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Mechanical strain attenuates cytokine-induced ADAMTS9 expression via transient receptor potential vanilloid type 1

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ABSTRACT

The synovial fluids of patients with osteoarthritis (OA) contain elevated levels of inflammatory cytokines, which induce the expression of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) and of the matrix metalloproteinase (MMP) in chondrocytes. Mechanical strain has varying effects on organisms depending on the strength, cycle, and duration of the stressor; however, it is unclear under inflammatory stimulation how mechanical strain act on. Here, we show that mechanical strain attenuates inflammatory cytokine-induced expression of matrix-degrading enzymes. Cyclic tensile strain (CTS), as a mechanical stressor, attenuated interleukin (IL)-1β and tumor necrosis factor (TNF)-α-induced mRNA expression of *ADAMTS4*, *ADAMTS9*, and *MMP-13* in normal chondrocytes (NHAC-kn) and in a chondrocytic cell line (OUMS-27). This effect was abolished by treating cells with mechano-gated channel inhibitors, such as gadolinium, transient receptor potential (TRP) family inhibitor, ruthenium red, and with pharmacological and small interfering RNA-mediated TRPV1 inhibition. Furthermore, nuclear factor κB (NF-κB) translocation from the cytoplasm to the nucleus resulting from cytokine stimulation was also abolished by CTS. These findings suggest that mechanosensors such as the TRPV protein are potential therapeutic targets in treating OA.

1. Introduction

Osteoarthritis (OA) is the most common chronic disorder affecting the joints and constitutes a major burden on public health. It is believed that the articular cartilage—which consists of chondrocytes and extracellular matrix (ECM) molecules including aggrecan, collagen, and hyaluronan (HA) [1,2]—in patients with OA is destroyed by the aberrant up-regulation of matrix-degrading proteinases; however, it has been demonstrated that the synovial fluid contains elevated levels of inflammatory cytokines [3] that induce expression and activity of matrix metalloproteinase (MMP), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) [4–7]. ADAMTS4, ADAMTS5, and ADAMTS9 cleave aggrecan, which holds water via its charged chondroitin sulfate and keratan sulfate chains; thereby conferring physical strength and stress resistance to cartilage [8]. In cartilage affected by OA, aggrecan and collagen are degraded [5]. We recently reported that inflammatory cytokines induce the expression of ADAMTS9 to a greater extent than that of ADAMTS4 and of ADAMTS5 in chondrocytes [6], suggesting that ADAMTS9 plays an important role in developing OA. We also found that HA treatment decreased

Abbreviations: ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; BAPTA-AM, (1,2-bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester); CTS, cyclic tensile strain; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; ER, endoplasmic reticulum; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HA, hyaluronan; IL, interleukin; MMP, matrix metalloproteinase; NFATc1, nuclear factor of activated T cells 1, cytoplasmic, calcineurin-dependent 1; NF-κB, nuclear factor κB; OA, osteoarthritis; qRT-PCR, quantitative reverse transcription PCR; siRNA, small interfering RNA; TNF-α, tumor necrosis factor α; TRP, transient receptor potential

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ADAMTS9 levels in human and rat chondrocytes, it protected articular cartilage in a rat model with OA by down-regulating aggrecanase [6].

Mechanical strain can induce the expression of ECM proteins including collagen and aggrecan; however, excessive amounts of mechanical stress can actually stimulate proteases in chondrocytes instead [9–11]. Various molecules, including integrins, function as sensors that transduce mechanical stress [12]. Integrins are transmembrane glycoproteins composed of α and β subunits, each with an extracellular domain that directly binds ECM proteins such as collagen, fibronectin, and vitronectin [13], as well as with a cytoplasmic domain that interacts with the cytoskeleton via focal adhesion molecules. Transient receptor potential (TRP) channels were first identified in *Drosophila* as six-transmembrane proteins that function in phototransduction [14]. The TRP family is subdivided into seven subfamilies: TRPC, TRPV, TRPM, TRPP, TRPML, TRPA, and TRPN; these channels are expressed in a variety of tissues and cell types and are mostly permeable to calcium, some also presumably to act as mechanosensors [15,16].

Currently, it is unknown whether mechanical strain can suppress or attenuate inflammatory cytokine-induced cellular responses. To address these questions, the present study investigated changes in gene expression in chondrocytes subjected to uniaxial cyclic stretching. Our results show that mechanical strain attenuates inflammatory cytokine-induced expression of matrix-degrading enzymes. Moreover, we determined that calcium channels function as mechanosensors and that mechanical strain inhibits the release of cytokine-induced calcium in chondrocytes; furthermore, it also blocks translocation of nuclear NF-κB from the cytoplasm to the nucleus induced by cytokine stimulation. These findings can provide a molecular basis for developing novel strategies for regenerating cartilage and treating OA.

2. Methods

2.1. Reagents

Recombinant human interleukin (IL)-1β and tumor necrosis factor (TNF)-α, purchased from R&D Systems (Minneapolis, MN, USA), was stored at −80 °C and diluted in culture medium immediately before use. Mechanosensor inhibitors (gadolinium, ruthenium red, capsazepine, tranilast, and HC067047) and the NF-κB inhibitor (1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester) [BAPTA-AM]) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cells and cell culture

Normal human articular chondrocytes from knee cells (NHAC-kn) were purchased from Lonza (Walkersville, MD, USA) and cultured at 37 °C in chondrocyte basal medium (Lonza) containing chondrocyte growth medium, fetal bovine serum (FBS), transforming growth factor β, R^3 insulin-like growth factor, transferrin, insulin, gentamicin, and amphotericin-B (CDM Bullet Kit; Lonza) as previously described [6]. The medium was changed every 3 days, only cells at passages 3–6 were used for all experiments.

OUMS-27 chondrosarcoma cells were prepared as previously described [6,17,18]. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and penicillin/streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. Cells were passaged to cell ratio of 1:2 or 1:4 using trypsin with ethylenediaminetetraacetic acid every 7–10 days, with medium replacement every 3 days. Only cells at passages 7–12 were used for experiments; 2.5×10^5 cells were seeded in 6-well plates for 2 days, and the medium was replaced with serum-free medium for 24 h before cytokine stimulation, after which the cells were cultured in the presence of IL-1β and TNF-α (both at 10 ng/mL).

2.3. Mechanical stimulation

Cells (1.5×10^5) were seeded in a stretch chamber coated with 0.1 mg/mL collagen and cultured for 2 days, then transferred to serum-free DMEM for 24 h. Cells were exposed to cycles of uniaxial stretching in the presence of IL-1β and TNF-α (both at 10 ng/mL) using a ShellPa mechanical stretch system (Menicon Life Science, Aichi, Japan) (n = 6 each), which allowed for uniform stretching of the entire silicone membrane.

2.4. Real-time quantitative reverse transcription PCR (qRT)-PCR

Following cytokine stimulation, cells were washed twice with phosphate-buffered saline (PBS) and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, and reverse transcribed into cDNA as previously described [19–21]. Briefly, genomic DNA was removed by treatment with 5 U DNase I (Roche Diagnostics, Lewes, UK) at 37 °C for 15 min, followed by enzyme inactivation at 65 °C for 10 min; 2 μg total RNA were reverse transcribed with random primers (Toyobo, Osaka, Japan). qRT-PCR was carried out on a StepOnePlus system (Applied Biosystems, Foster City, CA, USA) as previously reported [22–24], with slight modifications. Briefly, each reaction mixture contained 5 μl TaqMan Fast Advanced Master mix, 0.5 μl TaqMan Gene Expression assay for the target genes (*ADAMTS4*, *ADAMTS9*, *MMP-13*, *TRPV1*, *TRPV2*, and *TRPV4*) and the endogenous control (glyceraldehyde 3-phosphate dehydrogenase; *GAPDH*), and 4 μl cDNA. Cycling conditions were as follows: 95 °C for 20 s; and 40 cycles at 95 °C for 1 s and 60 °C for 20 s. All samples were analyzed in triplicate. TaqMan primers and probes (human *ADAMTS4*: assay ID Hs00192708_m1 based on RefSeq NM_005099.4; human *ADAMTS9*: assay ID Hs00172025_m1 based on Ref Seq NM_182,920.1; human MMP-13: assay ID Hs00233992_m1 based on RefSeq NM_002427.3; human *TRPV1*: assay ID Hs0021912_m1 based on Ref seq NM_018,727.5; human *TRPV2*: assay ID Hs00901648_m1 based on Ref seq NM_016,113.4; human *TRPV4*: assay ID Hs01099348_m1 based on Ref seq NM_001177428.1; human GAPDH: assay ID Hs02758991_g1 based on Ref Seq NM_001256799.1) as well as the TaqMan Fast Advanced Master Mix were purchased from Applied Biosystems. GAPDH was used to normalize the levels of target RNAs with the comparative Ct (ΔΔCT) method as previously described [25–27]. Values obtained from the untreated cells served as the control.

2.5. Treatment with mechanosensor inhibitors

Cells (1.5×10^5) were seeded in a stretch chamber coated with 0.1 mg/mL collagen and cultured for 2 days, then transferred to serum-free DMEM for 24 h. Cells were pretreated with the following mechanosensor inhibitors for 1 h: the integrin-binding motif peptide GRGDS (50 μg/mL; Sigma-Aldrich) and its analog GRADSP (50 μg/mL; Calbiochem, Nottingham, UK), gadolinium (10 μM), ruthenium red (10 μM), capsazepine (10 μM), tranilast (50 μM), and HC-067047 (5 μM). The cells were then subjected to cycles of uniaxial stretching (5%, 0.5 Hz) in the presence of IL-1β and TNF-α (both at 10 ng/mL) using the ShellPa mechanical stretch system.

2.6. RNA interference

To silence TRPV1, TRPV2, and TRPV4, we used predesigned small interfering siRNAs (Silencer Select Pre-designed siRNAs; Ambion, Foster City, CA, USA) as previously described [28]. OUMS-27 cells were seeded at 1.5×10^5 cells/well in a collagen-coated stretch chamber and transfected with human TRPV1-1 (assay ID, s14819 based on RefSeq NM_018,727.5), human TRPV1-2 (assay ID, s14817 based on RefSeq NM_018,727.5), human TRPV2-1 (assay ID, s28081 based on RefSeq NM_016,113.4), human TRPV2-2 (assay ID, s28082 based on RefSeq NM_016,113.4), human TRPV2-3 (assay ID, s28083 based on RefSeq

NM_016,113.4), human TRPV4-1 (assay ID, s531655 based on RefSeq NM_001177428.1), human TRPV4-2 (assay ID, s531654 based on RefSeq NM_001177428.1), and human TRPV4-3 (assay ID, s531656 based on RefSeq NM_001177428.1) at a final concentration of 10 nM with Lipofectamine RNAiMAX transfection reagent, (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Silencer Select Negative Control No. 1 siRNA (4390843) was used as negative control. Cells were subsequently incubated 24 h before being exposed to CTS stimulation in the presence of IL-1β and TNF-α (both at 10 ng/mL) using the ShellPa mechanical stretch system before they were collected for analysis. Target gene knockdown efficiency was confirmed by qRT-PCR.

2.7. Visualization of intracellular calcium

OUMS-27 cells (1.5×10^5) were seeded in collagen-coated glass-bottomed plates and cultured for 2 days, then washed twice with PBS and treated with 5 μM Fluo-4 AM in DMEM for 1 h. After washing the cells in PBS, the nuclei were stained with Hoechst 33258 (Sigma-Aldrich; 1:5000). The samples were mounted with coverslips and stored at 4 °C in the dark. Images were obtained with a microscope (BZ-X700; KEYENCE, Oosaka, Japan) [29–31].

2.8. Evaluation of NF-κB translocation

Cells (1.5×10^5) were seeded in a stretch chamber coated with 0.1 mg/mL collagen and cultured for 2 days before being transferred to serum-free DMEM for 24 h. After treatment with IL-1β and TNF-α (both at 10 ng/mL), with or without 0.5-Hz cycles of uniaxial stretching (n = 6 each), the cells were treated with cold methanol for 30 min, followed by cold acetone treatment for 10 min for fixation with permeabilization. After washing the cells in PBS, samples were blocked with 3% bovine serum albumin/PBS for 2 h, washed in PBS, and incubated overnight at 4 °C with anti–NF–κB p65 antibody (Santa Cruz Biotechnology, Dallas, TX, USA; 1:500, sc-372). After PBS washes, cells were incubated for 1 h at 20 °C with Alexa 488-conjugated anti-rabbit secondary antibody (Invitrogen), then washed in PBS. The nuclei were stained with Hoechst 33,258 (1:5000) and the samples were mounted with coverslips and stored in the dark at 4 °C. Images were obtained with a microscope (BZ-X700; KEYENCE).

2.9. BAPTA-AM treatment

Cells (2.5×10^5) were seeded in a 6-well plate and cultured for 2 days, then transferred to serum-free DMEM for 24 h. The cells were treated IL-1β and TNF-α (both at 10 ng/mL) for 6 h with or without a 1 h pretreatment with 30 μM BAPTA-AM. *ADAMTS9* transcript levels were evaluated relative to levels in unstimulated cells by qRT-PCR.

2.10. Statistical analysis

Data are expressed as the mean ± SD. Differences among groups were evaluated by analysis of variance followed by Bonferroni's test. P values < 0.05 were considered statistically significant.

3. Results

3.1. Mechanical strain attenuates inflammatory cytokine-induced expression of ADAMTS and MMP in OUMS-27 cells

First, we examined whether mechanical strain attenuates expression of *ADAMTS4*, *ADAMTS9*, and *MMP13* induced by IL-1β and TNF-α in OUMS-27 cells. Application of 5% CTS at a frequency of 0.5 Hz resulted in the downregulation of *ADAMTS4*, *ADAMTS9*, and *MMP13* transcript levels in OUMS-27 cells stimulated with cytokines (Fig. 1).

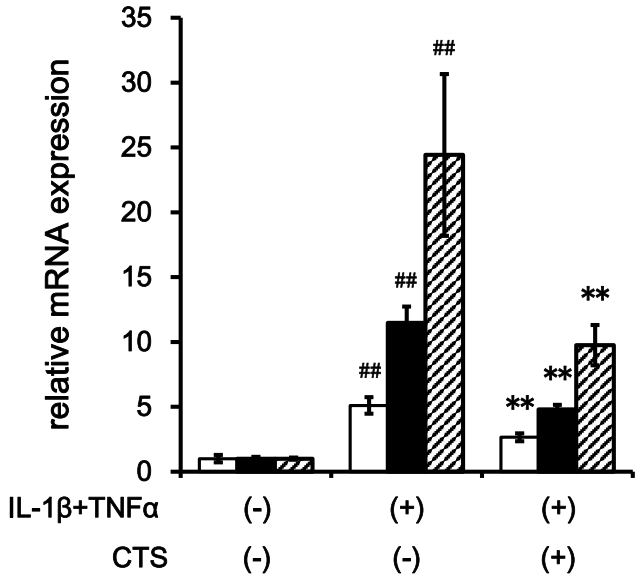

Fig. 1. Cytokine-induced expression of *ADAMTS4*, *ADAMTS9*, and *MMP*-13 mRNA in OUMS-27 cells is attenuated by CTS. Cells were subjected to 5% tensile strain at a frequency of 0.5 Hz and treated with 10 ng/mL IL-1β and TNF-α for 6 h. Levels of *ADAMTS4*, *ADAMTS9*, and *MMP*-13 mRNA were measured relative to the levels of mRNA found in the unstimulated control cells by qRT-PCR. Open bars; ADAMTS4, closed bars; ADAMTS9, hatched bars; MMP-13. Values represent mean ± SD (n = 6 per group). $^{\#\#}P < 0.01$ vs. control; $^{**}P < 0.01$ vs. cytokine-treated group.

3.2. Mechanical strain attenuates inflammatory cytokine-induced expression of ADAMTS9 in human chondrocytes

We examined whether mechanical strain attenuates expression of *ADAMTS9* mRNA induced by IL-1β and TNF-α in chondrocytes from non-OA patients. As expected, treatment of NHAC-kn cells with 5% tensile strain at 0.5-Hz frequency reduced cytokine-induced expression *ADAMTS9* mRNA in these cells (Fig. 2).

3.3. Mechanical strain conditions

We examined the effects of different mechanical strain conditions on expression *ADAMTS9* mRNA induced by cytokines in OUMS-27 cells. The levels of *ADAMTS9* mRNA in cells treated with IL-1β and TNF-α decreased as the frequency increased (0.16, 0.33, and 0.5 Hz at 5% tensile strain; Fig. 3A) and tensile strain (2.0%, 3.0%, and 5.0% at 0.5-Hz frequency; Fig. 3B).

3.4. Pretreatment with integrin binding motif peptide does not influence response to mechanical strain

Previous studies have shown that integrin functions as a mechanosensor [10]. Integrin binds to the ECM and associates with cytoskeletal actin filaments via linker proteins to physically connect intra- and extracellular structures. We found here that pretreatment with the integrin binding motif sequence GRGDS and its analog GRDSP did not alter expression of *ADAMTS9* mRNA in OUMS-27 cells (Fig. 4), indicating that integrin binding is unlikely involved in the cellular response to mechanical strain.

3.5. Mechanosensor inhibitors abrogate the impact of mechanical strain

Pretreatment with gadolinium (a broad-spectrum mechanosensor inhibitor) reversed the negative effects of mechanical strain on cytokine-induced *ADAMTS9* upregulation (Fig. 5A), whereas gadolinium on its own had no effect on the expression of *ADAMTS9* mRNA. Similar

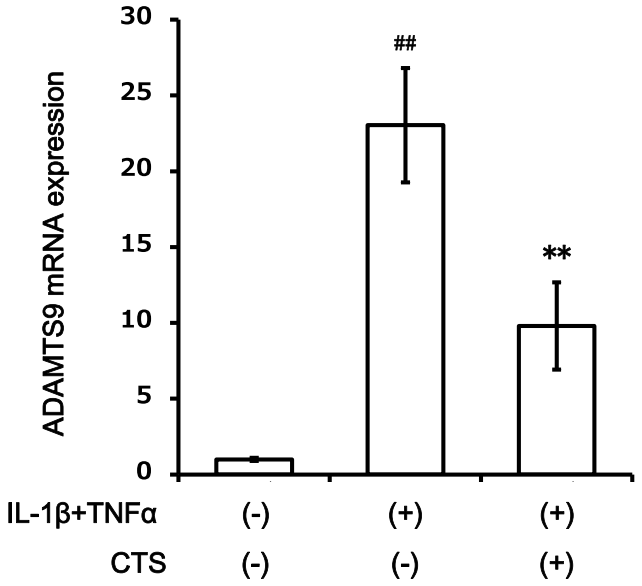

Fig. 2. CTS attenuates cytokine-induced expression of *ADAMTS9* mRNA in NHAC-kn cells. NHAC-kn cells were subjected to 5% tensile strain at a frequency of 0.5 Hz and treated with 10 ng/mL IL-1β and TNF-α for 6 h. The number *ADAMTS9* transcripts was measured relative to the levels of mRNA found in unstimulated the control cells by qRT-PCR. Values represent mean ± SD (n = 6 per group). $^{\#\#}$P < 0.01 vs. control; **P < 0.01 vs. cytokine-treated group.

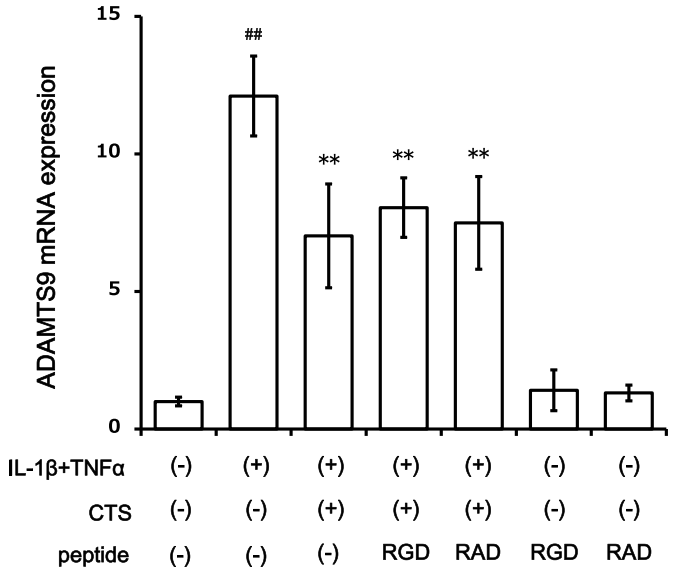

Fig. 4. Integrin binding motif peptide does not inhibit the effects of mechanical strain. Cells were pretreated with the integrin-binding peptide GRGDS (5 μg/mL) or its analog GRADSP (5 μg/mL) for 1 h and then cultured in the presence of IL-1β and TNF-α (both at 10 ng/mL) with cyclic tensile strain (5%, 0.5 Hz) for 6 h. The number of *ADAMTS9* transcripts was measured relative to the levels of mRNA found in unstimulated the control cells by qRT-PCR. Values represent mean ± SD (n = 6 per group). $^{\#\#}$P < 0.01 vs. control; **P < 0.01 vs. cytokine-treated group.

effects were observed with ruthenium red (TRPV family inhibitor) (Fig. 5B) and capsazepine (TRPV1 inhibitor) pretreatment. On the other hand, tranilast (TRPV2 inhibitor) and HC-067047 (TRPV4 inhibitor) pretreatment did not downregulate strain-induced expression of *ADAMTS9* mRNA in cytokine-treated cells (Fig. 5B).

3.6. TRPV silencing abolishes the effects of mechanical strain

To identify which TRPV family molecules mediate the effects of mechanical strain, we knocked down the expression of TRPV1, TRPV2, and TRPV4. Each of the siRNAs—but the not the scrambled control siRNAs—reduced the level of its target mRNA (Fig. 6B). Pretreatment with siRNAs (V1-1 and V1-2) against TRPV1 abolished the impact of mechanical strain in OUMS-27 cells (Fig. 6A); however, pretreatment with siRNAs against TRPV2 (V2-1, V2-3, and V2-3) and TRPV4 (V4-1, V4-2, and V4-3) did not alter the effects of mechanical strain (Fig. 6A). None of the siRNAs or the scrambled control RNAs used in this experiment affected cell viability or expression of *ADAMTS9* mRNA (data

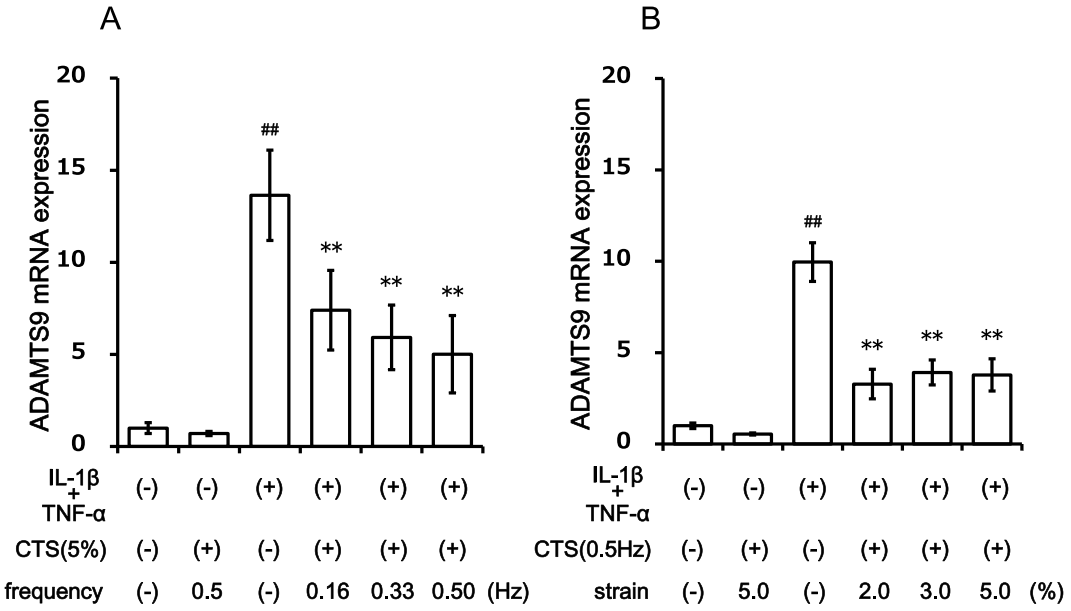

Fig. 3. CTS attenuates cytokine-induced expression of *ADAMTS9* mRNA in OUMS-27 cells. (A) Effect of frequency. Cells were subjected to 5% tensile strain at a frequency of 0, 0.16, 0.33, and 0.5 Hz and treated with 10 ng/mL IL-1β and TNF-α for 6 h. (B) Effect of tensile strain. Cells were treated 0.5 Hz of frequency with 0, 3.0 and 5.0 tensile strain along with 10 ng/mL IL-1β and TNF-α for 6 h. The number of *ADAMTS9* transcripts was measured relative to levels of mRNA number in the unstimulated cells by qRT-PCR. Values represent mean ± SD (n = 6 per group). $^{\#\#}$P < 0.01 vs. control; **P < 0.01 vs. cytokine-treated group.

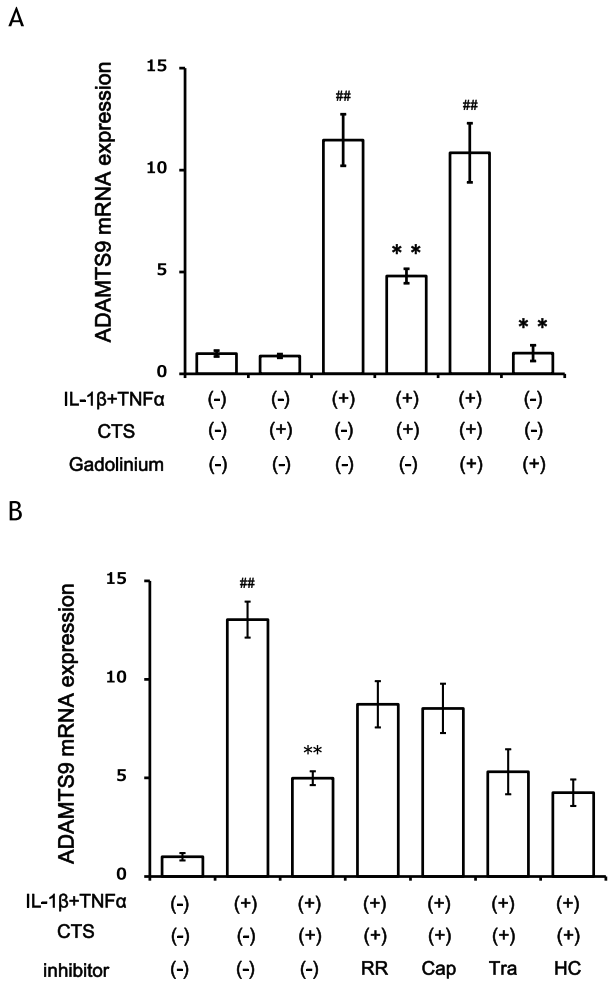

Fig. 5. Mechano-gated channel inhibition abrogates CTS attenuation. (A) Cells were pretreated with gadolinium (10 μM) for 1 h and then cultured in the presence of IL-1β and TNF-α (both at 10 ng/mL) with cyclic tensile strain (5%, 0.5 Hz) for 6 h. (B) Cells were pretreated with ruthenium red (10 μM), capsazepine (10 μM), tranilast (50 μM), or HC-067047 (5 μ M) for 1 h and then cultured in the presence of IL-1β and TNF-α (both at 10 ng/mL) with cyclic tensile strain (5%, 0.5 Hz) for 6 h. The number of *ADAMTS9* transcripts was measured relative to the levels of mRNA found in the unstimulated control cells by qRT-PCR. Values represent mean ± SD (n = 6 per group). $^{\#\#}P < 0.01$ vs. control; $^{**}P < 0.01$ vs. cytokine-treated group.

not shown).

3.7. Effects of inflammatory cytokines on intracellular calcium concentration in OUMS-27 cells

We examined whether inflammatory cytokines can influence intracellular calcium concentrations in OUMS-27 cells using Fluo-4 AM (Fig. 7). Treatment with IL-1β and TNF-α (both at 10 ng/mL) increased intracellular calcium content after 10 and 30 min of cytokine stimulation; however, after 60 min, the concentration declined.

3.8. BAPTA-AM suppresses expression of ADAMTS9 in OUMS-27 cells

We examined whether NF-κB inhibition by BAPTA-AM alters the expression of *ADAMTS9* mRNA and found that BAPTA-AM pretreatment decreased cytokine-induced expression of *ADAMTS9* after 6 h (Fig. 8).

3.9. Mechanical strain blocks cytokine-induced NF-κB nuclear translocation in OUMS-27 cells

We explored the intracellular mechanisms of mechanical strain by analyzing nuclear translocation of NF-κB—a key transcription factor involved in signal transduction of inflammatory cytokines—by immunofluorescence analysis. Rapid nuclear translocation of NF-κB was observed within 10 min of IL-1β and TNF-α treatment (Fig. 9); on the other hand, NF-κB was retained in the cytoplasm under mechanical strain for 6 h with IL-1β and TNF-α treatment (data not shown). In contrast, cells treated simultaneously with mechanical strain and cytokines exhibited strong suppression of IL-1β- and TNF-α-induced NF-κB nuclear translocation (Fig. 9).

3.10. Mechanical strain inhibits the cytokine-induced calcium increase in OUMS-27 cells

We investigated intracellular calcium kinetics by immunofluorescence analysis. Rapid calcium upregulation was observed within 10 min of adding IL-1β and TNF-α (Fig. 10). In contrast, cells subjected to CTS with simultaneous cytokine treatment showed strong suppression of the IL-1β- and TNF-α-induced increase in calcium content (Fig. 10). CST alone caused a slight elevation in intracellular calcium.

4. Discussion

In this study, we examined the effects of mechanical strain on inflammatory cytokine-induced expression of *ADAMTS9* mRNA and the underlying mechanisms in chondrocytes. We found that intracellular calcium and translocation of the NF-κB nuclear factor increased in the presence of cytokines, whereas mechanical strain reversed this effect via TRPV1, resulting in downregulation of *ADAMTS9*.

Cultured chondrocytes have been exposed to CTS stimulation at a wide range of strain magnitudes and durations with various systems [32–34]. Xu et al. (2000) reported that CTS acts as an antagonist of IL-1β actions in chondrocytes [32]. Although the mechanism has not yet been elucidated, they reported that CTS attenuates the induction of IL-1β-induced inflammatory responses. Interestingly, CTS had lower effect on inflammatory signals when inflammatory cytokines were not associated with it. Hayashi et al. (2015) reported that p21 plays a role in the expression of *MMP13* mRNA in response to CTS [10]. CTS increased *p21* expression and this effect was mediated by signal transducer and activator of transcription 3 (STAT3). Recently, Lohberger et al. (2019) reported that moderate tensile strain most effectively reduces *ADAMTS5* and *MMP13* expression levels in chondrocytes compared to strong tensile strain [33]. They also demonstrated that moderated tensile strain significantly decreases the expression of *IL-6*, a notable inflammatory marker. Their results suggested that different loading conditions cause different effects on the expression of inflammatory markers and MMPs/ADAMTSs in chondrocytes. Recently, Papadopoulou et al. (2019) reported that CTS immediately activates extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) in human periodontal ligament fibroblasts [34]. They also demonstrated that CTS induces *c-fos* activation. These accumulating evidences led to the hypothesis that CTS attenuates IL-1β-induced *ADAMTS9* expression. Notably, the effect of CTS is altered depending on its conditions such as magnitudes, durations, and frequency. Our data suggests that CTS (0.16–0.50 Hz with 5% elongation or 0.5 Hz with 2.0%–5.0% elongation) reduces levels of *ADAMTS9* mRNA in cytokine-stimulated chondrocytes (Fig. 3B). The duration of cytokine exposure and mechanical strain treatment was an important factor; the degree of attenuation was reduced by a shorter duration of mechanical strain (data not shown). Based on these data, we considered that the CTS condition used in the present study demonstrated the antagonistic effects in IL-1β stimulated chondrocytes via TRPV.

Previous in vitro and in vivo studies revealed that there is a stronger

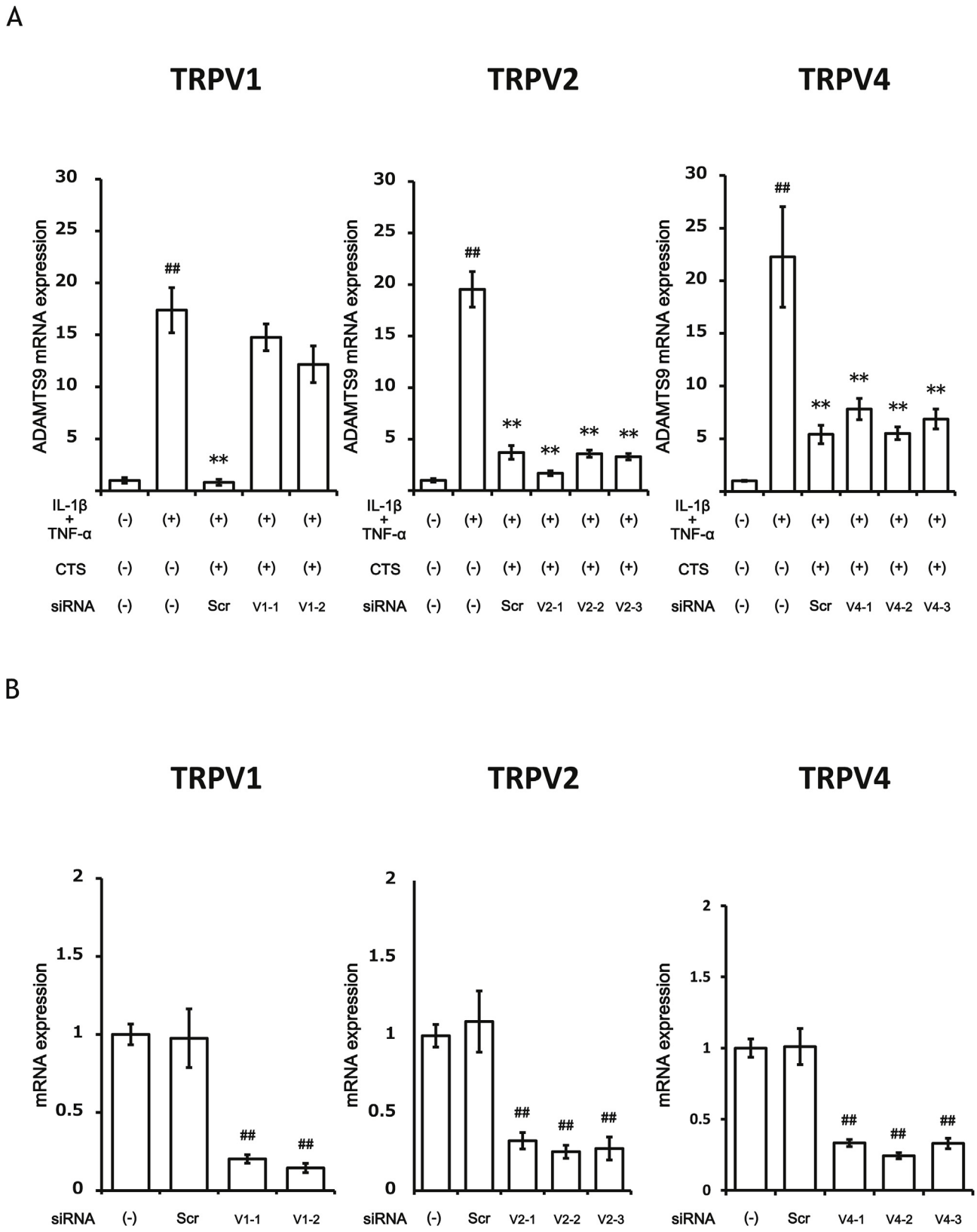

Fig. 6. TRPV knockdown abrogates CTS attenuation. Cells were transfected with siRNAs against TRPV1 (V1-1 and V1-2), V2 (V2-1, V2-2, and V2-3), and V4 (V4-1, V4-2 and V4-3) for 24 h and then cultured in the presence of IL-1β and TNF-α (both at 10 ng/mL) with cyclic tensile strain (5%, 0.5 Hz) for 6 h. The number of *ADAMTS9* transcripts was measured relative to the levels of mRNA number found in the unstimulated control cells by qRT-PCR. Values represent mean ± SD (n = 6 per group). $^{\#\#}P < 0.01$ vs. control; $^{**}P < 0.01$ vs. cytokine-treated group.

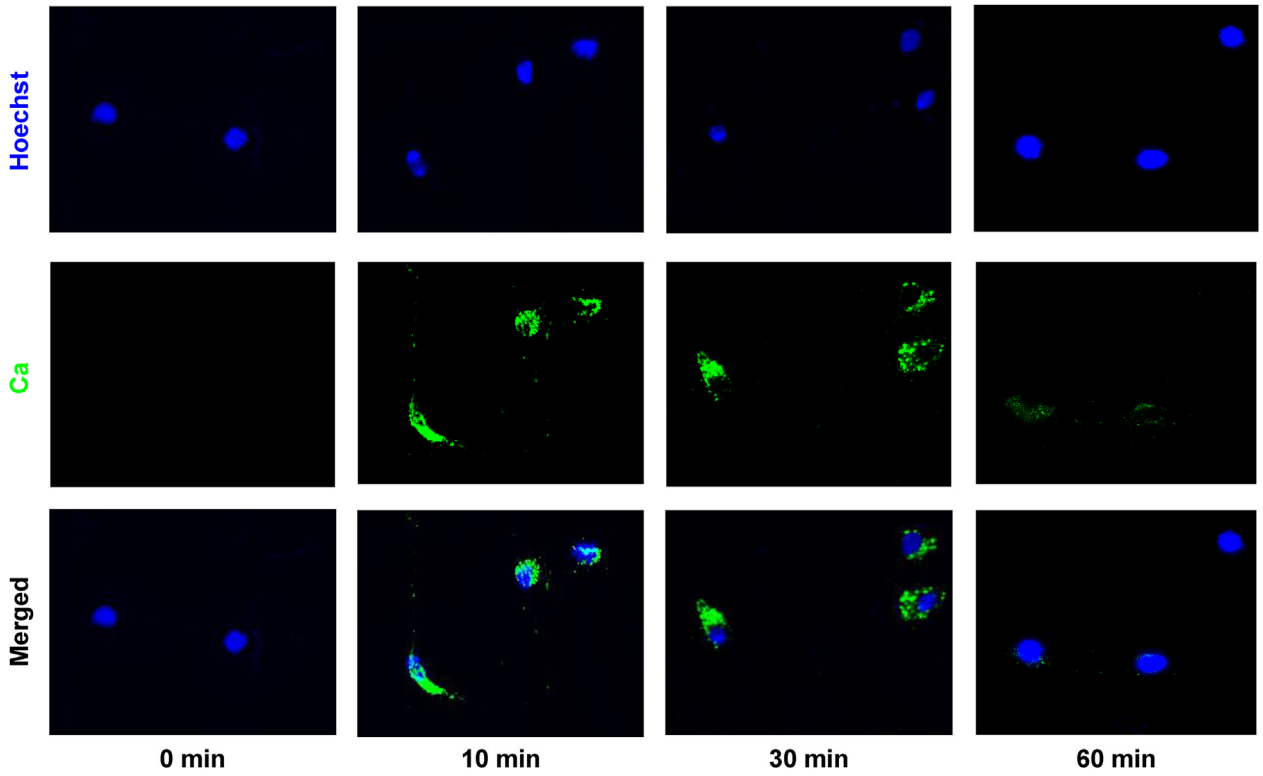

Fig. 7. Cytokine treatment increases intracellular calcium levels. OUMS-27 cells were seeded in glass-bottomed dishes for 48 h. After culturing cells overnight serum starvation, the cells were treated for 1 h with 5 μM Fluo-4 AM to visualize intracellular Calcium (green), followed by IL-1β and TNF-α (both at 10 ng/mL) for 10, 30, and 60 min. Cells were fixed by placing them in cold methanol for 30 min and in cold acetone for 10 min. After washing, the nuclei were stained with Hoechst 33258 (blue).

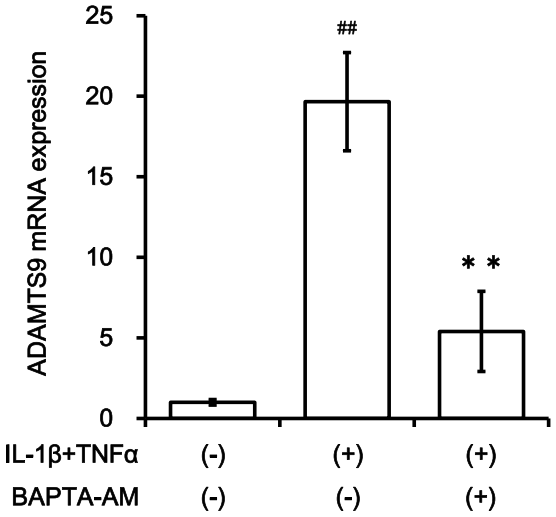

Fig. 8. BAPTA-AM attenuates cytokine-induced *ADAMTS9* expression in chondrocytes. OUMS-27 cells were treated with IL-1β and TNF-α (both at 10 ng/mL) for 6 h with or without 30 μM BAPTA-AM. The number of *ADAMTS9* transcripts was measured relative to the levels of mRNA found in the unstimulated control cells by qRT-PCR. Values represent mean ± SD (n = 6 per group). $^{\#\#}P < 0.01$ vs. control; $^{**}P < 0.01$ vs. cytokine-treated group.

expression of ADAMTS9 in chondrocytes than ADAMTS4 and ADAMTS5, indicating that it is more likely involved in articular cartilage degradation in OA. We have previously reported that nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1 (NFATc1) regulates expression of ADAMTS9 in chondrocytes via IL-1β, and that a treatment with the NFATc1-specific inhibitor reduces levels of ADAMTS9 [35], implying that expression of ADAMTS9 is regulated by different mechanisms. It is well known that NF-κB is widely used as transcription factor for matrix degradative molecules (ADAMTS4, 5, 9, 18, MMP-1, 2, 3, 8, 9, 13) under inflammatory stimulation [36]. It appears that NF-κB regulation is important for OA therapy. Yang et al. (2019) reported that CTS alleviates the chondrocyte damage induced by IL-1β by activating AMP-activated protein kinase (AMPK) phosphorylation and suppressing nuclear translocation of NF-κB [37]. They proposed that moderate AMPK, activated by moderated CTS, reduces intracellular ROS production, which inhibits nuclear translocation of NF-κB. Their data were consistent with our results; therefore, a similar underlying mechanism is suggested.

In order to analyze the molecular mechanism of OA, we measured the effects of mechanical strain on expression of *ADAMTS9* using NHAC-kn and OUMS-27 cell lines, which exhibit particular chondrocyte properties such as expression of the chondrocyte-specific ECM genes type II, IX, and XI collagen and aggrecan; moreover, treatment with IL-1β and TNF-α induced expression of *ADAMTS4* and *ADAMTS9* after 6 h in both cell lines, and with similar kinetics [6,17]. Aggrecan cleavage by ADAMTS was also observed in OUMS-27 cells (data not shown), suggesting that these cells can serve as a model for investigating the effects of cytokines on expression of ADAMTS.

Chondrocytes are surrounded by ECM. Integrins are transmembrane receptors that facilitate cell–ECM adhesion by binding specific integrin subtypes to activate signal transduction [11]. There is evidence that integrins function as mechanosensors [38]. CTS increased expression of collagen (*COL1A1* and *COL3A1*) via integrin αVβ3, which binds to the Arg-Gly-Asp (RGD) motif [11]. Integrins transduce mechanical signals in annulus fibrosus cells, whereas the RGD peptide suppresses phosphorylation of focal adhesion kinase induced by severe CTS (10%,

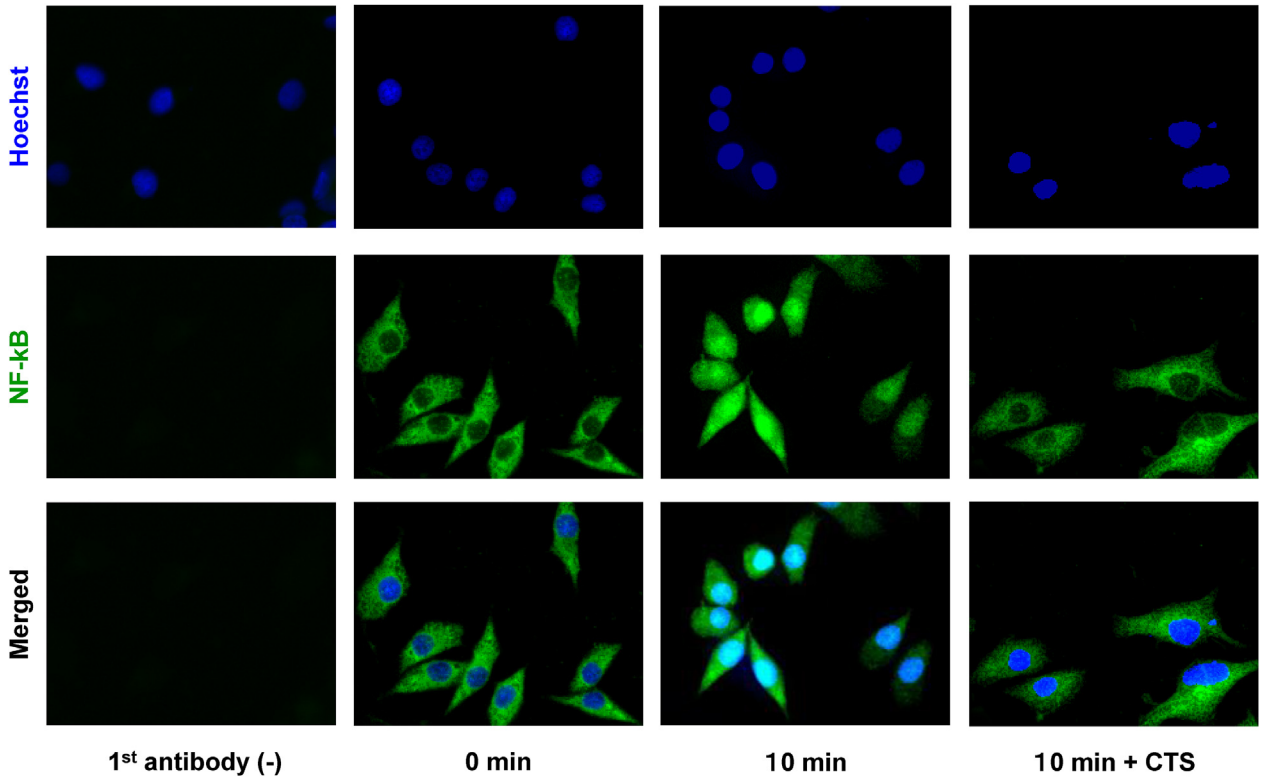

Fig. 9. Mechanical strain inhibits translocation of cytokine-induced NF-κB (p65) from cytoplasm to nucleus. OUMS-27 cells were seeded on a collagen-coated stretch chamber. After an overnight serum starvation, the cells were treated with IL-1β and TNF-α (both at 10 ng/mL) for 10 min with or without uniaxial strain (0.5-Hz cycles of stretch). Afterwards, the cells were fixed with cold methanol for 30 min followed by cold acetone for 10 min. After washing and blocking, NF-κB p65 (green) was detected by immunocytochemistry and nuclei were stained with Hoechst 33258 (blue).

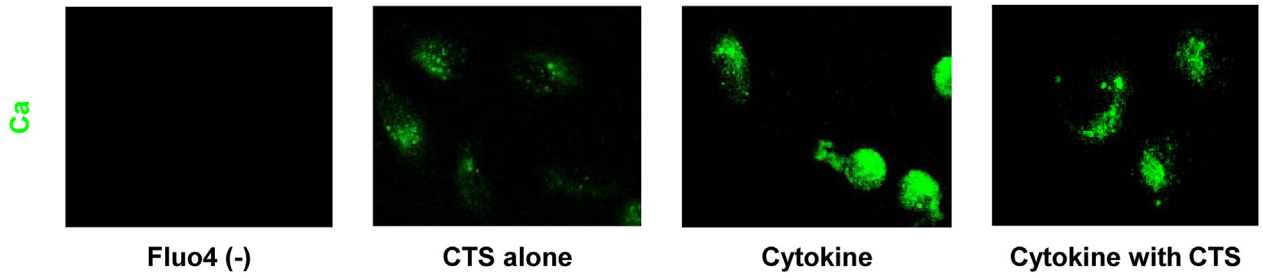

Fig. 10. Mechanical strain inhibits cytokine-induced calcium concentration in the cytoplasm. OUMS-27 cells were seeded on the collagen-coated stretch chamber. After an overnight starvation treatment, cells were treated with 5 μM Fluo-4 AM for 1 h to visualize intracellular Calcium (green), followed by IL-1β and TNF-α (both at 10 ng/mL) for 10 min with or without uniaxial strain (0.5-Hz cycles of stretch). The cells were fixed with cold methanol for 30 min, and cold acetone for 10 min.

1.0 Hz) [39]. Mechanical strain also induced hyperpolarization of the membrane in primary chondrocytes via α5β1 integrin, but could be reversed by RGD peptide [40]. On the other hand, we found that the RGD peptide did not attenuate cytokine-induced expression of *ADAMTS9* in OUMS-27 cells, implying that this integrin does not participate in this mechanism. On the other hand, pretreatment with gadolinium, which inhibits several types of Ca^+ channels [41], abrogated the impact of mechanical strain on expression of *ADAMTS9* mRNA, implying that calcium signaling is involved. We, therefore, focused on the function of calcium channels as possible mechanosensors using a variety of TRPV-specific inhibitors [42–46], and demonstrated that pretreatment with ruthenium red, a well-known TRPV family inhibitor [47], as well as capsazepine, a TRPV1 inhibitor, abolished the effects of mechanical stress. However, tranilast and HC-067047 (inhibitors of TRPV2 and TRPV4, respectively) did not have this effect. TRPV4 is highly expressed in both bone and cartilage [48,49]. Similarly, knocking down TRPV1 abrogated the effect of mechanical strain, whereas silencing other TRPV types (TRPV2 and TRPV4), on the other hand, did not. Based on these results, we conclude that TRPV1 mediates the effects of mechanical strain on expression of *ADAMTS9*.

Intracellular calcium levels are tightly controlled in cells. In their resting state, cells maintain a 20,000-fold gradient of calcium concentration between the cytosol (100 nM) and the extracellular space (mM) [50]. The rapid increase in calcium in the cytoplasm is achieved through a calcium influx from the extracellular environment or through its release from intracellular calcium stores such as the endoplasmic reticulum (ER). The ER is also a major intracellular calcium reservoir [51]. In chondrocytes, both mechanisms are critical for regulating physical stimulus-induced responses [52]. Even a single cycle of mechanical stress was found to instantly increase cytoplasmic calcium concentrations in OUMS-27 cells (data not shown). Our results clearly demonstrated that CTS decreased intracellular levels of cytokine-induced calcium and blocked cytokine-induced translocation of NF-κB from the cytoplasm to the nucleus in OUMS-27 cells. NF-κB translocation needs several steps; intracellular calcium up-regulation, Calcium/calmodulin-dependent protein kinase II (CaMKII) activation, IκB

phosphorylation and release from complex, NF-κB delivery to the nuclear membrane via molecular motor and few more steps [53]. Therefore, we propose the following hypothetic model to explain our results: translocation of NF-κB requires high concentrations of calcium for CaMKII activation; cytokines stimulate the release of calcium from the ER but mechanical strain activates TRPV1 in the plasma membrane, resulting in the release of calcium into the culture medium, NF-κB inactivation, attenuation of cytokine induced *ADAMTS9* expression.

In conclusion, mechanical strain attenuates cytokine-induced expression of *ADAMTS* and *MMP* in chondrocytes; the calcium channel and mechanosensor TRPV1 mediates the effect of mechanical strain on the expression of matrix-degrading enzymes. Our findings provide insight into the regulation of *ADAMTS9* mRNA expression in chondrocytes and suggest that mechanosensors such as TRPV can serve as therapeutic targets for treating OA.

Conflicts of interest disclosure

The authors have no conflicts of interest to disclose in relation to this manuscript.

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