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Original

Sclerostin derived from EMT-promoted human oral squamous cell carcinoma cells induces IL-6- and IL-17A-mediated M1 to M2 polarization shift in THP-1-derived macrophages

Yuudai ISHIKAWA[1,2], Taifu HIRANO[2], Hiroyuki YAMADA[2], Akira ISHISAKI[1], and Masaharu KAMO[1]

[1]Division of Cellular Biosignal Sciences, Department of Biochemistry, Iwate Medical University (Chief: Prof. Akira ISHISAKI)

[2]Division of Oral and Maxillofacial Surgery, Department of Reconstructive Oral and Maxillofacial Surgery, Iwate Medical University School of Dentistry (Chief: Prof. Hiroyuki YAMADA)

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Abstract: Squamous cell carcinoma（SCC）is the most common cancer in the oral cavity. Epithelial–mesenchymal transition（EMT）plays an important role in SCC cell metastasis process. We have previously demonstrated that transforming growth factor-β1（TGF-β1）induces EMT in HSC-4, a human oral SCC（hOSCC）cell line. However, the molecular mechanisms of metastasis after EMT-promoted cancer are poorly understood. On the other hand, tumor-associated macrophages（TAMs）that coexist with cancer tissue participate in permeation metastasis. However, whether TAMs affect hOSCC cell metastasis in the tumor microenvironment（TME）remains uncertain.

Here, we investigated the expression status of inflammatory and anti-inflammatory cytokines and M2 polarization status in macrophages（Mϕs）cocultured with EMT-promoted HSC-4 cells. Under coculture of EMT-promoted HSC-4 cells and M1-Mϕs, we found that the expression of tumor necrosis factor-α（TNF-α）, an M1-polarization inducer, was clearly suppressed in M1-Mϕ, while the expression of M2-polarization promoter TGF-β1 and M2-Mϕ marker CD163 was significantly reinforced. These results indicate that M1-Mϕ polarization into M2-Mϕ was promoted by coculture with EMT-promoted HSC-4 cells. In the next step, we tried to identify the factors derived from EMT-promoted HSC-4 cells that promoted M2-Mϕ polarization. We found that 1）TGF-β1 induces sclerostin expression through the Smad signal transduction pathway in HSC-4 cells, 2）sclerostin reinforces interleukin-6（IL-6）, TGF-β1, and IL-17A expression in M1-Mϕs, 3）IL-6 promotes IL-17A expression in M1-Mϕs and M1-Mϕ polarization into M2-Mϕ, and 4）IL-17A reinforces M1-Mϕ

1) Division of Cellular Biosignal Sciences, Department of Biochemistry, Iwate Medical University (Chief: Prof. Akira ISHISAKI)

2) Division of Oral and Maxillofacial Surgery, Department of Reconstructive Oral and Maxillofacial Surgery, Iwate Medical University School of Dentistry (Chief: Prof. Hiroyuki YAMADA)

1) 1-1-1, Idaidori, Yahaba-cho, Iwate 028-3694, Japan

2) 1-3-27 Chuo-dori, Morioka, Iwate 020-8505, Japan

1) 岩手県紫波郡矢巾町医大通 1-1-1（〒 028-3694）

2) 岩手県盛岡市中央通 1-3-27（〒 020-8505）

polarization into M2-Mϕ.

These results strongly suggest that the EMT-promoted hOSCC cells retained the ability to promote polar changes from M1-Mϕs to M2-Mϕs through sclerostin production in the TME.

Abbreviations: ADSCs, adipose-derived mesenchymal stem cells; EMT, epithelial–mesenchymal transition; hOSCC, human oral squamous cell carcinoma; IL, interleukin; Mϕ, macrophage; PMA, phorbol 12-myristate 13-acetate; RT-qPCR, quantitative real-time reverse transcription polymerase chain reaction; TAM, tumor-associated macrophage; TGF-β, transforming growth factor-β; TME, tumor microenvironment; TNF-α, tumor necrosis factor-α.

Key words: epithelial-mesenchymal transition, IL-17A, M1-macrophage, sclerostin, transforming growth factor-β

Introduction

Epithelial–mesenchymal transition (EMT) plays an important role in cancer metastasis[1]. It induces the loss of epithelial characteristics and the gain of mesenchymal characteristics in differentiated epithelial cells, which leads to increased cell migration and invasion[2]. Thus, it is an important process involved in not only development, adult tissue maintenance, and reproduction[3), 4)], but also cancer and desmoplasia in diseases[3), 5)]. Cadherin switch, a switch from E-cadherin to N-cadherin, plays an important role in the malignant transformation of cancer cells during EMT[6), 7)]. Transforming growth factor-β (TGF-β) is a crucial EMT inducer[8), 9), 10)]. TGF-β possesses a contradictory dual-faceted nature as it acts both as a tumor suppressor during the initial stages of tumorigenesis, as well as an activator in tumor progression. During tumor progression, TGF-β induces cancer invasion and metastasis through EMT, escapes from the immune system, and facilitates angiogenesis[11)]. TGF-β binds to TGF-β receptor type I and type II, which are transmembrane serine/threonine kinases. Both Smad2 and Smad3 phosphorylated by the TGF-β receptor bind to Smad4 and then translocate to the nucleus. Smad2/3/4 complex in cooperation with other cofactors regulates the transcription of several target genes[12), 13)].

Squamous cell carcinoma (SCC) is the most common cancer of the oral cavity[14)]. The mechanism underlying regulation of the cadherin switch in human oral SCC (hOSCC) cells remains to be elucidated. However, studies have reported changes in the expression of various genes related to the cadherin switch in many types of SCC cells other than hOSCC cells[15)]. We have previously demonstrated that TGF-β1 induces EMT in the hOSCC cell line HSC-4. We also showed that integrin α3β1/FAK activation promotes the migratory activity of HSC-4 cells[16)]. In addition, we found that TGF-β1-induced Slug expression upregulation positively regulates the migratory activity of HSC-4 cells. TGF-β1 also stimulates the invasion ability of HSC-4 cells through the Slug/Wnt-5b/MMP-10 signaling axis[17)]. In contrast, we demonstrated that Sox9 participates in upregulating N-cadherin expression[7)]. These results suggest that Slug and Sox9 are important EMT-related

transcription factors that promote the metastasis of hOSCC cells.

Macrophages (Mϕs), particularly tumor-associated macrophages (TAMs) in cancer tissue, are an important cell type in the tumor microenvironment (TME)[18]. TAMs progress cancer invasion and metastasis and protect against the immune system, similar to M2 type Mϕs. TAMs induce tumor progression and immune dysfunction, allowing cancer cell survival[18]. TAMs allow non-small cell lung cancer metastasis by promoting TGF-β secretion-induced EMT[19]. However, the effect of TAMs on the metastatic activity of hOSCC cells remains to be clarified. TAMs produce several cytokines and chemokines, depending on their phenotype[18]. We previously found that TGF-β1 stimulation upregulates the expression of CXCL14, which suppresses cancer progression, in HSC-4 cells[20, 21]. CXCL14 expression in HSC-4 cells was downregulated by coculture with Mϕs. In contrast, we found that CCL20 suppressed TGF-β1-induced CXCL14 expression. These data strongly suggest that TAM-derived CCL20 abrogated TGF-β1-induced CXCL14 expression in HSC-4 cells, activating the metastatic activity in HSC-4 cells[20]. However, it is not yet elucidated how hOSCC affects the immune regulatory characteristics of TAMs, particularly the M1-Mϕ character in the TME. M1-Mϕs participate in inflammatory and anti-tumor responses[22]. M1-Mϕs produce pro-inflammatory cytokines, such as TNF-α, whereas M2-Mϕs secrete anti-inflammatory cytokines, such as IL-10[22]. In general, CXCL9 and CXCL10 are upregulated in M1-Mϕs, whereas CD163, CD206, and CCL13 are upregulated in M2-Mϕs[23, 24]. Besides, IL-17A induces the polarization of Mϕs into M2-like Mϕs[25, 26]. In addition, IL-17A was vigorously expressed in differentiated Th17 cells derived from CD4$^+$ T cells treated with TGF-β and IL-6, or IL-1β and IL-23[25]. However, whether IL-17A expression is upregulated in Mϕs cocultured with EMT-promoted hOSCC cells remains to be clarified.

Sclerostin, encoded by the *SOST* gene and widely known to be secreted by osteocytes, is an antagonist of canonical Wnt signaling pathway[27]. There are contradicting reports that sclerostin induces metastasis in breast cancer[28] but inhibits invasion in prostate cancer[29]. Sclerostin is also upregulated by TGF-β in an osteoblast cell line[30]. However, whether TGF-β upregulates sclerostin expression in hOSCC cells remains to be clarified. In contrast, Chen *et al.* reported that sclerostin induces IL-6 and TGF-β expression in adipose-derived mesenchymal stem cells (ADSCs). In addition, they found that differentiation of CD4$^+$ T cells into Th17 cells was induced by coculture with sclerotin-overexpressing ADSCs[31]. However, whether sclerostin derived from hOSCC cells promotes IL-6, TGF-β, and IL-17A expression in Mϕs in the TME remains to be clarified.

Here, we attempted to elucidate the molecular mechanism underlying hOSCC-induced M2-polarization in TME and investigated the factors produced in coculture between EMT-promoted OSCC cells and Mϕs promoted the polarization of Mϕs into M2 phenotypes. In particular, we examined whether M1-Mϕs cocultured with the EMT-promoted HSC-4 cells by TGF-β1-treatment exhibited M2-Mϕ character; TGF-β1 promoted sclerostin expression in HSC-4 cells; sclerostin upregulated the expression of M2 polarization promoters IL-6, TGF-β1, and IL-17A in M1-Mϕs; and IL-6, TGF-β1, and IL-17A promoted M2-Mϕ polarization in M1-Mϕs.

Materials and Methods

Materials

Cultured cell lines were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Recombinant human TGF-β1, IL-6, IL-17A, interferon-γ (IFN-γ), and sclerostin were obtained from PeproTech (Rocky Hill, NJ, USA). The SIS3 inhibitor was provided by Selleck Chemicals (Houston, TX, USA). The SB4131542 inhibitor was purchased from Merck-Millipore (Frankfurt, Germany). The NF-κB kinase-2 (IKK-2) inhibitor TPCA-1 was purchased from R&D Systems Inc. (Minneapolis, MN, USA). Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Lipopolysaccharide from *Porphyromonas gingivalis* (LPS) was purchased from InvivoGen (San Diego, CA, USA). All other reagents were of analytical grade.

Cell culture

All cell lines were grown at 37 ℃ with 5% CO_2. Human HSC-4 SCC 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-3 cells (JCRB0623) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% FBS. The culture medium was removed and replaced with serum-free medium 24 h prior to the TGF-β1-stimulated experiments. For time-course experiments, 2.0×10^5 HSC-4 cells were cultured in 1000 or 500 µl medium without serum containing 10 ng/ml TGF-β1 for 1–48 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. Macrophage differentiation and THP-1 cell polarization were carried out according to the protocol reported by Baxter *et al.*[23]. Briefly, 2.0×10^5 cells in 12 well-tissue culture plates were treated with 5 ng/ml PMA for 24 h. After rinsing with phosphate-buffered saline (PBS) buffer to remove non-adherent cells, adherent cells (PMA-differentiated macrophages) were further cultured in RPMI 1640 medium supplemented with 10% FBS without PMA for 72 h to complete the differentiation of THP-1 cells (M0-Mϕ). M0-Mϕ was further polarized to M1 type Mϕ (M1-Mϕ) by treatment with 250 ng/ml LPS and 20 ng/ml IFN-γ for 48 h.

Coculture of HSC-4 cells with THP-1 cells or macrophage differentiation cells

HSC-4 cells (3.0×10^5 cells) were seeded into the upper chambers of 12 well-transwell (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 to RPMI 1640 medium supplemented with 10% FBS. In contrast, 3.0×10^5 THP-1 cells, PMA-treated THP-1 cells (M0-Mϕ), or M1 polarized M0-Mϕ treated with LPS and IFN-γ (M1-Mϕ) were cultured for 48 h in the lower chambers of 12 well-transwell with RPMI 1640 medium supplemented with 10% FBS.

Quantitative real time RT-PCR (RT-qPCR)

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 Inc.; Shiga, Japan). RT-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 (Table I). Target gene expression was normalized to an internal GAPDH reference and expressed in terms of fold-change relative to the control sample[32].

Enzyme-linked immunosorbent assay (ELISA)

Sclerostin secreted from HSC-4 cells was detected using an ELISA kit (R&D Systems Inc.) in accordance with the manufacturer's protocols. For ELISA experiments, 2.0×10^5 cells were cultured in a 12-well plate using serum-free MEM with or without 10 ng/ml TGF-β1 for 48 h. The culture medium (50 μl) was added to the ELISA microplates and incubated for 2 h at room temperature. After the plates were washed with buffer in kit, the samples were incubated with anti-sclerostin antibody for 2 h at room temperature. The absorbance at 450 nm was measured to estimate the added color reagent using a MPR-A4i II microplate reader (TOSOH, Tokyo, Japan). The assays were performed independently at least three times.

Table I. Sequence of primers used for RT-qPCR

Target mRNA	Oligonucleotide sequence (5′–3′)	Predicted size (bp)
CD163	(F) GCTCAATGAAGTGAAGTGCAAAG (R) CCAAGGATCCCGACTGCAA	199
CD206 (*MRC1*)	(F) GCCCGGAGTCAGATCACACA (R) AGTGGCTCAACCCGATATGACAG	178
CCL13	(F) AACGTCCCATCTACTTGCTGCTTC (R) CGGCCCAGGTGTTTCATATAATTCT	188
CXCL9	(F) AGGGTCGCTGTTCCTGCATC (R) TTCACATCTGCTGAATCTGGGTTTA	166
IL-1β	(F) CCAGGGACAGGATATGGAGCA (R) TTCAACACGCAGGACAGGTACAG	129
IL-6	(F) AAGCCAGAGCTGTGCAGATGAGTA (R) TGTCCTGCAGCCACTGGTTC	150
IL-10	(F) GAGATGCCTTCAGCAGAGTGAAGA (R) AGTTCACATGCGCCTTGATGTC	198
IL-17A	(F) GTTCTCGATTTCACATGCCTTCA (R) CTCAGGGCCATTATCTTATTTGCTC	118
Sclerostin (*SOST*)	(F) TCAGTGCCAAGGTCACTTCCAG (R) TCCAGGAGTTTGTCAGCCGTAAATA	134
TGF-β1	(F) GCGACTCGCCAGAGTGGTTA (R) GTTGATGTCCACTTGCAGTGTGTTA	143
TNF-α	(F) CTGCCTGCTGCACTTTGGAG (R) ACATGGGCTACAGGCTTGTCACT	132
GAPDH	(F) GCACCGTCAAGGCTGAGAAC (R) TGGTGAAGACGCCAGTGGA	89

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 sub-cultured 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 50 ng/ml recombinant human sclerostin (rhSclerostin) for 48 h. The cells were incubated with 10 μl CCK-8 solution at 37 ℃ in 5% CO_2 for 2 h. The absorbance at 450 nm was measured to estimate the reduced forms of the reagent using a MPR-A4i II microplate reader. The assays were performed independently at least three times.

Cell migration assay with Boyden chamber

Boyden chamber-based cell migration assays were performed as follows: HSC-4 cells were treated with 10 ng/ml TGF-β1 under serum-free conditions for 48 h. The TGF-β1-treated cells were further treated with or without 50 ng/ml rhSclerostin for 48 h. The treated cells (1.0×10^5 cells) were then plated into the upper chamber of a Boyden chamber apparatus in serum-free media and allowed to migrate into a medium containing 10% FBS in the lower chamber for 24 h at 37 ℃. After a 24 h incubation period, the cells on the filter were fixed in 4% paraformaldehyde and stained with DAPI for 10 min. After the upper side cells were wiped out with a cotton swab, 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 the 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.

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 sclerostin (SOST) expression in hOSCC cells as well as cytokine/chemokine expression in PTH-1/PTH-1-derived cells were analyzed using an unpaired two-tailed Student's *t*-test. On the contrary, 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 $^{**}P<0.01$.

Results

The expression of cytokines in M1-Mϕs was positively or negatively regulated by coculture with EMT-promoted HSC-4 cells

The expression of the pro-inflammatory cytokine IL-1β was significantly upregulated in monocytic THP-1 cells in coculture with TGF-β1-treated HSC-4 cells, but not in M0-Mϕs or M1-Mϕs (Fig. 1A). The expression of the pro-inflammatory cytokine TNF-α was significantly downregulated in monocytic THP-1, M0-Mϕs, and M1-Mϕs by coculture with TGF-β1-treated HSC-4 cells. On the contrary, the expression of the anti-inflammatory cytokine, TGF-β1, was upregulated in monocytic THP-1 and M1-Mϕs by coculture with TGF-β1-treated HSC-4 cells (Fig. 1C). In addition, the expression of the anti-

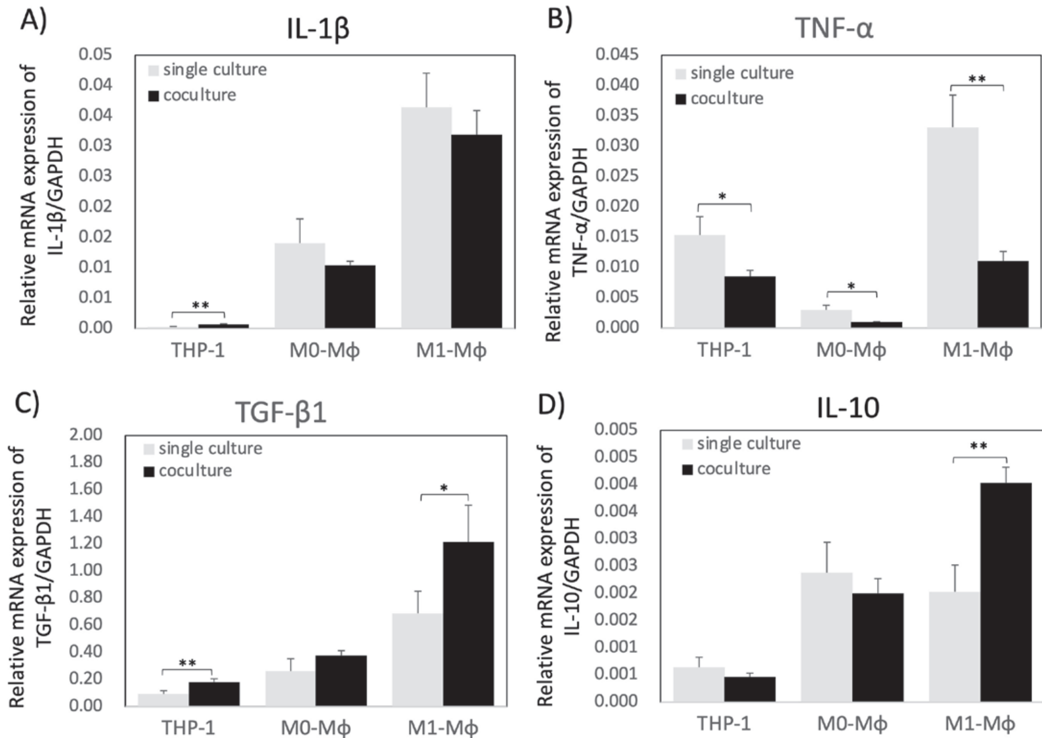

Figure 1: *The expressions of cytokines derived from M1-Mφs were increased by coculture with EMT-promoted HSC-4 cells*

THP-1 cells were stimulated with or without 5 ng/ml PMA for 24 h, and then PMA was removed for 72 h. The PMA-treated THP-1 cells were incubated with (M1-Mφ) or without (M0-Mφ) 20 ng/ml LPS and 250 ng/ml IFN-γ for 48 h. These cells were either single cultured (gray bars) or cocultured with HSC-4 cells treated with 10 ng/ml TGF-β1 (black bars) for 48 h. The mRNA expression levels of proinflammatory cytokines (A) IL-1β and (B) TNF-α, and anti-inflammatory cytokines (C) TGF-β1 and (D) IL-10 were then analyzed using RT-qPCR. Values have been normalized to GAPDH mRNA levels. Data are presented as the mean ± SD of quadruplicate experiments. Differences in values between single-cultured Mφs and Mφs cocultured with TGF-β1-treated HSC-4 cells were statistically analyzed using Student's *t*-test (** $P<0.01$ and * $P<0.05$).

inflammatory cytokine IL-10 was upregulated in M1-Mφs by coculture with TGF-β1-treated HSC-4 cells (Fig. 1D). These results indicated that M2 polarization in M1-Mφs was induced by coculture with EMT-promoted HSC-4 cells.

Expression of M1- and M2-Mφ markers in M1-Mφs cocultured with EMT-promoted HSC-4 cells

We examined whether coculture with TGF-β1-treated HSC-4 cells affected the status of M2-Mφ markers in M1-Mφ cells. The expression of M2-Mφ marker CD163 was significantly upregulated in M0-Mφs and M1-Mφs by coculture with TGF-β1-treated HSC-4 cells (Fig. 2A). The expression of the M2-Mφ marker CCL13 was significantly upregulated in monocytic THP-1, M0-Mφs, and M1-Mφs by coculture with TGF-β1-treated HSC-4 cells (Fig. 2B). Unexpectedly, CD206 expression was not increased in monocytic THP-1, M0-Mφs, and M1-Mφs, but

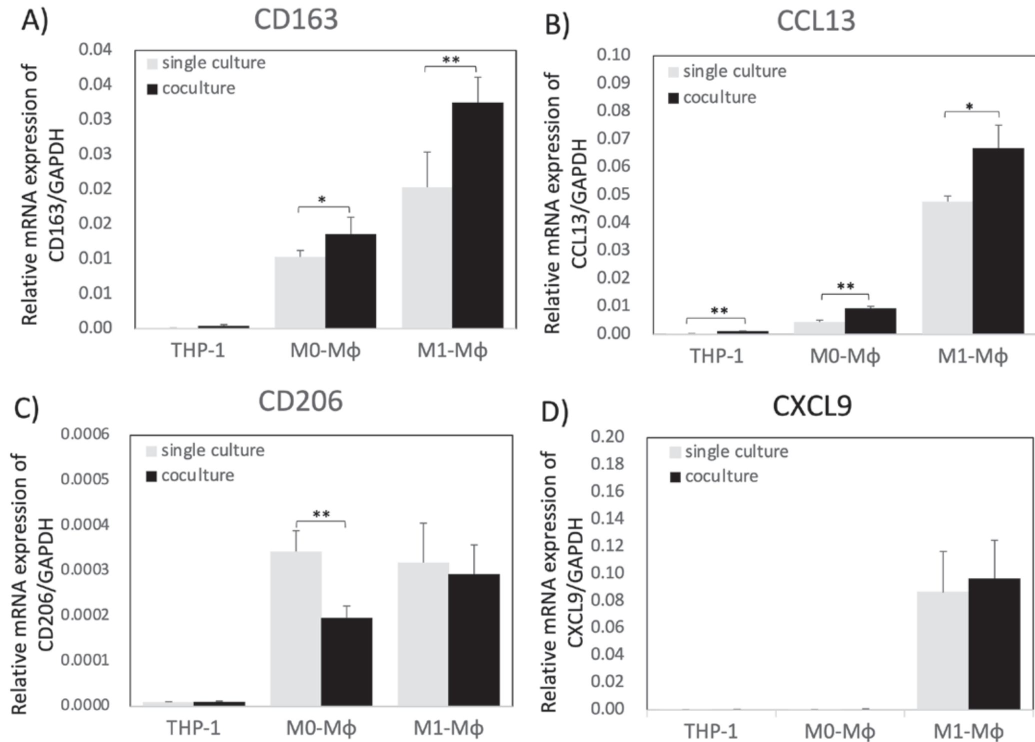

Figure 2: *Expressions of M1-, and M2-Mϕ markers in M1-Mϕ after coculture with EMT-promoted HSC-4 cells*
The mRNA expression levels of macrophage markers in THP-1 and THP-1-derived macrophages (M0-Mϕ and M1-Mϕ) were analyzed using RT-qPCR. M2-Mϕ markers, (A) CD163, (B) CCL13, (C) CD206, and M1-Mϕ marker (D) CXCL9 were analyzed in single-cultured Mϕs (gray bars) or those cocultured with 10 ng/ml TGF-β1-treated HSC-4 cells (black bars) for 48 h. Values were normalized to GAPDH mRNA levels. Data are presented as mean ± SD of quadruplicate experiments. Differences in values between single-cultured Mϕs and Mϕs cocultured with TGF-β1-treated HSC-4 cells were statistically analyzed using Student's *t*-test (**P<0.01, and *P<0.05).

downregulated in M0-Mϕs by coculture with TGF-β1-treated HSC-4 cells (Fig. 2C). In contrast, the expression status of the M1-Mϕ marker CXCL9 in monocytic THP-1, M0-Mϕs, and M1-Mϕs was not affected by coculture with TGF-β1-treated HSC-4 cells (Fig. 2D). These results indicated that M2 polarization in M1-Mϕs was partially induced by coculture with EMT-promoted HSC-4 cells.

Investigation of expression of the M2-Mϕ polarization inducer IL-17A and IL-17A production stimulator IL-6 in Mϕs cocultured with EMT-promoted HSC-4 cells

IL-17A expression in M1-Mϕ was increased by coculture with TGF-β1-treated HSC-4 cells (Fig. 3A). Furthermore, IL-6 expression in M1-Mϕ was also increased by coculture with the HSC-4 cells (Fig. 3B).

TGF-β1 induced sclerostin expression in HSC-4 cells

We attempted to identify the M2 type polarization-inducible liquid-related factors derived from EMT-promoted HSC-4 cells. We found that TGF-β1 significantly upregulated

sclerostin expression in HSC-4 cells after 48 h (Fig. 4A). In contrast, TGF-β1 marginally upregulated sclerostin expression 24 h after stimulation. ELISA revealed that the sclerostin protein level in conditioned medium from TGF-β1-treated HSC-4 cells also increased 48 h after stimulation (Fig. 4B). These data indicate that TGF-β1 stimulation induces sclerostin expression. We also examined whether TGF-β1 induced sclerostin expression in hOSCC cell lines other than HSC-4. Sclerostin expression was induced in SAS cells that exhibited EMT

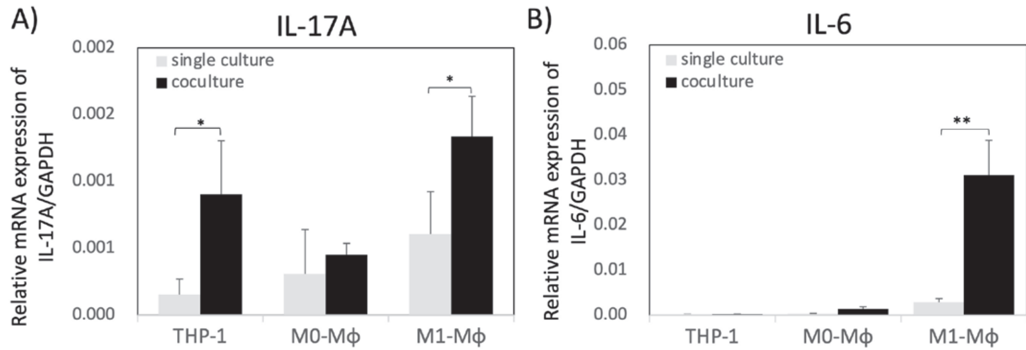

Figure 3: *Investigation of expressions of the M2-Mϕ polarization inducer IL-17A and IL-17A production stimulator IL-6 in Mϕs cocultured with EMT-promoted HSC-4 cells*

(A) IL-17A and (B) IL-6 expression was analyzed in single-cultured Mϕs (gray bars) or those co-cultured with 10 ng/ml TGF-β1-treated HSC-4 cells (black bars) for 48 h using RT-qPCR. Values were normalized to the GAPDH mRNA levels. Data are presented as mean ± SD of quadruplicate experiments. Differences in values between single-cultured Mϕs and Mϕs cocultured with TGF-β1 -treated HSC-4 cells were statistically analyzed using Student's *t*-test (** $P<0.01$ and * $P<0.05$).

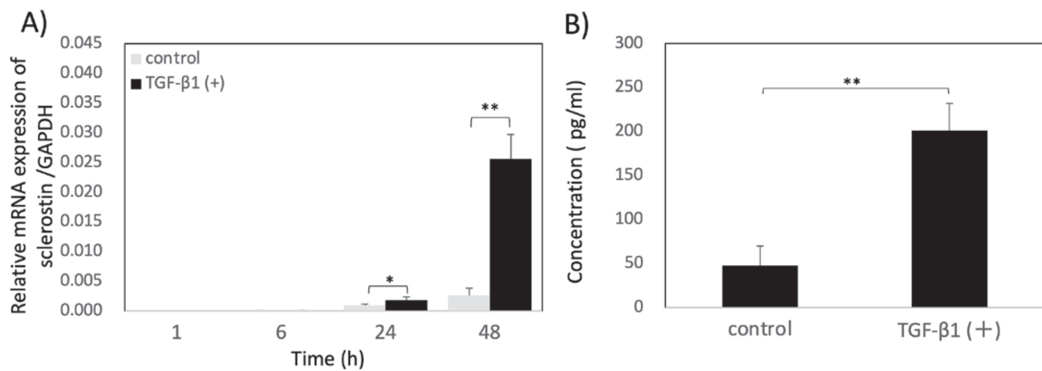

Figure 4: *TGF-β1 induced sclerostin expression in HSC-4 cells*

(A) Time course study of sclerostin mRNA expression in 10 ng/ml TGF-β1-stimulated HSC-4 cells (gray bar, control; black bar, TGF-β1 stimulation). Values were normalized to GAPDH mRNA levels in all RT-qPCR. Data are presented as mean ± SD of quadruplicate experiments. Differences in values between control and TGF-β1-treated cells at each time point were statistically analyzed using Student's t-test (** $P<0.01$, and * $P<0.05$). (B) Sclerostin protein levels in HSC-4 cells were analyzed after stimulation with or without 10 ng/ml TGF-β1 for 48 h using ELISA with anti-sclerostin antibody. Data are presented as mean ± SD of quadruplicate experiments. Differences in values between the two groups were statistically compared using an unpaired two-tailed Student's *t*-test (** $P<0.01$).

in response to TGF-β1 [16] (data not shown), which was not observed in HSC-3 cells that did not exhibit EMT in response to TGF-β1 [16] (data not shown). As sclerostin expression level in HSC-4 cells was relatively higher than that in SAS cells, we chose HSC-4 cells for subsequent experiments.

TGF-β1 upregulated sclerostin expression in HSC-4 cells through the Smad signaling pathway

We investigated whether sclerostin upregulation was dependent on the TGF-β receptor type I (ALK5)/Smad signaling pathway using specific TGF-β1-activated signal molecule inhibitors. RT-qPCR analysis revealed that the ALK5 inhibitor SB431542 and the specific Smad3 inhibitor SIS3 suppressed

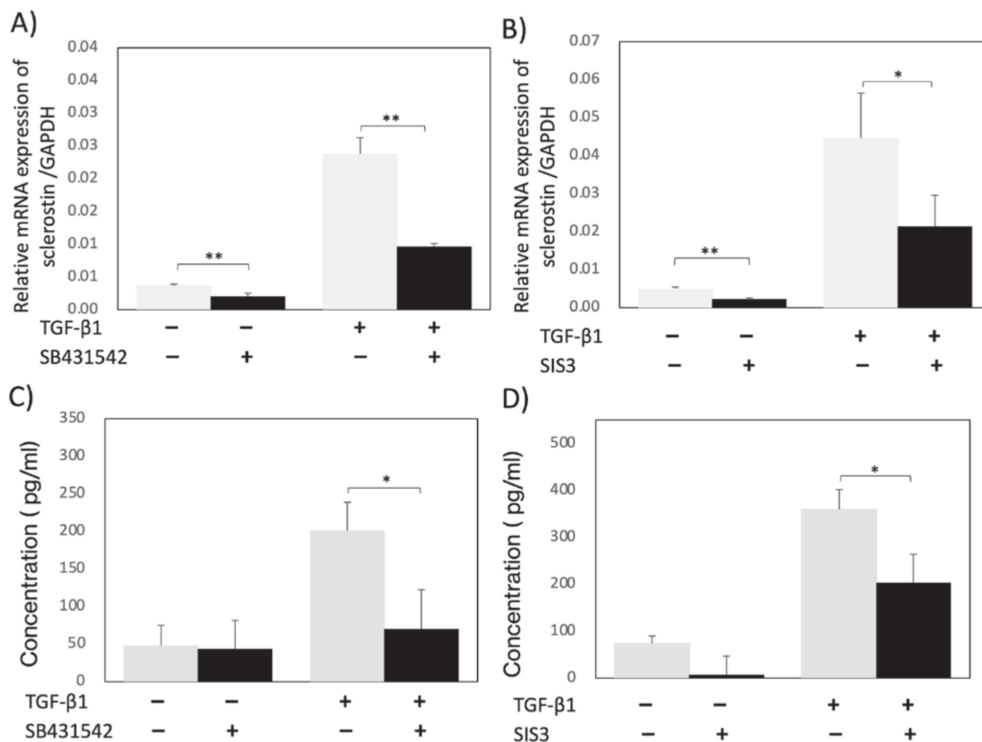

Figure 5: *Sclerostin expression was upregulated by TGF-β/Smad signaling pathway in HSC-4 cells*
(A and B) Sclerostin mRNA expression was evaluated in HSC-4 cells treated with or without 10 ng/ml TGF-β1 for 48 h. (A) The cells were treated with (black bars) or without (gray bars) 10 mM SB431542 for 30 min before TGF-β1 treatment. (B) The cells were treated with (black bars) or without (gray bars) 0.2 mM SIS3 for 30 min before 10 ng/ml TGF-β1 treatment. 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 and *P<0.05). (C and D) Sclerostin protein expression levels in TGF-β1-treated HSC-4 cells were assayed using ELISA with an anti-sclerostin antibody. The cells were then treated with 10 ng/ml TGF-β1 for 48 h. Some cells were treated with (C) 10 mM SB431542 or (D) 0.2 mM SIS3 30 min before TGF-β1 treatment. 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).

TGF-β1-induced sclerostin expression (Fig. 5A and B). ELISA revealed that both SB431542 and SIS3 also decreased sclerostin protein expression (Fig. 5C and D). These data indicated that the TGF-β1/ALK5/Smad3 signaling pathway upregulated sclerostin expression in HSC-4 cells.

Sclerostin increased the migratory and proliferative activities in HSC-4 cells

We examined whether sclerostin affected the migratory and proliferative activities of HSC-4 cells. Both the migratory and proliferative abilities of HSC-4 cells was significantly augmented by 50 ng/ml rhSclerostin stimulation (Fig. 6A and B). These results indicate that sclerostin increases cancer progression in HSC-4 cells.

Sclerostin derived from TGF-β1-treated HSC-4 cells increased the expression of polarization-related cytokines IL-6, TGF-β1, and IL-17A in M1-Mφs

We examined whether sclerostin induced the expression of polarization-related cytokines in M1-Mφs with rhSclerostin. The expression levels of IL-6 (Fig. 7A), TGF-β1 (Fig. 7B), and IL-17A (Fig. 7C) were dose-dependently upregulated in M1-Mφs by rhSclerostin treatment. These results indicated that sclerostin increased the expression of the polarization-related cytokines IL-6, TGF-β1, and IL-17A in M1-Mφ.

IL-6 derived from M1-Mφs cocultured with TGF-β1-treated HSC-4 cells increased the expression of polarization-related cytokines in M1-Mφs in autocrine and paracrine manners

We also investigated whether recombinant human IL-6 protein (rhIL-6) induced the expression of the M2-polarization promoter IL-17A and promoted the expression of M2-Mφ

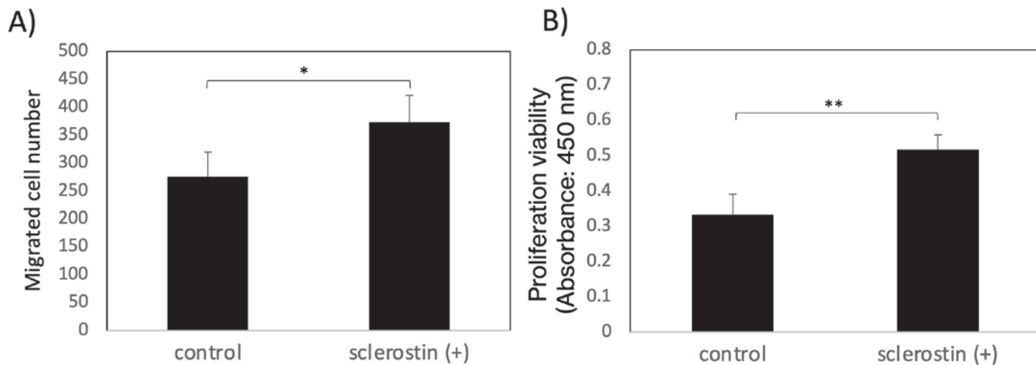

Figure 6: *Sclerostin increased the migratory-, and proliferative-activities in HSC-4 cells*
HSC-4 cells were stimulated with 10 ng/ml TGF-β1 for 48 h and then used for subsequent experiments. (A) Migratory ability was evaluated in cells stimulated with 100 ng/ml recombinant human sclerostin (rhSclerostin) using a Boyden chamber assay. After 24 h, the migrated cells were counted using DAPI staining. Data represent the mean ± SD of triplicate experiments. Differences in values between control and rhSclerostin-treated cells were statistically analyzed using Student's *t*-test (*$P<0.05$). (B) Proliferative ability was monitored in cells stimulated with or without 100 ng/mL rhSclerostin using the CCK-8 assay. Data are presented as mean ± SD of quadruplicate experiments. Differences in values between control and CXCL14-treated cells were statistically analyzed using the Student's *t*-test (**$P<0.01$).

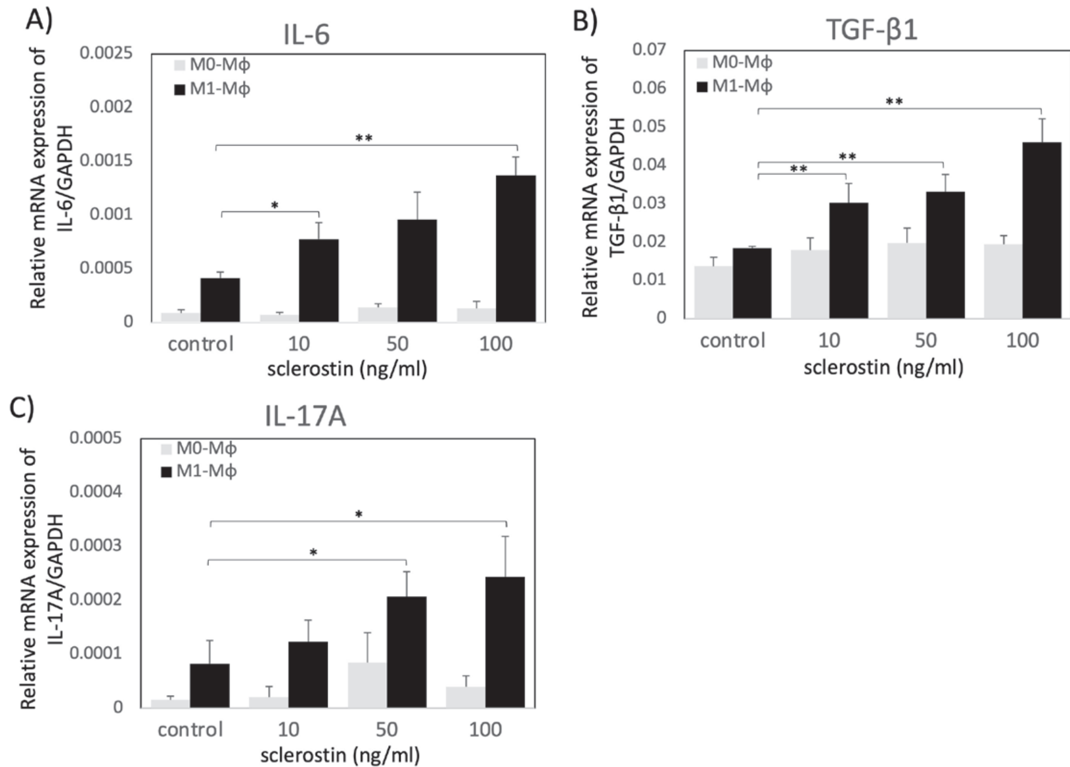

Figure 7: *Sclerostin derived from TGF-β1-treated HSC-4 cells increased the expression of polarization-related cytokines in M1-Mϕ cells*

The mRNA expression levels of cytokines/chemokines in THP-1-derived macrophages (M0-Mϕs and M1-Mϕs) were analyzed using RT-qPCR. THP-1 cells were stimulated with 5 ng/ml PMA for 24 h and incubated with (M1-Mϕs) or without (M0-Mϕs) 20 ng/ml LPS and 250 ng/ml IFN-γ for 48 h. Then, these cells were cultured with 0–100 ng/ml rhSclerostin for 48 h. (A) IL-6, (B) TGF-β1, and (C) IL-17A mRNA expression levels in M0-Mϕs (gray bars) or M1-Mϕs (black bars) were analyzed using RT-qPCR. Data are presented as mean ± SD of quadruplicate experiments. Values between every two groups were statistically compared using Tukey's multiple comparison test (** $P<0.01$ and * $P<0.05$).

markers CD163 and CCL13 in M1-Mϕs. IL-17A expression levels were significantly upregulated in M1-Mϕs following rhIL-6 treatment (Fig. 8A). Furthermore, CD163 (Fig. 8B) and CCL13 (Fig. 8C) expression levels were also increased in M1-Mϕs following rhIL-6 treatment.

IL-17A derived from M1-Mϕs cocultured with TGF-β1-treated HSC-4 cells increased M2 polarized marker expression in M1-Mϕs in autocrine and paracrine manners

We found that recombinant human IL-17A protein (rhIL-17A) induced the expression of M2-Mϕ markers in M1-Mϕ cells. CD163 (Fig. 9A) and CCL13 (Fig. 9B) expression levels were dose-dependently upregulated in M1-Mϕs after rhIL-17A treatment. IL-17A induces M2 polarization from M1-Mϕ through the NF-κB signaling pathway [26]. We then investigated whether the NF-κB kinase-2 (IKK-2) inhibitor TPCA-1 canceled M2 polarization in M1-Mϕs

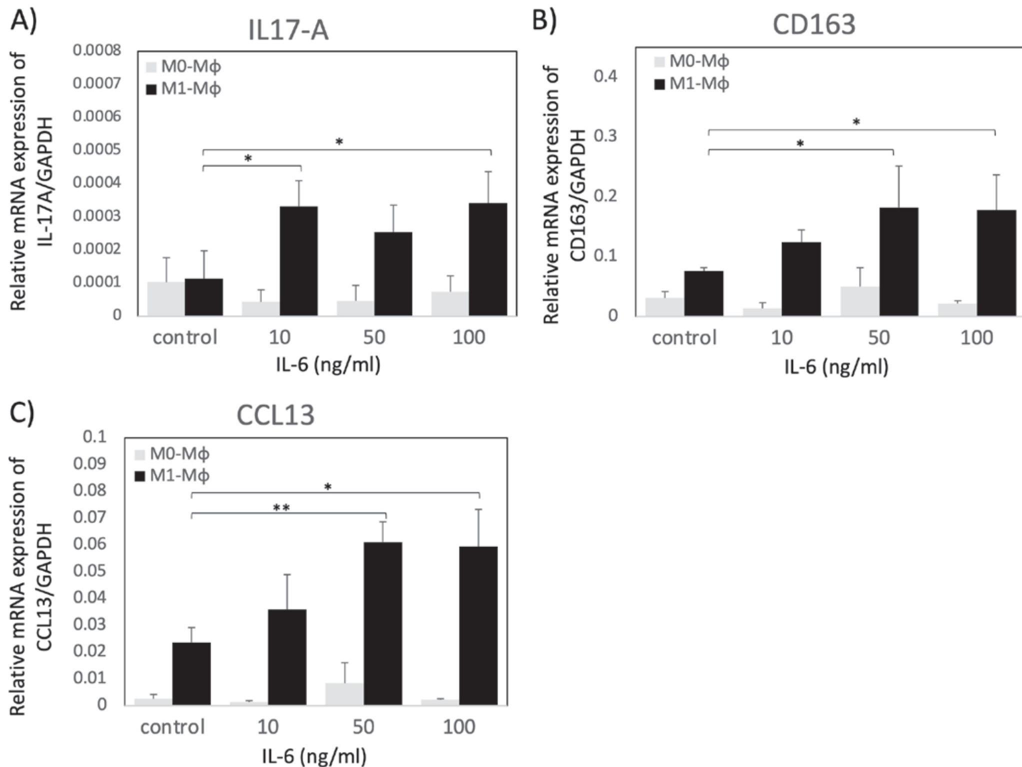

Figure 8: *IL-6 derived from M1-Mϕs cocultured with TGF-β1-treated HSC-4 cells increased the expression of polarization-related cytokines in M1-Mϕ cells*
The mRNA expression levels of chemokine and macrophage markers in THP-1-derived macrophages (M0-Mϕs and M1-Mϕs) were analyzed using RT-qPCR. THP-1 cells were stimulated with 5 ng/ml PMA for 24 h and incubated with (M1-Mϕs) or without (M0-Mϕs) 20 ng/ml LPS and 250 ng/ml IFN-γ for 48 h. Then, these cells were cultured with 0–100 ng/ml rhIL-6 for 48 h. (A) IL-17A, (B) CD163, and (C) CCL13 mRNA expression levels in M0-Mϕs (gray bars) or M1-Mϕs (black bars) were analyzed using RT-qPCR. Data are presented as mean ± SD of quadruplicate experiments. Values between every two groups were statistically compared using Tukey's multiple comparison test (**$P<0.01$ and *$P<0.05$).

by IL-17A stimulation. TPCA-1 (10 μM) abrogated IL-17A-induced upregulation of CD163 and CCL13 expression in M1-Mϕs (Fig. 9C and 9D).

Discussion

Several studies have reported the transformation of human acute monocytic leukemia THP-1 cells into Mϕs, among which many studies have used PMA to differentiate THP-1 cells into Mϕs. However, the properties of the Mϕs differentiated from THP-1 cells were dependent on the PMA concentration or stimulation period. Here, THP-1 cells were differentiated by PMA and then polarized into M1-Mϕ by LPS plus IFN-γ treatment according to the standard method reported by Baxer *et al.*[23].

First, we investigated whether EMT-promoted HSC-4 cells induced the polarization of Mϕs into M2-Mϕs. We found that the expression level of pro-inflammatory cytokines strongly decreased (Fig. 1B), but that of anti-

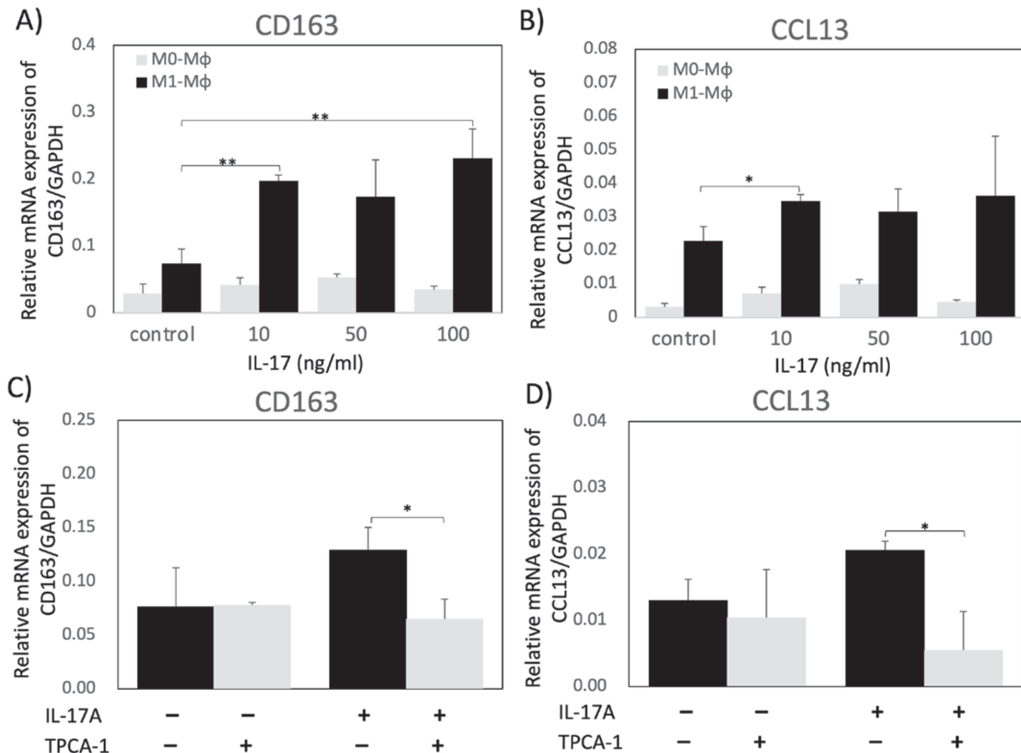

Figure 9: *IL-17A derived from M1-Mϕs cocultured with TGF-β1-treated HSC-4 cells increased the expression of M2 polarized markers in M1-Mϕ cells*

THP-1 cells were stimulated with 5 ng/ml PMA for 24 h and incubated with (M1-Mϕ) or without (M0-Mϕ) 20 ng/ml LPS and 250 ng/ml IFN-γ for 48 h. Then, these cells were cultured with recombinant human IL-17A (rhIL-17A) for 48 h. (A) CD163 and (B) CCL13 mRNA expression levels in M0-Mϕs (gray bars) or M1-Mϕs (black bars) treated with 0–100 ng/ml rhIL-17A for 48 h were analyzed using RT-qPCR. (C and D) Some cells were pretreated with 10 μM TPCA-1 for 30 min before rhIL-17A treatment. (C) CD163 and (D) CCL13 mRNA expression levels with (gray bars) or without (black bars) 10 μM TPCA-1 treatment for 48 h were analyzed using RT-qPCR. Values were normalized to the GAPDH mRNA levels. Data are presented as 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).

inflammatory cytokines/chemokines increased in M1-Mϕs by coculture with EMT-promoted HSC-4 cells (Fig. 1C and D). In addition, the expression levels of M2-Mϕ markers, CD163 and CCL13, were upregulated in M1-Mϕs by coculture with EMT-promoted HSC-4 cells (Fig. 2A and B). Interestingly, the expression of the M2-Mϕ polarization inducer IL-17A and IL-17A production stimulator IL-6 was also upregulated in M1-Mϕs by coculture with EMT-promoted HSC-4 cells (Fig. 3A and B). However, M2-Mϕ marker CD206 expression was not increased in M1-Mϕs by coculture with EMT-promoted HSC-4 cells (Fig. 2C), implying that EMT-promoted hOSCC cells might not fully but partially induce M2-polarization in M1-Mϕs.

On the contrary, the expression levels of

IL-1β and TNF-α in M1-Mϕ single cultures were higher than those in M2-Mϕ polarized by IL-4, which was consistent with the experimental results reported previously [23] (data not shown). Unexpectedly, the expression levels of TGF-β1 and IL-10 in M1-Mϕs were almost similar to those in M2-Mϕs single culture (data not shown). The expression level of CD206 in M2-Mϕ single culture was expectedly higher than that in M1-Mϕ single culture, but unexpectedly suppressed by co-culture with EMT-promoted HSC-4 cells (data not shown). These results suggest that M1-Mϕs derived from THP-1 cells did not fully retain M2-characteristics in comparison with native M2-Mϕs *in vivo*.

Next, we investigated the factors produced in coculture of EMT-promoted hOSCC cells and Mϕs, which promoted polarization of Mϕs into M2 phenotypes, and examined whether EMT-promoted hOSCC cells produced the tumor metastasis regulator sclerostin. Sclerostin plays an important role in bone metastasis in cancer [28), 33)]. Upregulation of sclerostin expression has been observed in breast cancer, which induces tumor growth, thus promoting tumor progression [28)]. However, sclerostin expression also serves as a tumor suppressor in prostate cancer [29)]. We found that TGF-β1 clearly promoted sclerostin mRNA and protein expression in HSC-4 cells (Fig. 4A and B). In addition, we found that TGF-β1 induced sclerostin expression through Smad3-dependent signal transduction (Fig. 4). Sclerostin expression was also upregulated in the TGF-β1 responsible hOSCC cells SAS than HSC-4 cells in a Smad3-dependent manner (data not shown). Moreover, sclerostin significantly promoted migratory and proliferative activities in HSC-4 cells, which suggests that sclerostin might positively regulate hOSCC metastasis in an autocrine- or paracrine-manner, thus forming a positive feedback loop in TGF-β1-promoted EMT in hOSCC cells. We also found that sclerostin expression was significantly upregulated by TGF-β1 stimulation after 24 h, but not 1–6 h, in HSC-4 cells (Fig. 4A). Thus, TGF-β1 did not induce sclerostin mRNA expression early after administration, possibly because it might not be a direct target gene for TGF-β1-activated Smad3-mediated signaling. We also demonstrated that MMP-10 and CXCL14 were not expressed early after TGF-β1 stimulation [17), 20)]. However, we found that FBS components suppressed sclerostin expression because sclerostin expression with FBS was lower than that without FBS (data not shown). However, we have not yet identified the factor(s) in FBS.

Interestingly, we also found that sclerostin increased the expression of IL-17A inducer IL-6, sclerostin inducer TGF-β1, and M2-polarization promoter IL-17A in M1-Mϕ dose-dependently (Fig. 7A, B, and C, respectively). In addition, IL-6 increased IL-17A expression in M1-Mϕs (Fig. 8A) and upregulated the expression levels of M2-Mϕ markers, CD163 and CCL13, in M1-Mϕs (Fig. 8B and C, respectively). Furthermore, we found that IL-17A upregulated the expression levels of M2-Mϕ markers, CD163 and CCL13, in M1-Mϕ in an NF-κB-dependent manner (Fig. 9). These results suggest that the interaction between hOSCC cells and Mϕs in the TME is advantageous for the acquisition of invasion and metastasis activities in hOSCC.

Taken together, we found that 1) TGF-β1 induced sclerostin expression through the Smad signal transduction pathway in HSC-4 cells, 2) sclerostin reinforced IL-6, TGF-β1, and IL-17A expression in M1-Mϕs; 3) IL-6 promoted IL-17A expression and M2-Mϕ

markers CD163 and CCL13 in M1-Mφs, and 4) IL-17A reinforced the expression of M2-Mφ markers CD163 and CCL13 in M1-Mφs.

These results strongly suggest that sclerostin derived from EMT-promoted hOSCC cells induced IL-6- and IL-17A-mediated polarization shift from M1 to M2 phenotype in macrophages in the TME (Fig. 10).

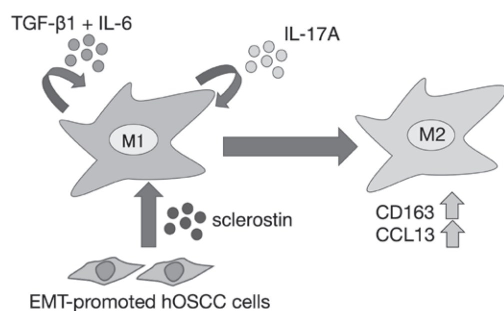

Figure 10: Cytokines and chemokines secreted by hOSCC cells and Mφs.

Recently, romosozumab, a humanized anti-sclerostin monoclonal antibody is available as a therapeutic drug of the osteoporosis. Romosozumab binds to sclerostin and abrogated sclerostin-promoted suppression of canonical Wnt signaling pathway through inhibition of binding of sclerostin to LPR5/6 [34]. Besides, this reagent is expected to possess anti-tumor effect in the future, as sclerostin induced bone metastasis and anti-sclerostin antibody reduced invasion of breast cancer cells [28]. Additionally, we think that the measurements of sclerostin concentration and immunohistochemistry of sclerostin in tumors are significant as a marker of tumor progression from the data in this study.

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Competing interests

The authors declare that they have no competing interests.

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