Original research:

Hyaluronan metabolism in overloaded temporomandibular joint

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Abstract

The present study aimed to examine hyaluronan (HA) metabolism in relation to the onset and progression of temporomandibular joint osteoarthritis (TMJ-OA) induced by mechanical overloading. Two-month-old and 6-month-old C57BL/6N mice were divided into experimental and untreated control groups (n = 5/group). A sliding plate was attached to the maxillary incisors of the experimental mice for 10 days to overload the condylar cartilage in TMJ. In experimental group, profound cartilage degradation was detected in hematoxylin-eosin, Safranin-O-Fast Green stained sections. It was also shown that the cartilage degradation was greater in older mice in both the control and the experimental groups. The number of HABP positive cells was decreased by mechanical overloading and with age. The reduction of HA expression was correlated with the progression of cartilage degradation induced by mechanical overloading. The absolute quantification of the mRNA expression related to HA synthesis and HA degradation was also performed in each group. The mRNA expression levels of HA synthase (HAS) 2 and 3 were lower in the experimental group compared with the control group in the younger mice. In contrast, the mRNA expression levels of the HA degradation gene, HYAL2 and KIAA1199, were higher in the experimental group compared with the control group in the older mice. Thus, mechanical overload differently affected the balance of HA degradation and HA synthesis in the older and younger mice, respectively. In conclusion, mechanical overloading affects HA metabolism and it might initiate or amplify the condylar cartilage degradation.

Keywords

Hyaluronan, temporomandibular joint, osteoarthritis

Background

The temporomandibular joint (TMJ), like other synovial joints, enables relatively large movements between two separate bones, the temporal bone and the mandibular condyle (1). However, bilateral TMJs function in tandem and the range of motion has a fixed endpoint in the dentition. Therefore, mandibular condylar cartilage plays a crucial role in mandibular movement by facilitating articulation with the TMJ disc and reducing mechanical loads on the underlying bone (2). Mandibular condylar cartilage is composed of chondrocytes and a large number of extracellular matrix (ECM) macromolecules, including proteoglycans, glycosaminoglycans (GAGs), and type II, IX, and XI collagens. Among the GAGs, hyaluronic acid (HA), chondroitin, and keratan sulfate are the main polysaccharides (3). HA is synthesized in the cytoplasmic space of the plasma membrane by three HA synthases, HAS1, HAS2, and HAS3 (4). HAS1 and HAS2 polymerize HA chains of similar lengths (e.g., up to 2×10^3 kDa), whereas HAS3 produces shorter chains of HA (e.g., 200–300 kDa) (4). Conversely, HA undergoes degradation by hyaluronidase (HYAL), and this process plays a key role in the development of osteoarthritis (OA) (5). Among the three HYALs known to date, HYAL1 and HYAL2 have been found to be widely expressed in mammalian tissues (6) and are the principal mediators of HA catabolism (7). More recently, the unique HA binding protein, KIAA1199, has been described and it facilitates the degradation of HA in a subset of tissues, including articular cartilage.

However, it does not share homology with the active sites of other known mammalian HYALs, and it remains unclear whether it directly or indirectly depolymerizes HA (8).

It has been hypothesized that HA degradation via free radical de-polymerization of the HA chain (9) occurs in OA joints of the TMJ, and this would potentially lead to a decrease in the molecular weight of HA in the synovial fluid (10). In addition, HA degradation could affect the viscoelastic properties of the synovial fluid in OA joints, thereby impairing joint lubrication (11). Fragmentation of HA and cartilage destruction could also manifest as a result of enhanced expression of metalloproteinases (MMPs) and up-regulation of CD44 (12). However, the role(s) of HA in condylar cartilage homeostasis remain unclear.

Therefore, the present study aimed to examine HA metabolism in relation to the onset and progression of TMJ-OA induced by mechanical overloading.

Methods

Animal experiments

Two-month old and six-month-old C57BL/6N male mice were randomly divided into four groups: two control groups (2-month-old and 6-month-old) and two experimental groups (2-month-old and 6-month-old). In the experimental groups, the TMJs were repeatedly overloaded for 10 consecutive days with a sliding plate that was applied to the maxillary incisors to keep the mandible anterior-superior position by biting (13). The sliding plate was carefully bonded onto the upper incisors with photo-curing resin while the mice were under anesthesia induced by an intraperitoneal injection of 40 mg/kg pentobarbital (Somnopentyl; Kyoritsu

Seiyaku, Tokyo, Japan). In contrast, the control groups remained untreated. All of the mice were maintained at a constant ambient temperature (22–24 °C) and 12 h light/dark cycle. All of the mice also received a stock diet with water provided *ad libitum*.

Tissue preparation

After 10 days of the experimental periods, all mice were killed with an overdose of anesthesia. TMJs were resected from all four groups and fixed in 4% paraformaldehyde for 24 h at 4°C, then were decalcified with 10% EDTA for 30 d. After dehydration with ethanol, the fixed TMJs were embedded in paraffin and serial sections (7 μm) of the sagittal plane were cut using a microtome (Carl Zeiss HM360, Jena, Germany). The sections were subsequently stained with hematoxylin and eosin (HE) for histologic assessments, and were stained with 0.1% Safranin-O and 0.02% Fast Green to detect cartilage and proteoglycans, respectively.

After deparaffinization of the sections with xylene, the sections were rehydrated in ethanol and then were incubated with 3% peroxide in methanol for 20 min to block endogeneous peroxidase activity. The sections were subsequently incubated overnight at 4°C with an anti-HABP antibody (ab174953; Abcam, Cambridge, UK) diluted 1:100 in 0.1% bovine serum albumin/phosphate-buffered saline (PBS). After the sections were washed with PBS, they were incubated with an appropriate secondary antibody for 30 min. To visualize antibody binding, the Dako Liquid DAB + Substrate Chromogen System (Dako, Glostrup, Denmark)

was used. Mounted sections were analyzed with a BioReVo BZ-9000 microscope (KEYENCE, Osaka, Japan).

RNA extraction and PCR amplification

Mandibular condyles were carefully debrided under a microscope before total RNA was extracted from the head with Nucleo Spin RNA II kits (Macherey-Nagel, Duren, Germany), according to the manufacturer's protocol. Total RNA samples were then converted to cDNA with a High-Capacity RNA to cDNA Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. Absolute quantification was performed with the QuantStudio™ 3D Digital PCR System (Life Technologies). Each reaction mixture contained 8 μL of 2-fold diluted QuantStudio™ 3D Digital PCR Master Mix (Life Technologies), 0.8 μL 20-fold Taq-Man Assay-by-Design primer-probe mix (Table 1), 1.6 μL diluted cDNA (10 ng/μL), and 5.6 μL nuclease-free water (Qiagen). Samples were loaded into the QuantStudio™ 3D Digital PCR Chip Loader and were amplified with a GeneAmp PCR System 9700 (Applied Biosystems) with the following cycling parameters: 96°C for 10 min, 42 cycles at 60°C for 2 min, 98°C for 30 s, and 60°C for 2 min. After thermocycling, the chips were measured with the QuantStudio™ 3D Analysis Suite and the raw data were analyzed with QuantStudio™ 3D Analysis Suite™ Cloud Software.

Statistical analysis

All data are presented as the mean ± standard deviation (SD). Statistically significant differences among the groups were evaluated with one-way analysis

of variance (ANOVA) with a post-hoc comparison between groups performed by using Tukey's test. A P-value less than 0.05 was considered statistically significant.

Results

Histology

HE staining of the TMJs that were resected from the 2-month-old (2M) control mice showed that the surface of the condylar cartilage was smooth and the cell layers were ordered (Fig. 1A). In contrast, some condylar cartilage of the TMJs resected from the 6-month-old (6M) control mice and the 2M experimental mice exhibited irregular cell arrangements, acellular areas under the surface, unclear borders for the cell layers, and vacuolation of the subchondral bone (Fig. 1A). In addition to these observations that were also made in the 6M experimental sections, a vertical cleft on the surface of the condylar cartilage was frequently observed (Fig. 1A).

Safranin-O stained sections showed that proteoglycans were abundant in the deep layer of the cartilage (Fig 1B). However, the amount of proteoglycan significantly decreased at 6M comparing to 2M in both the control and the experimental groups. ($P < 0.01$) (Fig. 1B, C). Furthermore, when proteoglycan expression was compared between the control and experimental sections from mice of the same age, the experimental sections exhibited significantly lower levels of proteoglycans ($P < 0.01$) (Fig. 1B, C). Histological grading according to Modified Mankin score showed that the values of 6M mice were significantly higher than the values of 2M mice in the control and the experimental groups,

respectively. ($P < 0.01$) (Fig. 1D). And, there were significant differences between the control and the experimental groups at both time points ($P < 0.01$) (Fig. 1D).

Immunohistochemical analysis

In the immunohistochemical studies that were performed to detect HABP-positive cells in the condylar cartilage of TMJs, the number of HABP-positive cells was markedly decreased between the 2M and the 6M mice in both the control and the experimental groups. (Fig. 2A, B). Specifically, HABP-positive cells were present in all layers of the condylar cartilage in the 2M control group, while a decrease in the number of HABP-positive cells was observed in the hypertrophic and mature layers of the condylar cartilage in the 6M control group (Fig. 2A). When the number of HABP-positive cells was compared between the control and experimental groups at both time points, significantly fewer number of HABP-positive cells was detected in the experimental group compared with the control group ($P < 0.01$) (Fig. 2A, B).

The mRNA expression of HA metabolism-related enzymes

To measure the mRNA copy numbers of both HA synthesis and HA degradation enzymes in mandibular condyles, we conducted Digital PCR using gene specific FAM™ probes (Table 2). Significant decreases in the mRNA expression levels of HA synthesis enzymes, HAS2 and HAS3, were detected between the 2M control and 2M experimental group ($P < 0.05$) (Fig. 3). In contrast, the mRNA expression levels of HA degradation enzymes, HYAL2 and KIAA1199, were significantly higher in the 6M experimental group compared with the 6M control group ($P <$

0.05) (Fig.3).

Discussion

In the present study, the area of proteoglycan expression and the number of HABP-positive cells in the condylar cartilage of the control and experimental groups were found to decrease with age between the 2M and the 6M mice, while the mRNA expression levels of both HA synthesis and HA degradation enzymes in the condylar cartilage were not significantly changed between the 2M and the 6M mice.

When compared the control and the experimental groups in the mice of same age, the area of proteoglycan expression and the number of HABP-positive cells were decreased in the experimental group at both time point. Furthermore, it was shown that mechanical overload to the TMJ significantly decreased the mRNA expression levels of HAS2 and HAS3 at 2M. On the other hand, the mRNA expression levels of HYAL2 and KIAA1199 were significantly increased by mechanical overload at 6M.

These results imply that mechanical overload to the TMJ affected HA synthesis in the younger mice, while in the aged mice, the mechanical overload affected the degradation of HA.

In previous studies, HA in articular cartilage has been shown to undergo various changes with age. For example, the molecular weight of HA in human articular cartilage was reported to decrease from 2000 kDa to 300 kDa in individuals varying in age from 2.5 and 86 years (14). Since HA in cartilage is essential for maintaining viscosity, such a decrease in molecular weight could potentially lead

to a reduction in the biological properties. Furthermore, both the frequency and severity of cartilage breakdown appear to increase with aging. Correspondingly, degenerative joint disease typically occurs in the fifth and sixth decades of life when articular cartilage starts to lose its cellular density and adaptive capacity (15). The amount of GAGs in TMJ cartilage has also been found to increase markedly with the growth of newborns into mature adults, and then decrease thereafter (16). A decrease in GAGs with age may lead to increased osmotic swelling pressure and cartilaginous stiffness, thereby resulting in very weak and fragile characteristics of the mandibular condylar cartilage in aged specimens. The results of the present study are consistent with these data.

HYALs are common and ubiquitously important hyaluronidases (17). In particular, maxillofacial skeletal defects have been observed in HYAL2$^{-/-}$ mice (18), thereby indicating that HYAL2 is necessary for normal growth of skeletal elements. We previously demonstrated that expression of HYAL2 in articular chondrocytes is enhanced in response to excessive mechanical stimuli, and consequently, HA catabolism was observed in articular cartilage (19). Taken together, these results suggest that HAYLs play an essential role in the accumulation of both low-molecular weight and high-molecular weight HA, and a decrease in high-molecular weight HA can lead to an inflammatory condition (20).

In the present study, the data from the aged group are consistent with the possibility that inflammation induced by the application of mechanical overload enhances the degradation of high-molecular weight HA by HYAL2 (which was up-regulated).

Recently, a new HA degradation mechanism has been reported which is related

to the clathrin-coated pit pathway (21). Specifically, the transfection of KIAA1199 cDNA into cells was found to confer the ability to catabolize HA via the clathrin-coated pit pathway (21). Previously, KIAA1199 was shown to be essential for the endogenous degradation of HA in human skin fibroblasts (8).

In the present study, the mRNA expression levels of KIAA1199 markedly increased 10 days after mechanical overload was applied to the mandibular condylar cartilage in 6M mice. While the function of KIAA1199 in relation to HA, as well as the physiological role of KIAA1199, remain unknown, this unique HA binding protein has been reported to play a key role in both RA and OA (22). Furthermore, it has been confirmed that KIAA1199 is an important factor in HA catabolism via cartilage degradation.

The HA synthesizing enzyme, HAS2, provides a major source of HA during early organogenesis in the mouse embryo and also provides HA that is necessary for the normal growth of skeletal elements (23). Accordingly, defects associated with the longitudinal growth of HAS2-deficient skeletal elements may result from impaired progression of chondrocyte maturation (24).

In the present study, the 2M mice exhibited a significant decrease in HAS2 mRNA expression, indicating that the application of excessive mechanical stress to mandibular condylar cartilage affects normal mandibular growth. Since HAS2-mediated production of HA has also been shown to be necessary for the cavitation process that results in the formation of the synovial joint cavities necessary for joint mobility (24), HAS2 appears to be essential for mandibular condylar growth. The mRNA expression level of HAS2 has also been found to be concomitantly upregulated at the onset of hypertrophic maturation in the

chondrocytic cell line, ATDC5 (25). Taken together, these findings support an important role for HA in chondrocyte maturation and condylar growth.

Conclusions

Mechanical overloading affects HA metabolism and it might initiate or amplify the condylar cartilage degradation. Further investigations are needed to confirm these results and to develop novel diagnostic and therapeutic strategies based on HA metabolism for repressing articular cartilage degradation in TMJ-OA.

Ethical approval and disclosure

All of the experimental procedures performed were approved by the Ethics Committee of Tokushima University for Animal Research (No. 12134), and the all authors state that they have no conflicts of interest.

Acknowledgments

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Figure Legends

Figure 1 Representative histological changes of murine TMJ cartilage. (A) Serial sections of TMJ cartilage were stained by HE. Compared with the 2M control section (a), the others (b-d) showed OA-like lesions, irregular cell arrangements, acellular areas under the surface, unclear cell layer borders, and vacuolation of the subchondral bone. The arrow in (d) indicates the vertical cleft that characterized the 6M experimental sections. Scale bars = 100 μm. (B) Serial sections of TMJ were stained with Safranin O and fast green. Compared with the 2M control section (a), the others (b-d) exhibited a decrease in the area of proteoglycan staining. Scale bars = 100 μm. (C) The amount of proteoglycan significantly decreased at 6M comparing to 2M in both the control and the experimental groups ($P < 0.01$). Furthermore, when proteoglycan expression was compared between the control and experimental sections from mice of the same age, the experimental sections exhibited significantly lower levels of proteoglycans ($P < 0.01$). (D) Histological grading according to Modified Mankin score showed that the values of 6M mice were significantly higher than the values of 2M mice in the control and the experimental groups, respectively ($P < 0.01$). And, there were significant differences between the control and the experimental groups at both time points ($P < 0.01$).

Figure 2 Immunohistochemical staining of HABP in condylar cartilage. (A) The distribution of HABP in representative sections of TMJ cartilage. Compared with the 2M control section (a), the others (b-d) exhibited a

decrease in the area of $HABP^+$ staining. Scale bars = 100 μm. (B) The number of $HABP^+$ cells in the stained condylar cartilage sections. When the number of HABP-positive cells was compared between the control and experimental groups at both time points, significantly fewer number of HABP-positive cells was detected in the experimental group compared with the control group. * $P < 0.05$, ** $P < 0.01$.

Figure 3 The mRNA expression of HA synthases (HAS1, HAS2, and HAS3) and HA degradation enzymes (HYAL1, HYAL2, and KIAA1199) were detected in total RNA samples collected from mandibular condyles. Significant decreases in the mRNA expression levels of HA synthesis enzymes, HAS2 and HAS3, were detected between the 2M control and 2M experimental group ($P < 0.05$). In contrast, the mRNA expression levels of HA degradation enzymes, HYAL2 and KIAA1199, were significantly higher in the 6M experimental group compared with the 6M control group ($P < 0.05$).

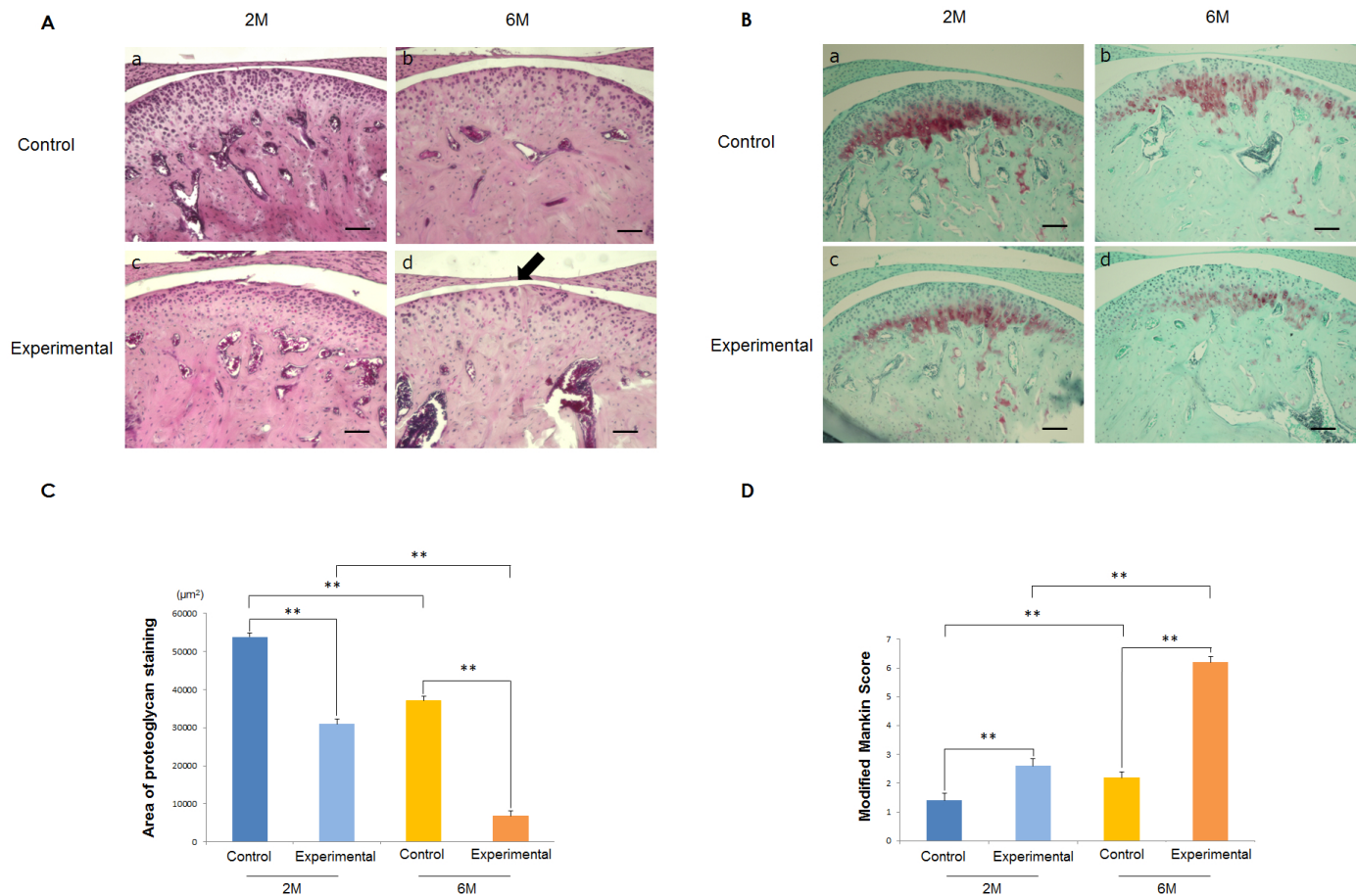
Table Legends

Table 1　Taqman® probes that were used to quantitate murine cDNA levels.

Gene	Abbreviation	Inventory number
Hyaluronan synthase 1	HAS1	Mm00472921_m1
Hyaluronan synthase 2	HAS2	Mm00515089_m1
Hyaluronan synthase 3	HAS3	Mm00515092_m1
Hyaluronoglucosaminidase 1	HYAL1	Mm00476207_g1
Hyaluronoglucosaminidase 2	HYAL2	Mm01230689_g1
Cell migration inducing protein, hyaluronan binding	KIAA1199	Mm00472921_m1

Table 2 The mRNA expression levels of the enzymes known to mediate the synthesis and degradation of HA as detected with the Digital PCR absolute quantification system.

Assay	Target	Sample	Copies/microliter	CI Copies/microliter	Chips
HAS1	FAM	2M WT	41.47	22.92-60.02	5
	FAM	2M AP	34.84	18.39-51.30	5
	FAM	6M WT	28.33	7.75-48.92	5
	FAM	6M AP	21.48	16.06-26.89	5
HAS2	FAM	2M WT	62.23	40.25-84.21	5
	FAM	2M AP	27.05	13.61-40.49	5
	FAM	6M WT	35.30	26.38-44.22	5
	FAM	6M AP	21.05	12.98-29.12	5
HAS3	FAM	2M WT	97.97	39.11-156.83	5
	FAM	2M AP	17.65	12.80-22.50	5
	FAM	6M WT	92.47	83.65-101.28	5
	FAM	6M AP	64.37	50.09-78.65	5
HYAL1	FAM	2M WT	57.83	56.38-59.29	5
	FAM	2M AP	51.87	48.74-54.99	5
	FAM	6M WT	41.35	23.75-58.96	5
	FAM	6M AP	45.60	36.94-54.26	5
HYAL2	FAM	2M WT	45.56	17.77-73.34	5
	FAM	2M AP	63.07	28.58-97.56	5
	FAM	6M WT	14.02	9.81-18.22	5
	FAM	6M AP	99.07	64.67-133.46	5
KIAA1199	FAM	2M WT	20.59	13.97-27.21	5
	FAM	2M AP	33.17	26.88-39.46	5
	FAM	6M WT	15.97	8.00-23.93	5
	FAM	6M AP	58.00	33.57-82.43	5


A
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Area of proteoglycan staining
60000
50000
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Control
Experimental
Control
Experimental
2M
6M
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7
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Figure 1

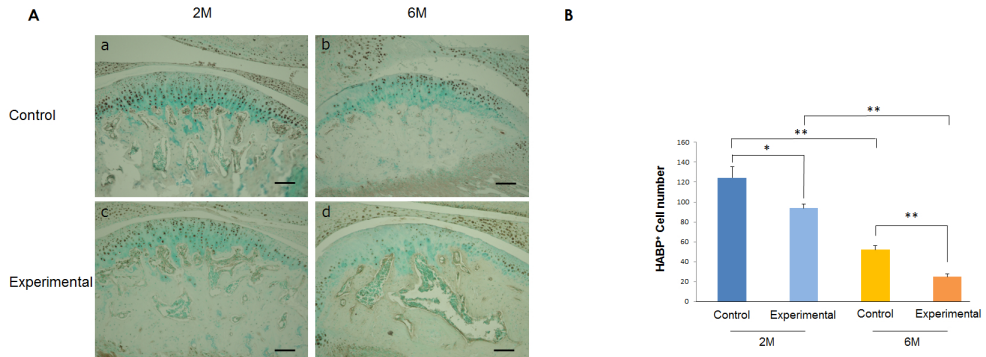
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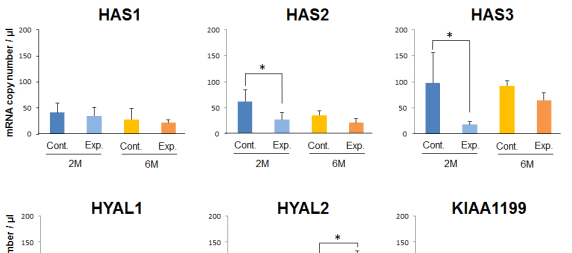
Figure 2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HAS2
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mRNA copy number / µl
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Figure 3