# Histone deacetylase inhibitors suppress mechanical stress-induced expression of RUNX-2 and ADAMTS-5 through the inhibition of the MAPK signaling pathway in cultured human chondrocytes

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**Abstract**

***Objective:*** To investigate the inhibitory effects and the regulatory mechanisms of HDAC inhibitors on mechanical stress-induced gene expression of RUNX-2 and ADAMTS-5 in human chondrocytes

***Methods:*** Human chondrocytes were seeded in stretch chambers at a concentration of 5 × $10^4$ cells/chamber. Cells were pre-incubated with or without HDAC inhibitors (MS-275 or Trichostatin A; TSA) for 12 h, followed by uniaxial cyclic tensile strain (CTS) (0.5 Hz, 10% elongation), which was applied for 30 min using the ST-140-10 system (STREX, Osaka, Japan). Total RNA was extracted and the expression of RUNX-2, ADAMTS-5, MMP-3, and MMP-13 at the mRNA and protein levels were examined by real-time PCR and immunocytochemistry, respectively. The activation of diverse mitogen-activated protein kinase (MAPK) pathways with or without HDAC inhibitors during CTS was examined by western blotting.

***Results:*** HDAC inhibitors (TSA: 10 nM, MS-275: 100 nM) suppressed CTS-induced expression of RUNX-2, ADAMTS-5, and MMP-3 at both the mRNA and protein levels within 1 h. CTS-induced activation of p38, ERK, and JNK MAPKs was downregulated by both HDAC inhibitors.

***Conclusion:*** The CTS-induced expression of RUNX-2 and ADAMTS-5 was suppressed by HDAC inhibitors via the inhibition of the MAPK pathway activation in human chondrocytes. The results of the current study suggested a novel therapeutic role for HDAC inhibitors against degenerative joint disease such as osteoarthritis.

## Introduction

The main extracellular matrix (ECM) macromolecules of the articular cartilage are type II collagen and aggrecan [1, 2]. The disease progression of osteoarthritis (OA) is a highly complicated process involving multiple events, including aggrecan and type II collagen degradation that is caused by increased cleavage due to the activation of proteolytic enzymes, such as matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs). MMPs are induced in chondrocytes in response to various stimuli, such as proinflammatory cytokines or mechanical load, and then cleave a variety of ECM components, including proteoglycans, collagens, and procollagens [3]. Currently, ADAMTS-5 is the most efficient in terms of its proteolytic activity, as previous studies have suggested that ADAMTS-5 may play a pivotal role in the OA pathogenesis [4-6]. Loss of type II collagen and aggrecan degradation are two of the earliest events in the course of OA following mechanical injury of collagen fibrils [7].

Runt-related transcription factor (RUNX) family members regulate gene expression involved in cellular differentiation and cell cycle progression. RUNX-2 plays a key role in bone mineralization by stimulating osteoblast differentiation [8] and contributes to OA pathogenesis through chondrocyte hypertrophy and matrix breakdown after the initiation of joint instability [9]. Mitogen-activated protein kinase (MAPK) pathways play essential regulatory roles in early osteoblast differentiation in response to mechanical stress via the activation of RUNX-2 [10-13]. Several MAPKs, such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK (p38), have been reported to play a role in osteoblast differentiation [14-16]. We

previously reported that mechanical stress-induced expression of RUNX-2 and ADAMTS-5 is regulated by p38 in a SW1353 human chondrosarcoma cell line [17].

Recent reports have shown that the epigenetic regulation of gene expression may be a novel therapeutic approach for arthritis [18, 19]. Histone deacetylase (HDAC) inhibitors have emerged as a promising new class of anticancer drugs based on their ability to activate a variety of genes implicated in the regulation of cell survival, proliferation, and apoptosis [20-23]. We previously showed that the modification of histone acetylation by HDAC inhibitors can successfully ameliorate synovial inflammation via the upregulation in synovial fibroblasts of cell cycle regulators in an animal arthritis model [24]. Interestingly, the expression of MMP-3 and MMP-13 were effectively downregulated, leading to the abrogation of cartilage destruction in mouse models [25]. However, whether HDAC inhibitors directly contribute to the prevention of cartilage matrix degradation has not been fully elucidated.

In the current study, the effect of HDAC inhibitors on the mechanical stress-induced gene expression of RUNX-2, ADAMTS-5, and MMP-3 was examined in vitro using human chondrocytes. Our findings provide further evidence that HDAC inhibitors may have a role in the suppression of cartilage degeneration through the inhibition of mechanical stress-induced proteolytic enzymes.

## Materials and methods

*Cells and cell culture*

Normal human articular chondrocytes (NHAC-kn cells) obtained from a 45-year-old male were purchased from Lonza (Walkersville, MD, USA). Cells were cultured in 15 mL of chondrocyte basal medium (CBM; Lonza) containing supplements and several growth factors [Revitropin - long R3 insulin-like growth factor (R3-IGF-1), basic fibroblast growth factor (bFGF), tranferrin, insulin, fetal bovine serum (FBS), and gentamicin/amphotericin-B; CGM$^{TM}$ singleQuots®, Lonza] and were then subcultured at split ratios of 1:3 using trypsin plus ethylenediaminetetraacetic acid (EDTA) every 6−7 days. The medium was changed every 3 days. The cells were subcultured for two passages and cells at the third passage were used for experiments within 2 weeks after starting the cell cultures. For all experiments, human chondrocytes were transferred to serum-free α-modified minimum essential medium (MEMα, Wako, Osaka, Japan) for 12 h before exposure to different stimuli.

*Cyclic tensile strain on chondrocytes cultured in monolayer*

Human chondrocytes were seeded in stretch chambers coated with fibronectin at a concentration of $5 \times 10^4$ cells/chamber; each chamber had a culture surface of 2 × 2 cm. Mechanical stresses were applied using the ST-140-10 mechanical stretch system (STREX, Osaka, Japan). The chamber was attached to the stretching apparatus, which has one fixed side opposite a movable side that can be driven by a computer-controlled motor. By using this apparatus, the entire silicon membrane area and almost all cells on the stretch chambers can be stretched uniformly [26, 27]. After culturing for 48 h, the cells increased to 60% confluence in the chamber. In the current study, a cyclic tensile

115 strain (CTS; 0.5 Hz, 10% elongation) was applied for 30 min according to our previous
116 study [17]. To apply mechanical stress, cells were cultured in stretch chambers, and set
117 on the ST-140-10 system in an incubator (Supplemental Fig. 1). Cells without mechanical
118 stress were seeded on the same chambers, and used as controls.

119 *Treatment with HDAC inhibitors*

120 We used two HDAC inhibitors: trichostatin A (TSA; Sigma-Aldrich, Oakville,
121 Ontario, Canada), a general HDAC inhibitor, and MS-275 (Cayman Chemical, Ann
122 Arbor, MI, USA), a class I HDAC specific inhibitor. All inhibitors were used at various
123 concentrations for 12 h before CTS. TSA and MS-275 were dissolved in dimethyl
124 sulfoxide (DMSO) and then diluted with phosphate buffered saline (PBS) to a working
125 concentration up to 500 μM.

126 *Cell proliferation assay*

127 Cells were incubated with or without TSA and MS-275 at various concentrations
128 (10, 100, 500 nM) for 12 h before CTS. Cell viability was evaluated at 1 h after CTS,
129 using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
130 bromide, a yellow tetrazole) assay (Chemicon, Temecula, CA, USA) according to the
131 manufacturer's instructions. The experiments were repeated 6 times.

132 *RT-PCR and real-time PCR analysis*

133 We examined the effect of CTS and HDAC inhibitors on RUNX-2, MMP-3,
134 MMP-13, and ADAMTS-5 expression in human chondrocytes by reverse transcription
135 polymerase chain reaction (RT-PCR) and real-time PCR. The half-life of the type II

collagen α1 chain (COL2A1) mRNA is reported to be approximately 15 h [28, 29]; therefore, we used COL2A1 expression using real-time PCR at 15 h after CTS as a positive control.

After stimulation, the cells were washed with PBS, and total RNA was extracted using ISOGEN reagent (Nippon Gene, Toyama, Japan), according to the manufacturer's protocol. The concentration and purity of total RNA were assayed by spectrophotometry. To make complementary DNA (cDNA), 1 μg of total RNA was reverse transcribed using ReverTra Ace, a Moloney murine leukemia virus reverse transcriptase, with Oligo-dT primers, according to the manufacturer's instructions (TOYOBO, Tokyo, Japan). The cDNA was PCR-amplified using 10 pmol of each specific primer and ExTaq DNA polymerase (TAKARA BIO, Shiga, Japan). The sequences of the oligonucleotide primers are shown in Table 1 (COL2A1, RUNX-2, MMP-3, MMP-13, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and ADAMTS-5). For all RT-PCR fragments, the reactions were allowed to proceed for 35 cycles (30 cycles for G3PDH) in a T3000 thermocycler (Biometra, Göttingen, Germany).

Real-time PCR was performed using an Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA, USA) with Brilliant III Ultra-Fast SYBR Green QPCR Master Mix. The PCR mixture was in a total volume of 20 μL and consisted of 1× SYBR Green PCR Master Mix, which included DNA polymerase, SYBR Green dye, dNTPs (including dUTP), PCR buffer, 10 pmol each of the forward and reverse primers, and cDNA of the samples. Amplification of a housekeeping gene, G3PDH, was used for normalizing the efficiency of cDNA synthesis and the amount of RNA. We calculated the final expression levels by dividing the expression level of RUNX-2, MMP-3, MMP-13,

and ADAMTS-5 by the expression level of G3PDH. Each value obtained for the control cells (un-stretched cells without HDAC inhibitors) was set to 1.

*Immunocytochemistry*

Immunocytochemistry was used to observe the mechanical-stress induced expression and localization of RUNX-2 and ADAMTS-5. Cells were loaded for 30 min by CTS (0.5 Hz, 10% elongation) with or without HDAC inhibitors (TSA: 10 nM, MS-275: 100 nM) and then fixed with 1% paraformaldehyde solution. The chambers were incubated with anti-RUNX-2 antibody (10 mg/mL, ab76956, Abcam, Cambridge, UK) and anti-human ADAMTS-5 antibody (10 mg/mL, R&D Systems, Minneapolis, MN, USA) for 120 min at room temperature. Bovine serum albumin-containing solutions without primary antibodies were used as negative controls. We used Alexa Fluor 488-conjugated antibody (10 mg/mL, anti-mouse), Alexa Fluor 568-conjugated phalloidin (2 mg/mL, Molecular Probes, Eugene, OR, USA), and Hoechst 33342 (1 mg/mL, ICN Biomedicals, Aurora, OH, USA) as secondary antibodies. Samples were evaluated under a fluorescence microscope (Leica, Wetzlar, Germany), and protein expression was evaluated by the positive cell ratio of RUNX-2 or ADAMTS-5 (number of positive cells / all cells). The cell number counting was done in 4 fields, at ×100 magnification, and averaged.

*Western blot analysis and protein kinase inhibitor assay*

All cells were incubated in 1 mL of CBM containing 10% FBS on 2 × 2 cm stretch chambers. After 24 h, the medium was changed to serum-free MEMα, and the cells were incubated overnight. For western blot analysis, cells were stretched by CTS

(0.5 Hz, 10% elongation) for 30 min using the ST-140-10 system with or without HDAC inhibitors (TSA: 10 nM, MS-275: 100 nM). Cell lysates (10 μg of total protein/lane, 15 μg/lane for RUNX-2 and phospho-RUNX-2) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% gel and then transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were incubated with blocking reagent (TOYOBO**)** and incubated overnight at 4°C with anti-p38, ERK1/2, JNK MAPK, RUNX-2 (Abnova, Taipei, Taiwan), anti-phospho-p38, p44/42, JNK MAPK (Cell Signaling Technology, Beverly, MA, USA), anti-phospho-RUNX-2 (Cell Signaling Technology), histone H3, and acetyl-histone H3 (Lys9) (Cell Signaling Technology) antibodies at a dilution of 1:1000 (1:2000 for phospho-p38, histone H3, and acetyl-histone H3) in CanGet Signal Immunoreaction Enhancer Solution (TOYOBO). After washing, the membranes were stained with the appropriate horseradish peroxidase-conjugated anti-mouse secondary antibody (diluted 1:10000, R&D Systems) or anti-rabbit secondary antibody (diluted 1:10000, Bethyl Laboratories, Montgomery, TX, USA) at room temperature for 1 h. We detected immunoreactive proteins using the Enhanced Chemiluminescence (ECL) Detection System (GE Healthcare, Buckinghamshire, UK).

*Statistical analysis*

All data were expressed as the means ± 95% confidence intervals (CI). All experiments were repeated at least 5 times and similar results were acquired. Differences among groups were analyzed using a one-way analysis of variance (ANOVA) with Bonferroni post-hoc test (SPSS Inc., Chicago, IL, USA). A P-value $< 0.05$ was considered statistically significant.

## Results

*HDAC inhibitors have an effect on the viability of cultured human chondrocytes*

Both TSA and MS-275, at concentrations of 500 nM, inhibited chondrocytic proliferation to about 50% (Table. 2). MS-275 concentrations of 10 and 100 nM and TSA concentration of 10 nM did not reduce cell proliferation. The results of western blotting confirmed that histone H3 was acetylated by TSA or MS-275 at 10, 100, 500 nM concentrations without CTS (Supplemental Fig. 2).

*HDAC inhibitors upregulate COL2A1 and downregulate CTS-induced RUNX-2, ADAMTS-5, and MMP-3 gene expression*

The human chondrocytes used in this study continued to express type II collagen, even though they were grown in monolayers. COL2A1 expression in human chondrocytes was decreased by CTS and increased after treatment with 10 nM of TSA and 100 nM of MS-275. These effects were not noted after 100 nM or 500 nM of TSA treatment, and COL2A1 expression was downregulated by 500 nM of MS-275. RUNX-2, ADAMTS-5, MMP-3, and MMP-13 expressions were upregulated by CTS and CTS-induced expression of RUNX-2, ADAMTS-5, and MMP-3 were downregulated by TSA or MS-275 at concentrations of 10, 100, 500 nM (Fig. 1). CTS-induced expression of MMP-13 was downregulated by MS-275 at concentrations of 10, 100, 500 nM. These results lead us to perform subsequent experiments with TSA at 10 nM and MS-275 at 100 nM.

The results of real-time PCR showed that COL2A1 expression was increased by 10 nM of TSA and 100 nM of MS-275, regardless of CTS addition. CTS-induced upregulation of RUNX-2, ADAMTS-5, and MMP-3 were significantly downregulated by

the treatment with 10 nM of TSA and 100 nM of MS-275. Treatment of the cells by 10 nM of TSA and 100 nM of MS-275 without CTS did not affect the expression of RUNX-2, ADAMTS-5, and MMP-3 (Fig.2A-D). These results were confirmed using human chondrocyte-like cells (SW1353) and NHAC-kn cells from an 18-year-old male, and obtained similar results (data not shown). MMP-13 expression tended to be upregulated by CTS and downregulated by HDAC inhibitors, however, the differences were not significant (Fig. 2E).

*HDAC inhibitors suppress CTS-induced increases in RUNX-2 and ADAMTS-5 protein expression*

RUNX-2 expression was upregulated and localized to the nucleus following CTS without HDAC inhibitors, but was not upregulated by incubation with HDAC inhibitors (Fig. 3A, RUNX-2, green signals). Similarly, ADAMTS-5 expression was upregulated and localized in the cytoplasm after CTS without HDAC inhibitors, but was not upregulated with HDAC inhibitors (Fig. 3A, ADAMTS-5, green signals). The positive cell ratios for RUNX-2 and ADAMTS-5 after CTS without HDAC inhibitors were 70.3 ± 10.5% and 67.4 ± 10.6%, respectively. The positive cell ratios in the other models were < 9.1 ± 7.1%.

*HDAC inhibitors regulate the activation of RUNX-2 in human chondrocytes*

We investigated the change in RUNX-2 phosphorylation with and without HDAC inhibitors by western blotting. RUNX-2 phosphorylation increased after CTS and was significantly suppressed by HDAC inhibitors ($P < 0.001$) (Fig. 4).

*HDAC inhibitors regulate the activation of MAPK in human chondrocytes*

Mechanical stress can activate stress response signaling pathways**,** such as MAPK**.** Thus, we investigated MAPK phosphorylation (p38, JNK, and ERK) with and without HDAC inhibitors after CTS by western blotting. CTS significantly increased the phosphorylation of ERK and p38 at 60 min compared to the non-stretched controls ($P < 0.001$) (Fig. 5A, C). HDAC inhibitors significantly inhibited the mechanical stress-induced phosphorylation of ERK and p38 ($P < 0.001$). JNK phosphorylation tended to increase compared to non-stretched controls ($P = 0.158$) and was inhibited by HDAC inhibitors (TSA: $P < 0.001$, MS-275: $P = 0.534$) (Fig. 5B).

## Discussion

Chondrocytes are responsive to mechanical stress at both the protein and mRNA levels. During normal movement, articular cartilage experiences compression loads of up to 15%, which results in up to a 5% increase in chondrocytic elongation [30]. Although chondrocytes are directly compressed during the loading of normal cartilage, it is very probable that the matrix components of the ECM network, which are connected to the chondrocytes, stretch the cells during compression of cartilage [31].

It is clear that the protein catabolic enzymes, such as MMPs and ADAMTSs, play important roles in the degradation of cartilage. MMPs and ADAMTS inhibitors do not have an obvious beneficial effect on OA due to side effects, such as ostealgia, myalgia, and tendovaginitis [32]. Therefore, the upstream regulators of these enzymes are potentially key candidates for the targeted OA therapy. However, the regulatory mechanisms of mechanical stress on these enzymes are largely unknown.

The ADAMTS-5 promoter has a RUNX-2 binding site [33], suggesting that ADAMTS-5 is a potential downstream target of RUNX-2. Our report has shown that RUNX-2 is an upstream regulator of the mechanical stress-induced ADAMTS-5, suggesting that RUNX-2 could be a target gene in matrix degradation [17].

HDAC inhibitors have been investigated as anti-cancer compounds, largely by virtue of their influence on the cell cycle and apoptosis in transformed cells [34]. Recent reports suggested the efficacy of HDAC inhibitors as a therapy for arthritis. The action of HDAC inhibitors on cytokine-induced chondrocyte gene expression was first demonstrated by Young et al. [35], who reported that the interleukin (IL)-1α/oncostatin

M (OSM)-induced expressions of MMPs and ADAMTSs were suppressed by TSA in human chondrosarcoma cell lines and human chondrocytes. MMP-13 expression was reportedly controlled by MAPK through RUNX-2 activation [17, 36]. Furthermore, several reports showed that nuclear factor (NF)-κB pathways activated by IL-1 and tumor necrosis factor (TNF)-α regulated MMP-13 expression [37, 38]. In our study, CTS-induced MMP-13 expression was not significantly downregulated by either HDAC inhibitors, nevertheless MAPK and RUNX-2 activation were suppressed. MMP-13 expression was not upregulated significantly within 1 h after CTS, which is consistent with our previous result that significant MMP-13 upregulation was seen 12-24 h after CTS [17]. Hence, MMP-13 expression might be influenced by CTS-activated NF-κB pathways or other cytokines, and our experimental protocol in the current study to examine the early response to CTS might have failed to detect the later changes of MMP-13 and the influence of HDAC inhibitors.

In the present study, CTS-induced RUNX-2, ADAMTS-5, and MMP-3 mRNA expression were downregulated by treatment with HDAC inhibitors. In contrast, treatment with HDAC inhibitors led to an upregulation of COL2A1 mRNA expression. Therefore, treatment with HDAC inhibitors can both decrease catabolic effects and increase anabolic effects. Huh et al. [39] reported HDAC-induced COL2A1 suppression in rabbit chondrocytes. Whereas, Furumatsu et al. [40] showed that HDAC enhanced COL2A1 expression in human chondrocytes. This discrepancy may be caused by species difference. We used human chondrocytes and our results were consistent with those of Furumatsu et al., who demonstrated that histones H3/H4 around the COL2A1 enhancer region were highly acetylated by HDAC inhibitor treatments by chromatin immunoprecipitation assays. HDAC inhibitor at concentrations of 500 nM decreased cell

proliferation by approximately half (Table 2). Hence, COL2A1 might be upregulated by TSA at a concentration of 10 nM or MS-275 at a concentration of 100 nM, while downregulated at higher concentrations probably by decreasing cell proliferation and viability.

The MAPK pathway, involving p38, JNK, and ERK activity, has been shown to be modulated by diverse external stimuli, such as cytokines and physical stresses, which are transduced to the intracellular environment by mechanoreceptors [13, 41]. RUNX-2 regulation by mechanical stress is thought to be mediated by specific MAPK pathways [16, 17, 42-44]. In particular, MEK/ERK signaling showed a strong correlation between cell surface integrin activation and subsequent stimulation of RUNX-2-dependent transcription [11-13]. RUNX-2 activity is controlled by phosphorylation; another group demonstrated that RUNX-2 was phosphorylated by ERK1/2 and p38 in response to mechanical stress [16, 17]. We previously reported that p38 was phosphorylated by mechanical stress in a human chondrosarcoma cell line (SW1353) [17]. Here, we demonstrated that ERK1/2, p38, and JNK were phosphorylated by mechanical stress in human chondrocytes. This discrepancy may be explained by cell type differences.

Previous studies suggested that HDAC inhibitors regulate the MAPK pathway in several cancer cell lines [45-48]. It was reported that phosphorylation of ERK and JNK was decreased following the treatment of human K562 leukemia cells with butyrate [48]. Another report showed that valproic acid (VPA) and TSA blocked ERK and Akt activation in mouse C3H10T1/2 fibroblasts and that TSA also downregulated JNK phosphorylation [47]. In Ras-transformed 10T1/2 cells, Fecteau et al. [49] showed that the HDAC inhibitor FR901228 also suppressed the ERK and p38 pathways. Therefore,

HDAC inhibitors can modulate a number of intracellular signaling cascades in response to mechanical stress in human normal chondrocytes. The current study demonstrated that HDAC inhibitors reduced CTS-induced phosphorylation of ERK1/2, p38, and JNK in human chondrocytes. These findings suggested that HDAC inhibitors suppress RUNX-2 and ADAMTS-5 expression by downregulating MAPK signaling. Therefore, the current study is the first to demonstrate that HDAC inhibitors decrease mechanical stress-induced MAPK phosphorylation and the resulting catabolic effects; however, the precise mechanism of this decrease remains unknown. Previous reports have suggested that HDAC inhibitors induce apoptosis in several types of tumor cells through cell cycle arrest mediated by the cyclin-dependent kinase (CDK) inhibitor $p21^{WAF1/Cip1}$ [20-22, 50-52]. We previously showed that HDAC inhibitors increase the expression of the CDK inhibitors p16 and p21 [24]. Other groups have reported that p21 suppressed p38 activity and reduced the secretion of proinflammatory cytokines in Toll-like receptor-stimulated macrophages [53] and that p21 suppressed JNK activity and the IL-1-triggered activation of IL-6, IL-8, MCP-1, MIP-3α, MMP-1 and -3 in rheumatoid synovial fibroblasts [54]. These results suggested that the activation of CDK inhibitors by HDAC inhibitors may contribute to the suppression of the MAPK pathway.

There are several limitations in the current study. First, the stretch system was a simple experimental model for OA in vitro experiments using cells in a monolayer; however, it remains unknown if HDAC inhibitors influence chondrocytes cultured in three dimensions and the efficacy of HDAC inhibitors for articular cartilage protection in vivo. Second, it was not clear which HDAC is the therapeutic target for cartilage degradation. Based on the result that both TSA and MS-275 showed inhibitory effects on the mechanical stress-induced expression of catabolic enzymes, it is reasonable to

consider that the therapeutic candidates are included in class I HDACs, such as HDAC 1, 2, 3, and 8. Third, further investigations are required to demonstrate the efficacy of HDAC inhibitors using in vivo experiments with an animal OA model of cartilage destruction.

In conclusion, the results of the current study demonstrated that HDAC inhibitors may function as potent repressors of the expression of matrix-degrading proteases, such as ADAMTS-5 and MMP-3, induced by mechanical stress via the inhibition of RUNX-2 and the activation of MAPK in chondrocytes.

## Author contributions

Conception and study design: Nishida K,

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All other authors contributed to writing and revising the manuscript for scientific content and approved the final version before submission.

## Conflict of interest

The authors declare no conflicts of interest.

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Table 1

| *Gene* | | Nucleotide sequence | NCBI Gene No. | Annealing Tm |
|---|---|---|---|---|
| *COL2A1* | F | AAT TCC TGG AGC CAA AGG AT | NM_ 001844 | 55˚C |
| | R | AGG ACC AGT TGC ACC TTG AG | | |
| *RUNX-2* | F | CTC TAC CAC CCC GCT GTC TT | NM_ 004348 | 55˚C |
| | R | CAC CTG CCT GGC TCT TCT TAC | | |
| *ADAMTS-5* | F | TAT GAC AAG TGC GGA GTA TG | NM_ 007038 | 60˚C |
| | R | TTC AGG GCT AAA TAG GCA GT | | |
| *MMP-3* | F | ATG CCC ACT TTG ATG ATG ATG AAC | NM_ 002422 | 60˚C |
| | R | CCA CGC CTG AAG GAA GAG ATG | | |
| *MMP-13* | F | ACC CTG GAG CAC TCA TGT TTC CTA | NM_ 002427 | 60˚C |
| | R | TGG CAT CAA GGG ATA AGG AAG GGT | | |
| *G3PDH* | F | CAT CAA GAA GGT GGT GAA GCA G | NM_ 002046 | 60˚C |
| | R | CGT CAA AGG TGG AGG AGT GG | | |

Table 2

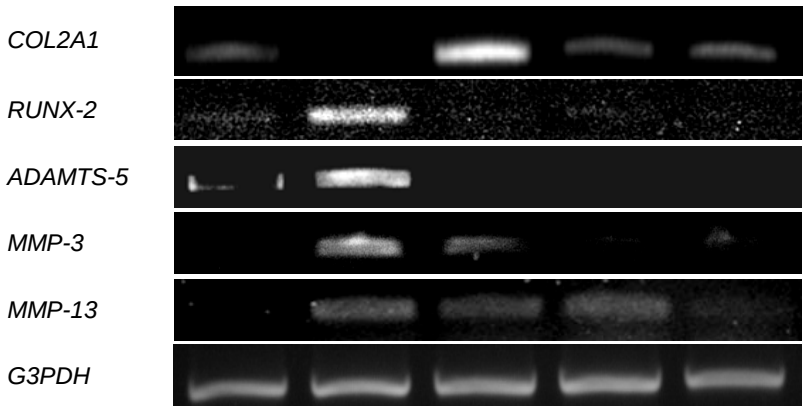
The effects of TSA and MS-275 on the viability of human chondrocytes as determined by the MTT assay

| CTS | - | + | + | + | + | + | + | + |
|---|---|---|---|---|---|---|---|---|
| TSA | - | - | 10 | - | 100 | - | 500 | - |
| MS-275 | - | - | - | 10 | - | 100 | - | 500 |
| Mean folds of control | 1.00 | 1.23* | 1.11* | 1.33* | 0.88* | 1.37* | 0.54* | 0.56* |
| 95% CI | | 1.16 - 1.30 | 1.03 - 1.18 | 1.25 - 1.40 | 0.80 - 0.95 | 1.29 - 1.44 | 0.46 - 0.61 | 0.49 - 0.63 |

The data are presented as the mean with 95% confidence intervals (CI) of 6 times determinations. *P < 0.01, relative to CTS (-) and HDAC inhibitors (-).

Figure 1

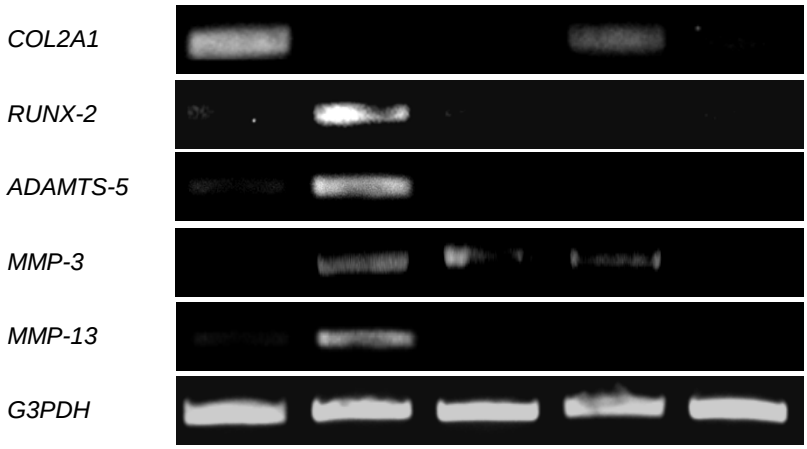
A




| | | | | | |
|---|---|---|---|---|---|
| CTS | - | + | + | + | + |
| TSA | - | - | 10 | 100 | 500 |

(nM)

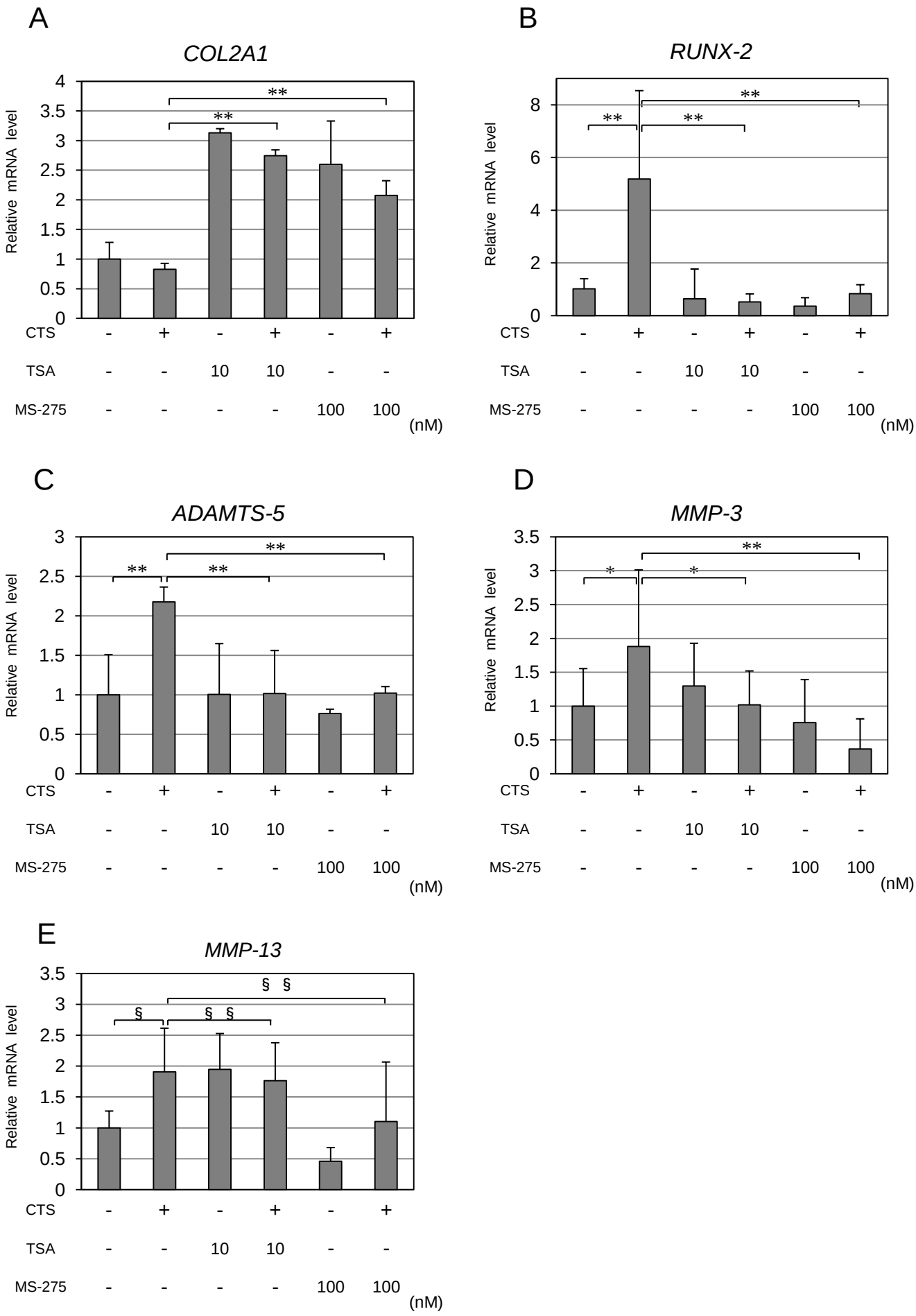
B




| | | | | | |
|---|---|---|---|---|---|
| CTS | - | + | + | + | + |
| MS-275 | - | - | 10 | 100 | 500 |

(nM)

Figure 2


A
COL2A1
Relative mRNA level
4
3.5
3
2.5
2
1.5
1
0.5
0
**
**
CTS - + - + - +
TSA - - 10 10 - -
MS-275 - - - - 100 100
(nM)
B
RUNX-2
Relative mRNA level
8
6
4
2
0
**
**
**
CTS - + - + - +
TSA - - 10 10 - -
MS-275 - - - - 100 100
(nM)
C
ADAMTS-5
Relative mRNA level
3
2.5
2
1.5
1
0.5
0
**
**
**
CTS - + - + - +
TSA - - 10 10 - -
MS-275 - - - - 100 100
(nM)
D
MMP-3
Relative mRNA level
3.5
3
2.5
2
1.5
1
0.5
0
*
*
**
CTS - + - + - +
TSA - - 10 10 - -
MS-275 - - - - 100 100
(nM)
E
MMP-13
Relative mRNA level
3.5
3
2.5
2
1.5
1
0.5
0
§
§ §
§ §
CTS - + - + - +
TSA - - 10 10 - -
MS-275 - - - - 100 100
(nM)

# Figure 3

A

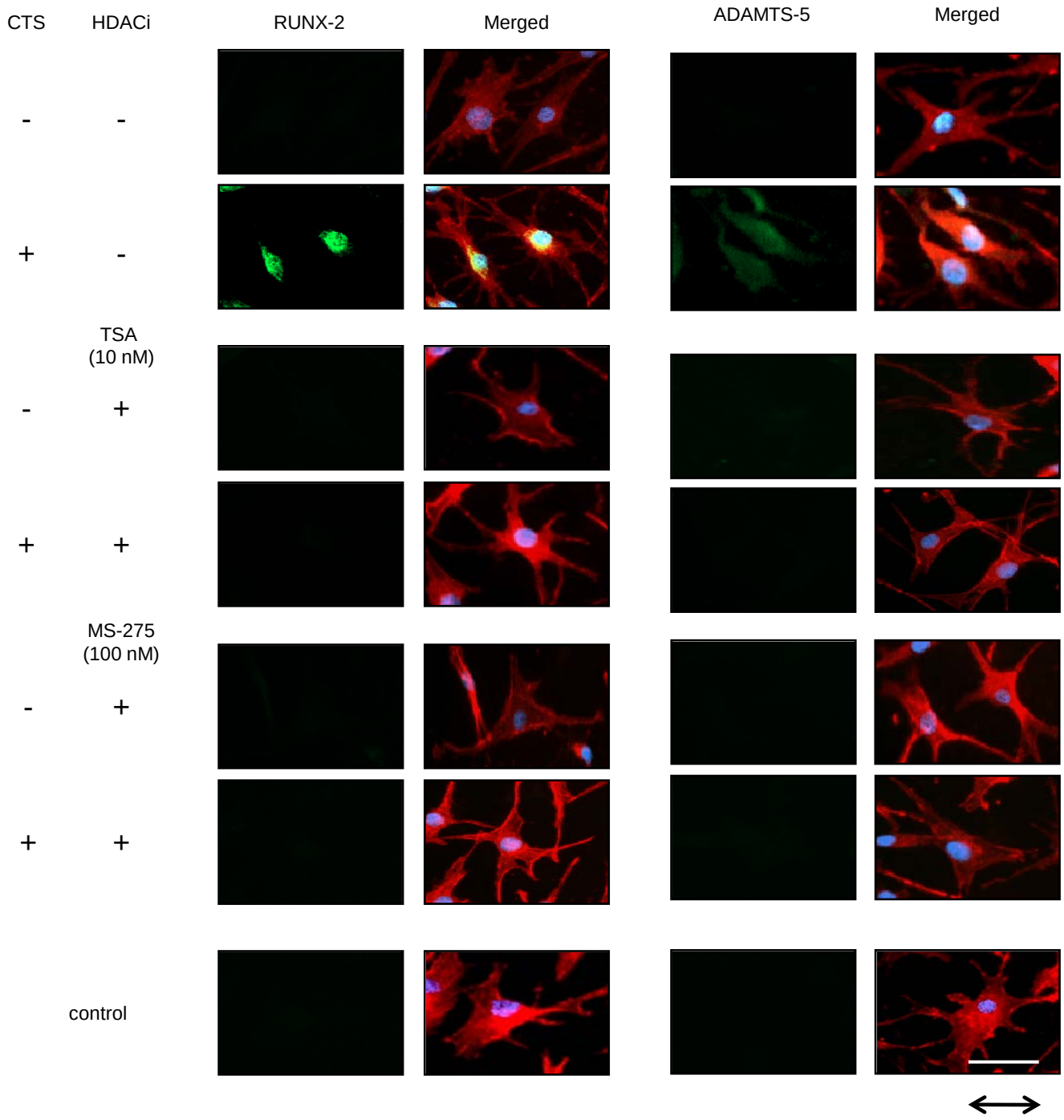
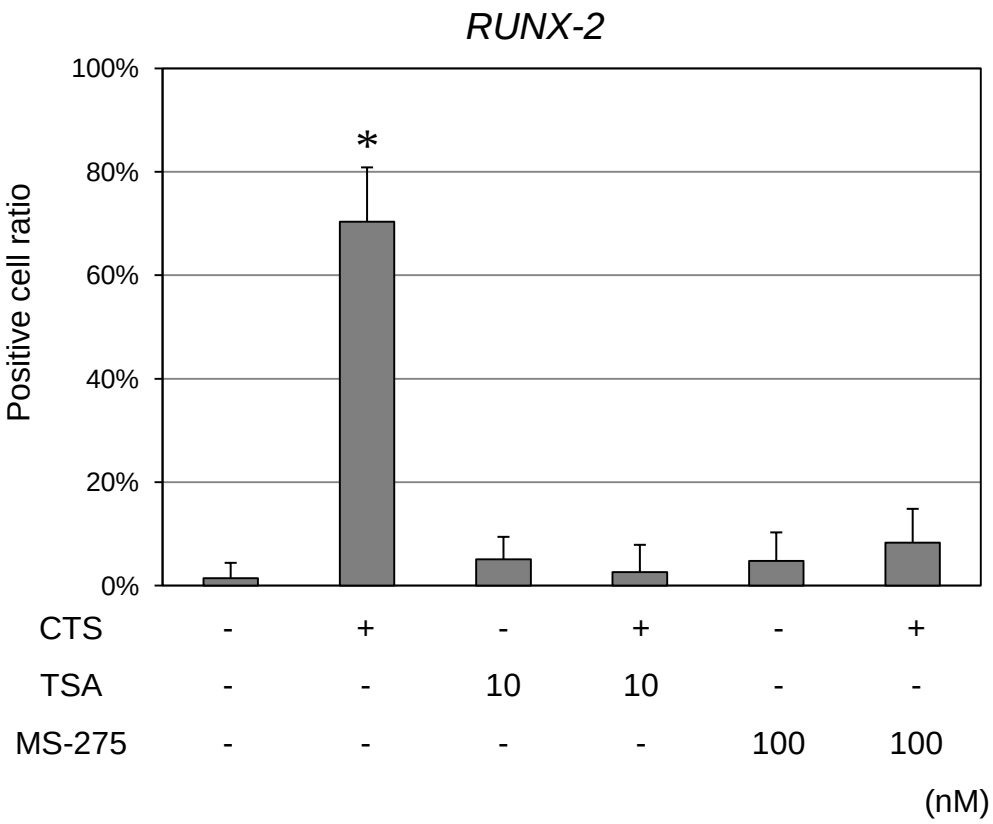
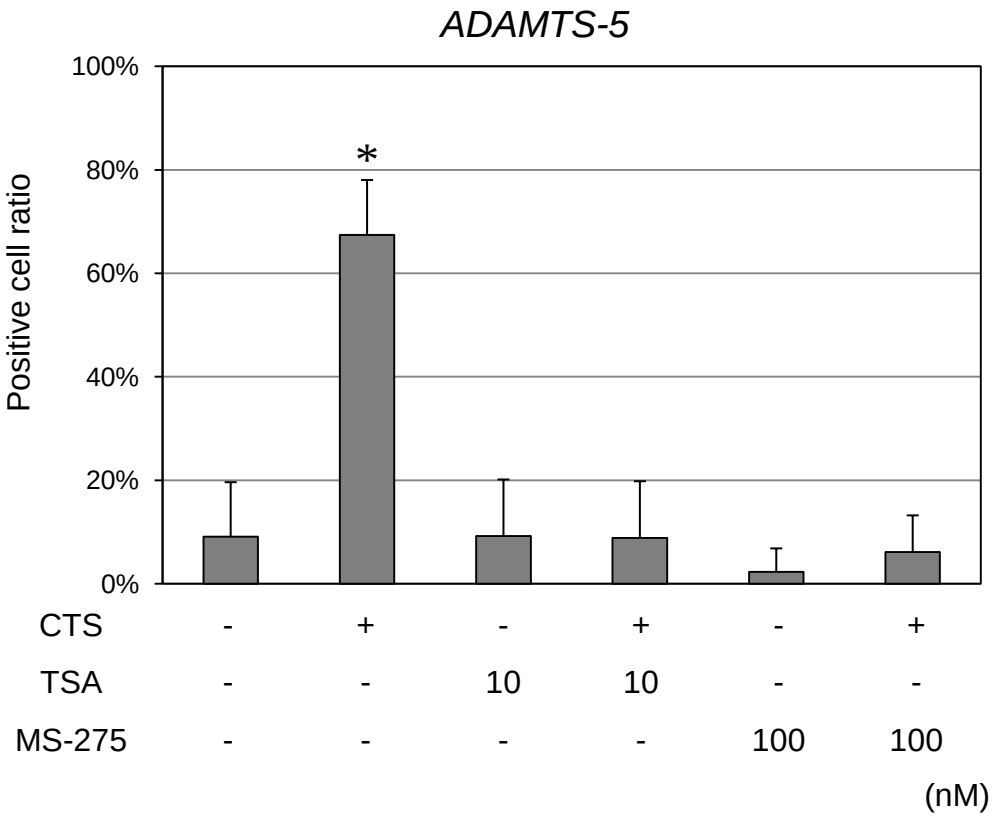

CTS
HDACi
RUNX-2
Merged
ADAMTS-5
Merged
-
-
+
-
TSA
(10 nM)
-
+
+
+
MS-275
(100 nM)
-
+
+
+
control

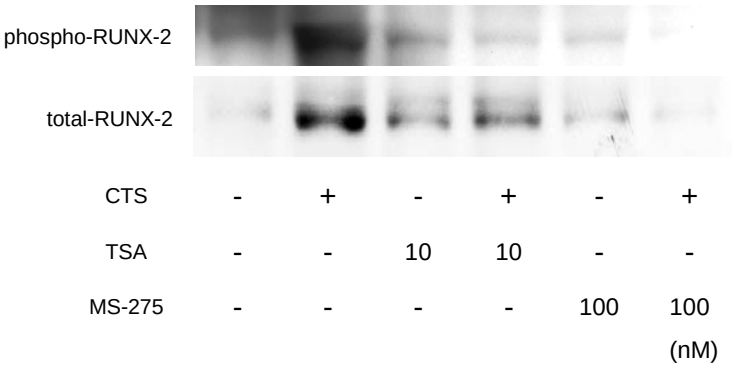
Figure 3

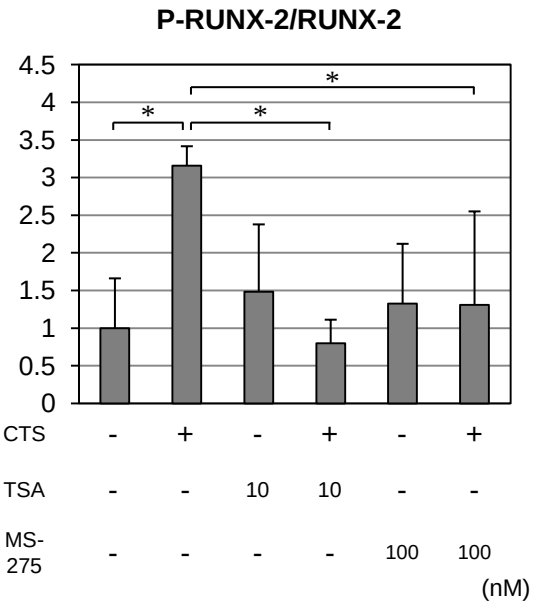
B


RUNX-2
100%
80%
60%
40%
20%
0%
Positive cell ratio
*
CTS - + - + - +
TSA - - 10 10 - -
MS-275 - - - - 100 100
(nM)

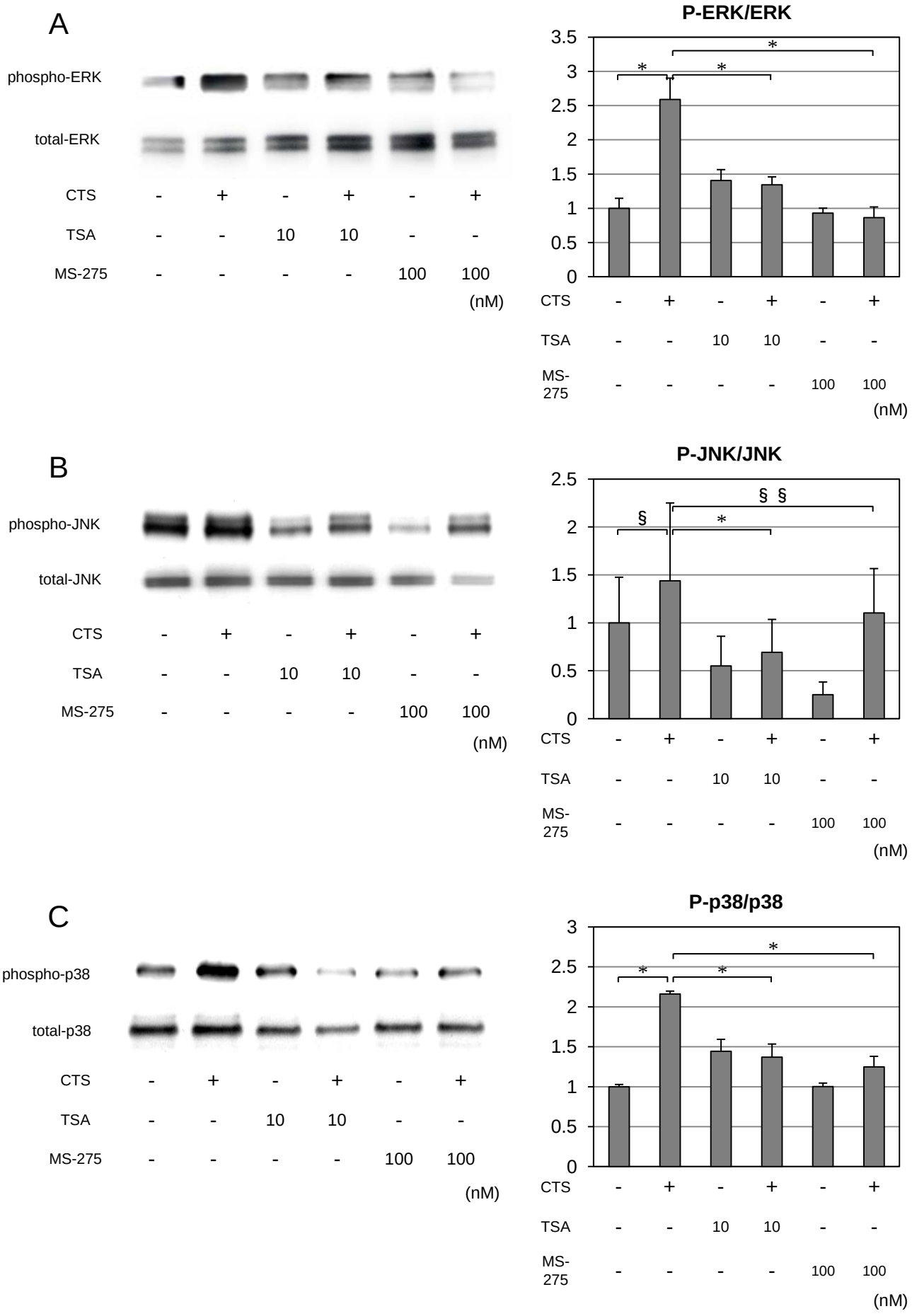

ADAMTS-5
100%
80%
60%
40%
20%
0%
Positive cell ratio
*
CTS - + - + - +
TSA - - 10 10 - -
MS-275 - - - - 100 100
(nM)

# Figure 4


phospho-RUNX-2
total-RUNX-2
CTS - + - + - +
TSA - - 10 10 - -
MS-275 - - - - 100 100
(nM)


P-RUNX-2/RUNX-2
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
CTS - + - + - +
TSA - - 10 10 - -
MS-275 - - - - 100 100
(nM)

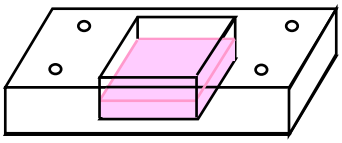
# Figure 5


A
phospho-ERK
total-ERK
CTS - + - + - +
TSA - - 10 10 - -
MS-275 - - - - 100 100
(nM)
P-ERK/ERK
3.5
3
2.5
2
1.5
1
0.5
0
* * *
CTS - + - + - +
TSA - - 10 10 - -
MS-275 - - - - 100 100
(nM)
B
phospho-JNK
total-JNK
CTS - + - + - +
TSA - - 10 10 - -
MS-275 - - - - 100 100
(nM)
P-JNK/JNK
2.5
2
1.5
1
0.5
0
§ § * §
CTS - + - + - +
TSA - - 10 10 - -
MS-275 - - - - 100 100
(nM)
C
phospho-p38
total-p38
CTS - + - + - +
TSA - - 10 10 - -
MS-275 - - - - 100 100
(nM)
P-p38/p38
3
2.5
2
1.5
1
0.5
0
* * *
CTS - + - + - +
TSA - - 10 10 - -
MS-275 - - - - 100 100
(nM)

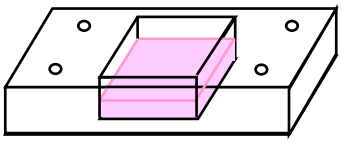
Supplemental Figure 1

HDAC inhibitor

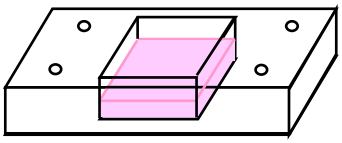
no-mechanical stress models



-

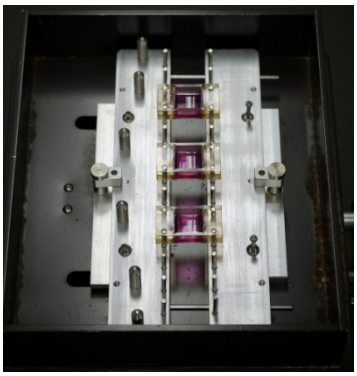


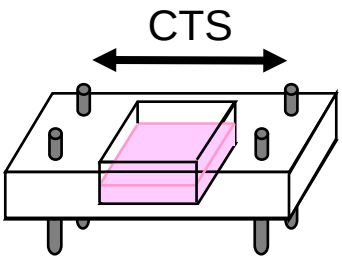
+TSA
(10, 100, 500 nM)



+MS-275
(10, 100, 500 nM)

mechanical stress models



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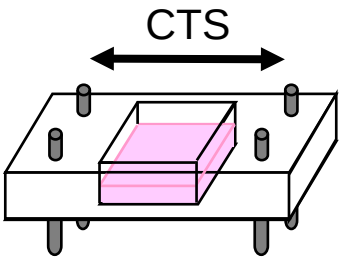
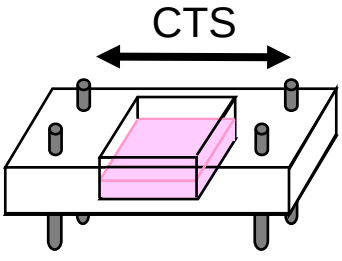
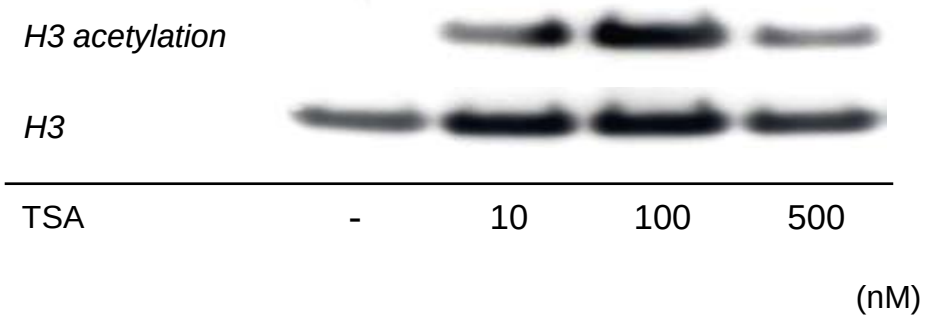



+TSA
(10, 100, 500 nM)




+MS-275
(10, 100, 500 nM)

# Supplemental Figure 2




| TSA | - | 10 | 100 | 500 |
|---|---|---|---|---|

(nM)


| MS-275 | - | 10 | 100 | 500 |
|---|---|---|---|---|

(nM)

# Supplemental Figure 3