages derived from THP-1 cells*
(A and B) THP-1 cells were stimulated with [PMA (+)] or without [PMA (-)] 50 ng/ml PMA for 96 h. The mRNA expression levels of macrophage markers (A) CD163 and (B) CD206 were then analyzed using RT-qPCR. (C) HSC-4 cells treated with (black bars) or without (gray bars) 10 ng/ml TGF-β1 were either single cultured (control) or cocultured with PMA-differentiated macrophages derived from monocytic THP-1 cells [PMA (+)], or with the non-differentiated monocytic THP-1 cells [PMA (-)]. Values have been normalized to GAPDH mRNA levels. Data are presented as the mean ± SD of quadruplicate experiments. Values between two groups were statistically compared using Tukey's multiple comparison test (**$P<0.01$).

cells, but not by coculture with non-differentiated monocytic THP-1 cells (Fig 4C). These results indicated that both CD163 and CD206-positive macrophages differentiated from monocytes retained suppressive activity against the TGF-β1-promoted expression of CXCL14 mRNA in HSC-4 cells, but non-differentiated monocytes did not.

CCL20 derived from PMA-differentiated macrophages possibly suppressed TGF-β1-promoted expression of CXCL14 in HSC-4 cells in PI3K-, ERK1/2-, and NF-κB-dependent manners, resulting in the promotion of the metastatic activity of HSC-4 cells

The PMA (50 ng/ml) -differentiated macrophages derived from monocytic THP-1 cells more abundantly expressed CCL20

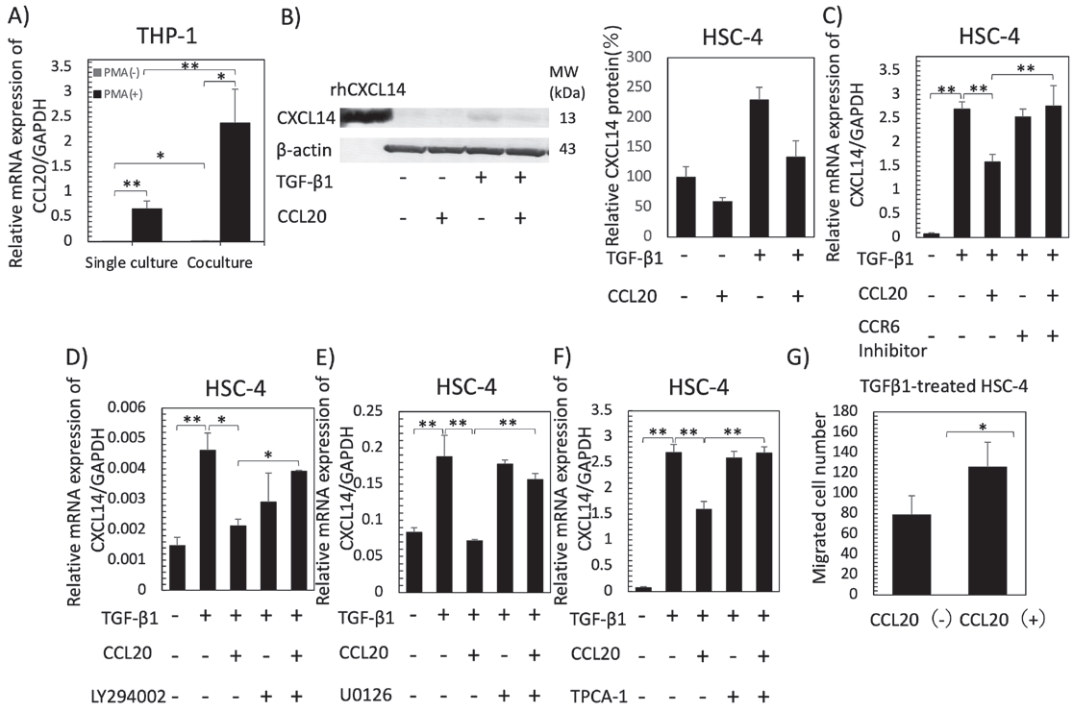

Figure. 5 : *CCL20 derived from PMA-differentiated macrophages possibly suppressed the TGF- β1-promoted expression of CXCL14 in HSC-4 cells, resulting in the promotion of the metastatic activity of HSC-4 cells*

(A) The mRNA expression levels of CCL20 in THP-1 cells were analyzed using RT-qPCR. The cells were stimulated with [PMA (+), black bars] or without [PMA (-), gray bars] 50 ng/ml PMA for 96 h. Then, the cells were single cultured (single culture), or cocultured with the TGF-β1-treated HSC-4 cells for 48 h (coculture). Data are presented as the mean ± SD of quadruplicate experiments. Values between every two groups were statistically compared using Tukey's multiple comparison test (**P<0.01 and *P<0.05). (B) Protein expression level of CXCL14 by CCL20 treatment in HSC-4 cells was examined using western blotting with an anti-CXCL14 antibody. First, the cells were treated with or without 10 ng/ml TGF-β1 for 48 h. The cells were then subsequently treated with or without 20 ng/ml CCL20 for 48 h. 0.3 μg rhCXCL14 was loaded as a positive control. β-actin in the cell lysate was used as a loading control (left panel). The intensities of CXCL14 bands were evaluated by using ImageJ software (right panel). Data are presented as the mean ± SD of triplicate experiments. The band intensities between two samples were statistically compared using Tukey's multiple comparison test (**P<0.01 and *P<0.05). (C to F) The mRNA expression level of CXCL14 in HSC-4 cells stimulated with 10 ng/ml CCL20 and/or 10 ng/ml TGF-β1 was analyzed using RT-qPCR. Some cells were pretreated with signal inhibitors 30 min before TGF-β1 treatment (C, 1 μM CCR6 Inhibitor 1; D, 1 μM LY294002; E, 5 μM U0126; F, 10 μM TPCA-1). Values have been normalized to GAPDH mRNA levels. Data are presented as the mean ± SD of quadruplicate experiments. Values between every two groups were statistically compared using Tukey's multiple comparison test (**P<0.01 and *P<0.05). (G) First, HSC-4 cells were treated with 10 ng/ml TGF-β1 for 48 h. The TGF-β1-treated cells were subsequently stimulated with 10 ng/ml CCL20 for 48 h, following which they were transferred to a Boyden chamber. Migratory activities in the cells treated with or without 10 ng/ml CCL20 plus 10 ng/ml TGF-β1 were evaluated by using a Boyden chamber assay. Data represent the mean ± SD of triplicate experiments. Differences in values between control and CXCL14-treated cells were statistically analyzed using Student's t-test (*P<0.05).

mRNA than the non-differentiated monocytic THP-1 cells (Fig. 5A, left graph). Intriguingly, the expression levels of CCL20 mRNA in non-differentiated monocytes, and differentiated macrophages were significantly upregulated by coculture with TGF-β1 (10 ng/ml) -stimulated HSC-4 cells (Fig. 5A, right graph), suggesting that the hOSCC cells in EMT retained an ability to stimulate monocytes/macrophages to express chemokines such as CCL20. The TGF-β1-incuced expression of CXCL14 protein was significantly downregulated by CCL20 (20 ng/ml) (Fig. 5B). Treatment with CCR6 inhibitor-1 (1 μM) abrogated the suppressive effect of CCL20 on TGF-β1-promoted expression of CXCL14 mRNA (Fig. 5C). PI3K inhibitor LY294002 (1 μM) abrogated the suppressive effect of CCL20 on TGF-β1-promoted expression of CXCL14 mRNA in HSC-4 cells (Fig. 5D). MEK1/2 inhibitor U0126 (5 μM) abrogated the suppressive effect of CCL20 on TGF-β1-promoted expression of CXCL14 mRNA in HSC-4 cells (Fig. 5E). NF-κB kinase-2 (IKK-2) inhibitor TPCA-1 (10 μM) abrogated the suppressive effect of CCL20 on TGF-β1-promoted expression of CXCL14 mRNA in HSC-4 cells (Fig. 5F). These results indicated that the CCL20/CCR6-mediated signal abrogated TGF-β1-incuced expression of CXCL14 in PI3K-, MEK1/2-, and NF-κB-dependent manners. Next, we investigated whether CCL20 positively regulated the migratory activity of TGF-β1-treated HSC-4 cells; we have previously demonstrated that HSC-4 cells stimulated by TGF-β1 exhibited higher migrate ability than unstimulated HSC-4 cells[11]. The migratory activity of TGF-β1 -treated HSC-4 cells was significantly enhanced by CCL20 stimulation (Fig 5G).

Discussion

Nakayama *et al.* reported that differential epigenetic gene silencing mechanisms caused diversity of expression level of CXCL14 in hOSCC cells[31]. Meng *et al.* reported that TGF-β/Smad signaling regulates the extent of renal fibrosis through epigenetic-correlated mechanisms[32]. Here, we found that the TGF-β1 -induced expression level of CXCL14 mRNA was higher in HSC-4 cells than in SAS cells (Fig. 1A and B). Therefore, TGF-β1 may participate in the epigenetic modification of *CXCL14* genes in hOSCC cells. On the other hand, a time course estimation of mRNA expression level revealed that CXCL14 expression was significantly upregulated at 24 to 48 h, but not at 0.5 to 6 h after TGF-β1 stimulation (Fig. 1E). We also found that TGF-β1 increased the expression of CXCL14 in hOSCC cells through the Smad3 signaling pathway (Fig. 2B and C). TGF-β1 did not induce CXCL14 mRNA expression within 6 h, possibly because *CXCL14* might not be a direct target gene for the TGF-β1-activated Smad3-mediated signal; we have previously demonstrated that TGF-β1 increased the mRNA expression of EMT-related transcription factors Slug and Wnt5b within a few hours in HSC-4 cells in a Smad3-dependent manner. Then, MMP-10 mRNA expression increased at 24 to 48 h after TGF-β1 stimulation in these cells in a Slug/Wnt5b-dependent manner[28].

CXCL14 plays important roles in the regulation of immune and inflammatory-responses[33], glucose metabolism[25], and antimicrobial activity[34]. The upregulation of CXCL14 expression has been observed in hOSCC and melanoma, colon, liver and lung cancer suppressed the tumor growth, resulting in suppression of the tumor progression[23].

However, CXCL14 expression has also been found to serve as a tumor promoter in breast and prostate cancer[23]. Ozawa *et al.* reported the anti-tumor activity of CXCL14 in HSC-2, HSC-3 and HSC-4 cell lines; however, it remained to be clarified that what kinds of molecular mechanisms regulated CXCL14 expression in hOSCC cells[35]. In the present study, we found that TGF-β1 upregulated CXCL14 expression in SAS cells and HSC-4 cells (Fig. 1A, and B). Intriguingly, CXCL14 inhibited the proliferative and migratory activities of HSC-4 cells (Fig. 3A and 3B). These results suggest that TGF-β1-induced expression of CXCL14 suppressed the metastatic activity of hOSCC cells in an autocrine- or paracrine-manner, resulting in the formation of a negative feedback loop in TGF-β1-promoted EMT in HSC-4 cells.

PMA-differentiated macrophages retained the ability to suppress the TGF-β1-promoted expression of CXCL14 in HSC-4 cells (Fig. 4B). We also found that the expression level of CXCL14 was also downregulated in HSC-3 cells by coculture with PMA-differentiated macrophages (data not shown), while the expression level of CXCL14 mRNA was not affected by TGF-β1 stimulation (Fig. 1D). These data suggest that the expression levels of CXCL14 in hOSCC are downregulated by some factors derived from the PMA-differentiated macrophages. Ozawa *et al.* reported that EGF abrogates CXCL14 expression in hOSCC cells other than HSC-4 cells in an ERK1/2-dependent manner[26]. We also found that EGF (10 ng/ml) strongly downregulated CXCL14 mRNA expression in HSC-4 cells with or without TGF-β1 stimulation (data not shown). In addition, we confirmed that HSC-4 cells did not retain the ability to stimulate the non-differentiated monocytes, or PMA-differentiated macrophages to increase EGF mRNA expression through cell-to-cell interaction in a coculture system (data not shown).

CCL20 mRNA was more highly expressed in PMA-differentiated macrophages than the non-differentiated monocytic THP-1 cells (Fig. 5A). Kadomoto *et al.* also reported that PMA-differentiated macrophages derived from THP-1 cells vigorously expressed CCL20 mRNA by coculturing with renal cell carcinoma cells[36]. We found that the TGF-β1-induced expression of CXCL14 protein was suppressed by CCL20 treatment in HSC-4 cells (Fig. 5B). We also confirmed the CCL20-induced suppression of TGF-β1-induced CCCL14 mRNA expression using RT-qPCR analysis (data not shown). In addition, EGF more vigorously suppressed CXCL14 mRNA expression than CCL20 (data not shown).

We found that the CCL20/CCR6-activated signal clearly suppressed TGF-β1-incuced mRNA expression of CXCL14 in HSC-4 cells in PI3K-, ERK1/2-, and NF-κB-dependent manners (Fig. 5C, D, E, and F). On the other hand, Muscella *et al.* reported that the CCL20/CCR6-activated signal induces the expression of MMP-9 through the PKC/Akt/NF-κB signaling pathway in cancerous breast epithelial cells[22]. Kang *et al.* reported that PMA induces the expression of the metastasis-promoting enzyme MMP-9 expression partially through the ERK1/2-, and NF-κB-signal pathways[37]. We also found that PMA inhibits the expression of CXCL14 (data not shown). These results suggest that CCL20/CCR6-induced signaling suppresses the metastatic activity of hOSCC cells through the PKC/Akt/NF-κB-, and PKC/ERK/NF-κB-mediated signaling pathways.

The migratory activity of TGF-β1-stimulated HSC-4 cells was significantly enhanced by

CCL20 stimulation (Fig 5G). We also found that EGF enhanced the migratory activity of HSC-4 cells (data not shown), however, we did not confirm whether the EGF-induced suppression of CXCL14 expression resulted in suppression of the migration ability of HSC-4 cells. Muscella *et al.* reported that CCL20 promotes cell migration and invasion of cancerous breast epithelial cells through enhancement of MMP-2 and MMP-9 expressions[22]. Kadomoto *et al.* reported that CCL20 enhances cell migration and EMT in renal cell carcinoma cells[36]. Intriguingly, the expression of CCL20 receptor CCR6 was clearly upregulated by TGF-β1 treatment in HSC-4 cells (data not shown), possibly resulting in the enhancement of the CCL20-activated signal mediated through CCR6.

Taken together, these results suggest that the TGF-β1-induced CXCL14 expression in hOSCC cells, which exhibits suppressive effects on the migratory and proliferative activities of these cells, was possibly abrogated by CCL20 derived from TAMs, resulting in EMT progression in hOSCC.

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Competing interests

The authors declare that they have no competing interests.

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