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Tensile strain increases expression of CCN2 and COL2A1 by activating TGF-β-Smad2/3 pathway in chondrocytic cells

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Abstract

Physiologic mechanical stress stimulates expression of chondrogenic genes, such as multifunctional growth factor CYR61/CTGF/NOV (CCN) 2 and α1(II) collagen (COL2A1), and maintains cartilage homeostasis. In our previous studies, cyclic tensile strain (CTS) induces nuclear translocation of transforming growth factor (TGF)-β receptor-regulated Smad2/3 and the master chondrogenic transcription factor Sry-type HMG box (SOX) 9. However, the precise mechanism of stretch-mediated Smad activation remains unclear in transcriptional regulation of CCN2 and COL2A1. Here we hypothesized that CTS may induce TGF-β1 release and stimulate Smad-dependent chondrogenic gene expression in human chondrocytic SW1353 cells. Uni-axial CTS (0.5 Hz, 5% strain) stimulated gene expression of CCN2 and COL2A1 in SW1353 cells, and induced TGF-β1 secretion. CCN2 synthesis and nuclear translocalization of Smad2/3 and SOX9 were stimulated by CTS. In addition, CTS increased the complex formation between phosphorylated Smad2/3 and SOX9. The CCN2 promoter activity was cooperatively enhanced by CTS and Smad3 in luciferase reporter assay. Chromatin immunoprecipitation revealed that CTS increased Smad2/3 interaction with the CCN2 promoter and the COL2A1 enhancer. Our results suggest that CTS epigenetically stimulates CCN2 transcription via TGF-β1 release associated with Smad2/3 activation and enhances COL2A1 expression through the complex formation between SOX9 and Smad2/3.

Introduction

Articular cartilage is subjected to various biomechanical stresses such as static/dynamic loads, compressive force, shear strain, hydrostatic pressure, fluid flow, and osmotic stress (Wong et al., 2010). During normal daily activity, the knee joint cartilage compresses 2-5% of the overall thickness, and intense exercise adds another 2-3% compression of the cartilage (Eckstein et al., 2006). Tensile stiffness of articular cartilage plays an essential role even in compressive properties by increasing transient fluid pressures during physiological loading [Charlebois et al., 2004]. Physiologic mechanical stress stimulates chondrogenic gene expression and maintains cartilage homeostasis. On the other hand, repetitive excess mechanical stress can alter cartilaginous composition and metabolism, leading to osteoarthritis. Abnormal joint loading inhibits extracellular matrix (ECM) synthesis of cartilage via induction of proteolytic enzymes including matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) (Patwari et al., 2003). We have previously reported that immoderate cyclic tensile strain (CTS, 0.5 Hz, 10% strain) induces expression of catabolic factors, such as MMP-13 and ADAMTS-4/5/9, and inhibits expression of cartilage-specific ECM molecule α1(II) collagen (COL2A1) in human chondrocytic SW1353 cells (Tetsunaga et al., 2011). In addition, excessive CTS (10% strain) stimulates expression of MMP-3/13 and ADAMTS-5 in human articular chondrocytes (Saito et al., 2013). On the other hand, appropriate stretching force (5-6% strain) increases expression of anabolic factors, such as COL2A1 and multifunctional growth factor CYR61/CTGF/NOV (CCN) 2, in human fibrochondrocytes derived from the inner meniscus and chondrocytic HCS-2/8 cells (Nishida et al., 2008; Kanazawa et al., 2012; Furumatsu et al., 2012). However, mechanical stretch-mediated transcriptional regulation of chondroprotective genes remains unclear.

CCN2 has a multifunctional role in cellular proliferation, differentiation, ECM synthesis, and tissue regeneration (Kubota and Takigawa, 2007). Ccn2-null mice are perinatal lethal, showing severe chondrodisplasia characterized by deficient ECM production, impaired endochondral ossification, and reduced growth plate angiogenesis (Ivkovic et al., 2003). These phenotypes indicate that CCN2 plays an

essential role in chondrogenesis and skeletal development. In addition, transforming growth factor (TGF)-β, which organizes chondrogenic differentiation, and its receptor-regulated Smad2/3 are considered as central inducers of CCN2 expression via Smad2/3 association with the Smad-binding element (SBE), which contains GTCT and its complement AGAC motifs, on the CCN2 promoter .[10,12] Mechanical stretch also modulates expression of CCN2 and TGF-β in ligament-derived cells (Kim et al., 2002). In rat endplate-derived chondrocytes, cyclic strain enhances release of TGF-β1 in the conditioned medium, and the optimum TGF-β1 secretion is observed under a stretching frequency of 0.5 Hz (Xu et al., 2011). Dynamic compression (0.5 Hz, 20-40 kPa, 5-15 min) promotes the phosphorylation (activation) of TGF-β-related Smad2/3, but does not activate that of bone morphogenetic protein-regulated Smad1/5/8, in three-dimensional-cultured chondrocytes derived from mouse embryonic costal cartilage (Bougault et al., 2012). We have previously demonstrated that CTS (0.5 Hz, 5% strain) induces the nuclear translocation of phosphorylated Smad2/3 and enhances Smad3-dependent CCN2 expression in inner meniscus cells (Furumatsu et al., 2012). However, the precise mechanism of stretch-mediated Smad activation is not fully elucidated. Here we hypothesized that physiological stretching stimulus may induce immediate TGF-β1 release, followed by Smad2/3 activation in chondrocytic cells.

Smad3 directly associates with Sry-type HMG box (SOX) 9, and also activates SOX9-dependent transcription on chromatin during chondrogenesis (Furumatsu et al., 2005A). SOX9, which encodes a high-mobility group DNA-binding domain, has been identified as the master transcription factor in chondrogenesis. SOX9 regulates the expression of its target genes through the association with the SOX9-binding sequences (WWCAAWG) on promoters or enhancers of cartilage-specific genes, such as COL2A1 and aggrecan (Bell et al., 1997). During the epigenetic regulation of chondrogenesis, SOX9-dependent COL2A1 expression is synergistically activated by co-activator p300, which has an intrinsic histone acetyltransferase activity, and Smad3 (Furumatsu et al., 2005B, 2009). In addition, the nuclear translocalization of phosphorylated SOX9 is increased by CTS treatment (0.5 Hz, 5% strain) in inner meniscus cells (Kanazawa et al., 2012). These findings prompted

us to investigate the mechanical stretch-induced transcriptional complex formation between Smad2/3 and SOX9 on chromatin.

In the present study, we examined expression of CCN2 and COL2A1 responding to mechanical stretch in chondrocytic SW1353 cells, and further investigated the mechanism of stretch-mediated Smad2/3 activation and the interplay between Smad2/3 and their target gene expression.

Methods

Cells and cell culture: Institutional Review Board approval was obtained before all experimental studies. A human chondrosarcoma cell line (SW1353), well-characterized cells for investigating chondrocytic behaviors during chondrogenesis, was used as chondrocytic cells that maintain a sufficient potential to induce SOX9-regulated chondrogenesis (Tsuda et al., 2003; Barksby et al., 2006; Furumatsu et al., 2005A and 2010). SW1353 cells were maintained with Dulbecco's modified Eagle's medium (DMEM, Wako, Osaka, Japan) containing 10% fetal bovine serum (FBS, HyClone, South Logan, UT), 1% penicillin/streptomycin (Sigma, St. Louis, MO), and 0.17 mM ascorbate (Sigma).

Quantitative real-time PCR: RNA samples were obtained from cultured SW1353 cells. Total RNAs were isolated using ISOGEN reagent (Nippon Gene, Toyama, Japan). RNA samples (500 ng) were reverse-transcribed with ReverTra Ace (Toyobo, Osaka, Japan). The cDNAs underwent PCR amplification in the presence of specific primers for CCN2, COL2A1, SOX9, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (Tetsunaga et al., 2009; Furumatsu et al., 2011). Quantitative real-time PCR analyses were performed using a LightCycler ST-300 instrument (Roche Diagnostics, Mannheim, Germany) and FastStart DNA Master SYBR Green I kit (Roche Diagnostics) according to the manufacturer's protocol. The PCR mixture consisted of 1 x STBR Green PCR Master Mix, which included DNA polymerase, SYBR Green I dye, dNTPs, PCR buffer, 10 pmol of forward and reverse primers, cDNA samples, in a total volume of 15μL. Real-time PCR validation was carried out using the $2^{-\Delta\Delta Ct}$ method. The cycle number at which the amount of the amplified gene of interest reached a fixed threshold was determined. Amplification data of G3PDH were used for normalization. Relative

mRNA levels were normalized with the level of unstretched or untreated cells.

Cyclic tensile strain (CTS): Polydimethylsiloxane stretch chambers (STREX, Osaka, Japan) were coated with 100 μg/mL of rat tail type I collagen (BD Biosciences, Bedford, MA). SW1353 cells were seeded onto stretch chambers, having a culture surface of 1, 4, or 10 cm^2, at 15,000 cells/cm^2. Cells were incubated on chambers for 24 h in FBS-supplemented DMEM, followed by 12 h in serum-free DMEM before stretching. Uni-axial CTS (0.5 Hz, 5% strain) was applied using a STB-140 system (STREX) for an indicated duration. Unstretched cells on chambers were used as controls. Cells, RNAs, proteins, and conditioned media were immediately collected after stretching experiments.

Immunohistochemistry: The distribution of CCN2 protein in SW1353 cells was assessed by immunohistochemical analyses using a goat anti-CCN2 antibody (R&D Systems, Minneapolis, MN, working dilution 1:100 for 1 h incubation) as described (Miyake et al., 2011). Cells seeded on type I collagen-coated chambers were fixed with 4% paraformaldehyde solutions after CTS treatments. The localization of Smad2/3, SOX9, and TGF-β1 in SW1353 cells was assessed by rabbit anti-Smad2/3 antibody (Millipore, Temecula, CA), mouse anti-SOX9 antibody (Sigma), and mouse anti-TGF-β1 antibody (R&D Systems) under a fluorescence microscope, respectively. A bovine serum albumin solution without the primary antibody was used as a negative control. Alexa Fluor 568 phalloidin and Hoechst 33342 (Invitrogen, Carlsbad, CA) were used as a counter staining.

Nuclear extraction, Western blot (WB), and immunoprecipitation (IP): Whole cell lysates, cytoplasmic fractions, and nuclear extracts of SW1353 cells were prepared as described (Furumatsu et al., 2009). Protein concentrations were measured by BCA protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of proteins were applied (20 μg/lane). WB analyses were performed using anti-Smad2/3, anti-SOX9, and goat anti-phosphorylated Smad2/3 antibody (P-Smad2/3, Santa Cruz, Santa Cruz, CA) antibodies (1:1000 for 1 h). Cell extracts (1 x 10^6 cells) were prepared with IP buffer and then sonicated. Supernatants were incubated for 1 h with anti-SOX9 antibody (1:200) and protein G beads (Sigma). IP fraction was assessed by WB using an anti-P-Smad2/3 antibody.

Luciferase reporter assay: SW1353 cells were seeded on type I collagen-coated stretch chambers (1 cm^2).

Reporter plasmid pTS589, which contains the human CCN2 promoter (ranged from -802 to +22 base pair), and Smad2/3 constructed in pcDEF3-FLAG(N) (100 ng each) were transfected using Lipofectamine 2000 (Invitrogen) (Furumatsu et al., 2005A, 2012). pRL-SV40 (10 ng, Promega, Madison, WI) was used as an internal control. Cells were cultured for 12 h in serum-free DMEM prior to CTS treatments (0.5 Hz, 5% strain, 2 h). Luciferase activities were analyzed using Dual-Luciferase Reporter Assay System (Promega).

Chromatin immunoprecipitation (Chromatin IP): Chromatin IP assays were performed as described (Furumatsu et al., 2005A). SW1353 cells were starved on chambers for 12 h before stretching. CTS (0.5 Hz, 5% strain) was performed for 2 h, and the sonicated cell lysates were suspended in nuclear lysis buffer. The 10% volume of supernatant was stocked as an input sample. The half of each remaining sample was incubated for 2 h with anti-Smad2/3 antibody and protein G beads. The remaining supernatant was incubated with mouse IgG and protein G beads as a control. Input fraction DNAs, DNA fragments immunoprecipitated with Smad2/3, and DNAs in IgG controls were purified. PCR reactions were performed using the following primer set and were allowed to proceed for 30 cycles. Primer sets; (-754F, -595R) and (-243F, -83R) for amplifying specific regions of the human CCN2 promoter (Furumatsu et al., 2012), (-723F, -558R) and (+2311F, +2465R) to amplify specific regions of the human COL2A1 promoter and enhancer (Matsumoto et al., 2012).

Enzyme-linked immunosorbent assay (ELISA): The concentration of TGF-β1 in conditioned media was measured by Quantikine ELISA human TGF-β1 immunoassay kit (R&D Systems). SW1353 cells seeded on chambers were maintained in FBS-free DMEM for 12 h. CTS treatment (0.5 Hz, 5% strain) was performed for an indicated duration (15, 30, 60, and 120 min). Each conditioned medium was collected for TGF-β1 ELISA.

CCN2 treatment and cell proliferation assay: Cells were cultured on type I collagen-coated plates for 12 h in serum-free DMEM before the treatment with recombinant human CCN2 (BioVender, Candler, NC). CCN2 was added into serum-free DMEM at indicated concentrations (1, 10, 50, and 100 ng/mL). Cells (5,000 cells/well) were incubated for 48 h prior to the addition of a cell proliferation reagent, water

soluble tetrazolium (WST)-1 (Roche Diagnostics), on 96-well plates. Optical density (OD) was measured at evaluation and control wavelengths of 450 nm and 630 nm, respectively. Data obtained by subtracting 630-nm readings from 450-nm readings were used for evaluation (n = 5). For PCR analysis, CCN2 treatment (10 and 50 ng/mL) was performed for 4 h before the preparation of RNA samples.

Statistical analysis: Quantitative real-time PCR (4 reactions for each cDNA sample), immunohistochemistry (3 chambers for each detection), Western blot (2 membranes per each whole cell lysate), luciferase assay (5 chambers for each group), ELISA (5 wells per each sample), and proliferation assay (5 wells for each treatment) were repeated at least three times independently and similar results were obtained. Chromatin IP analysis was repeated four times. Data were expressed as means with standard deviations. Mean values were compared with a one-way ANOVA. Post hoc comparisons were performed using the Tukey test. Significance was set at $p < 0.05$.

Results

Mechanical stretch stimulates gene expression of CCN2 and COL2A1 in chondrocytic cells

CTS treatments increased CCN2 expression in SW1353 cells up to a 1.7-fold level of unstretched controls (Fig. 1A, 4 h). In addition, COL2A1 gene expression was enhanced up to a 2.1-fold level of controls by 4-h CTS in SW1353 cells (Fig. 1B). However, expression of the master chondrogenic transcription factor SOX9 was not significantly increased in stretched SW1353 cells (Fig. 1C).

Mechanical stretch increases protein production of CCN2 and induces nuclear translocalization of Smad2/3 and SOX9 in chondrocytic cells

CCN2 deposition in the cytoplasm of SW1353 cells was stimulated by CTS (Fig. 2A). CTS induced nuclear translocalization of Smad2/3 and SOX9 in SW1353 cells (Fig. 2A). Total amounts of endogenous Smad2/3 and SOX9 were not influenced by 2-h CTS treatment (Fig. 2B). However, Smad2/3 localized to the nucleus was increased by CTS (Fig. 2C). In addition, IP analyses revealed that CTS increased the complex formation between phosphorylated (or activated) Smad2/3 and SOX9 in the nuclear fraction of stretched SW1353 cells (Fig. 2C).

Mechanical stretch and Smad3synergistically activate the CCN2 promoter in chondrocytic cells

CTS treatment (2 h) enhanced the luciferase activity of the human CCN2 promoter-containing pTS589 in SW1353 cells (Fig. 3A). In addition, transient transfection of Smad2 increased relative luciferase activity compared with untransfected controls (Fig. 3A). However, CTS did not influence the pTS589 activity of Smad2-transfected cells (Fig. 3A). On the other hand, Smad3 transfection and CTS cooperatively enhanced the activity of pTS589 up to a 2-fold level of control (Fig. 3A).

Mechanical stretch promotes Smad2/3 association with the CCN2 promoter and the COL2A1 enhancer in chondrocytic cells

Chromatin IP analysis revealed that CTS treatment increased the association between endogenous Smad2/3 and the Smad-binding element located at the CCN2 promoter 2 in SW1353 cells (Fig. 3B). PCR fragment derived from the CCN2 promoter 2 was increased in the chromatin IP fraction using an anti-Smad2/3 antibody under 2-h stretched condition (Fig. 3B). The CCN2 promoter 1 that did not contain the Smad-binding element was not amplified in chromatin IP analysis (Fig. 3B). In addition, CTS increased the association between Smad2/3 and the COL2A1 enhancer on chromatin in SW1353 cells (Fig. 3C). In stretched cells, DNA fragment that contained the COL2A1 enhancer was coimmunoprecipitated with Smad2/3, probably via complex formation among Smad2/3-SOX9-the SOX9-binding site, and was amplified by PCR using specific primers for the COL2A1 enhancer (Fig. 3C). However, no amplicon of the COL2A1 promoter was observed in SW1353 cells even under a stretched condition (Fig. 3C).

Mechanical stretch induces TGF-β1 secretion in chondrocytic cells

TGF-β1 was secreted from stretched SW1353 cells in a time-dependent manner (Fig. 4A). The concentration of secreted TGF-β1 reached a mean of 22.7 pg/mL in serum-free conditioned media of 2-h-stretched cells (Fig. 4A). TGF-β1 concentration was increased up to 1.9- and 2.5-fold levels of unstretched control by 60- and 120-min CTS, respectively (Fig. 4A). In addition, the cytoplasmic deposition of TGF-β1 was decreased in SW1353 cells stretched for more than 60 min (Fig. 4B).

CCN2 stimulates chondrocytic cell proliferation and COL2A1 expression

CCN2 treatments (10, 50, and 100 ng/mL) stimulated the proliferation of SW1353 cells (Fig. 5A). In addition, recombinant CCN2 increased COL2A1 expression up to a 1.8-fold level of untreated control (Fig. 5C). However, neither endogenous CCN2 nor SOX9 gene expression was influenced by 48-h CCN2 treatment (Fig. 5B and D).

Discussion

Cellular responses to mechanical stress underlie many critical functions such as development, morphogenesis, and wound healing. In experimental studies, physiological force has been mimicked by various mechanical stimuli, including stretch, compression, shear stress, and bending torque (Orr et al., 2006). Cell stretch would induce mechanical extension of cytoplasmic macromolecules, activation of ion channels, and phosphorylation of mechanotransducers (Orr et al., 2006). However, cellular behavior is not uniform under comparable stretching condition in vitro. CCN2 expression is increased by cyclic stretch (no more than 15% strain) in bladder smooth muscle cells and chondrocytic cells (Nishida et al., 2008; Yang et al., 2008). On the other hand, 20% length of cellular stretch rather decreases CCN2 expression in skin fibroblasts (Kanazwa et al., 2009). In our experiments, 5% strain of stretching force stimulated gene expression of CCN2 and COL2A1 in chondrocytic cells (Fig. 1). In addition, recombinant CCN2 treatment stimulated cellular proliferation and COL2A1 expression (Fig. 5). Our results suggest that the syntheses of CCN2 and cartilaginous ECM molecules are induced under a physiological stretching force (< 10% strain) and by a stretching frequency showing a slow gait pattern (0.5 Hz) (Oberg et al., 1993; Shirazi et al., 2008). Considering the molecular property of CCN2 that promotes physiological process of tissue development and regeneration under multiple molecular interactions, mechanical stretch has more important roles in furnishing the microenvironment that supports type II collagen production and cartilage homeostasis. In our previous studies, CTS (0.5 Hz, 5-10% strain) does not influence COL2A1 expression in SW1353 chondrocytic cells cultured on fibronectin-coated stretch chambers (Tetsunaga et al., 2011). In addition, CTS increased expression of catabolic genes, such as MMP-3/13 and ADAMTS-4/5/9, in SW1353 cells and human articular

chondrocytes on fibronectin (Tetsunaga et al., 2011; Saito et al., 2013). Several authors have demonstrated that fibronectin, localized mainly in the articular surface, stimulated MMP production and induced proteoglycan leakage (chondrolysis) in chondrocytes and organ-cultured cartilage (Homandberg et al., 1992; Long et al., 2013). On the other hand, the attachment and growth of chondrocytes are superior in type I collagen-coated scaffolds rather than in uncoated scaffolds (Honda et al., 2003). Chondrocytes seeded on type I collagen sponges show higher COL2A1 expression than those in type II atelocollagen gels (Freyria et al., 2009). However, passaged chondrocytes cultured in type II collagen matrix exhibit better cartilaginous features than those in type I collagen matrix (Chiu et al., 2011). Further investigations will be required to understand differential effects of ECM molecules on chondrocytic cells.

SOX9 is necessary for mesenchymal condensation and sequential chondrogenic differentiation. Several transcription partners such as Sox5/6, c-Maf, TRAP230, p300, Barx2, Smad3, PIAS, β-catenin, and Scleraxis/E47 can modify SOX9-dependent transcription in chondrogenesis and sex determination (Furumatsu and Asahara, 2010). Co-activator p300 acts as a bridging factor for connecting DNA-binding transcription factors to the transcriptional apparatus, and as a protein scaffold to form multicomponent transcriptional complexes. We have previously reported that the histone acetyltransferase activity of p300 facilitates SOX9-dependent transcription on chromatin by histone modification (Furumatsu and Ozaki, 2010). COL2A1 transactivation on chromatin is directly regulated by the epigenetic function of SOX9-related transcriptional complex, which is composed of SOX9, Smad3, and p300. In the present study, CTS increased nuclear translocation of SOX9 and Smad2/3 in chondrocytic SW1353 cells (Fig. 2A). In addition, CTS enhanced the complex formation between SOX9 and Smad2/3 without increasing total amounts of SOX9 and Smad2/3 (Fig. 2B and C), and stimulated association of Smad2/3 with COL2A1 enhancer, probably mediated by SOX9, on chromatin (Fig. 3C). Dynamic compression load also increases the nuclear translocation and phosphorylation of SOX9 in hydrogel-embedded human articular chondrocytes (Haudenschild et al., 2010). These findings suggest that mechanical stretch epigenetically regulates COL2A1 expression in chondrocytic cells via structural change of SOX9-related

transcriptional complex on chromatin (Fig. 6). Although Smad2 forms a complex with SOX9 in a manner similar to Smad3, COL2A1 expression induced by SOX9/Smad2 is lower than that by SOX9/Smad3 (Furumatsu et al., 2005A). Co-activator p300 stimulates Smad-dependent transactivation by binding to the MH2 domain of Smads and shows stronger affinity against Smad3 rather than Smad2 (Pouponnot et al., 1998). In our studies, CTS-dependent up-regulation of the CCN2 promoter was enhanced by Smad3, but was not by Smad2, in SW1353 cells (Fig. 3A) and inner meniscus cells (Furumatsu et al., 2012). We consider that the distinct function between Smad2 and Smad3 in chondrogenesis may depend on their abilities to associate with p300 and to stabilize SOX9/p300 transcriptional complex. Further examinations that investigate Smad/p300-mediated histone acetylation will be required to evaluate the precise role of Smad2/3 in response to mechanical stretch.

The TGF-β ligands bind to a heteromeric TGF-β receptor complex consisting of two type I and two type II serine/threonine kinase receptors (Ross and Hill, 2008). The constitutively active type II receptor phosphorylates the type I receptor that phosphorylates the intracellular mediators of the TGF-β signaling pathway, the receptor-regulated Smads (R-Smads) including Smad2/3 (Fig. 6). In this study, we demonstrated that CTS induced TGF-β1 release and nuclear translocation of Smad2/3 in chondrocytic cells (Fig. 2 and 4). However, it is unclear whether the phosphorylation of Smad2/3 is directly induced by stretch-released TGF-β1 alone in our experiments. Phosphatase activity in the nucleus can remove the C-terminal phosphate from the active R-Smads and unphosphorylated inactive R-Smads constantly shuttle between the nucleus and cytoplasm (Ross and Hill, 2008). The activation of TGF-β receptor complex is also influenced by the inhibitory Smads (I-Smads), E3-ligases, and Smad anchor for receptor activation (SARA). In addition to the Smad2/3 pathway, TGF-β activates the mitogen-activated protein kinase (MAPK) pathway, which stimulates expression of COL2A1 and SOX9, during chondrogenesis (Tuli et al., 2003). The small GTPase Rac supports the activation of MAPK pathway and regulates the formation of focal adhesion complexes associated with lamellipodia (Burridge and Wennerberg, 2004). Rac1 also stimulates CCN2 expression via TGF-β/Smad signaling pathway in mouse primary chondrocytes isolated from E15.5 long bones (Woods et al., 2009). These findings suggest that the other

mechanisms such as the I-Smads, SARA, MAPK, and Rac1 may have distinct roles in modulating stretch-dependent Smad2/3 activation in chondrocytic cells. We consider that the Rho family GTPases (RhoA, Rac1, and Cdc42) involved in organizing the actin cytoskeleton might have an important role in modulating Smad-dependent expression of CCN2 and COL2A1 during chondrogenesis. In our study, pTS589-derived luciferase activity did not show a dramatic change (Fig. 3A). The human CCN2 promoter-constructed pTS589 (from -802 to +22 base pair) includes many protein-binding sequences such as SBE, AP-1, SP-1, and TRENDIC (Eguchi et al., 2001 and 2008). pTS589 shows a 12-fold increase of luciferase activity compared with pDS4, which contains a shorter region of the CCN2 promoter (from -88 to +22 base pair, lacking SBE). However, TGF-β1 stimulation induces only 30% increase of pTS589 activity in chondrocytic HCS-2/8 cells (Eguchi et al., 2001). These findings suggest that TGF-β-Smad pathway has a critical role in stimulating CCN2 gene expression but the TGF-β-Smad-dependent up-regulation of the CCN2 promoter may require a cooperative interaction of the other factors to induce full activation of the CCN2 promoter. Our results would be useful to prepare un-dedifferentiated chondrocytes and better tissue-engineered cartilage for regeneration therapy in cartilage injury and osteoarthritis. However, the comparative study using primary articular chondrocytes will be required to assess a complicated role of tensile strain in chondrocyte homeostasis.

In conclusion, we demonstrate that mechanical stretch epigenetically stimulates CCN2 transcription via TGF-β1 release associated with Smad2/3 activation and enhances COL2A1 expression through the complex formation between SOX9 and Smad2/3. Our results suggest that stretch-induced Smad2/3 activation may have a crucial role in regulating chondrogenesis.

Conflict of interest statement

The authors have no conflicts of interest.

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Fig. 1. CTS stimulated gene expression of CCN2 and COL2A1 in chondrocytic SW1353 cells. In quantitative real-time PCR analyses, CTS treatments (0.5 Hz, 5% strain) increased CCN2 expression in SW1353 cells up to 1.5- and 1.7-fold levels of unstretched controls by 2-h and 4-h CTS, respectively (A). In addition, COL2A1 gene expression was enhanced up to a 2.1-fold level of controls by 4-h CTS in SW1353 cells (B). However, no significant increase of SOX9 expression was observed in stretched SW1353 cells (C). * $p < 0.05$.

Fig. 2. CTS increased the deposition of CCN2 (A, green) in the cytoplasm of chondrocytic SW1353 cells. In addition, CTS induced nuclear translocalization of Smad2/3 (A, green) and SOX9 (A, green) in SW1353 cells. Controls in the absence of primary antibodies showed no signals. Double-headed arrows represent the direction of stretching. Red, F-actin. Bars, 25 μm. In Western blot (WB) analysis, CTS treatment (2 h) did not influence total amounts of endogenous Smad2/3 and SOX9 in each whole cell lysate (B, 20 μg/lane). However, Smad2/3 detected in the nuclear fraction was increased by CTS (C, Nucleus, 20 μg/lane). In IP analysis using an anti-SOX9 antibody, CTS increased the association between phosphorylated Smad2/3 and SOX9 in the nuclear fraction derived from stretched SW1353 cells (C, IP). Arrows indicate an expected size for each protein.

Fig. 3. (A) CTS treatment (2 h) increased relative luciferase activity of pTS589, which contained the human CCN2 promoter, compared with a level of unstretched control in chondrocytic SW1353 cells. Smad2 transfection also stimulated pTS589 luciferase activity. No additional increase of the pTS589 activity was observed in Smad2-transfected cells under stretched condition. On the other hand, Smad3 and CTS synergistically enhanced the pTS589 activity up to a 2-fold level of control. * $p < 0.05$. (B) Chromatin IP analysis revealed that 2-h CTS treatment increased the association between Smad2/3 and the Smad-binding element located at the CCN2 promoter 2 in SW1353 cells. PCR fragment derived from the CCN2 promoter 2 was increased in the chromatin IP fraction using an anti-Smad2/3 antibody under a stretched condition (B, Promoter 2), compared with that in unstretched condition (B, Promoter 2). The CCN2 promoter 1 that did not contain the Smad-binding element was not amplified in chromatin IP analysis (B, Promoter 1). CTS also induced the association between Smad2/3 and the COL2A1 enhancer in SW1353 cells (C, Enhancer). The SOX9-binding site-free fragment was not detected in chromatin IP fraction (C, Promoter). Mouse IgG was used as the control for chromatin IP (IgG). Schemes involving

the promoters of human CCN2 and COL2A1 are shown (B and C). Gray box denotes the Smad-binding element on the CCN2 promoter (B). Filled box indicates the SOX9-binding site on the COL2A1 enhancer (C). Numbers indicate the distance from the transcription start site of the CCN2 gene (GenBank, AF316366) and the COL2A1 (GenBank, AC004801). Arrowheads and dotted lines denote the primer sets and expected PCR fragments in chromatin IP, respectively (B and C).

Fig. 4. CTS induced TGF-β1 secretion from stretched SW1353 cells in a time-dependent manner (A). The concentration of secreted TGF-β1 was 17.9 and 22.7 pg/mL in serum-free conditioned media of 60- and 120-min stretched cells, respectively (A). TGF-β1 concentration of unstretched cells was a mean of 9.3 pg/mL (A). * $p < 0.05$. TGF-β1 was evenly distributed in the cytoplasm under a CTS-free condition (B, green). However, the cytoplasmic deposition of TGF-β1 was decreased in SW1353 cells stretched for 60 and 120 min (B). Control in the absence of an anti-TGF-β1 antibody showed no signal. Blue, Hoechst staining. Bars, 25 μm.

Fig. 5. Recombinant CCN2 treatment (48 h) stimulated the proliferation of SW1353 cells (A; 10, 50, and 100 ng/mL). * $p < 0.05$. In real-time PCR analysis, CCN2 increased COL2A1 expression up to a 1.8-fold level of untreated control (C, 50 ng/mL). On the other hand, CCN2 did not influence endogenous gene expression pattern of CCN2 and SOX9 in SW1353 cells (B and D).

Fig. 6. Schematic illustration of mechanical stretch-mediated responses in chondrocytic cells. (A) In the absence of stretching force, sufficient amounts of SOX9, TGF-β1, and Smad2/3 are stored in the cytoplasm of chondrocytic cells. Gene expression of COL2A1 and CCN2 is maintained, but not excessively up-regulated, under unstretched condition. (B) Physiological stretching force induces TGF-β1 secretion from the cytoplasm of chondrocytic cells. Extracellular TGF-β1 would stimulate membrane-bound TGF-β receptors in an autocrine (or paracrine) fashion. Then, the nuclear translocalization of TGF-β receptor-regulated Smad2/3 is activated. In addition, mechanical stretch induces the nuclear translocalization of SOX9. Phosphorylated (P) Smad2/3 and SOX9 cooperatively enhance COL2A1 expression under stretched condition. CCN2 gene expression is also activated by nuclear translocated Smad2/3 in chondrocytic cells. Stretch-induced CCN2 would stimulate chondrocytic cell proliferation and COL2A1 expression.

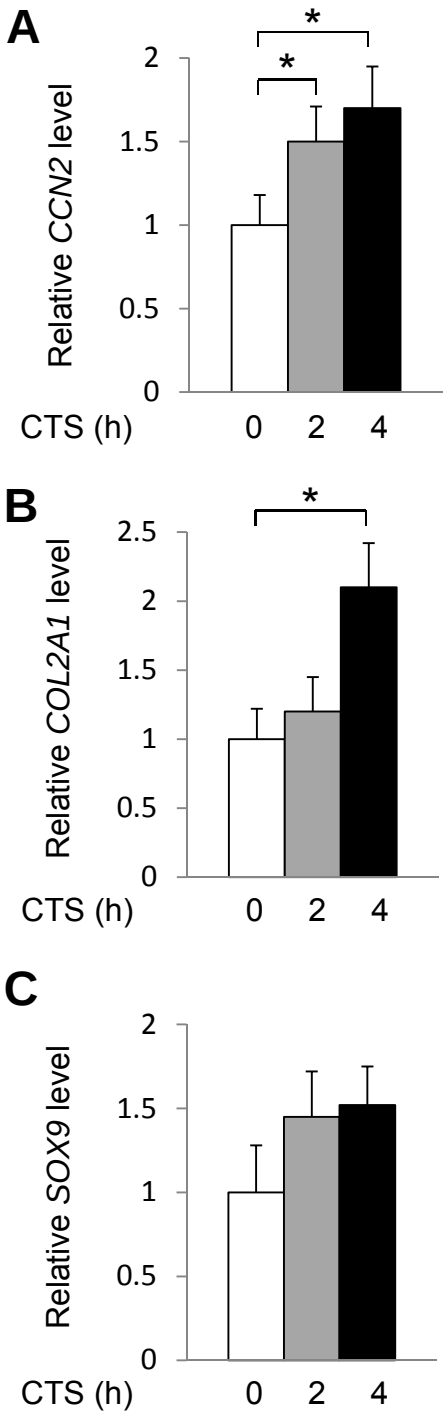

Figure 1
Furumatsu et al.

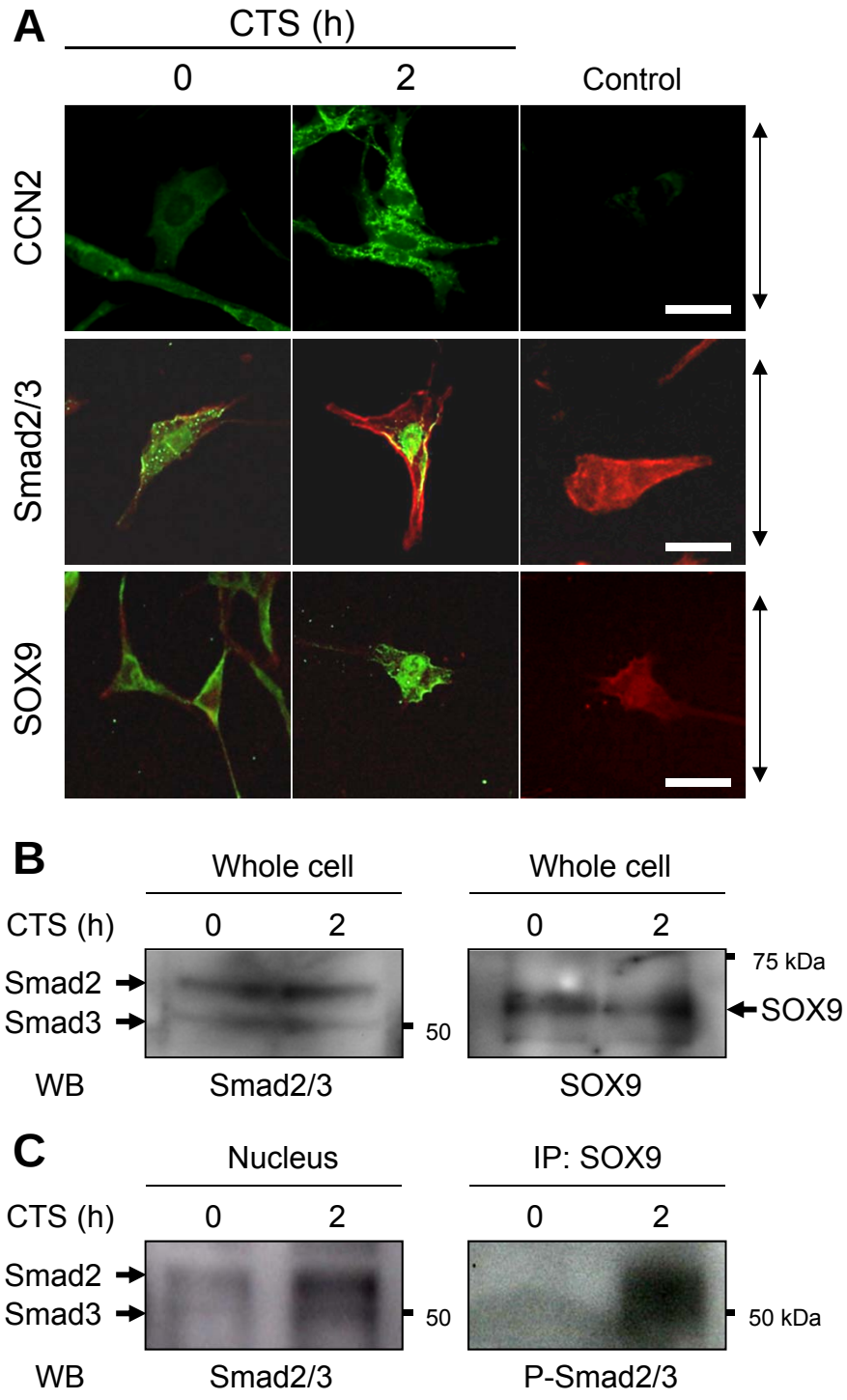

Figure 2
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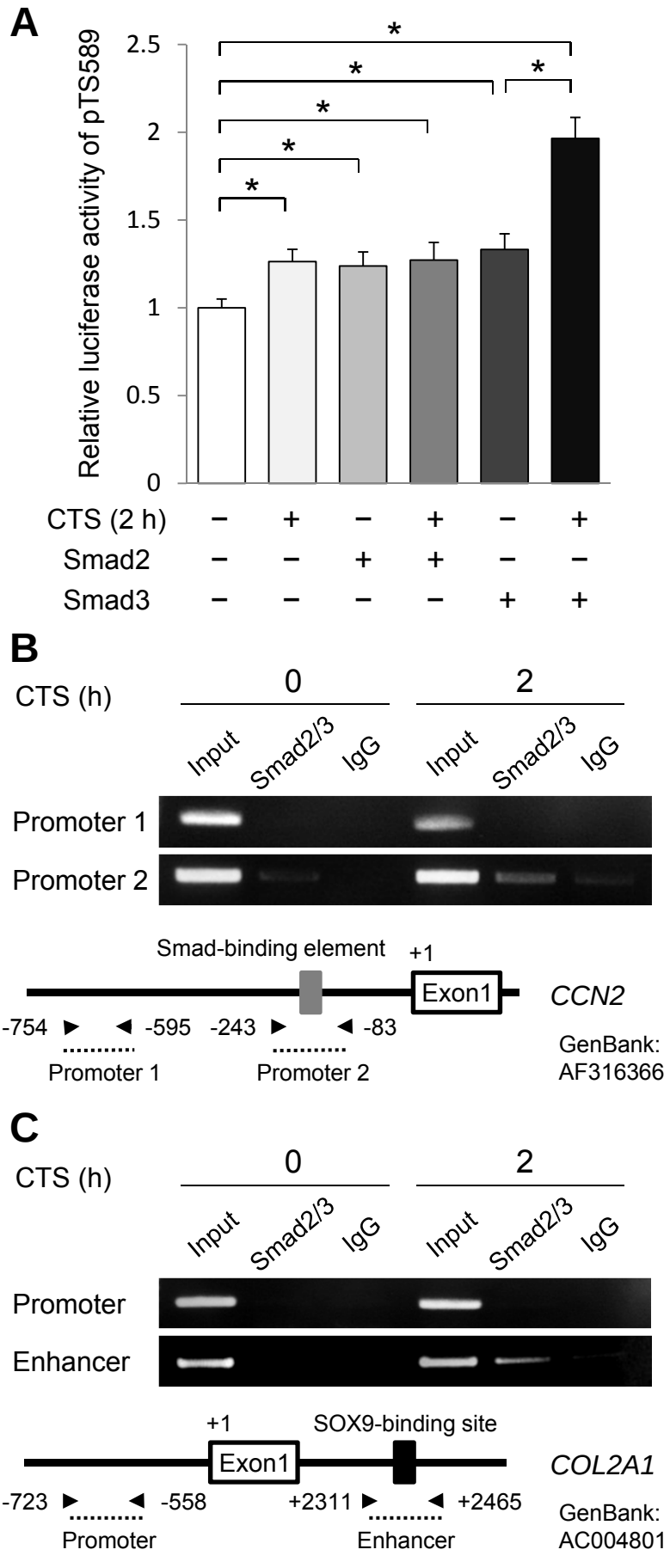

Figure 3 Furumatsu et al.

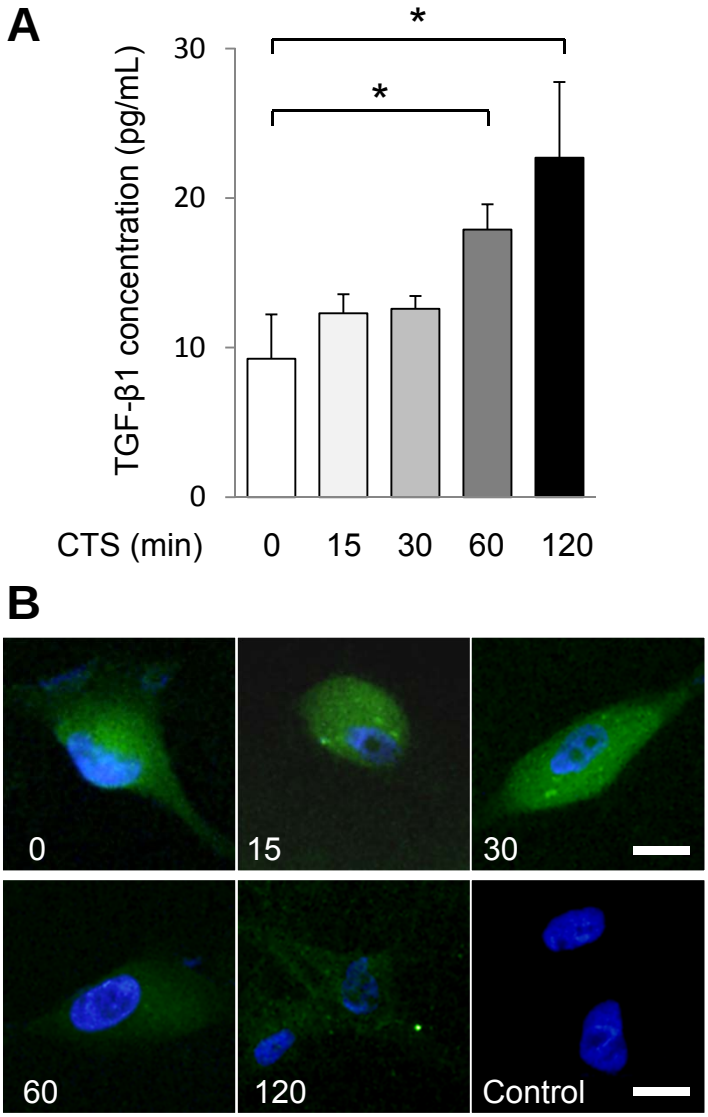

Figure 4
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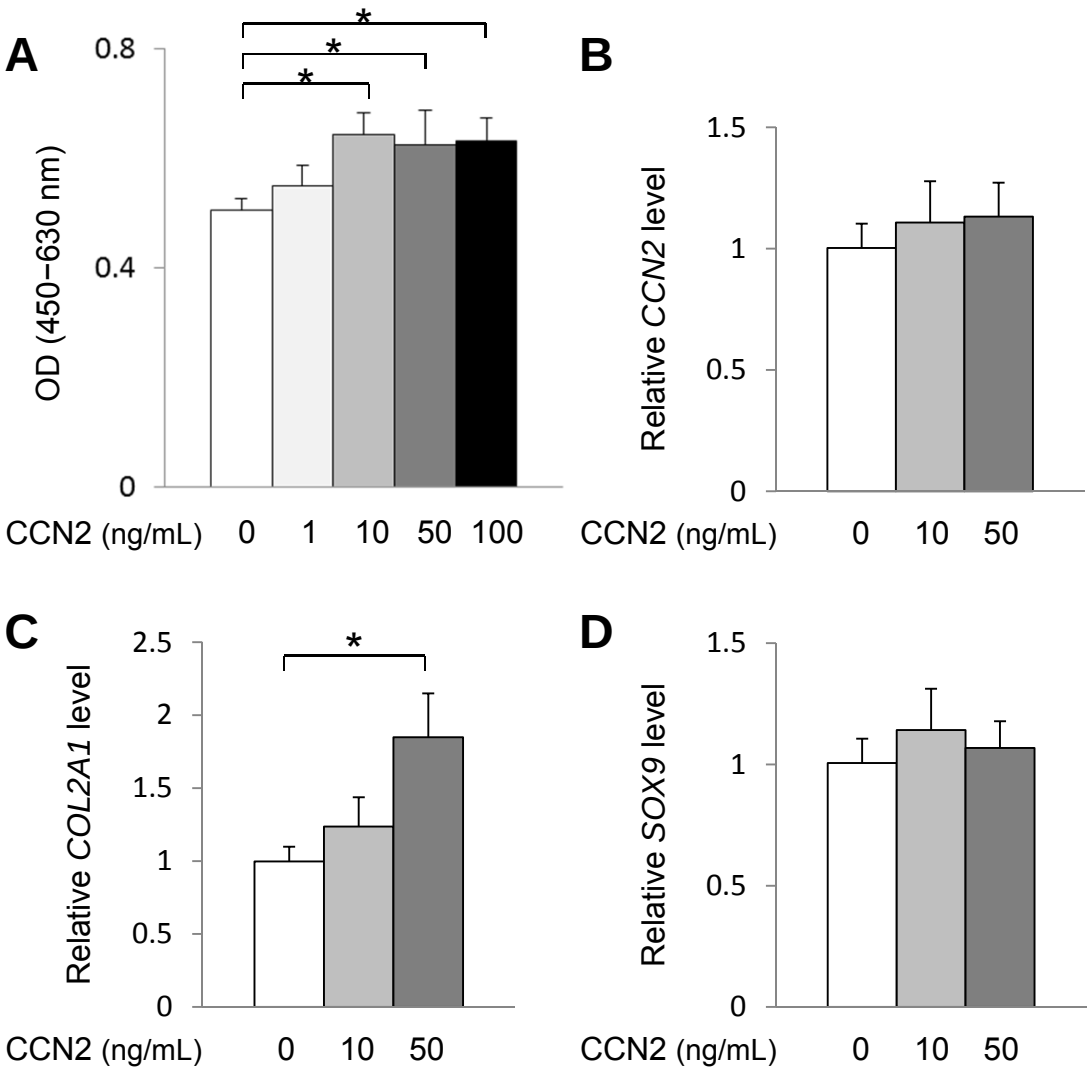

Figure 5
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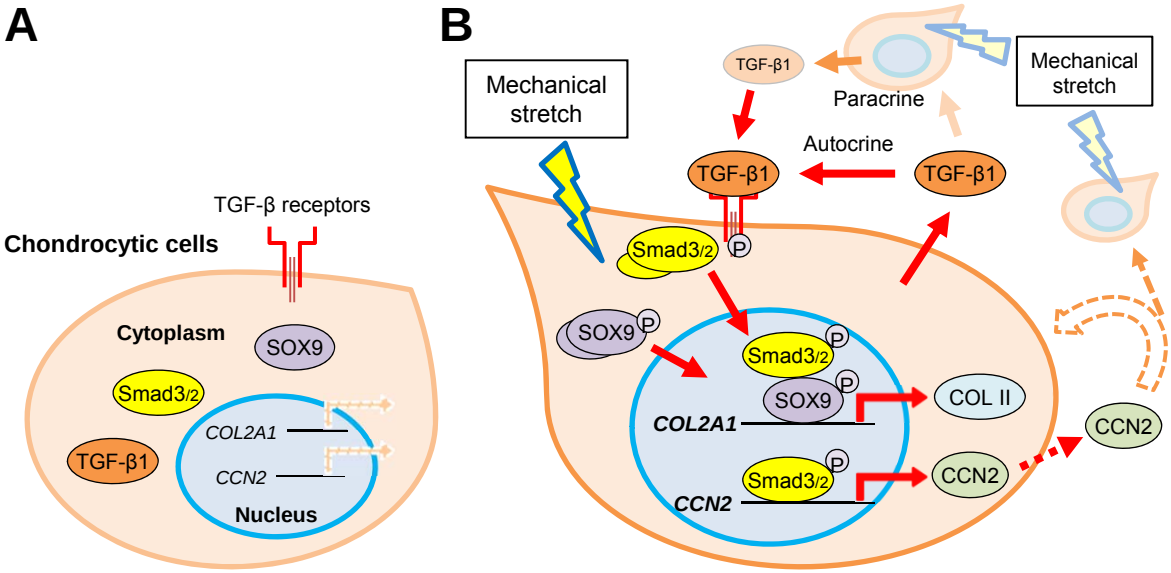

Figure 6
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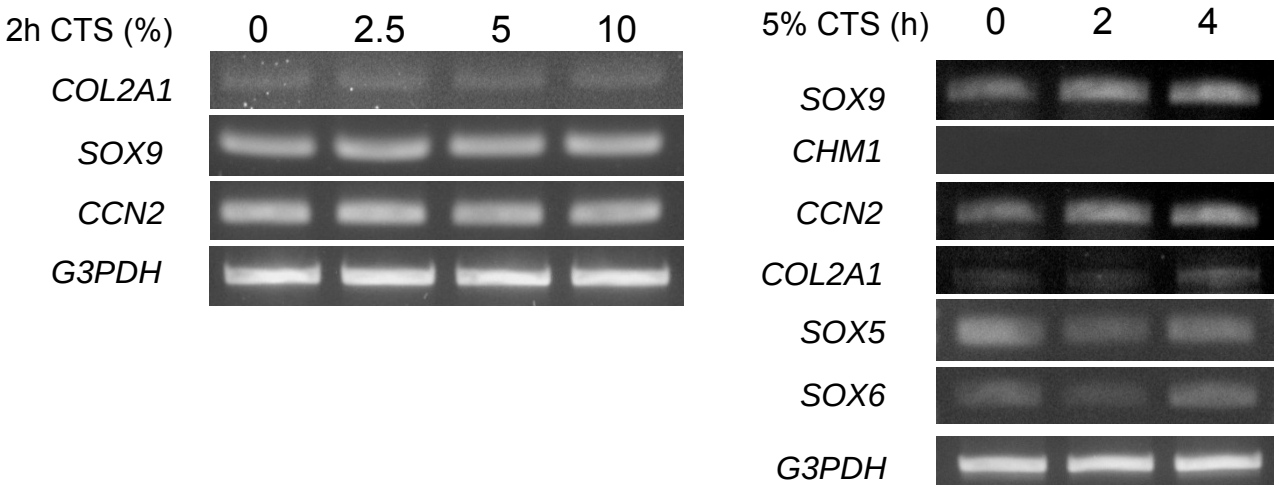

Supplemental Figure
Furumatsu et al.