Expression and activity of Runx2 mediated by hyaluronan during chondrocyte differentiation

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Running head; Effects of hyaluronan on the expression of Runx2 during chondrocyte differentiation

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Abstract

During endochondral ossification, the production of hyaluronan (HA) is strictly and selectively regulated by chondrocytes, with a temporal peak at the hypertrophic stage. This study was conducted to clarify the effects of HA on expression and activity of runt-related gene 2 (Runx2), a potent transcription factor for chondrocyte differentiation in hypertrophic chondrocytes. Immature chondrocytes from an ATDC5 cell line were cultured and differentiated in DMEM/Ham'sF12 with pre-defined supplements. Using real time PCR, the gene expressions of type II collagen, MMP-13, HAS2, and Runx2 in cultured chondrocytes were analyzed from day 0 to 18 of cell differentiation. The activity and expression of Runx2 in hypertrophic chondrocytes were analyzed after the treatment with HA oligosaccharide (HAoligo) using AML-3/Runx2 binding, real-time PCR and Western blot analysis. The effects of pre-incubation of anti-CD44 antibody on Runx2 expression were also examined. Expression of type X collagen and Runx2 mRNAs reached a maximum at the terminal differentiation of chondrocytes. The activity and expression of Runx2 was significantly inhibited in hypertrophic chondrocytes treated with HAoligo compared to the untreated controls. High molecular weight-HA did not affect the expression or activity of Runx2. The expression of Runx2 mRNA was significantly decreased in hypertrophic chondrocytes treated with anti-CD44 antibody. These results suggest that HAoligo may affect the terminal differentiation of chondrocytes during the endochondral ossification by inhibiting the expression and activity of Runx2.

Introduction

Vertebrate bone is formed through either intramembranous or endochondral ossification. The intramembranous bone is directly formed by osteoblasts, whereas the cartilaginous bone is initially formed by chondrocytes at the endochondral ossification. The cartilage is then replaced with bone by a repeated remodeling process mediated with osteoblasts and osteoclasts.[1] In endochondral ossification, chondrocytes undergo a process of proliferation and maturation. Maturation process of chondrocytes is defined by the expression of specific extracellular matrix genes. Prechondrogenic cells express type I collagen, and the induction of type II collagen occurs with the change of cellular phenotype from prechondrogenic cells to proliferating chondrocytes.[2] Hypertrophic chondrocytes then express various proteins such as type X collagen. Matrix metalloproteinase (MMP)-13 contributes to the calcification process of surrounding matrix.[3]

Runt-related gene 2 (Runx2), a potent transcription factor, belongs to the runt domain gene family.[4,5] It has been shown that endochondral ossification is completely blocked when chondrocyte maturation is disturbed in Runx2-deficient mice,[6,7] whereas Runx2 overexpression promoted maturation process of chondrocytes in Cbfa1 transgenic mice.[8] Expression of Runx2 mRNA was upregulated prior to the differentiation toward the hypertrophic phenotype, which indicated that Runx2 may be related to the maturation of hypertrophic chondrocytes in ATDC5 cells *in vitro*.[9] From these findings, Runx2 is assumed to be an essentially indispensable factor for chondrocyte differentiation or maturation.

In previous studies, a large amount of hyaluronan (HA) was detected around hypertrophic chondrocytes.[10,11] The pericellular matrix surrounding hypertrophic chondrocytes is composed of both HA and aggrecan, and attaches to the surfaces of chondrocytes through HA binding proteins.[12] Due to this structure, high molecular weight-HA (HMW-HA) is capable of absorbing a large amount of water, and its function allows it to exert hydrostatic pressure on the surrounding tissue.[13] In addition, HA function is enhanced by activation of HA receptors such as CD44.[14] It has also been shown that signal transduction of HMW-HA is performed through the activation of Smad proteins in the BMP-7 signaling pathway in chondrocytes.[15] Conversely, HA oligosaccharide (HAoligo), such as HA hexasaccharides (HA_6) induce MMP-3 via retinoid and/or NF-κB signaling pathway in chondrocytes.[16]

Therefore, HA may have a signaling function through chondrocyte differentiation that is dependent on its molecular weight. Although a crucial role of HA at the hypertrophic stage has been speculated, the effects of HA as a signaling molecule during chondrocyte differentiation remain unclear.

The aim of this study is to clarify the effects of HMW-HA and HAoligo on the expression and activity of Runx2 at the hypertrophic chondrocytes through the endochondral ossification process.

Materials and methods

Cell culture

A mouse chondrogenic cell line, ATDC5, was purchased from the RIKEN Cell Bank (Tsukuba Science City, Japan). ATDC5 cells were cultured in DMEM/Ham's F12 hybrid medium (Sigma, St. Louis, MO, USA) containing 5% fetal bovine serum (FBS) (Mitsubishi Kagaku, Tokyo, Japan), 10 μg/ml human transferrin (Sigma) and 3×10^{-8} M sodium selenite (Sigma) at 37˚C in a humidified atmosphere with 5% CO_2 in air. To induce chondrogenesis, ATDC5 cells were seeded at a density of 6×10^4 cells/well onto six well plates and cultured for 18 days in the above-mentioned medium supplemented with 10 μg/ml bovine insulin (Sigma).

Quantitative real-time PCR analysis

Total RNA was extracted from cultured ATDC5 cells using a Total RNA Extraction Kit^R (Pharmacia Biotech, Tokyo, Japan) every 3 days after the cells became confluent. The first strand cDNA was synthesized from 1 μg

total RNA using a ReverTra Ace-α first-strand cDNA synthesis kit (Toyobo, Osaka, Japan). Quantitative real-time PCR analysis was carried out using a Light Cycler Quick System 350S (Roche Diagnostics, Tokyo, Japan) under the following conditions: denaturation at 94˚C for 15 s, annealing at 60˚C for 30 s and primer extension at 72˚C for 10 s for 40 cycles. Primer sequences of type II collagen, type X collagen, Runx2, MMP-13, HAS2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are shown in Table 1. The gene expressions of type II and X collagens, MMP-13, Runx2, and HAS2 were analyzed during the overall experimental period from day 0 to 18.

Thermal cycling and fluorescence detection were performed, and the quantitative results of real-time PCR were assessed with a cycle threshold (Ct) value, which identifies a cycle when the fluorescence of given sample becomes significantly different from the base signal. Quantification of signals was performed by normalizing their signals relative to those of GAPDH. Normalized Ct values were expressed relative to that of day 0.

Alizarin red and alkaline phosphatase (ALP) staining

To detect calcium accumulation, ATDC5 cells cultured for 18 days were fixed with 2% neutralized formaldehyde solution and stained with 1% alizarin red (pH6.3) as previously described [17]. Alkaline phosphatase (ALP) staining was also performed for cultured ATDC5 cells on day 18 using fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (Sigma).

Immunostaining

Articular cartilage was removed from the TMJ of a 10-week-old rat. After fixation in 4% paraformaldehyde, cartilage tissue was embedded in paraffin and sliced into 3 μm sections. The sections were then incubated with a 1:250 dilution of anti-goat Runx2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4˚C. Following washes in phosphate buffered saline, the sections were incubated for 2 hrs with a 1:250 dilution of Donkey anti-goat antibody (Sigma). For staining, sections were preincubated with peroxidase anti-proxidase (Chemicon international, Carifornia, USA) for 2 hrs. The washed slides were then developed by the addition of 0.02% 3,3'-diaminobenzidine (DAB) for 5 min.

Preparation of HAoligo

HAoligo was refined according to the method established by Knudson *et al.*[18,19] HAoligo was generated by the cleavage of HA sodium salt from human umbilical cord (3.0-5.8×10^3 kDa; Sigma) by bovine testicular HAase (type Ⅰ-S; Sigma) at a ratio of 320 U/mg HA in 40 ml of 0.1M sodium acetate buffer with 0.15 M NaCl, pH 5.0. The sample was boiled for 15 min and precipitated with trichloroacetic acid (TCA; Katayama Chemical, Osaka, Japan). After centrifugation at 10,000 rpm for 15 min at 4˚C in a microfuge, the supernatant was dialyzed to remove small molecules. The HAoligo was freeze-dried, dissolved in phosphate buffered saline (PBS) and filtrated with 0.25μm filter (Millipore, Bedford, MA, USA). In this experiment, we used HA hexasaccharides (HA_6), which is the smallest fragment of HA capable of binding to cell surface receptors.[20] HA_6 was prepared by HAase digestion at 37˚C for 16 hrs.

Particle-exclusion assay

Visualization of HA-rich pericellular matrices was done by using a particle-exclusion assay.[21] ATDC5 cells were seeded at a density of 6×10^4 cells/well onto six-well plates and cultured 2 days. ATDC5 cells were treated with 250 μg/ml HAoligo for 1 hr before addition of 10% sheep red blood cells (Inter-cell technologies, Hopewell, NJ, USA).

Treatment with HMW-HA, HAoligo

ATDC5 cells were seeded at a density of 6×10^4 cells/well in six-well plates and cultured for 18 days (confluent on day 0). On day 18, which corresponds to the hypertrophic stage, ATDC5 cells were treated with phosphate buffered saline (PBS), serving as the control. These cells were then treated with 250 μg/ml purified HMW-HA of 1.2×10^5 Da (Seikagaku Corporation, Tokyo, Japan), 250 μg/ml HAoligo for 12 hrs with or without preincubation of 1.0 μg/ml anti-CD44-neutralizing antibody (IM7.8.1 mAb: ENDOGEN, Woburn, MA, USA) for 1 hr. The optimal treatment time of ATDC5 cells was defined by a preliminary experiment (data not shown).

Measurement of Runx2 activity

Nuclear proteins were extracted from the experimental and control cultures of ATDC5 cells on day 18 using a Nuclear Extraction Kit (Sigma). The cells were collected in ice-cold PBS in the presence of phosphatase inhibitors, and resuspended in hypotonic buffer. Cytoplasmic fractions were collected by centrifugation at 14,000 rpm for 30 s in a microfuge after the addition of detergent. Then, the nuclei were placed in a lysis buffer in the presence of the protease inhibitor and separated from insoluble materials by centrifugation. Nuclear extracts were quantified by bicinchoninic acid (BCA) protein assay and stored at -80 ˚C.

The activity of Runx2 was measured using a TransAM AML-3/Runx2 Kit (Sigma) according to the manufacturer's instructions. Briefly, 3 μg nuclear protein samples were incubated for 1 hr in a 96-well plate coated with an oligonucleotide containing a Runx2 consensus binding site, to which activated Runx2 in nuclear extracts specifically binds. After washing, Runx2 antibody was added to these wells and incubated for 1 hr. Following further incubation with a secondary HRP-conjugated antibody, specific binding was detected by colorimetric estimation at 450 nm with a reference wavelength of 655 nm.

Western blot analysis

ATDC5 cells were lysed in 50 mM Tris, pH 7.5, with 250 mM NaCl, 0.1% Triton-X-100 buffer, 1mM EDTA and 50 mM NaF with phosphatase inhibitors. 20 μg of protein was separated on 8.0% gel by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Whatman, Dassel, Germany). After blocking the membrane with 5% milk, the membrane was incubated with Runx2 monoclonal antibody (Medical & Biological Laboratories, Nagoya, Japan, dilution 1:1000) or β-actin peptide mAb (AC-15; Sigma, dilution 1:1000) detected with horse radish peroxidase (HRP)-conjugated secondary antibody (dilution 1:1000) and ECL reagents (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Statistical analysis

All assays were done in triplicate and reported with threee different samples. The data were compared by one-way analysis of variance (ANOVA).

Results

Gene expression of chodrogenic markers, HAS2 and Runx2 in cultured ATDC5 cells during the differentiation

Gene expression of type II collagen exhibited a significant increase by day 3 and reached a maximum on day 9, followed by a rapid decrease to the control level (day 0) (Fig. 1A). Type X collagen mRNA level was increased from day 0 to days 3, then decreased to the control level (Fig. 1B). From day 15, type X collagen mRNA increased again, and reached a significantly higher level on day 18. MMP-13 mRNA level exhibited an increase after day 3, reached a peak level on day 6, and then decreased gradually until day 15 (Fig. 1C). From day 15, the

MMP-13 mRNA level increased again, and reached a significantly higher level than the control. These findings indicated that the hypertrophic stage of ATDC5 cells occurred between days 15 and 18.

The Runx2 mRNA level increased from day 6, reached a maximum on day 12, and then rapidly decreased to the control level (Fig. 1D). From day 15 to day 18, defined as the hypertrophic stage, Runx2 mRNA increased to a significantly higher level.

The HAS2 mRNA level at the matrix–forming stage was relatively low (Fig. 1E). From day 12, the HAS2 mRNA level increased, and reached a maximum on day 15, defined as the pre-hypertrophic stage.

ALP activity and matrix calcification in cultured ATDC5 cells at the hypertrophic stage

We next examined whether ATDC5 cells in the hypertrophic stage expressed the hypertrophic phenotype, characterized by ALP activity and matrix calcification. ATDC5 cells on day 18 showed very high levels of ALP activity and matrix calcification, while the control cultures on day 0 showed very low levels of ALP activity and no matrix calcification (Fig. 2).

Distribution of Runx2 in temporomandibular joint (TMJ) condyle cartilage

Runx2 was highly expressed in the nuclear region of chondrocytes in the maturation and hypertrophic layers (Fig. 3B). The distribution of Runx2 in differentiating cartilage was coincident with gene expression of Runx2 in differentiating ATDC5 cells.

Effects of HAoligo on pericellular matrix around ATDC5 cells

A particle-exclusion assay was used to determine if ATDC5 cells were capable of producing and organizing HA-rich pericellular matrices. In this assay, the presence of a pericellular matrix can be visualized by the exclusion of fixed red blood cells from a clear, halo-like area adjacent to the cell (Fig. 4A). Pericellular matrix was removed by treatment with both HAoligo (Fig. 4B).

Effects of HMW-HA and HAoligo on Runx2 expression and activity in cultured ATDC5 cells at the hypertrophic stage

Runx2 mRNA expression was significantly suppressed in cultured ATDC5 cells at the hypertrophic stage by the treatment with HAoligo ($p< 0.01$) when compared to the untreated control (Fig. 5A). In particular, HAoligo decreased the expression of Runx2 mRNA most significantly, to less than half of the control level. Meanwhile, no significant effects of HMW-HA were found on Runx2 mRNA expression (Fig. 5A).

The expression of Runx2 protein was evaluated using Western blot analysis. An immunoreactive band of 55kDa was observed, which corresponding to Runx2 (Fig. 5B). The treatment of HAoligo prominently decreased Runx2 protein expression, while Runx2 protein exhibited a tendency to decrease by the treatment with HMW-HA.

Runx2 activity was significantly decreased in cultured ATDC5 cells at the hypertrophic stage by the treatment with HAoligo as compared to the untreated control ($p<0.05$). Meanwhile, Runx2 activity exhibited a tendency to decrease, but not significant, with the treatment of HMW-HA (Fig. 5C).

Effects of HMW-HA, HAoligo, and anti-CD44 neutralizing antibody on Runx2 expression in cultured ATDC5 cells at the hypertrophic stage

Runx2 mRNA expression significantly decreased in cultured ATDC5 cells at the hypertrophic stage by the treatment with anti-CD44 neutralizing antibody alone ($p<0.01$). Furthermore, anti-CD44 neutralizing antibody treatment, followed by addition of HAoligo and HMW-HA, decreased the expression of Runx2 mRNA (Fig. 6). The

addition of HMW-HA significantly restored the expression of Runx2 which decreased with the treatment of anti-CD44 neutralizing antibody ($p<0.05$)(Fig. 6).

Effects of HMW-HA, HAoligo, and anti-CD44 neutralizing antibody on chondrogenic markers gene expression in cultured ATDC5 cells at the hypertrophic stage

Type II collagen mRNA expression was significantly decreased by the treatment of anti-CD44 neutralizing antibody, while type II collagen mRNA expression exhibited a tendency to decrease, but not significantly, by the treatment with HMW-HA (Fig. 7A). Furthermore, type II collagen mRNA expression was unchanged by the treatment with HAoligo.

Type X collagen mRNA expression was significantly decreased by treatment with HAoligo and anti-CD44 neutralizing antibody treatment (Fig. 7B).

MMP-13 mRNA expression was significantly decreased by the treatment with HAoligo, while no significant effects of HMW-HA and anti-CD44 neutralizing antibody were found on Runx2 mRNA expression (Fig. 7C).

Discussion

Cultures of ATDC5 cells have been shown to be a useful *in vitro* model for examining the differentiation of chondrocytes. Cultured in the presence of insulin, ATDC5 cells form many spots of cartilage-like cellular condensation, and they are able to differentiate to hypertrophic chondrocytes.[22,23] During ATDC5 cell differentiation, type II collagen mRNA markedly increased from day 9 to 15. It has already been demonstrated that type II collagen is synthesized by proliferating chondrocytes after the differentiation from prechondrogenic cells.[2,9,24,25] Therefore, cells proliferating from day 9 to 15 in ATDC5 cultures are most likely chondrocytes. Meanwhile, we have showed that type X collagen mRNA markedly increased from day 15 to 18. Because type X collagen is synthesized specifically by hypertrophic chondrocytes, induction of type X collagen mRNA is regarded as a signal of the differentiation from proliferating to hypertrophic ones.[9,22]

It was also clarified that both MMP-13 and Runx2 increased during this period. Runx2 is well known to upregulate the expression of the gene promoters related to MMP-13 and bone matrix proteins including osteopontin (OPN) and bone sialoprotein (BSP).[1,26] These results indicated that the expression of both Runx2 and MMP-13 mRNAs in ATDC5 cells markedly increased from day 15, suggesting an initiation of the hypertrophic stage. In the hypertrophic stage, ATDC5 cells showed very high levels of ALP activity and matrix calcification.

We also investigated the distribution of Runx2 in mandibular condylar cartilage, and found that Runx2 was distributed widely in the maturation and hypertrophic layer coincident, with gene expression of Runx2 in cultured ATDC5 cells.

With respect to the effect of HA on Runx2 activity, we have shown that HMW-HA had no significant effects on gene expression and activity of Runx2. In process of chondrocyte differentiation, the gene expression and synthesis of HA are increased at the hypertrophic stage in cultured chondrocytes.[10] Magee *et al*.[27] reported that HA synthesis was elevated in the hypertrophic zone, along with the up-regulation of HAS2 mRNA and the activation of UDP-glucose pyrophosphorylase (UDPG-PPase) in the developing growth plate chondrocytes. The present study showed that the gene expression of HAS2, which is well known to lead to HMW-HA synthesis, was up-regulated after the matrix-forming stage and at the pre-hypertrophic stage in cultured ATDC5 cells. The up-regulation of the HAS2 gene generated a considerable increase in HMW-HA synthesis in hypertrophic chondrocytes.[10] From these findings, it would be reasonably assumed that HAS2 and HMW-HA exert anabolic functions for chondrocytes and the pericellular matrices, leading to no suppression of gene expression and activity of Runx2.

On the other hand, the treatment with HAoligo suppressed both gene expression and activity of Runx2. This

result supports a hypothesis that HAoligo is an active mediator for the response of chondrocyte by either transmitting a signal directly through CD44 or disrupting HA-CD44 interactions. Knudson *et al*.[18,28] revealed that the cell–matrix interactions in chondrocytes were inhibited by the treatment with excessive small HAoligo, such as HA_6. It is also shown that HAoligo induces MMP-3 via the retinoid and/or NF-$_\kappa$B signaling pathway in chondrocytes.[16] Furthermore, HAoligo induced the activation of NF-$_\kappa$B, (MAP)-kinases, protein kinase C (PKC), G-protein, and PI3 kinase in other cells.[29-33] From these results, it is suggested that HAoligo might have a signaling function through chondrocyte differentiation.

These results indicate have shown that Runx2 gene expression was significantly decreased in cultured ATDC5 cells at the hypertrophic stage treated with anti-CD44 neutralizing antibody alone. However, HAoligo had no synergistic effect of anti-CD44 neutralizing antibody on the down-regulation of Runx2 mRNA, comparing to anti-CD44 neutralizing antibody alone. Taking it into consideration that HAoligo down-regulates the expression and activity of Runx2, it is probable that HAoligo transduces some signals through CD44.

It has been previously shown that HA_6 was effective at preventing formation of HA-dependent pericellular matrices in other cells.[21] In this study we have shown that HAoligo decreased HA-dependent pericellular matrices in ATDC5 cells. Furthermore, anti-CD44 neutralizing antibody treatment followed by addition of HAoligo and HMW-HA decreased the expression of Runx2 mRNA. The addition of HMW-HA significantly restored the expression of Runx2, which decreased with the treatment of anti-CD44 neutralizing antibody. In addition, it has been reported that HAoligo competes for binding of HMW-HA to the cell surface.[34,35] Given that HMW-HA is bound to CD44 in this study, HMW-HA likely prevents the novel binding of low molecular weight-HA to CD44, and maintains chondrocyte differentiation of hypertrophic stage.

In this study, the expression of Type X collagen and MMP-13 mRNA in cultured ATDC5 cells at the hypertrophic stage significantly decreased with the treatment of HAoligo, while type II collagen mRNA expression was unchanged with the treatment of HAoligo. Previous studies have suggested that chondrocytes promote type X collagen mRNA expression and concomitantly decrease expression of type II collagen mRNA in the cell hypertrophy.[36] In addition, MMP-13 is associated with both chondrocyte hypertrophy and with matrix vesicles implicated in cartilage matrix mineralization.[37,38] It has also been reported that Runx2 is able to induce the expression of MMP-13.[36] Therefore, the decrease of Runx2 expression by HAoligo treatment might downregulate chondrocyte differentiation at the hypertrophic stage.

In conclusion, a new finding in this study was that HAoligo suppressed the gene expression and activity of Runx2 through CD44 in hypertrophic chondrocytes. In addition, HAoligo may contribute to the inhibition of endochondral ossification by blocking the hypertrophy of chondrocytes.

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Figure Legends

Fig.1. Changes in the gene expression of type II and X collagen, MMP-13, Runx2 and HAS2 during differentiation in cultured ATDC5 cells. Total RNA was extracted from ATDC5 cells on 0-18 days after the confluence. The mRNA expression levels of type II and (A) type X collagen (B), MMP-13 (C), Runx2 (D), and HAS2 (E) were determined by means of a real-time PCR analysis, normalized relative to the expression of GAPDH and depicted as the rate of change in gene expression. Error bars indicate a standard deviation.

Fig.2. Alizarin red and alkaline phosphatase (ALP) staining. To detect calcium accumulation, cultured ATDC5 cells for 18 days were fixed with 2% neutralized formaldehyde solution and stained with 1% Alizarin red (pH6.3). Alkaline phosphatase (ALP) staining was also performed for cultured ATDC5 cells on day 18 using fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets.

Fig.3. Distribution of Runx2 in mandibular condylar cartilage. Paraffin sections of articular cartilage from rat TMJ were incubated with control IgG (A) or Runx2 antibody (B). Runx2 distribution was visualized by 0.02% 3,3'-diaminobenzidine (DAB). Runx2 was highly expressed in the nuclear region of chondrocytes in the maturation and hypertrophic layer. Bar = 6μm.

Fig.4.Effects of HAoligo on pericellular matrix around ATDC5 cells. ATDC5 cells were incubated for 60 minutes in the absence (A) or presence of 250 μg/ml of HAoligo (B) and then examined with the particle-exclusion assay. The particle-exclusion assay was used to demonstrate the presence of the HA-dependent pericellular matrix, which was apparent as a clear zone around ATDC5 cells. Bar = 50 μm.

Fig.5. Effects of HMW-HA and HAoligo on Runx2 mRNA expression in cultured ATDC5 cells. On day 18, ATDC5 cells were treated with PBS (control), 250 μg/ml purified HMW-HA of 1.2×10^5 Da and 250 μg/ml of HAoligo. The expression levels of Runx2 mRNA was determined by means of a real-time PCR analysis, normalized relative to the expression of GAPDH and depicted as the rate of change in gene expression (A). Protein was prepared from each culture by using Triton X-100 buffer, and 20 μg of protein was separeted on 8.0% gel by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated with Runx2 monoclonal antibody and detected with HRP-conjugated secondary antibody and ECL reagents (B). After incubation for 12 hrs, proteins were extracted from the control and experimental cultures of ATDC5 cells for a TransAM analysis (C). Error bars indicate a standard deviation.

Fig.6. Effects of HMW-HA, HAoligo and anti-CD44 neutralizing antibody on Runx2 mRNA expression in cultured ATDC5 cells. On day 18, ATDC5 cells were treated with IgG (control), 250 μg/ml purified HMW-HA of 1.2×10^5 Da and 250 μg/ml of HAoligo for 12 hrs with or without preincubation of 1.0 μg/ml anti-CD44-neutralizing antibody for 1 hr. The expression levels of Runx2 mRNA was determined by means of a real-time PCR analysis, normalized relative to the expression of GAPDH and depicted as the rate of change in gene expression. Error bars indicate a standard deviation.

Fig.7. Effects of HMW-HA, HAoligo and anti-CD44 neutralizing antibody on Type II collagen, Type X collagen and MMP-13 mRNA expression in cultured ATDC5 cells. On day 18, ATDC5 cells were treated with PBS (control), 250 μg/ml purified HMW-HA of 1.2×10^5 Da, 250 μg/ml of HAoligo and 1.0 μg/ml anti-CD44-neutralizing antibody

for 12 hrs. The expression levels of Type II collagen (A), Type X collagen (B) and MMP-13 (C) mRNA were determined by means of a real-time PCR analysis, normalized relative to the expression of GAPDH, and depicted as the rate of change in gene expression. Error bars indicate a standard deviation.

Figure 1

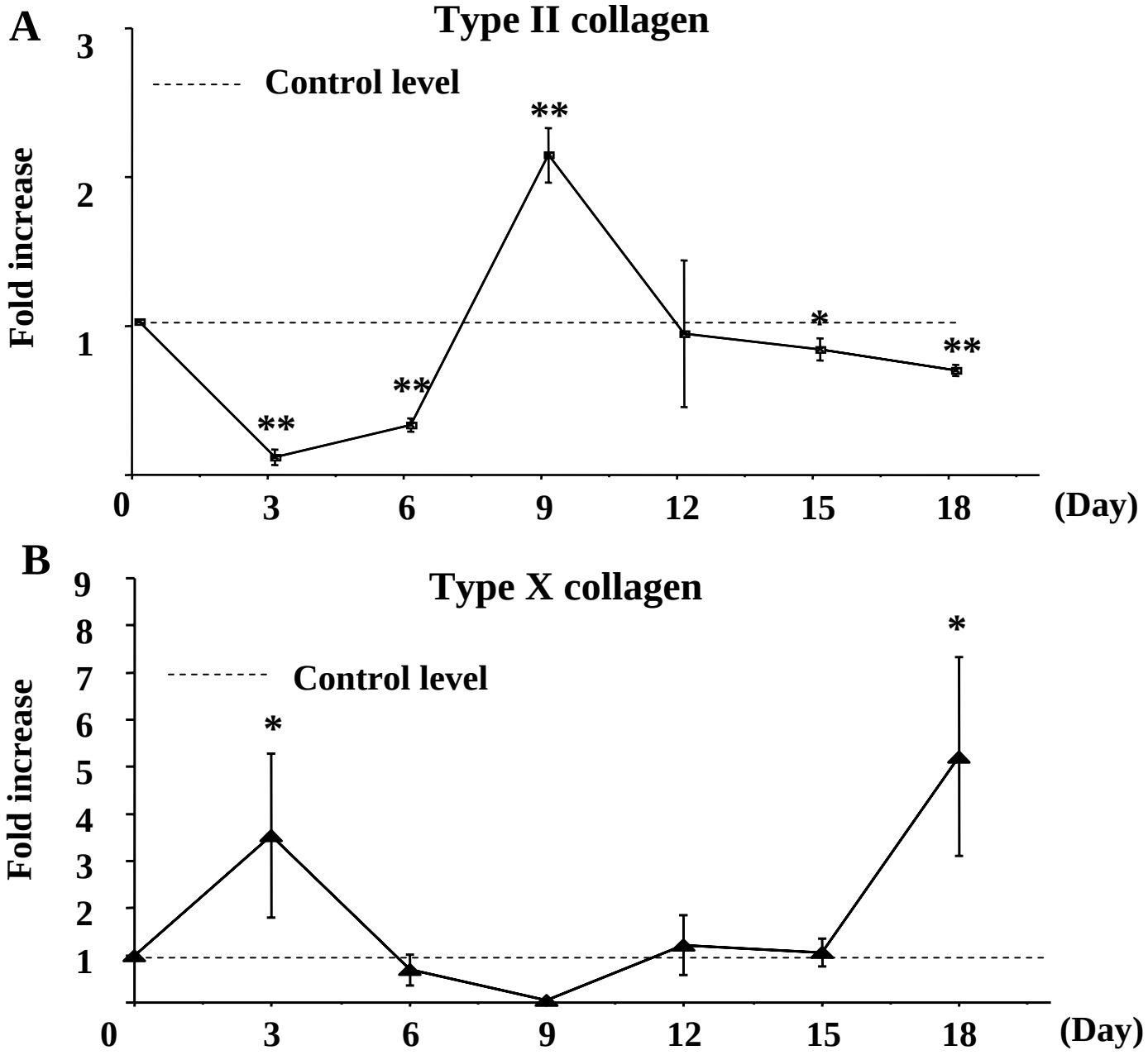
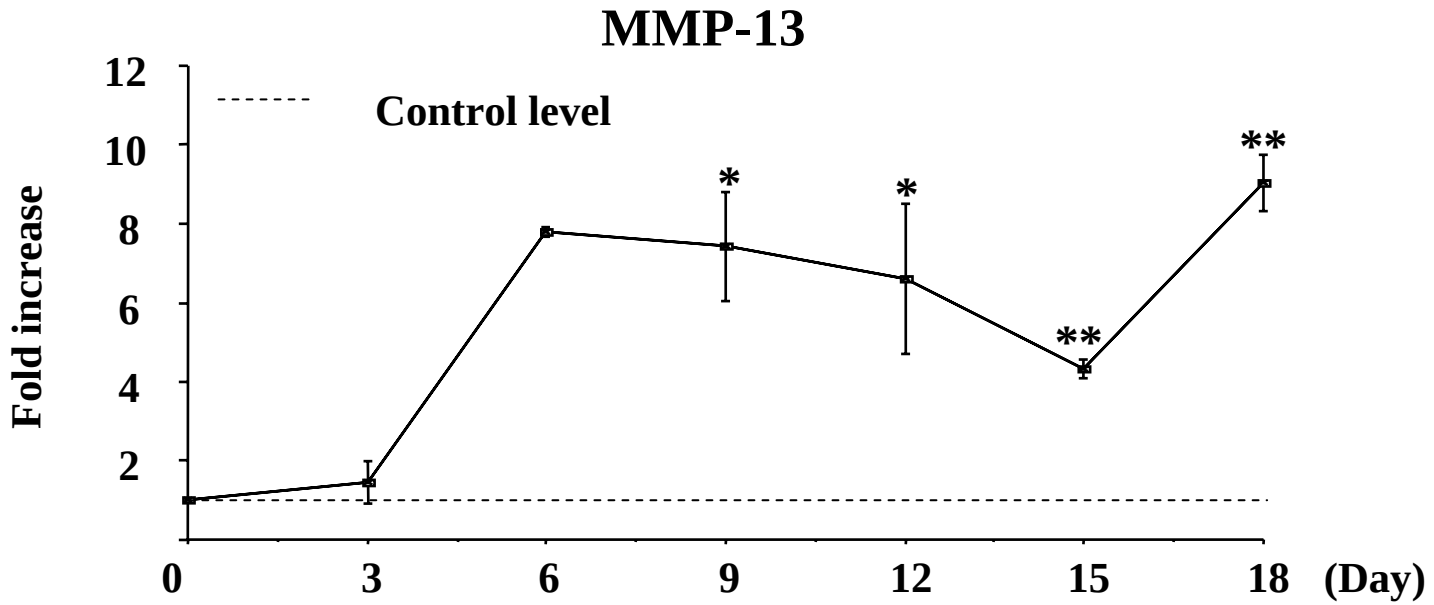
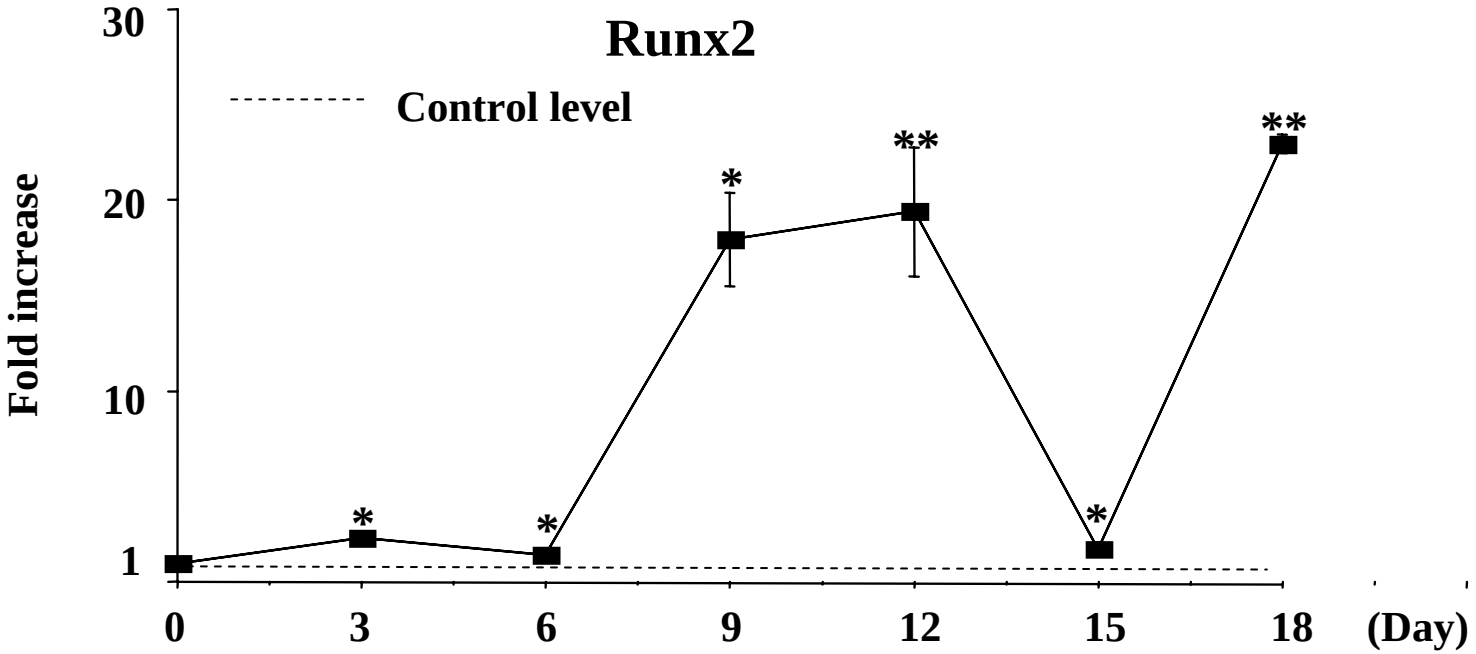

A
Type II collagen
Control level
Fold increase
3
2
1
**
**
**
*
**
0
3
6
9
12
15
18
(Day)
B
Type X collagen
Control level
Fold increase
9
8
7
6
5
4
3
2
1
*
*
0
3
6
9
12
15
18
(Day)

Figure 1

C


MMP-13
Control level
Fold increase
12
10
8
6
4
2
0
3
6
9
12
15
18
(Day)
*
*
**
**

D


Runx2
Control level
Fold increase
30
20
10
1
0
3
6
9
12
15
18
(Day)
*
*
*
**
*
**

E

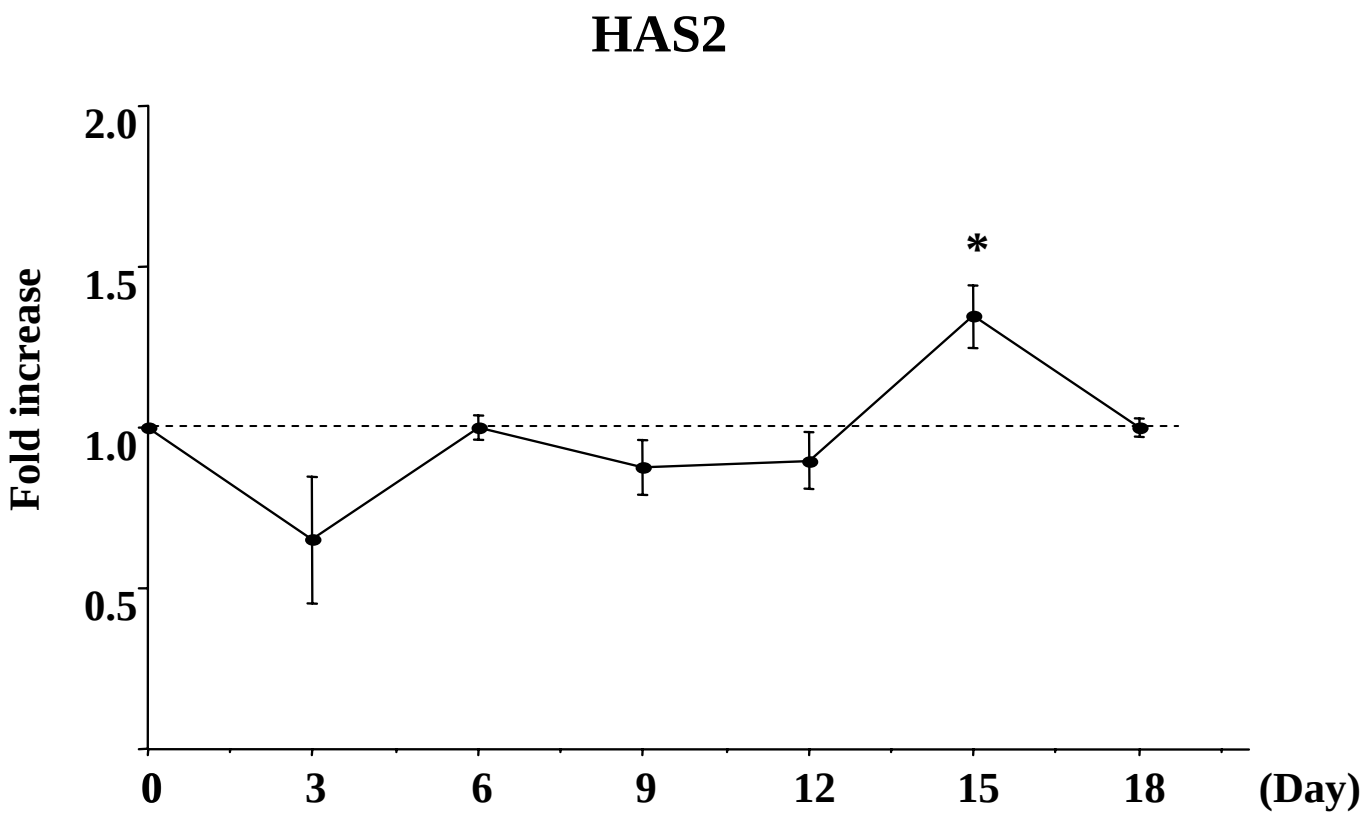


Figure 2

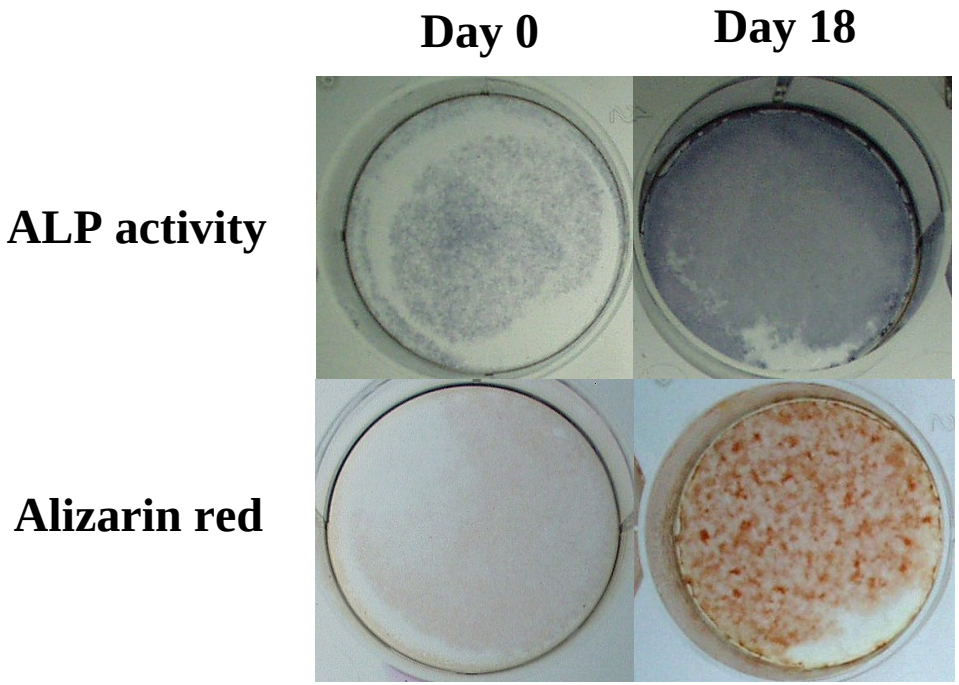


Figure 3

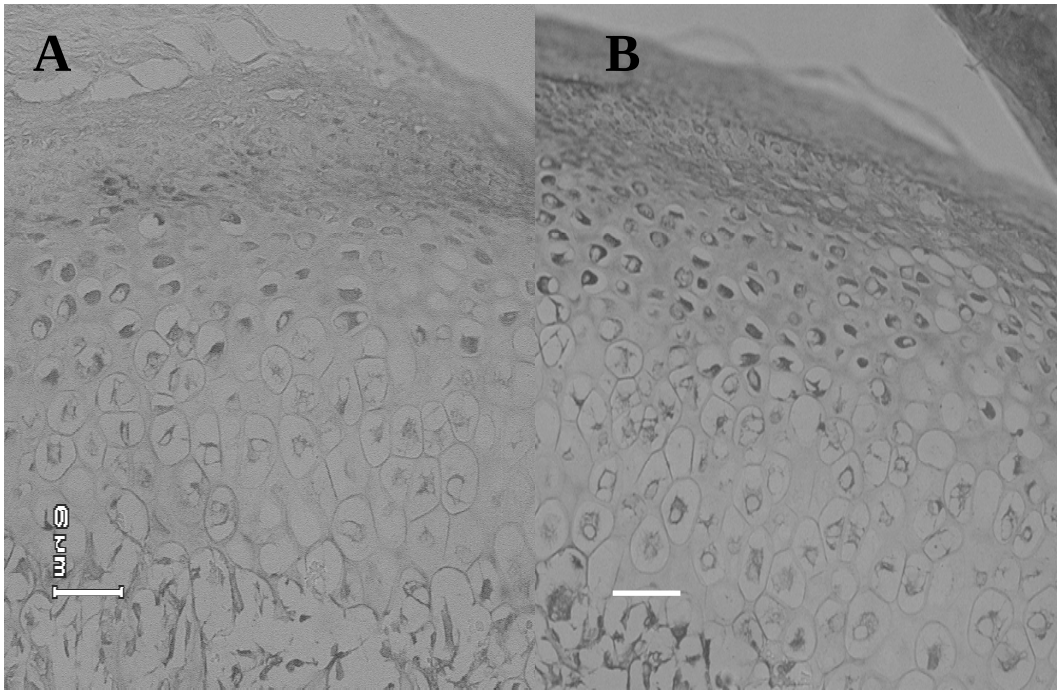


Figure 4

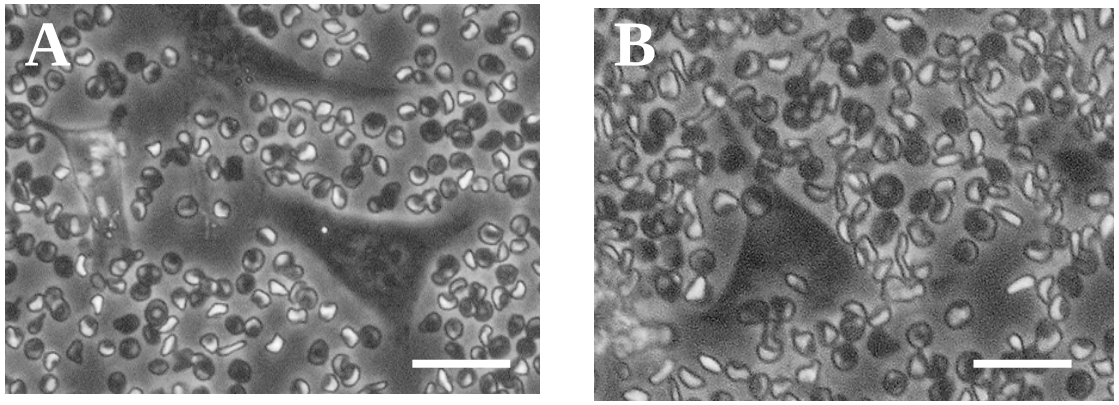


Figure 5

A

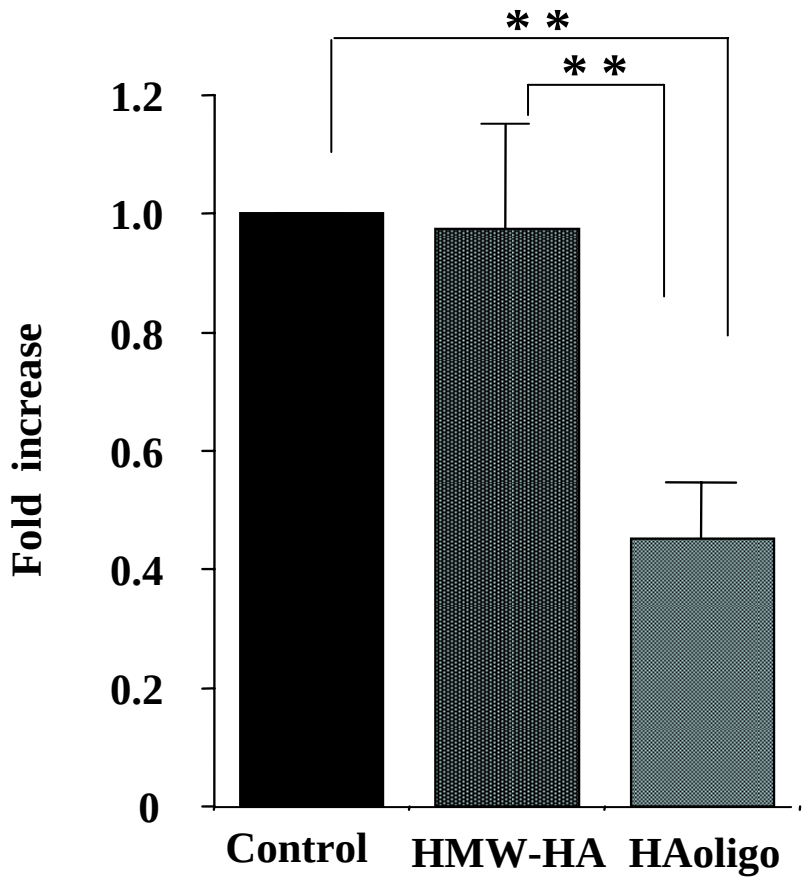
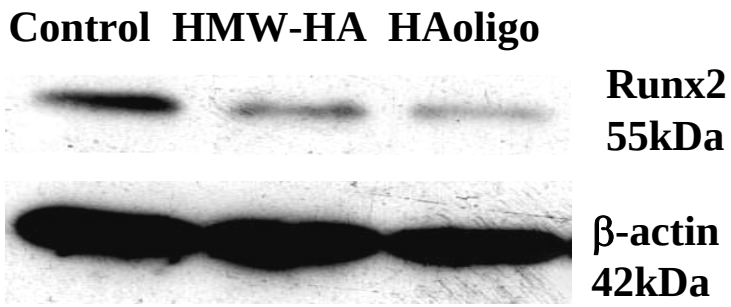


Figure 5

B


Control HMW-HA HAoligo
Runx2
55kDa
β-actin
42kDa

C

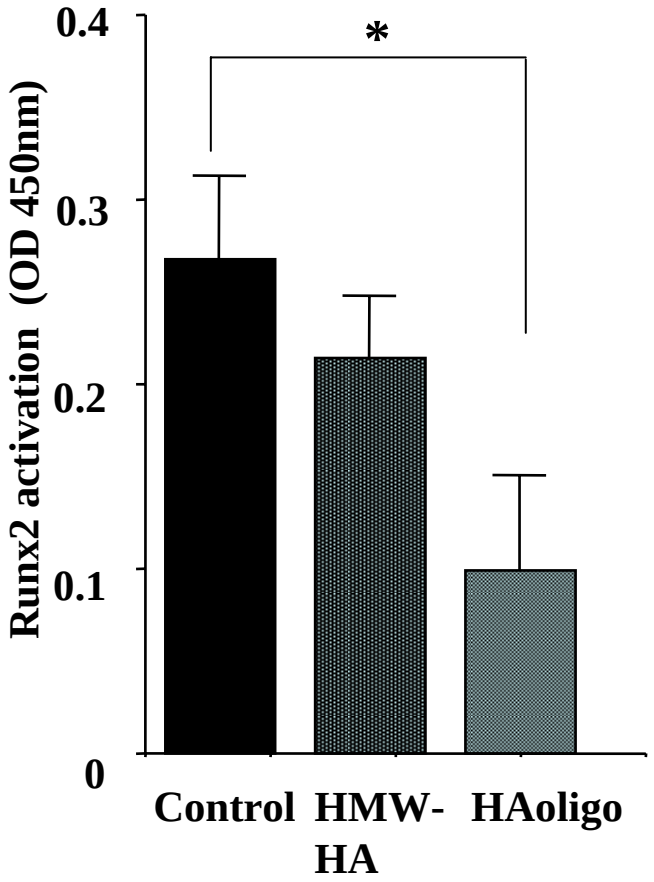

0.4
0.3
0.2
0.1
0
*
Runx2 activation (OD 450nm)
Control
HMW-HA
HAoligo

Figure 6

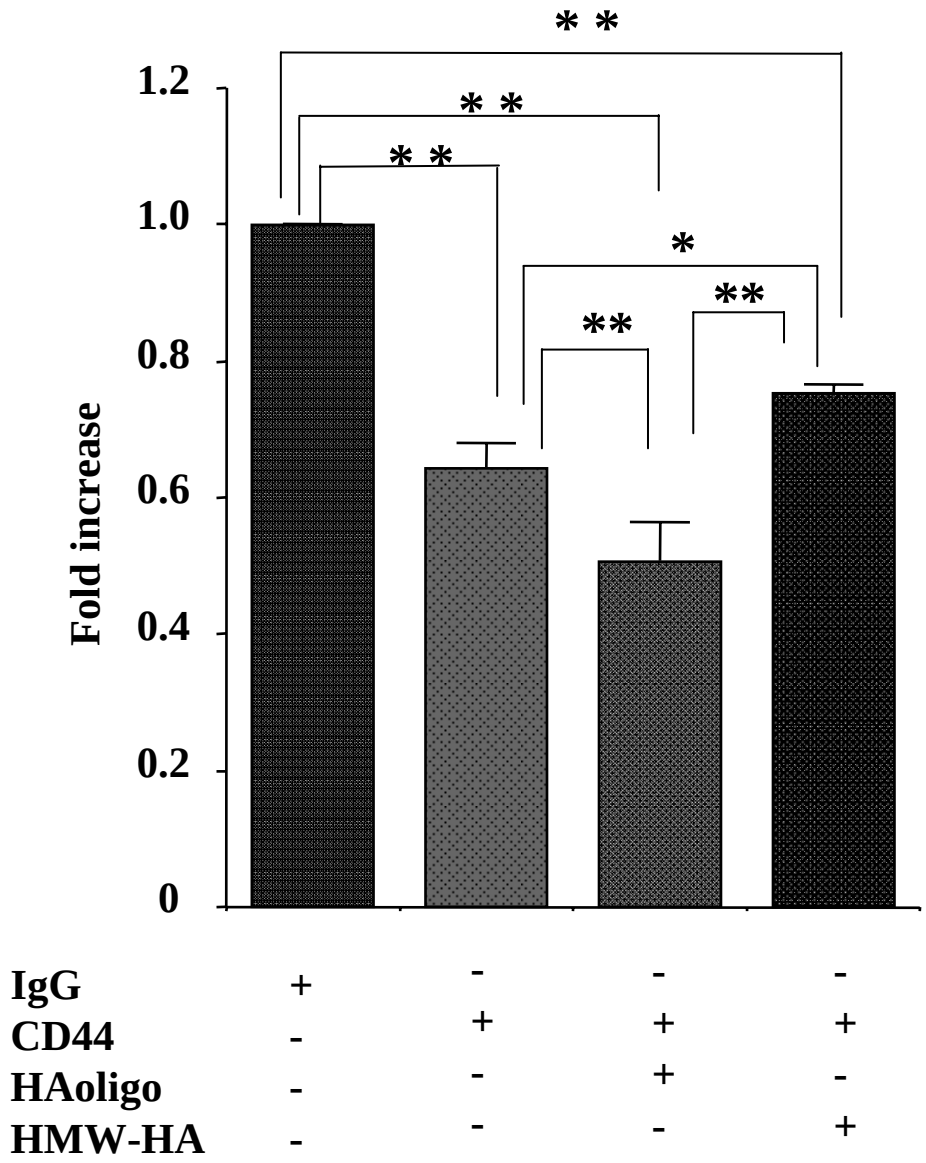


Figure 7

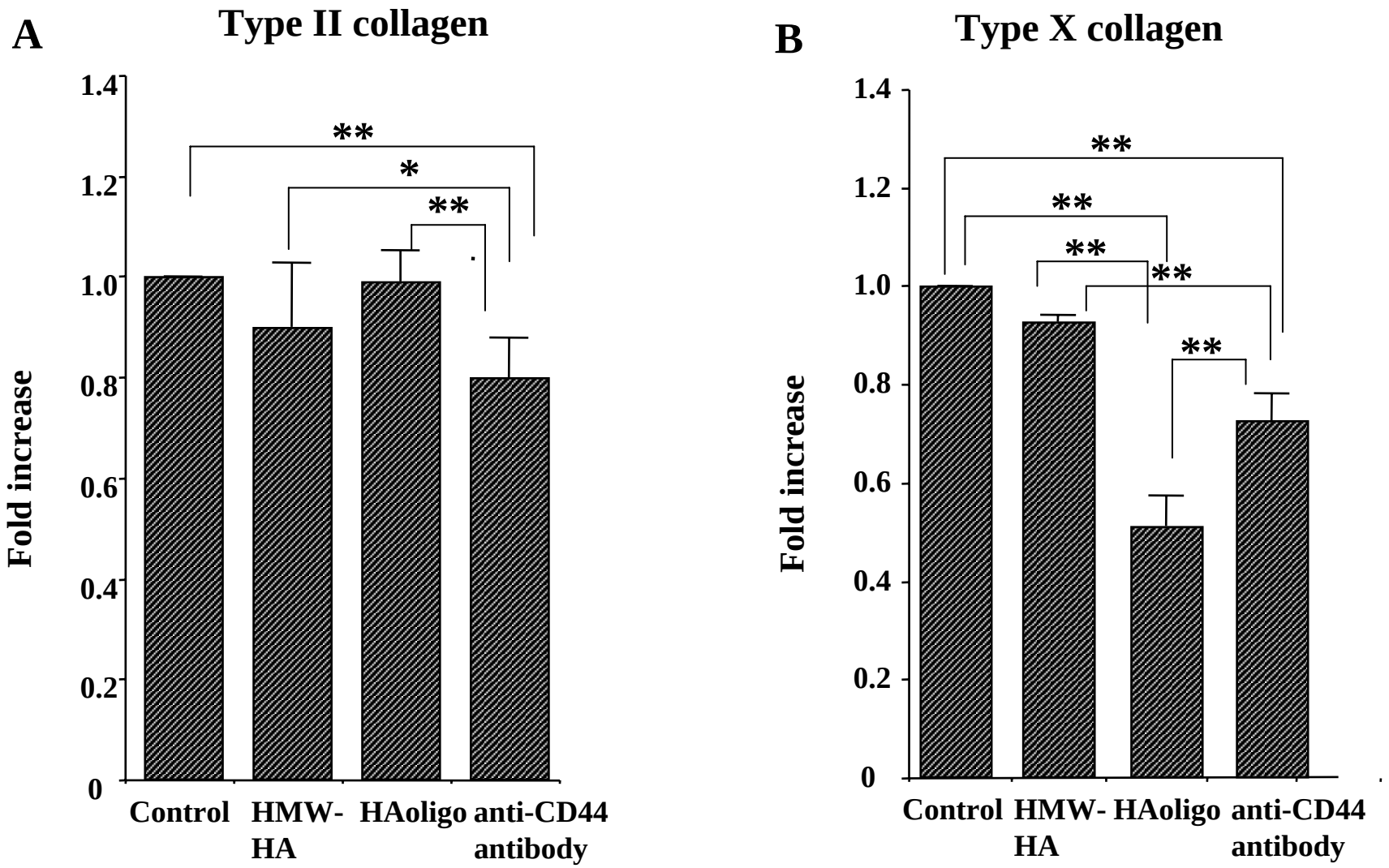
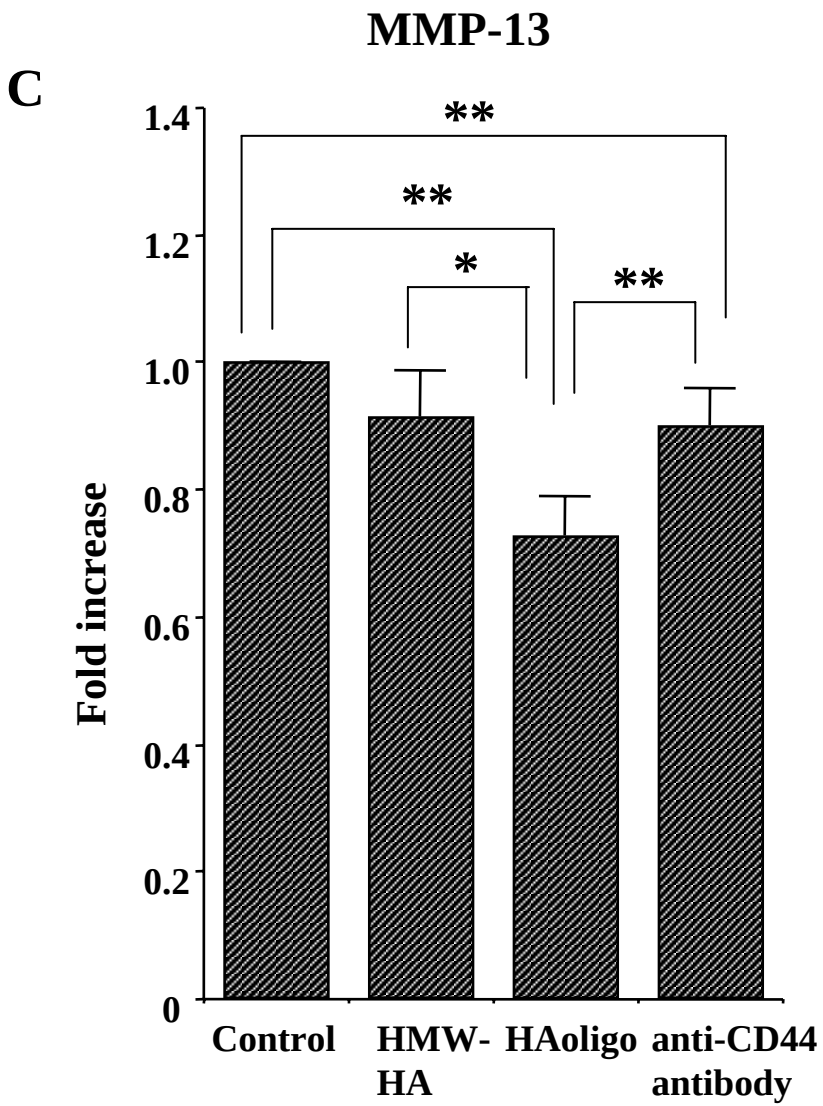

A
Type II collagen
Fold increase
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
*
**
Control
HMW-HA
HAoligo
anti-CD44 antibody
B
Type X collagen
Fold increase
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
**
**
**
Control
HMW-HA
HAoligo
anti-CD44 antibody

Figure 7


C
MMP-13
Fold increase
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
**
*
**
Control
HMW-HA
HAoligo
anti-CD44 antibody