Receptor tyrosine kinase ligands and inflammatory cytokines cooperatively suppress the fibrogenic activity in temporomandibular-joint-derived fibroblast-like synoviocytes via mitogen-activated protein kinase kinase/extracellular signal-regulated kinase

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Abstract. Osteoarthritis (OA)-related fibrosis is a possible cause of temporomandibular joint (TMJ) stiffness. However, the molecular mechanisms underlying the fibrogenic activity in fibroblast-like synoviocytes (FLSs) remain to be clarified. The present study examined the effects of receptor tyrosine kinase (RTK) ligands, such as fibroblast growth factor (FGF)-1 and epidermal growth factor (EGF), on myofibroblastic differentiation of the FLS cell line FLS1, which is derived from the mouse TMJ. The present study revealed that both FGF-1 and EGF dose-dependently suppressed the expression of the myofibroblast (MF) markers, including α-smooth muscle actin (α-SMA) and type I collagen, in FLS1 cells. Additionally, both FGF-1 and EGF activated extracellular signal-regulated kinase (ERK) in FLS1 cells. In addition, the mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) inhibitor U0126 abrogated the FGF-1- and EGF-mediated suppression of MF marker expression. On the other hand, inflammatory cytokines, such as interleukin-1β and tumor necrosis factor-α, also suppressed the expression of MF markers in FLS1 cells. Importantly, U0126 abrogated the inflammatory cytokine-mediated suppression of MF marker expression. Interestingly, RTK ligands and inflammatory cytokines additively suppressed the expression of type I collagen. These results suggested that RTK ligands and inflammatory cytokines cooperatively inhibited the fibrogenic activity in FLSs derived from the TMJ in a MEK/ERK-dependent manner. The present findings partially clarify the molecular mechanisms underlying the development of OA-related fibrosis in the TMJ and may aid in identifying therapeutic targets for this condition. Additionally, FGF-1 and EGF could be therapeutically utilized to prevent OA-related fibrosis around the inflammatory TMJ.

Introduction

The temporomandibular joint (TMJ) is a synovial joint that is composed of the mandibular fossa of the temporal bone and the mandibular condyle (1). TMJ-osteoarthritis (OA) symptoms include cartilage degeneration, subchondral bone remodeling, and synovitis, which result in TMJ dysfunction (2). Intriguingly, histological studies have shown the presence of extensive fibrosis in the TMJ-OA synovial tissue (3,4), suggesting that fibrotic tissue formation may be responsible for restricted joint movements (5).

We have previously established a fibroblast-like synoviocyte (FLS) cell line, FLS1, from fibroblastic cells derived from a mouse TMJ and found that these cells exhibit myofibroblast (MF)-like fibrogenic characteristics (6). We have also demonstrated that fibroblast growth factor (FGF)-1 alone significantly suppresses the MF differentiation markers α-smooth muscle actin (α-SMA) and type I collagen in FLS1 cells (6). The FGF family consists of 24 members that share 13-71% amino acid identity (7). Although FGF-11-15 are generally considered to belong to the FGF family, they do not activate any FGF receptor (FGFR) (8). However, four FGFRs belong to the receptor tyrosine kinase (RTK) family (9). In general, FGF-1 binds to FGFR1-4 (10) to activate various intracellular signaling factors, including phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 MAPK (11). Intriguingly, human synovial fibroblasts derived from the knee synovial tissues

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express FGF-1 and FGF-R1 proteins (12). However, it remains to be determined which FGF-1-induced intracellular signaling pathway negatively controls the fibrogenic activity in FLSs.

Epidermal growth factor (EGF) was first purified from the mouse salivary gland as a soluble factor that accelerated corneal wound healing (13); however, EGF was soon after found to be a general growth factor that affected various cellular functions involving cell proliferation and differentiation (14). The EGF receptor (EGFR) family consists of four members, namely EGFR/ErbB1/HER1, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4, all of which belong to the RTK family (15). EGFR dimerizes upon its association with EGF. The cytoplasmic tyrosine kinase domains of EGFR can be autophosphorylated to relay extracellular signals to various intracellular signaling proteins. The carboxy terminal tyrosine residues on EGFR, Tyr1068 and Tyr1173, are the major sites of the autophosphorylation, which occurs as a result of EGF-binding and converts the extracellular EGF signal to intracellular signals (16,17). Autophosphorylated EGFR activates various types of intracellular signaling molecules, including MAPKs and PI3K/Akt (18). Intriguingly, EGF has been detected in the human knee synovial fluid (19). In addition, the EGFR signaling is critical for maintaining the superficial layer of the articular cartilage and preventing OA initiation (20). However, whether EGF-induced intracellular signaling affects the fibrogenic activity in FLSs remains elusive. Furthermore, the mechanism whereby EGF affects the FGF-1-mediated suppression of the fibrogenic activity in FLSs derived from the TMJ synovial tissues warrants investigation.

It is worth noting that inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are important factors participating in the pathogenesis of OA. However, the roles of IL-1β and TNF-α in the onset of OA have not been comprehensively studied yet (21). IL-1β binds to the cell membrane receptors IL-1R1 (IL-1RI and CD121a) and IL-1R2 (IL-1RII and CD121b) (22), whereas TNF-α binds to the cell membrane receptors TNF-R1 (p55, CD120a, and TNFRSF1a) and TNFR-2 (p75, CD120b, and TNFRSF1b) (23). In general, IL-1β and TNF-α use similar signal transduction mechanisms to activate nuclear factor-kappa B (NF-κB) and MAPKs, including ERK1/2, JNK, and p38 MAPK. Interestingly, IL-1β and TNF-α both induce MF differentiation in mesenchymal cells in an NF-κB-dependent manner (24,25). In addition, transforming growth factor-β1 (TGF-β1) promotes MF differentiation in an NF-κB-dependent manner as well (26), suggesting that NF-κB-mediated signals positively regulate MF differentiation in mesenchymal cells. Interestingly, IL-1β promotes TGF-β1-induced MF differentiation in nasal fibroblasts in MAPK/ERK kinase (MEK)/ERK-, JNK-, and p38 MAPK-dependent manners (24), whereas TNF-α attenuates TGF-β1-induced MF differentiation in pulmonary fibroblasts in a MEK/ERK-dependent manner (27). These results suggest that MAPK-mediated signals positively or negatively regulate the MF differentiation of mesenchymal cells in a cell-type-specific manner.

Here, we examined the mechanisms whereby the RTK ligands FGF-1 and EGF affect the fibrogenic activity in the myofibroblastic FLS cell line FLS1. We also investigated the effects of FGF-1 and EGF on the activity of PI3K/Akt and MAPKs, such as ERK, JNK, and p38 MAPK, in FLS1 cells and examined whether FGF-1-, or EGF-activated PI3K/Akt or MAPKs affected the status of myofibroblastic differentiation in FLS1 cells. In addition, we examined the cooperative and non-cooperative effects of RTK ligands and inflammatory cytokines, such as IL-1β and TNF-α, on the myofibroblastic differentiation in FLS1 cells. Our study clarified the molecular mechanisms underlying the development of OA-related fibrosis in TMJ and may aid in identifying new therapeutic targets for this condition.

Materials and methods

Reagents. Recombinant mouse EGF was purchased from PeproTech, Inc. Recombinant human IL-1β and TNF-α were obtained from Miltenyi Biotec, GmbH (Bergisch Gladbach). The MEK inhibitors U0126 and PD98059, and the EGFR inhibitor PD153035 were purchased from Calbiochem (Merck KGaA). The NF-κB inhibitor BAY 11-7085 was obtained from Cayman Chemical. Recombinant human FGF-1 and the NF-κB kinase-2 (IKK-2) inhibitor TPCA-1 were purchased from R&D Systems, Inc. The FGFR1 inhibitor SU-5402 was obtained from Wako Pure Chemical Industries, Ltd. We confirmed that dimethyl sulfoxide (DMSO), the vehicle used for the U0126, PD98059, PD153035, BAY 11-7085, TPCA-1, and SU-5402 treatments, did not affect the expression of the MF markers α-SMA and type I collagen (data not shown). Heparin sodium salt was obtained from Merck KGaA. Heparin was included to achieve the optimal FGF-1 activity (28).

Cell culture. The FLS cell line FLS1 was previously established and reported (6): Briefly, to prepare FLSs derived from the mouse TMJ, TMJ synovial tissue was obtained from eight-week-old female mice (C57BL/6J). The tissue was then immersed in digestion solution composed of 20 ml of Ham's F-12 containing 2 mg/ml collagenases consisting of class I and class II collagenases (Collagenase NB4; Wako), at 37˚C for 30 min with continuous vigorous rocking. The cells released from the tissue were transfected with pBABE-puro-simian virus 40 large T antigen (SV40LT) expression plasmid (cat. no. 13970) obtained from Addgene, Inc., with Lipofectamine LTX Reagent (ThermoFisher Scientific, Inc.) according to the manufacturer's protocol. The immortalized FLSs, FLS1 cells were maintained in culture with Ham's F-12 supplemented with 2 mM glutamine, 10% FBS, and penicillin-streptomycin (Invitrogen). These cells were then sub-cultured at a ratio of 1:4 when they reached sub-confluency.

RNA isolation and RT-qPCR. FLS1 cells were seeded into 12-well tissue culture plates at a density of $1x10^5$ cells/well in FLS1 growth medium and maintained for 24 h. The growth medium was replaced with Ham's F-12 containing 0.5% FBS for 24 h for cell starvation. Subsequently, the cells were cultured with or without FGF-1, heparin, EGF, IL-1β, or TNF-α for the indicated periods. Total RNA was isolated from FLS-1 cells using ISOGEN reagent (Nippon Gene) according to the manufacturer's protocol. First-strand cDNA was synthesized from total RNA using the PrimeScript RT

reagent Kit (Takara-Bio). PCR was subsequently performed on a Thermal Cycler Dice Real Time System (Takara-Bio) using SYBR Premix Ex Taq II (Takara-Bio), with the following specific oligonucleotide primers: Mouse *α-SMA*, 5'-CAGATG TGGATACAGCAAACAGGA-3' (forward) and 5'-GACTTA GAAGCATTTGCGGTGGA-3' (reverse); mouse α1 chain of collagen type I (*colIα1*), 5'-GACATGTTCAGCTTTGTG GACCTC-3' (forward) and 5'-GGGACCCTTAGGCCATTG TGTA-3' (reverse); and mouse *GAPDH*, 5'-TGTGTCCGTCGT GGATCTG-3' (forward) and 5'-TTGCTGTTGAAGTCGCAG GAG-3' (reverse). The mRNA levels of *α-SMA* and *colIα1* were normalized to *GAPDH* mRNA levels, and the relative expression levels were calculated as the fold increase or decrease relative to the control.

Western blot analysis. Cells were seeded into 6-well tissue culture plates at a density of $2x10^5$ cells/well in FLS1 growth medium and maintained for 24 h. Afterward, the cells were starved for 24 h as indicated above and cultured with or without FGF-1 plus heparin, EGF, IL-1β, or TNF-α for the indicated periods. Eventualy, the cells were lysed in RIPA buffer [Sigma; 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS] or lysis buffer [20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100] containing protease and phosphatase inhibitor cocktails (Sigma). The protein contents of the cell extracts were measured using BCA reagent (Pierce). Extracts containing equal amounts of protein were separated on 10% SDS-polyacrylamide gels and transferred onto polyvinylidenedifluoride membranes (Millipore). After blocking the membranes with 1% BSA or 1% skim milk in T-TBS (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, and 0.05% Tween 20), they were incubated with the appropriate primary antibody. The primary antibodies used included rabbit anti-p44/42 (ERK1/2; cat. no. 9102), rabbit anti-p38 MAPK (cat. no. 9212), rabbit anti-SAPK/JNK (cat. no. 9252), rabbit anti-Akt (cat. no. 9272), rabbit anti-phospho-p44/42 (ERK1/2, Thr202/Tyl204; cat. no. 9101), rabbit anti-phospho-p38 MAPK (Thr180/Tyr182; cat. no. 9211), rabbit anti-phospho-SAPK/JNK (Ther183/185; cat. no. 9251), rabbit anti-phospho-Akt (Ser473; cat. no. 9271) polyclonal antibodies (1:1,000; Cell Signaling Technology), and anti-β-actin antibody (cat. no. sc-47778, 1:1,000; Santa Cruz Biotechnology). The blots were then incubated with the appropriate alkaline phosphatase-conjugated secondary antibody, and signals were detected using an alkaline phosphatase substrate kit (BCIP/NBT Substrate Kit; Vector Laboratories Inc.). Especially, β-actin blots ware obtained from the same membrane as the total ERK1/2 blots after stripping anti-total ERK1/2 antibody from the membranes according to the manufacturer's protocol.

Statistical analysis. Data were presented as mean ± standard deviation (SD; n=4) and statistically analyzed by Tukey's multiple comparison test except for the data analysis in Fig. S1. In Fig. S1, the data were statistically analyzed by Student's t-test. Values of $^{*}P<0.01$ and $^{**}P<0.05$ were considered to indicate a statistically significant difference. The results shown in all the experiments are representatives of at least two separate experiments.

Results

FGF-1 suppressed the expression of myofibroblast markers in FLSs. As shown in Fig. 1A, FGF-1 (0.1-1 ng/ml) with heparin (15 μg/ml) significantly downregulated the *α-SMA* mRNA level in FLS1 cells in a dose-dependent manner. In addition, FGF-1 (0.01-1 ng/ml) with heparin (15 μg/ml) significantly downregulated the *colIa1* mRNA level in FLS1 cells in a dose-dependent manner (Fig. 1B). Importantly, we confirmed that the FGFR1 inhibitor SU-5402 (5 μM) significantly abrogated this FGF-1-mediated suppression of *α-SMA* and *colIa1* expression (Fig. 1A and B, respectively). We also confirmed that 15 μM of heparin alone did not significantly affect the mRNA levels of the MF markers α-SMA and type I collagen (Fig. S1) relative to the control cells.

EGF suppressed the expression of myofibroblast markers in FLSs. As shown in Fig. 2A, EGF (0.01-0.1 ng/ml) significantly downregulated the *α-SMA* mRNA level in FLS1 cells in a dose-dependent manner. In addition, EGF (0.1-1 ng/ml) significantly downregulated the *colIa1* mRNA level in FLS1 cells (Fig. 2B). Importantly, we confirmed that the EGFR inhibitor PD153035 (0.5 μM) significantly abrogated this EGF-mediated suppression of *α-SMA* and *colIa1* expression (Fig. 2A and B, respectively).

FGF-1 and EGF promoted phosphorylation of ERK1/ERK2 in FLSs. We used western blotting to evaluate the phosphorylation statuses of ERK1/2, p38 MAPK, JNK, and AKT after the stimulation of FLS1 cells with EGF or FGF-1. As shown in Fig. 3A, strong phosphorylation of ERK1/2 was observed between 5 and 60 min after stimulation with FGF-1 (10 ng/ml) with heparin (15 μg/ml). On the other hand, strong phosphorylation of ERK1/2 was observed at 5-15 min after EGF (10 ng/ml) treatment (Fig. 3B). However, phosphorylated p38 MAPK, JNK, or AKT were not at detectable levels even after treatment with FGF-1 (10 ng/ml) and heparin (15 μg/ml) or EGF (10 ng/ml) alone (data not shown). We also confirmed that β-actin expression was unaffected by the administrations of FGF-1 (10 ng/ml) with heparin (15 μg/ml) or EGF (10 ng/ml) alone at any time points of the treatments (Fig. 3A and B).

FGF-1 and EGF downregulated the mRNA levels of the myofibroblast markers α-SMA and colIa1 in FLSs in a MEK-dependent manner. As shown in Fig. 4A, the MEK inhibitor U0126 (1 μM) partially and significantly reversed the FGF-1 (1 ng/ml) plus heparin (15 μg/ml)-mediated suppression of *α-SMA* (left graph) and *colIa1* (right graph) expression in FLS1 cells, respectively. In addition, U0126 (1 μM) partially and significantly reversed the EGF (5 ng/ml)-mediated suppression of *α-SMA* (left graph) and *colIa1* (right graph) expression, respectively, in FLS1 cells (Fig. 4B). We also found that the MEK inhibitor PD98059 similarly abrogated the FGF-1 (0.25 ng/ml) and heparin (15 μg/ml)-, or EGF (5 ng/ml)-mediated suppression of MF marker expression at concentrations of 5 and 1 μM, respectively (data not shown). Interestingly, FGF-1 (0.1 ng/ml) plus heparin (15 μg/ml) and EGF (0.1 ng/ml) additively downregulated the mRNA levels of *α-SMA* (left graph) and *colIa1* (right graph) in FLS1 cells (Fig. 4C).

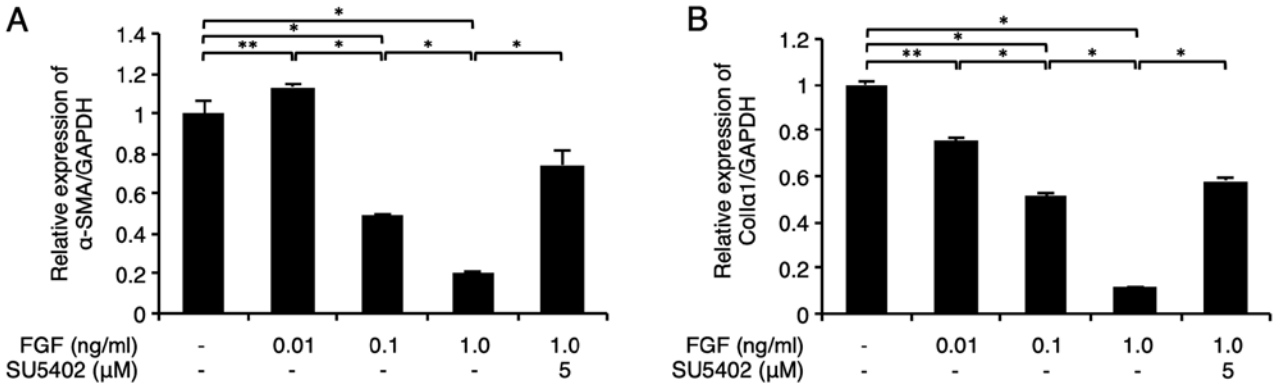

Figure 1. FGF-1 suppresses the expression of myofibroblast markers in fibroblast-like synoviocytes. Cells were starved for 24 h and then treated with FGF-1 at the indicated concentrations and heparin (15 μg/ml) for 24 h. Some cells were pretreated with the specific fibroblast growth factor receptor 1 inhibitor SU5402 (5 μM) for 30 min prior to the stimulation. The relative expression levels of the myofibroblast markers (A) α-SMA and (B) colIα1 were evaluated using reverse transcription-quantitative PCR. Data are presented as the mean ± SD (n=4). *P<0.01, **P<0.05. α-SMA, α-smooth muscle actin; colIα1, α1 chain of collagen type I; FGF, fibroblast growth factor.

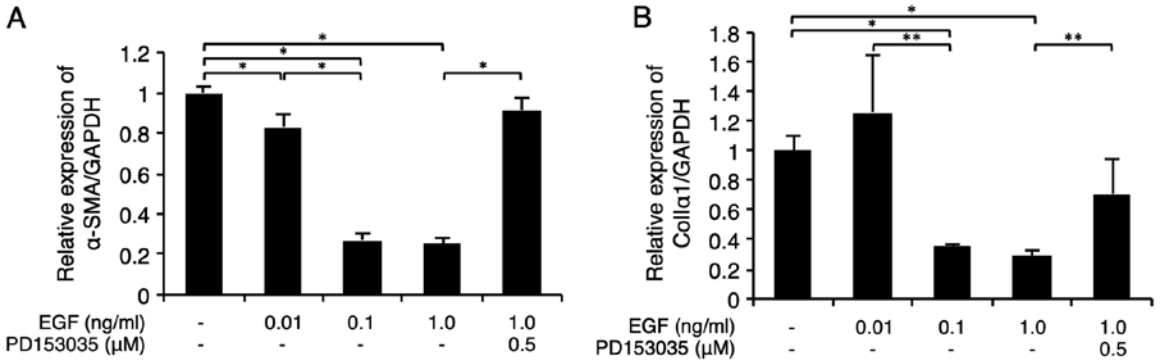

Figure 2. EGF suppresses the expression of myofibroblast markers in fibroblast-like synoviocytes. Cells were starved and then treated with EGF for 24 h at the indicated concentrations. Some cells were pretreated with the specific epidermal growth factor receptor inhibitor PD153035 (0.5 μM) for 30 min prior to the stimulation. The relative expression levels of the myofibroblast markers (A) α-SMA and (B) colIα1 were evaluated using reverse transcription-quantitative PCR. Data are presented as the mean ± SD (n=4). *P<0.01, **P<0.05. α-SMA, α-smooth muscle actin; colIα1, α1 chain of collagen type I; EGF, epidermal growth factor.

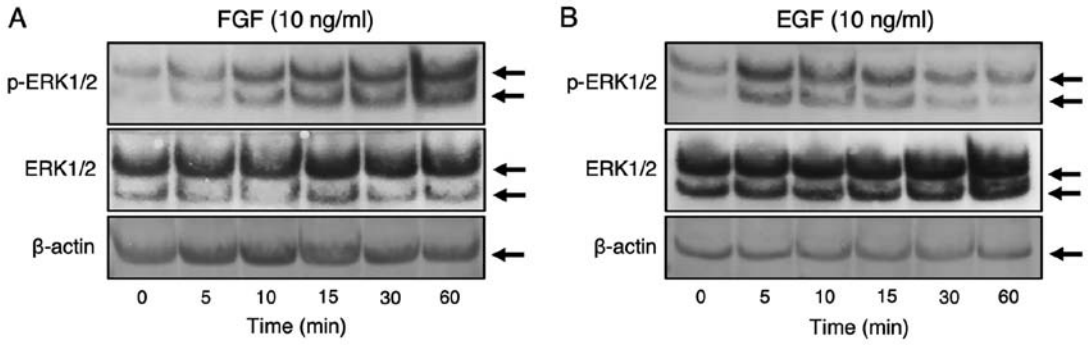

Figure 3. FGF-1 and EGF promoted phosphorylation of ERK1/ERK2 in fibroblast-like synoviocytes. Cells were starved and then (A) treated with FGF-1 (10 ng/ml) and heparin (15 μg/ml) or (B) with EGF (10 ng/ml) for the indicated times. ERK1/2 phosphorylation was evaluated using western blot analysis. α-SMA, α-smooth muscle actin; colIα1, α1 chain of collagen type I; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; p-, phosphorylated.

RTK ligands and inflammatory cytokines cooperatively inhibited the fibrogenic activity in FLSs in a MEK/ERK-dependent manner. As shown in Fig. 5A, the inflammatory cytokines IL-1β (10 ng/ml) and TNF-α (10 ng/ml) significantly suppressed *α-SMA* (left graph) and *colIa1* (right graph) expression in FLS1 cells. Interestingly, the suppression of *α-SMA* expression by the combination of the inflammatory cytokines IL-1β (10 ng/ml) and TNF-α (10 ng/ml) was evidently abrogated by U0126 (1 μM) or PD98059 (5 μM; Fig. 5B, left graph). In addition, the suppression of *colIa1* expression by the combinatorial

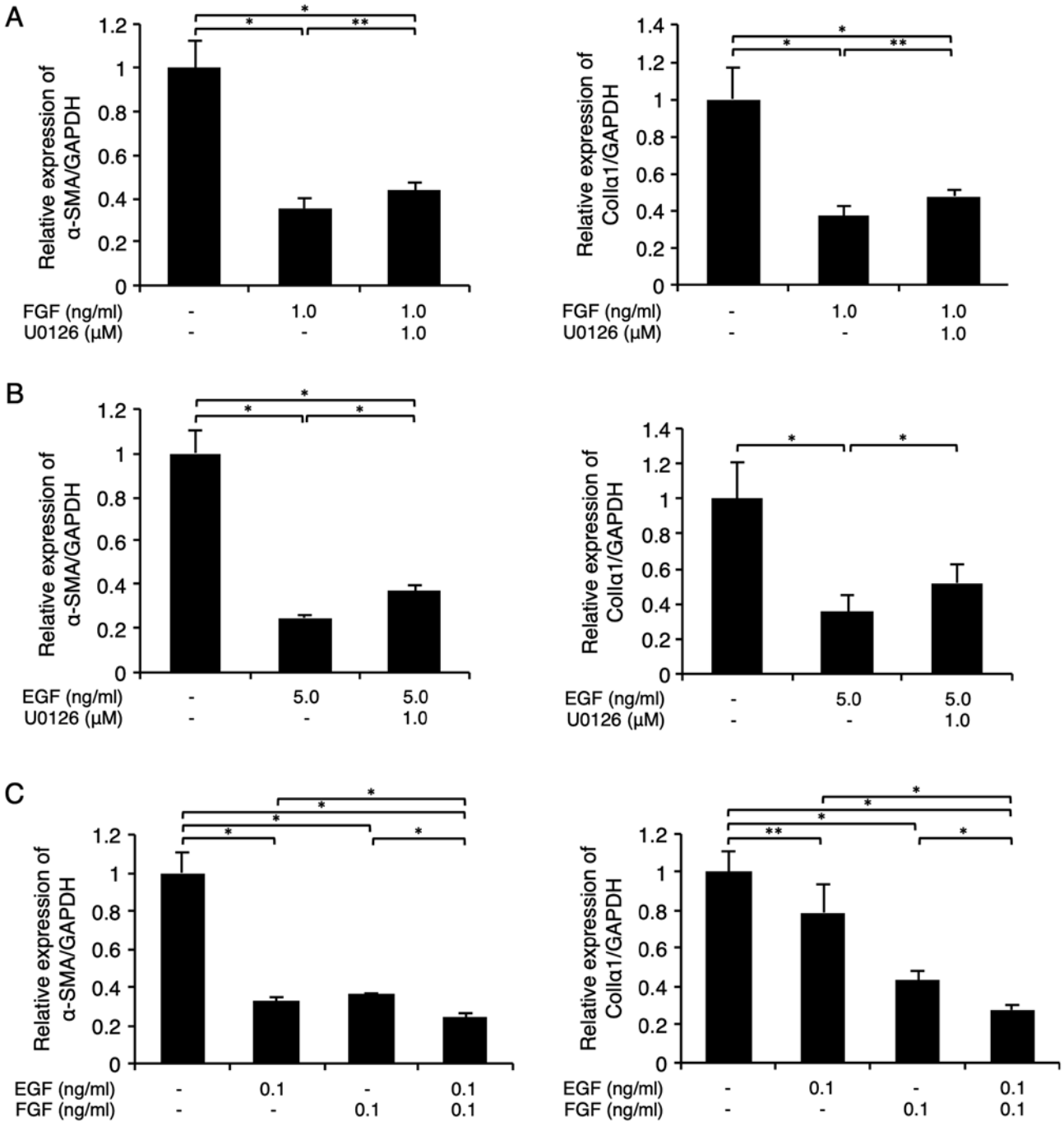

Figure 4. FGF-1 and EGF suppress the mRNA expression of the myofibroblast markers α-SMA and colIα1 in fibroblast-like synoviocytes in a MEK-dependent manner. Cells were starved and then cultured with (A) FGF-1 (1 ng/ml) and heparin (15 μg/ml) or (B) EGF (5 ng/ml) for 24 h. Some cells were pretreated with the specific MEK inhibitor U0126 (1 μM) for 30 min prior to the stimulation. The relative expression levels of the myofibroblast markers α-SMA (left) and colIα1 (right) were evaluated using RT-qPCR. (C) Cells were starved and then cultured with FGF-1 (0.1 ng/ml) and heparin (15 μg/ml) and/or EGF (0.1 ng/ml) for 24 h. The relative expression levels of the myofibroblast markers α-SMA and colIα1 were then evaluated using RT-qPCR. Data are presented as the mean ± SD (n=4). *P<0.01, **P<0.05. α-SMA, α-smooth muscle actin; colIα1, α1 chain of collagen type I; EGF, epidermal growth factor; FGF, fibroblast growth factor; MEK, mitogen activated protein kinase; RT-qPCR, reverse transcription-quantitative PCR.

stimulation of the inflammatory cytokines was partially but significantly abrogated by U0126 (1 μM), but not by PD98059 (5 μM; Fig. 5B, right graph). However, the NF-κB inhibitor BAY 11-7085 (5 μM) or the IKK-2 inhibitor TPCA-1 (5 μM) did not abrogate the suppression of MF marker expression caused by the combination of the inflammatory cytokines and instead, further downregulated the levels of these MF markers (Fig. S2). As shown in Fig. 5C (left panels), strong phosphorylation of ERK1/2 was observed at 5-15 min after IL-1β (10 ng/ml) stimulation. In addition, strong phosphorylation of ERK1/2 was observed at 30 min after TNF-α (10 ng/ml) stimulation (Fig. 5C, right panels). We also confirmed that β-actin expression was unaffected by the administration of IL-1β (10 ng/ml) or TNF-α (10 ng/ml) at any time point after the stimulation (Fig. 5C). Interestingly, FGF-1 (10 ng/ml) plus heparin (15 μg/ml)- or EGF (10 ng/ml)-mediated suppression

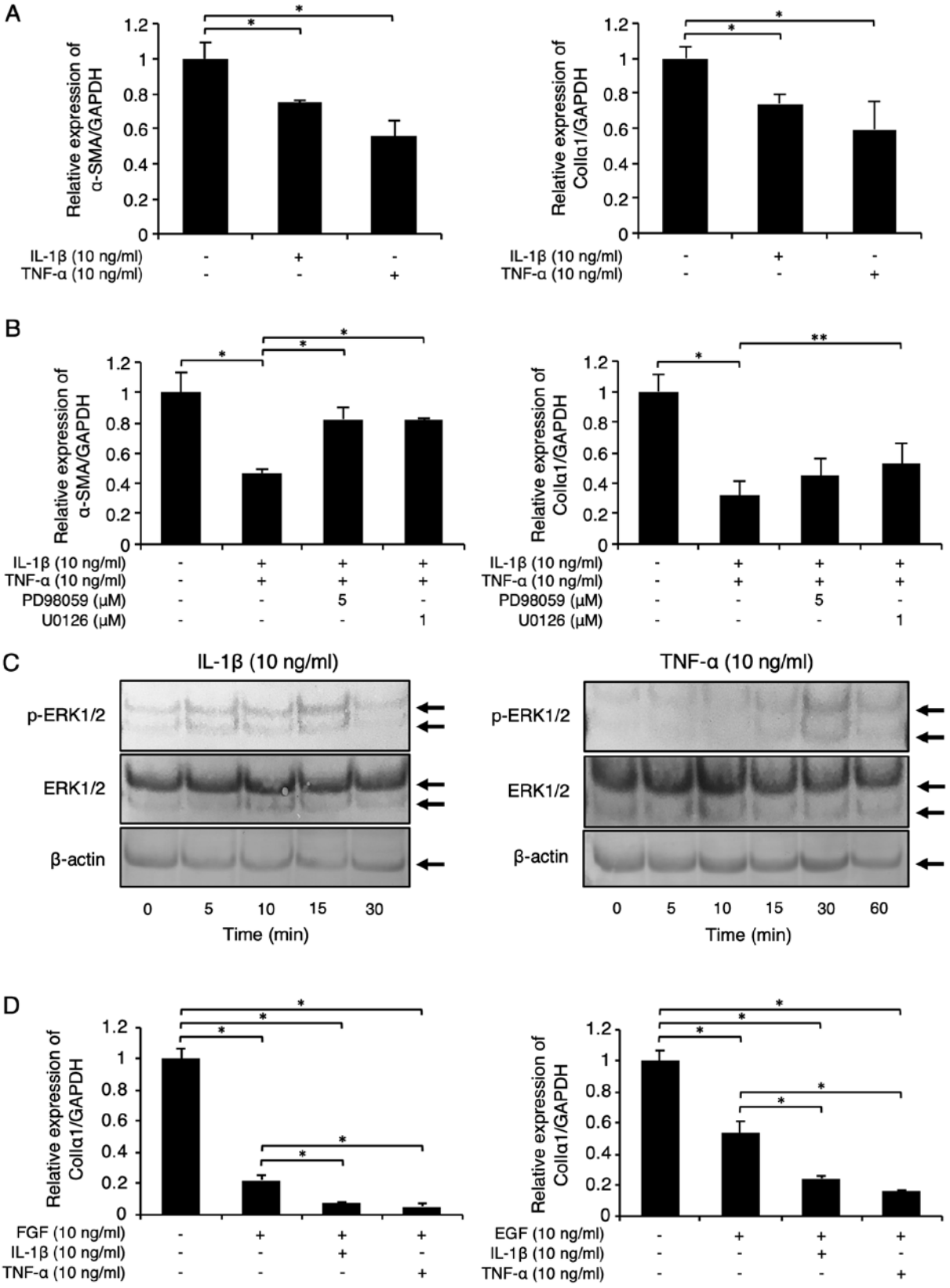

Figure 5. Receptor tyrosine kinase ligands and inflammatory cytokines cooperatively inhibit the fibrogenetic activity in fibroblast-like synoviocytes in a MEK/ERK-dependent manner. (A and B) Cells were starved and then cultured with IL-1β (10 ng/ml) and/or TNF-α (10 ng/ml) for 24 h. (B) Some cells were pretreated with the specific MEK inhibitors PD98059 (5 μM) or U0126 (1 μM) for 30 min prior to stimulation. The relative expression levels of the myofibroblast markers α-SMA and colIα1 were then evaluated using RT-qPCR. (C) Cells were starved and then treated with IL-1β (10 ng/ml; left) or TNF-α (10 ng/ml; right) for the indicated times. ERK1/2 phosphorylation was evaluated using western blot analysis. (D) Cells were starved and then cultured with IL-1β (10 ng/ml) or TNF-α (10 ng/ml), with or without FGF-1 (10 ng/ml) and heparin (15 μg/ml) or EGF (10 ng/ml) for 24 h. The relative expression levels of the myofibroblast markers α-SMA and colIα1 were then evaluated using RT-qPCR. Data are presented as the mean ± SD (n=4). *P<0.01, **P<0.05. α-SMA, α-smooth muscle actin; colIα1, α1 chain of collagen type I; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; IL, interleukin; MEK, mitogen activated protein kinase; p-, phosphorylated; RT-qPCR, reverse transcription-quantitative PCR; TNF-α, tumor necrosis factor α.

of *colIa1* expression was further enhanced by the administration of IL-1β (10 ng/ml) or TNF-α (10 ng/ml) (Fig. 5D; left and right graphs, respectively).

Discussion

Bernasconi *et al* (29), have investigated the morphological modifications occurring in synovial tissue after severe derangement of the articular structure, with dislocation or perforation of the TMJ disks. After histological examination, they have reported remarkable hyperplasia of the synovial tissue, with an increase in the number of myofibroblastic fibroblast-like cells (29). Interestingly, post-traumatic joint stiffness is characterized by an increase in the number of MFs in the joint capsules (30,31), suggesting that MFs retain crucial roles in the pathogenesis of joint stiffness.

MFs are the cells primarily responsible for inducing fibrosis in scleroderma, renal fibrosis, pulmonary fibrosis, and liver fibrosis (32). MFs retain contractile properties and produce a large quantity of extracellular molecules, such as type I collagen (33). The most widely recognized molecular marker of differentiated and activated MFs is the *de novo* expression of α-SMA (34). We have previously established an FLS cell line, FLS1, which is derived from a mouse TMJ. We have reported that FLS1 cells express higher levels of MF marker molecules, such as α-SMA and type I collagen than mouse NIH3T3 embryonic fibroblasts, which are frequently used as a standard fibroblast control (6). Thus, the FLS1 cell line is a suitable experimental model for the investigation of the molecular mechanisms underlying the extensive fibrosis observed in the TMJ-OA synovial tissue. We have previously demonstrated that Rho-associated coiled-coil-forming kinase (ROCK)-mediated actin-polymerization, which induces the translocation of myocardin-related transcription factor (MRTF) from the cytoplasm to the nucleus, promotes MF differentiation in FLS1 cells (6). However, it remains to be clarified what intra-cellular signals other than the ROCK-mediated signal affect the fibrogenic activity in FLS1 cells.

Here, we demonstrated that FGF-1 and EGF dose-dependently downregulated the mRNA levels of the MF differentiation markers *α-SMA* and type I collagen in FLS1 cells (Figs. 1 and 2). In addition, we found that ERK1/2-mediated signaling played an important role in these anti-fibrogenic effects of FGF-1 and EGF (Figs. 3 and 4). These results strongly suggested that FGF-1 and EGF suppressed the fibrogenic activity in FLSs in a MEK/ERK-dependent manner. However, U0126 only partially abrogated the FGF-1- or EGF-mediated downregulation of MF differentiation markers in FLS1 cells, suggesting that signaling pathways other than MEK/ERK play important roles (Fig. 4). Interestingly, we have previously demonstrated that EGF suppresses the expression of MF differentiation markers in periodontal ligament-derived endothelial progenitor cells (EPCs, SCDC2) through MEK- and JNK-dependent signaling pathways (35), whereas the activation of JNK was not detected after the EGF stimulation of FLS1 cells (data not shown). These results suggested that EGF differentially induced intracellular signals in EPCs and FLSs and had a negative effect on myofibroblastic differentiation. On the other hand, the combination of the inflammatory cytokines IL-1β and TNF-α suppressed the expression of the MF markers α-SMA (Fig. 5B, left graph) and type I collagen (Fig. 5B, right graph), and this effect was significantly abrogated by the MEK1/2 inhibitor U0126. In contrast, PD98059, which is known as a MEK1 inhibitor, but not a MEK1/2 inhibitor, in a cell-type specific manner (36), significantly abrogated the suppression of *α-SMA* expression by the combinatorial treatment with the inflammatory cytokines (Fig. 5B, left graph), but did not abrogate the suppression of type I collagen expression (Fig. 5B, right graph), suggesting that MEK2-mediated intracellular signaling played an important role in the suppression of type I collagen expression in FLSs after stimulation with inflammatory cytokines. Importantly, neither an NF-κB inhibitor nor an IKK-2 inhibitor abrogated the IL-1β- and TNF-α-mediated suppression of MF marker expression (Fig. S2), suggesting that these effects of the inflammatory cytokines in FLSs were not mediated by NF-κB. Interestingly, the NF-κB and IKK-2 inhibitors further decreased the expression levels of the MF markers in FLS1 cells treated with the inflammatory cytokines (Fig. S2), suggesting that the NF-κB-mediated signaling possibly positively regulated the MF marker expression in FLSs. In addition, we confirmed that IL-1β and TNF-α induced ERK1/2 phosphorylation in FLS1 cells (Fig. 5C). Moreover, these inflammatory cytokines further enhanced the FGF-1- or EGF-mediated suppression of type I collagen expression (Fig. 5D). These results strongly suggested that RTK ligands and inflammatory cytokines cooperatively inhibited the fibrogenic activity in FLSs in a MEK/ERK-dependent manner. Interestingly, Ma *et al* (37) have previously reported that EGF and IL-1β synergistically promote ERK1/2-mediated invasive breast ductal cancer cell migration and invasion. In addition, Ziv *et al* (38) and Kakiashvili *et al* (39) have reported that TNF-α activates ERK1/2 through EGFR activation in epithelial cells. However, it remains to be determined whether IL-1β and TNF-α activate ERK1/2 through EGFR activation in FLSs.

Our findings partially clarify the molecular mechanisms underlying the development of OA-related fibrosis in the TMJ and may aid in identifying novel therapeutic targets for this condition. In addition, FGF-1 and EGF may be used to prevent OA-related fibrosis around inflammatory TMJs.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SM, SY, NC, SK, HK, MK and KS performed RT-qPCR and western blotting. SM and AI designed the present study. AI was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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