Original

Adenosine 5′-triphosphate strengthens receptor tyrosine kinase-mediated suppression of fibrogenic activity in fibroblast-like synoviocytes derived from mouse temporomandibular joints possibly through $P2Y_2$, $P2Y_4$, and $P2Y_{13}$ purinergic receptors

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Abstract：Osteoarthritis (OA) -related fibrosis is a plausible cause of temporomandibular joint (TMJ) stiffness. We previously demonstrated that receptor tyrosine kinase (RTK) ligands, fibroblast growth factor (FGF) -1, and epidermal growth factor (EGF), suppressed fibrogenic activity in a fibroblast-like synoviocytes (FLS) cell line, FLS1, derived from mouse TMJ. However, the molecular mechanisms underlying the suppression of fibrogenic activity in FLS1 cells by RTK ligands remained to be clarified. In particular, in inflamed TMJ, it remained unclear how extracellular adenosine 5′-triphosphate (eATP), which is a danger associated molecular pattern (DAMP), affected the RTK-ligand-induced suppression of fibrogenic activity in FLS1 cells. Here, we found that FLS1 cells vigorously expressed the purinergic receptors, $P2X_3$, $P2X_7$, $P2Y_2$, $P2Y_4$, $P2Y_{12}$, and $P2Y_{13}$. We found that ATP significantly decreased the expression of the fibrogenic marker, alpha-smooth muscle actin (α-SMA), in FLS1 cells. In addition, ATP strengthened the RTK-ligand-induced suppression of α-SMA expression. Since ATP typically binds to $P2X_3$, $P2X_7$, $P2Y_2$, $P2Y_4$, and $P2Y_{13}$, and to determine which purinergic receptor mediates the suppressive effects of ATP on the fibrogenic marker expression, we examined the effects of $P2Y_{12}$ and $P2Y_{13}$ agonist, adenosine 5′-diphosphate (ADP), and $P2Y_2$ and $P2Y_4$ agonist, uridine 5′-triphosphate (UTP), and found that either ADP or UTP significantly decreased α-SMA expression and strengthened the RTK-

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ligand-induced suppression of α-SMA expression. Importantly, $P2X_3$ antagonist, RO-3, and $P2X_7$ antagonists, A-438079 and A-804598, did not reverse the ATP-induced suppressive effects on the α-SMA expression. Taken together, these results strongly suggested that eATP strengthened the RTK-ligand-induced suppression of fibrogenic activity in FLSs possibly through $P2Y_2$, $P2Y_4$, or $P2Y_{13}$.

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

Keywords : adenosine 5′-triphosphate, fibroblast-like synoviocytes, temporomandibular joint, fibroblast growth factor, epidermal growth factor, myofibroblast differentiation.

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) presents symptoms including cartilage degeneration, subchondral bone remodeling, and synovitis, which results in TMJ dysfunction [2]. Intriguingly, histological studies have shown the presence of extensive fibrosis in TMJ-OA synovial tissues [3, 4], suggesting that fibrotic tissue formation may be responsible for restricted joint movements [5].

We previously demonstrated that fibroblast growth factor (FGF) -1, and epidermal growth factor (EGF) suppressed expression of the fibrogenic markers, alpha-smooth muscle actin (α-SMA) and type I collagen, in fibroblast-like synoviocytes (FLS) -1 in a mitogen-activated protein kinase (MAPK) kinase (MEK) -dependent manner (unpublished observation). However, it remains unclear how extracellular nucleotides, which are danger-associated molecular patterns (DAMPS) [6], affects the FGF-1-, and EGF-induced suppression of fibrogenic activity in the inflamed TMJ. Extracellular adenosine 5′-triphosphate (eATP) derived from necrotic cells binds to the purinergic receptors $P2X_{1\text{-}7}$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_{11}$, and $P2Y_{13}$ [7]. In general, P2X receptors are ligand-gated, cation-selective channels permeable to Na^+, K^+, or Ca^{2+}, whereas P2Y receptors are G protein-coupled receptors that increase cytoplasmic Ca^{2+} and modulate intra-cellular cyclic adenosine 5′-monophosphate (cAMP) levels [7]. Caporali et al. demonstrated that human synoviocytes derived from rheumatoid arthritis patients expressed $P2X_1$, $P2X_2$, $P2X_4$, $P2X_5$, $P2X_6$, $P2X_7$, $P2Y_1$, $P2Y_4$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$, but did not express $P2X_3$, $P2Y_2$, and $P2Y_6$ receptors [8]. Intriguingly, they demonstrated that the ATP analog 2′-3′-O-(4-benzoylbenzoyl) ATP (BzATP) induced interleukin (IL) -6 expression in human synoviocytes. In addition, Klein et al. reported that ATP induced expression of brain-derived neurotrophic factor, which is produced in the synovial fluid of arthritic patients [9], in synovial fibroblasts derived from OA patients through the $P2X_4$ receptor in a p38 MAPK-dependent manner [10].

The FGF family consists of 24 members that share 13–71% amino acid identity [11]. It is generally known that FGF-11 to -15 are homologs of the FGF family, but do not activate any FGF receptor [12]. On the other

hand, FGF receptors (FGFRs) consist of 4 members that belong to the receptor tyrosine kinase (RTK) family [13]. In general, FGF-1 binds to FGFR1–4 [14] to activate various intracellular signaling molecules, including phosphoinositide 3-kinase (PI3K) /Akt and MAPKs, such as extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 MAPK [15].

EGF was first purified from mouse salivary glands as a soluble factor that accelerates corneal wound healing [16]; however, EGF was soon found to be a general growth factor that affects various cellular functions involved in cell proliferation and differentiation [17]. The EGF receptor (EGFR) family consists of 4 members, including EGFR/ErbB1/HER1, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4, which belong to the RTK family [18]. EGF binds to EGFR and induces dimerization of the receptors. The cytoplasmic tyrosine kinase domains of EGFR can be autophosphorylated to mediate intracellular signals to various signaling proteins. The carboxy terminal tyrosine residues on EGFR, Tyr1068 and Tyr1173, are the major sites of autophosphorylation, which occurs as a result of EGF binding and transduces the EGF-induced signals [19, 20]. Autophosphorylated EGFR activates various types of intracellular signaling molecules, including MAPKs and PI3K/Akt [21]. Intriguingly, EGF protein has been detected in the human knee synovial fluid [22]. In addition, EGFR signaling is critical for maintaining the superficial layer of articular cartilage and preventing OA initiation [23].

Intriguingly, Arthur et al. demonstrated that the ATP analog, adenosine 5′-O-(3-thio) triphosphate (ATPγS) -induced $P2Y_2$ receptor-mediated signal activated the nerve growth factor (NGF) receptor, TrkA, which belongs to the RTK family, in root ganglion neurons, enhancing NGF-induced neurite extension in an MEK/early response kinase (ERK) - and p38 MAPK-dependent manner [24]. However, it remained unclear how eATP affected the FGF receptor- or EGF receptor-mediated suppression of fibrogenic activity in fibroblast-like synoviocytes (FLSs). Here, we evaluated whether ATP enhanced the FGF receptor- or EGF receptor-mediated suppression of the fibrogenic marker, α-SMA in an FLS cell line FLS1 [25]. In addition, we tried to elucidate the signal transduction mechanisms underlying the suppressive effects of ATP on fibrogenic marker expression in FLS1 cells.

Materials and methods

Reagents

Recombinant mouse EGF was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). ATP, ATPγS, adenosine 5′-diphosphate (ADP), uridine 5′-triphosphate (UTP), and heparin sodium salt were purchased from Merck KGaA (Darmstadt, Germany). Heparin was included to achieve optimal FGF-1 activity [26]. We confirmed that heparin itself did not decrease expression of the fibrogenic marker, α-SMA (data not shown). The $P2X_3$ antagonist, RO-3, and the $P2X_7$ antagonists, A-438079 and A-804598, were obtained from R&D Systems (Minneapolis, MN, USA). We confirmed that dimethyl sulfoxide (DMSO), the vehicle for RO-3, A-438079, and A-804598 treatments, did not affect the expression of the fibrogenic marker, α-SMA (data not shown).

Cell culture

The fibroblast-like synoviocyte (FLS) cell line, FLS1 was established, as previously described [25]. FLS1 cells were maintained in

culture with Ham's F-12 supplemented with 2 mM glutamine, 10% fetal bovine serum (FBS), and penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). These cells were then subcultured at a 1:4 ratio after the cells reached subconfluency. Mesenchymal stem cells (MSCs) were obtained from mouse bone marrow and cultured as previously reported [27]. NIH3T3 mouse embryonic fibroblasts were obtained from Riken Cell Bank (Tsukuba Science City, Japan). NIH3T3 cells were maintained in a growth medium consisting of minimum essential medium Eagle's α-modification (α-MEM) (Wako Pure Chemical Industries Ltd.) supplemented with 10% FBS and penicillin-streptomycin (Invitrogen Life Technologies). For the evaluation of mRNA expression level of fibrogenic marker, α-SMA, some FLS1 cells were treated with ATP, ATP γS, ADP, and UTP for indicated times. Especially for the evaluation of effects of purinergic receptor antagonists RO-3, A-438079, and A-804598 on the ATP-induced suppression of the fibrogenic marker expression, FLS1 cells were pretreated with these purinergic receptor antagonists at 30 min before the ATP administration.

RNA isolation and Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from FLS1 cells using ISOGEN reagent (Nippon Gene, Toyama, Japan), according to the manufacturer's protocol. First-strand cDNA was synthesized from total RNA using a PrimeScript RT reagent Kit (Takara-Bio, Shiga, Japan). 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 *P2X2*, 5′-CCATCTTCAGGCTGGGCTTC-3′ (forward) and 5′-TTCAGACAAGTCCAGGTCACAGTTC-3′ (reverse);

mouse *P2X3*, 5′-CATAAACCATCATGGGCAGCA-3′ (forward) and 5′-ACACCTGGGCAATGGAGACA-3′ (reverse);

mouse *P2X4*, 5′-AGACTTGGCTGCTGCGTCTG-3′ (forward) and 5′-GCAACCCTGAGTATTTGTGGAGTG-3′ (reverse);

mouse *P2X5*, 5′-CCCACAGTCATCAACATTGGTTC-3′ (forward) and 5′-ATCTCGTTGGCCTCAACCTC-3′ (reverse);

mouse *P2X6*, 5′-ACTGGGATTGCAACCTGGAC-3′ (forward) and 5′-AAACGGATTCCATAGAGCTTGAG-3′ (reverse);

mouse *P2X7*, 5′-GCCACTTATGCAGCCATAAATCC-3′ (forward) and 5′-AGCTTATCGCTCACCAAAGCAA-3′ (reverse);

mouse *P2Y1*, 5′-CCAGGACACTAACCCATCGTGA-3′ (forward) and 5′-CTGTACAACTGAAGGCCCACAAAC-3′ (reverse);

mouse *P2Y2*, 5′-GACCTGGGCACGATGGACTTA-3′ (forward) and 5′-GGAGTCTCCAGGGACCTGACATTA-3′ (reverse);

mouse *P2Y4*, 5′-TCCCAATTCCTGGGATTAAAGACA-3′ (forward) and 5′-ACGCAGATTGGAATGAAGCATAGAG-3′ (reverse);

mouse *P2Y6*, 5′-GGCAGCTGTCTTTGCCATCA-3′ (forward) and 5′-TTGTAGGCAGCAGCGAAGGTC-3′ (reverse);

mouse *P2Y12*, 5′-GGTTCAGCCAAAGTTCCCAAGA-3′ (forward) and 5′-CCGAGTTTGGCTCAGGGTGTA-3′ (reverse);

mouse *P2Y13*, 5′-TTCCATAGCCTCAGAAGCCACAG-3′ (forward) and 5′-GTACAGTGCAGTGCGGACCAA-3′ (reverse);

mouse *P2Y14*, 5′-CTTGCTGTCCCAAACATCATCC-3′ (forward) and 5′-AGACGCCTTGTGCCACTTCC-3′ (reverse);

mouse *α-SMA*, 5′-CAGATGTGGATACAG-

CAAACAGGA-3′ (forward) and 5′-GACTTAGAAGCATTTGCGGTGGA-3′ (reverse); mouse *GAPDH*, 5′-TGTGTCCGTCGTGGATCTG-3′ (forward) and 5′-TTGCTGTTGAAGTCGCAGGAG-3′ (reverse).

The mRNA levels of *P2X2-7*, *P2Y1*, *P2Y2*, *P2Y3*, *P2Y6*, *P2Y12*, *P2Y13*, *P2Y14*, and *α-SMA* were normalized to *GAPDH* mRNA levels and the relative expression levels were calculated as the fold increase or decrease relative to the control.

Statistical analysis

Data are presented as mean ± standard deviation (SD; n = 4) and statistically analyzed by Tukey's multiple comparison test. Values of $^{*}P < 0.01$ and $^{**}P < 0.05$ were considered to be statistically significant. The results shown for all experiments are representative of at least two replicates.

Results

FLS1 cells strongly expressed the purinergic receptors P2X3, and P2X7

Jiang et al. demonstrated that mesenchymal stem cells (MSCs) expressed various kinds of P2X receptors such as $P2X_1$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, and $P2X_7$ [28]. Therefore, we used MSCs derived from mouse bone marrow as a positive control for the detection of P2X receptor mRNAs. In addition, mouse NIH3T3 embryonic fibroblasts were used as a standard fibroblast control. As shown in Fig. 1, FLS1 cells expressed 78.1 times more $P2X_3$ and 65.0 times more $P2X_7$ mRNAs than MSCs (Fig. 1). Thus, FLS1 cells strongly expressed the $P2X_3$ and $P2X_7$ receptors. In addition, NIH3T3 cells strongly expressed the $P2X_3$ receptor; NIH3T3 cells expressed 18.9 times more $P2X_3$ mRNA than MSCs (Fig. 1).

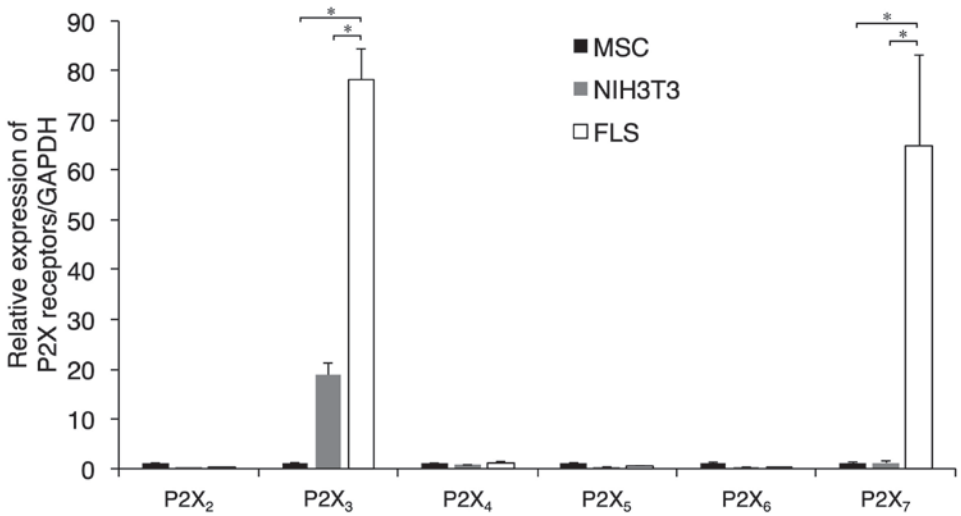

Fig. 1 : *Fibroblast-like synoviocytes (FLS) cell line, FLS1 cells strongly expressed the purinergic receptors, P2X3 and P2X7.* Mesenchymal stem cells (MSCs), NIH3T3 cells, and FLS1 cells were seeded onto 12-well tissue culture plates with FLS1 growth medium at a density of 1×10^5 cells/well and maintained for 24 h. The relative expression levels of the purinergic receptors, *P2X2-7*, were measured using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Bars show the mean ± SD (n = 4). $^{*}P < 0.01$.

FLS1 cells strongly expressed the purinergic receptors P2Y$_2$, and P2Y$_4$, P2Y$_{12}$, P2Y$_{13}$, and P2Y$_{14}$

Jiang et al. demonstrated that MSCs expressed various kinds of P2Y receptors such as P2Y$_1$, P2Y$_2$, P2Y$_4$, P2Y$_6$, P2Y$_{11}$, P2Y$_{12}$, P2Y$_{13}$, and P2Y$_{14}$ [28]. Therefore, we used MSCs derived from mouse bone marrow as a positive control for the detection of P2Y receptor mRNAs. In addition, mouse NIH3T3 embryonic fibroblasts were used as a standard fibroblast control. As shown in Fig. 2, FLS1 cells expressed 8.8 times more P2Y$_2$, 2.6 times more P2Y$_4$, 4.5 times more P2Y$_{12}$, and 3.6 times more P2Y$_{13}$ mRNAs than MSCs (Fig. 2). These results indicated that FLS1 cells strongly expressed the purinergic P2 receptors P2Y$_2$, P2Y$_4$, P2Y$_{12}$, and P2Y$_{13}$. We also found that FLS-1 cells expressed P2Y$_1$, P2Y$_6$, and P2Y$_{14}$ receptors as much as MSCs (Fig. 2). In addition, NIH3T3 cells strongly expressed the P2Y$_2$ receptor; NIH3T3 cells expressed 8.2 times more P2Y$_2$ mRNA than MSCs (Fig. 2).

ATP strengthened the FGF-1- and EGF-induced suppression of fibrogenic marker expression in FLS1 cells

As shown in Fig. 3A, ATP (100 μM) itself significantly suppressed mRNA expression of fibrogenic marker α-SMA. In addition, ATP (100 μM) significantly strengthened the FGF-1 (1 ng/mL) with heparin (15 μg/mL) -, and EGF (0.1-1 ng/mL) -induced suppression of mRNA expression of the fibrogenic marker. We also found that non-hydrolysable ATP analogue, ATPγS (100 μM) itself significantly suppressed the mRNA expression of fibrogenic marker α-SMA. In addition, ATP γS (100 μM) strengthened the FGF-1 (0.1 ng/mL) with heparin (15 μg/mL) -, and EGF (0.01-1 ng/mL) -induced suppression of mRNA expression of the fibrogenic marker (Fig. 3B).

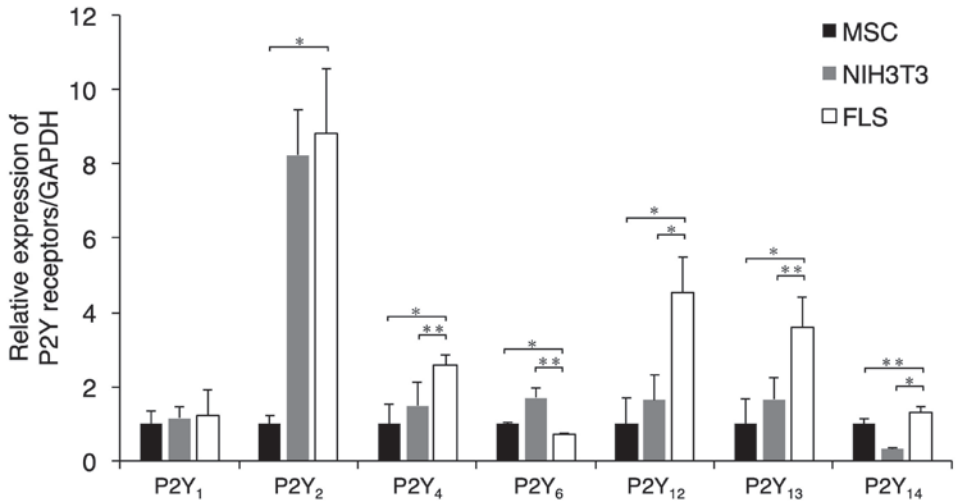

Fig. 2 : *FLS-1 cells strongly expressed the purinergic receptors, P2Y$_2$, P2Y$_4$, P2Y$_{12}$, P2Y$_{13}$, and P2Y$_{14}$.* MSCs, NIH3T3 cells, and FLS1 cells were seeded onto 12-well tissue culture plates with FLS1 growth medium at a density of 1×10^5 cells/well and maintained for 24 h. The relative expression levels of the purinergic receptors, *P2Y$_1$, P2Y$_2$, P2Y$_4$, P2Y$_6$, P2Y$_{12}$, P2Y$_{13}$, and P2Y$_{14}$* were measured using qRT-PCR. Bars show the mean ± SD (n = 4). $^*P < 0.01$, $^{**}P < 0.05$.

Fig. 3A

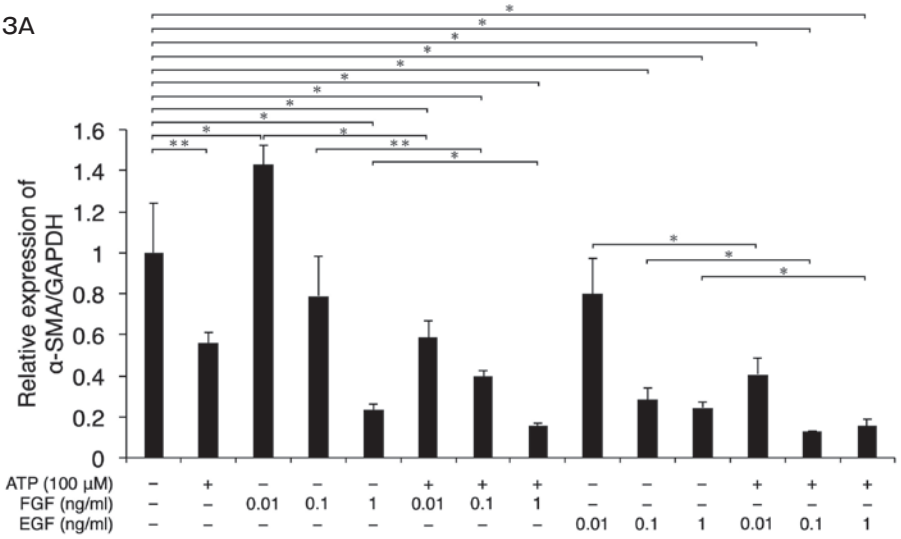

Fig. 3B

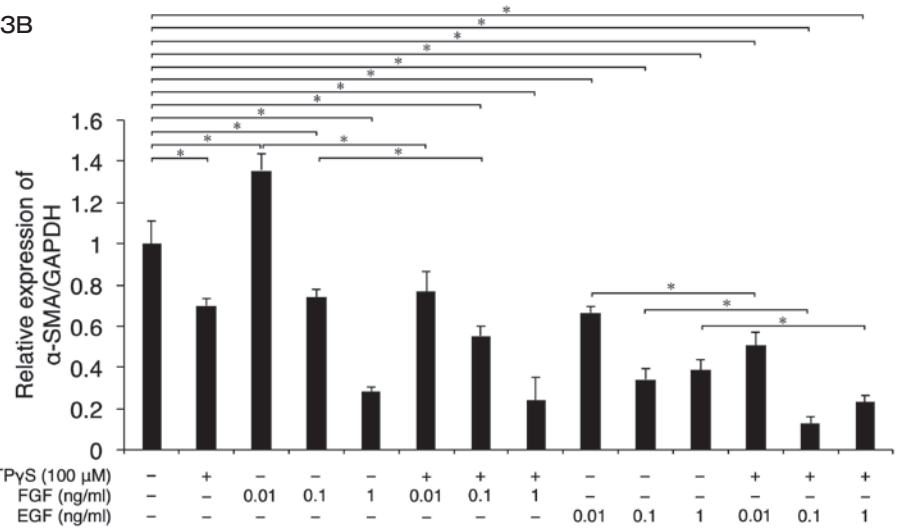

Fig. 3 : *Adenosine 5′ -triphosphate (ATP) and the non-hydrolyzable ATP analogue, 5′ -O- (3-thio) triphosphate (ATPγS), strengthened receptor tyrosine kinase (RTK) -induced suppression of fibrogenic activity in FLS1 cells.* (*A* and *B*) FLS1 cells were seeded onto 12-well tissue culture plates with FLS1 growth medium at a density of 1×10^5 cells/well and maintained for 24 h. The growth medium was replaced with Ham's F-12 containing 0.5% FBS for 24 h to induce cell starvation. (*A*) Cells were then cultured with or without ATP (100 μM), fibroblast growth factor (FGF) -1 (0.01–1 ng/mL) plus heparin (15 μg/mL), or epidermal growth factor (EGF) (0.01–1 ng/mL) for 24 h, as indicated. (*B*) Cells were then cultured with or without ATPγS (100 μM), FGF-1 (0.01–1 ng/mL) plus heparin (15 μg/mL), or EGF (0.01–1 ng/mL) for 24 h, as indicated. The relative expression level of the fibrogenic marker, alpha-smooth muscle actin (*α -SMA*), was measured using qRT-PCR. Bars show the mean ± SD (n = 4). $^*P < 0.01$, $^{**}P < 0.05$.

ATP suppressed the fibrogenic activity in FLS1 cells possibly through the purinergic receptors–$P2Y_2$, $P2Y_4$, and $P2Y_{13}$

As shown in Figs. 1, and 2, FLS-1 cells vigorously expressed the purinergic receptors $P2X_3$, $P2X_7$, $P2Y_2$, $P2Y_4$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$. Coddou et al. reported that ATP preferentially binds to the purinergic receptors $P2X_{1\text{-}7}$ [29]. On the other hand, Lu & Insel reported that ATP preferentially binds to $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_{11}$, and $P2Y_{13}$ [7]. Therefore, it is probable that the ATP-induced suppressive effect on fibrogenic activity in FLS1 cells operates through $P2X_3$, $P2X_7$, $P2Y_2$, $P2Y_4$, or $P2Y_{13}$. We found that the $P2X_3$ antagonist, RO-3, and the $P2X_7$ antagonists, A-438079 and A-804598, did not abrogate the ATP-induced suppressive effects on fibrogenic marker expression (data not shown), indicating that ATP did not suppress fibrogenic activity in FLS1 cells through the purinergic receptors $P2X_3$ and $P2X_7$. On the other hand, $P2Y_{12}$ or $P2Y_{13}$ agonists, ADP itself significantly suppressed mRNA expression of α-SMA (Fig. 4). In addition, ADP significantly strengthened the FGF-1 (0.1-1 ng/mL) with heparin (15 μg/mL) -, and EGF (0.01-1 ng/mL) -induced suppression of mRNA expression of the fibrogenic marker (Fig. 4). As shown in Fig. 5, $P2Y_2$, and $P2Y_4$ agonist UTP itself significantly suppressed mRNA expression of the fibrogenic marker α-SMA. In addition, UTP significantly strengthened the FGF-1 (0.1-1 ng/mL) with heparin (15 μg/mL) -, and EGF (0.01-1 ng/mL) -induced suppression of mRNA expression of the fibrogenic marker (Fig. 5).

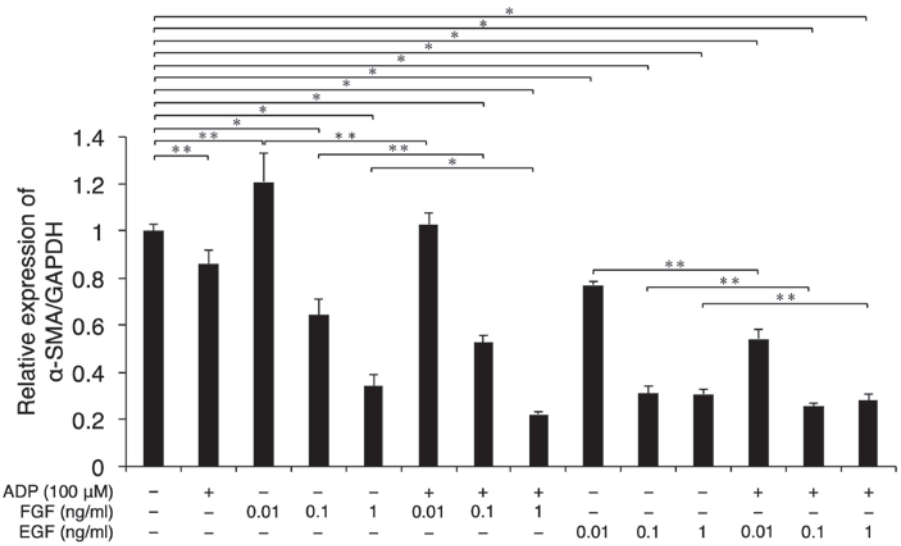

Fig. 4 : *$P2Y_{12}$ and $P2Y_{13}$ agonist, Adenosine 5′-diphosphate (ADP), strengthened RTK-induced suppression of fibrogenic activity in FLS1 cells.* FLS1 cells were seeded onto 12-well tissue culture plates with FLS1 growth medium at a density of 1×10^5 cells/well and maintained for 24 h. The growth medium was replaced with Ham's F-12 containing 0.5% FBS for 24 h to induce cell starvation. Cells were then cultured with or without ADP (100 μM), FGF-1 (0.01–1 ng/mL) plus heparin (15 μg/mL), or EGF (0.01–1 ng/mL) for 24 h, as indicated. The relative expression level of the fibrogenic marker, *α-SMA*, was measured using qRT-PCR. Bars show the mean ± SD (n = 4). $^*P < 0.01$, $^{**}P < 0.05$.

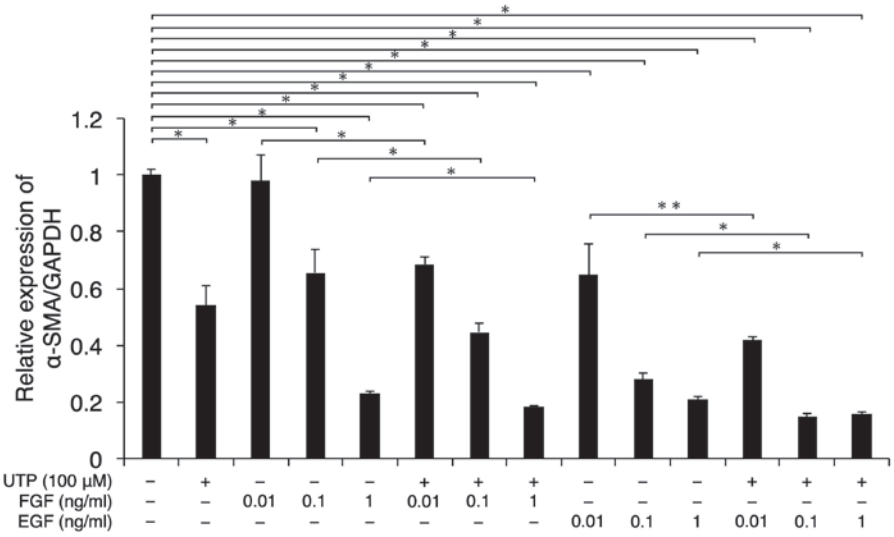

Fig. 5 : *The $P2Y_2$ and $P2Y_4$ agonist, uridine 5′-triphosphate (UTP), strengthened RTK-induced suppression of fibrogenic activity in FLS1 cells.* FLS1 cells were seeded onto 12-well tissue culture plates with FLS1 growth medium at a density of 1×10^5 cells/well and maintained for 24 h. The growth medium was replaced with Ham's F-12 containing 0.5% FBS for 24 h to induce cell starvation. Cells were then cultured with or without UTP (100 μM), FGF-1 (0.01–1 ng/mL) plus heparin (15 μg/mL), or EGF (0.01–1 ng/mL) for 24 h, as indicated. The relative expression level of the fibrogenic marker, α-SMA, was measured using qRT-PCR. Bars show the mean ± SD (n = 4). $^*P < 0.01$, $^{**}P < 0.05$.

Discussion

We demonstrated that the purinergic receptors, $P2X_3$, $P2X_7$, $P2Y_2$, $P2Y_4$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$, were strongly expressed in FLSs derived from mouse TMJ (Figs. 1 and 2). We found that ATP itself significantly decreased the expression of the fibrogenic marker, α-SMA, in FLS1 cells (Fig. 3A). In addition, ATP strengthened the RTK-ligand-induced suppression of the fibrogenic marker's expression (Fig. 3A). Since ATP typically binds to $P2X_3$, $P2X_7$, $P2Y_2$, $P2Y_4$, and $P2Y_{13}$, we tried to determine which purinergic receptor functionally mediated the suppressive effects of ATP on expression of the fibrogenic marker. We examined the effects of the $P2Y_{12}$ and $P2Y_{13}$ agonist, ADP, and the $P2Y_2$ and $P2Y_4$ agonist, UTP, on the fibrogenic marker expression. We found that ADP itself significantly decreased expressions of the fibrogenic marker, α-SMA. In addition, ADP strengthened the RTK ligands-induced suppression of the fibrogenic marker expression (Fig. 4). In general, EC50 of ADP against to purinergic receptors $P2Y_{12}$, and $P2Y_{13}$, both of which coupled with Gi proteins, were almost same value (60.7 μM, and 60.0 μM, respectively) [7], suggesting that $P2Y_{12}$, and $P2Y_{13}$ transduced similar bioactivities into cells after the ADP stimulation. We also found that UTP itself significantly decreased expressions of fibrogenic marker α-SMA. In addition, UTP strengthened the RTK ligands-induced suppression of the fibrogenic marker expression (Fig. 5). Importantly, the $P2X_3$ antagonist RO-3, and the $P2X_7$ antagonists, A-438079 and A-804598, did not abrogate the

ATP-induced suppressive effects on the fibrogenic marker expression (data not shown). Taken together, these results strongly suggested that eATP strengthened the RTK-ligand-induced suppression of fibrogenic activity in FLSs possibly through $P2Y_2$, $P2Y_4$, or $P2Y_{13}$.

Sequential hydrolysis of extracellular ATP catalyzed by ectonucleotidases (e.g. CD39 and CD73) is the main pathway for the generation of adenosine, which in turn activates P1 receptors [30]. Therefore, there was a possibility that hydrolyzed ATP, as adenosine, also might retain their suppressive effects on fibrogenic activity in FLS1 cells. As shown in Fig. 3B, the non-hydrolyzable ATP analogue, ATPγS (100 μM), significantly suppressed expression of the fibrogenic marker and strengthened the RTK-ligand-induced suppression of α-SMA expression, strongly suggesting that ATP itself retained its suppressive effect on fibrogenic activity in FLS1 cells.

We previously demonstrated that FGF-1 and EGF suppressed expression of the fibrogenic markers, α-SMA and type I collagen, in FLS1 cells, partially through a MEK/ERK-dependent signal (unpublished observation). On the other hand, Ortega et al. previously reported that the $P2Y_{13}$ agonist, 2-methylthioadenosine-5′-diphosphate (2MeSADP), exhibited MEK/ERK-dependent neurotrophic activity in granule neurons [31]. In addition, Mühleder et al. demonstrated that a $P2Y_2$-mediated signal phosphorylated vascular endothelial growth factor receptor-2, belonging to the RTK family, and ERK1/2 in endothelial cells, promoting their angiogenic activity [32]. Ito et al. also reported that ATP induced activation of a MEK/ERK-mediated signal through $P2Y_2$ in skeletal muscle cells, resulting in skeletal muscle hypertrophy [33]. These results implied that ATP and RTK ligands might additively suppress fibrogenic activity in FLS1 cells in a MEK/ERK-dependent manner. However, it remained unclear whether MEK-inhibitors abrogated the ATP-induced suppression of fibrogenic activity in FLS1 cells.

Thus, we demonstrated that ATP strengthened RTK-mediated suppression of fibrogenic activity in FLSs derived from mouse TMJs possibly through the purinergic receptors $P2Y_2$, $P2Y_4$, and $P2Y_{13}$. Our 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. In addition, FGF-1 and EGF could be utilized as drugs to prevent OA-related fibrosis around inflamed TMJs.

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Author contributions

S, M., S, Y., N, C., and S, K. performed the qRT-PCR analyses. S, M., and K, S. designed this study. S, M., and K, S. were major contributors in writing the manuscript.

Competing interests

The authors declare that there are no competing interests associated with this manuscript.