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Depletion of glycosphingolipids induces excessive response of chondrocytes under mechanical stress

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ABSTRACT

Glycosphingolipids (GSLs) are ubiquitous membrane components that play an indispensable role in maintaining chondrocyte homeostasis. To gain better insight into roles of GSLs, we studied the effects of GSL-deletion on the physiological responses of chondrocytes to mechanical stress. Mice lacking Ugcg gene ($Ugcg^{-/-}$) were genetically generated to obtain GSL-deficient mice, and their chondrocytes from the joints were used for functional analyses in vitro culture experiments. The cells were seeded in a three-dimensional collagen gel and subjected to 5%, 10% or 16% cyclic tensile strain for either 3 or 24 h. The gene expressions of chondrocyte anabolic and catabolic factors, and the induction of Ca^{2+} signaling were analyzed. Our results revealed that chondrocytes derived from GSL-deficient mice exhibited an elevation in the expression of catabolic factors (ADAMTS-5, MMP-13) following the exposure to strain with amplitudes of 10%. Likewise, applying cyclic tensile strain with these amplitudes resulted in an increased Ca^{2+} oscillation ratio in chondrocytes from GSL-deficient as compared to the ratio from control mice. These results demonstrated that deletion of GSL stimulated the catabolic responses of chondrocytes to mechanical stress via the augmentation of the sensitivity to mechanical stress that may lead to the cartilage deterioration. These findings suggest that the regulation of the physiological responses of chondrocytes by GSLs could be a potential target in a therapeutic intervention in osteoarthritis.

1. Introduction

Osteoarthritis (OA) is the most common form of arthritis affecting over 100 million people worldwide, and it involves inflammation and major structural changes of the joint, causing pain and functional disability (Bhatia et al., 2013; Cross et al., 2014). The pathogenesis of OA is multifactorial, involving genetic, molecular, environmental influences, and biomechanical stress (Krsticevic et al., 2017). In particular, altered joint loadings, such as with obesity, malalignment, trauma, and joint instability, are critical factors for joint degradation (Liu et al., 2016). Under normal physiological loading, articular chondrocytes maintain homeostasis of the cartilage matrix by regulating the balance between catabolic and anabolic events, whereas abnormal mechanical stimuli disrupt the balance, accelerate matrix loss, and induce degeneration of cartilaginous tissue (Chen et al., 2013; Sanchez-Adams et al., 2014).

Chondrocyte mechanotransduction is regulated by a number of key transduction mechanisms such as signaling through primary cilia, cytoskeletal molecules, and mechanosensitive ion channels (Phan et al., 2009; Wann et al., 2012). Once these transduction mechanisms are disrupted, loading-mediated cartilage metabolism, especially matrix synthesis, in chondrocytes decreases even under normal physiological conditions (Sanchez-Adams et al., 2014). Although the disruption of such mechanosensing elements in chondrocytes may lead to OA, the involvement of the mechanotransduction events in OA induction is not fully understood.

Glycosphingolipids (GSLs) are ubiquitous membrane components that consist of a hydrophilic sugar part and a lipophilic ceramide anchor. With their ceramide anchor they are integrated in the outer leaflet of the plasma membrane of all eukaryotic cells. The differences in the characteristics of lipids influences the mechanical properties of the cell membrane in a raft-like lipid bilayer (Wang et al., 2014). In articular cartilage, it has been

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reported that GSL expression of chondrocytes is decreased in human OA samples (David et al., 1993), and GSLs play a functional role in maintaining chondrocyte homeostasis (Seito et al., 2012). Application of excessive mechanical stress to articular cartilage in vivo enhances the progression of OA induction in a GSL-deficient mouse model (Seito et al., 2012), suggesting that mechanical stress may play a crucial role in the development of OA with GSL depletion. GSLs interact with key transmembrane receptors that trigger intracellular signaling cascades (Lingwood and Simons, 2010) and mediate the stress response (Degroote et al.,

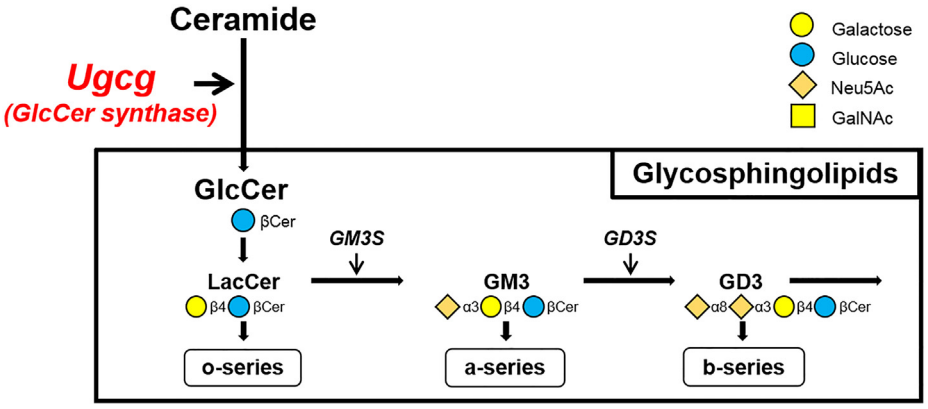

Fig. 1. Schematic diagram of the GSL synthetic pathway in mammalian cells. GSLs from ceramide through glucosylceramide (GlcCer) are synthesized by GlcCer synthase, encoded by the Ugcg locus.

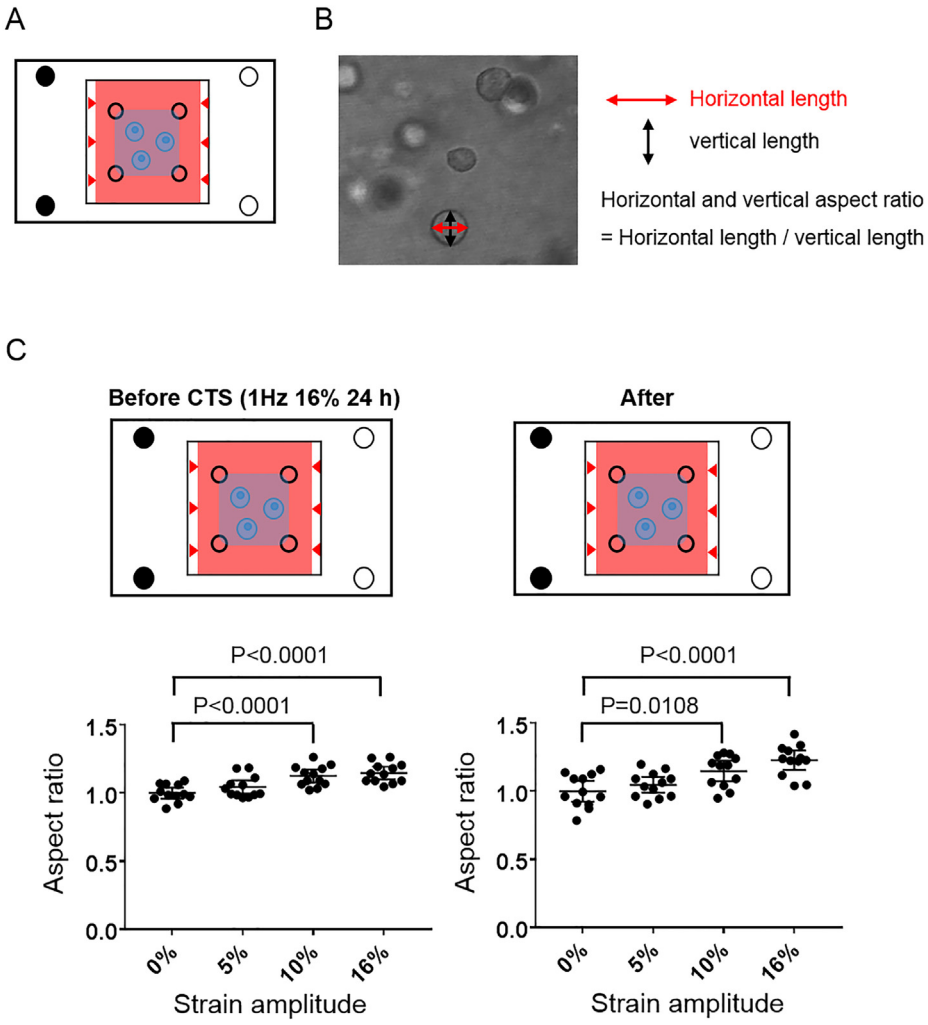

Fig. 2. A, The cells are seeded in the area surrounded by the four posts, and the gel is detached from the sidewalls before the application of CTS, while keeping the gel attached to the sidewalls parallel to the loading axis. B, The horizontal and vertical lengths of representative cells within a field of view are measured at each of the strain levels to calculate the cell aspect ratio. C, Cell aspect ratios before (left) and after CTS culture (right) with an amplitude of 16% at 1 Hz for 24 h. Values are the means and 95% CI (n = 12 in each of the experimental groups).

2004). It has also been demonstrated that GSLs are associated with the cytoskeleton (Jennemann and Grone, 2013), suggesting that GSLs may affect the mechanical properties of chondrocytes, possibly via alterations in the cytoskeletal structure.

Therefore, we hypothesized that GSLs regulate the physiological responses of chondrocytes to mechanical stress. To test this hypothesis, a three-dimensional (3D) culture model that can apply designated levels of mechanical stress to chondrocytes in vitro was developed. The responses in chondrocytes were then examined using chondrocyte-specific inactivated Ugcg mutant mice ($Ugcg^{flox/flox}$; Col2-Cre). The purpose of this study was to identify the role of GSLs in the sensitivity and responsiveness of chondrocytes to mechanical stress using a mechanical loading culture model.

2. Materials and methods

2.1. Animals

The subjects were C57BL/6 mice (wild-type mice) and mice with knockout of the chondrocyte-specific *Ugcg* gene, encoding the enzyme glucosylceramide synthase functioning in the first step of the GSL synthetic pathway [Fig. 1] (Col2-$Ugcg^{-/-}$ mice), and wild-type littermates in which the Col2a1-Cre transgene was absent (UgcgloxP/loxP mice) as controls (Sakai et al., 2001; Seito et al., 2012; Suzuki et al., 2015). Both types of knockout mice have already been established and well characterized in previous reports (Yamashita et al., 2005), and hence the Col2-Cre driver of isolated chondrocytes from these mice is thought to have been active throughout the experimental period. Wild-type mice, Col2-$Ugcg^{-/-}$ mice, and $Ugcg^{loxP/loxP}$ mice are referred to as WT mice, Ugcg mice, and Flox mice, respectively. All experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee of the Hokkaido University Graduate School of Medicine (Sapporo, Japan).

2.2. Cell isolation and passage

Immature mouse chondrocytes were obtained from the knee joints of 5-day-old mice, as previously described (Gosset et al., 2008; Salvat et al., 2005). Briefly, cartilage specimens, obtained from femoral and tibial condyles, were treated with 0.25% trypsin (Wako, Osaka, Japan) in sterile saline for 30 min. This was followed by digestion in 0.25% collagenase (ThermoFisher Scientific, Waltham, USA) in culture medium consisting of DMEM including 1% antibiotic solution (penicillin-streptomycin, Gibco-BRL, NY, USA) and 10% fetal bovine serum (FBS; Nichirei Biosciences Inc., Tokyo, Japan) for 4 h at 37 °C in a culture bottle. Approximately 5.0×10^5 chondrocytes could be harvested from a mouse.

Isolated primary mouse chondrocytes were defined as passage 0 (P0) and cultured for 6 days in a 100-mm-diameter culture dish in a humidified atmosphere of 5% CO_2 at 37 °C. The medium was changed every 3 days. On reaching 80% confluence, the cells were passaged by trypsinization using standard procedures. The cells were passaged repeatedly from passage 1 (P1) to passage 4 (P4) before they were used for subsequent experiments.

2.3. Mechanical loading on chondrocytes in the 3D culture model

Cellmatrix I-A COL1 gel (Nitta Gelatin, Osaka, Japan) was used for the 3D culture model. The stiffness of the collagen gel was evaluated, and Young's modulus was 2.118 ± 0.510 kPa. P1 chondrocytes from WT, Flox, or Ugcg mice were seeded in the gel, and the final cell density was adjusted to 1.0×10^6 cells/mL (Hirano et al., 2008). Before starting the experiment, the effect of the culture environment and passages of the cells was confirmed [See Supplementary Figs. 3 and 4] (Darling et al., 2009).

The cell-embedded gel was formed within a silicone chamber at a concentration of 2.0×10^6 cells/chamber, and 2 mL of culture medium were placed on top. The chamber possessed four cylindrical posts at the four corners of a square region (1.0 mm × 1.0 mm) in the center of the chamber [Fig. 2(A)]. After the chondrocyte-gel constructs were cultured in culture medium for 24 h, the medium was changed to the medium without FBS, and cyclic tensile strain (CTS) was applied to the constructs using a mechanical stretch system (ST-140, STREX, Osaka, Japan). The frequency was set at 1 Hz, the duration was set to 3 h or 24 h, and the amplitude was set to 5%, 10%, or 16%. The constructs cultured without CTS were used as controls. In the present study, the cells were seeded in the area surrounded by the four posts, and the gel was detached from the sidewalls before the application of CTS, while keeping the gel attached to the sidewalls parallel to the loading axis [Fig. 2(A)]. Cell deformation was evaluated by measurement of cell diameters [Fig. 2(B) and Supplementary Material 1]. This helped to apply CTS with a consistent magnitude throughout the CTS culture period [Fig. 2(C)]. For the details of this procedure, please see Supplementary Fig. 1.

2.4. Real-time quantitative PCR analysis

At the end of 3 h or 24 h of CTS culture, total RNA was extracted from chondrocytes in the gel surrounded by four posts with Trizol

Table 1
Primers used in the present study.

Col2a1-encoding gene	Forward	5′-AGGATGGCTGCACCAAACAC-3′
	Reverse	5′-TGTCCATGGGTGCGATGTC-3′
Aggrecan-encoding gene	Forward	5′-CCCTCACCCCAAGAATCAAG-3′
	Reverse	5′- GGATAGTTGGGGAGCGACAC-3′
ADAMTS-5-encoding gene	Forward	5′-GGAGCGAGGCCATTTACAAC-3′
	Reverse	5′-CGTAGACAAGGTAGCCCACTTT-3′
MMP-13-encoding gene	Forward	5′-TTGGCCACTCCCTAGGTCTG-3′
	Reverse	5′-GGTTGGGGTCTTCATCGC-3′
GAPDH-encoding gene	Forward	5′-ACTTTGTCAAGCTCATTTCC-3′
	Reverse	5′-TGCAGCGAACTTTATTGATG-3′

When collagen gel containing WT chondrocytes was injected into the square well of the stretch chamber, the gel attached to the four walls of the well (Figure A). The walls along the stretching direction are now termed "Parallel wall", and those perpendicular to the stretching direction termed "Transverse wall" (Figure B). In the initial, pilot experiment, the gel kept attached to the walls was stretch for 5, 10 or 16% tensile strain at 1 Hz for 24 h. At the end of the experiment, we observed that the gel in all samples stretched to 16% detached from transverse walls, while the gel remained attached to the parallel walls (Figure C). This happened approximately 50% in the 10% stretched samples, but did not happen in the 5% stretched samples. We also confirmed that, in the application of 16% stretch, the deformation induced in the cells in the gel detached from the transverse wall was not as much as that in the gel kept attached to the walls (Figure D). This was also in the case in the application of 10% stretch. Namely, before CTS culture, the aspect ratio of WT mice chondrocytes was 1.19 ± 0.09 and 1.29 ± 0.10 under 10% and 16% strain, respectively, which was significantly higher than that in 0% (1.00 ± 0.09) (both $P < 0.0001$). After CTS culture, the ratio was not significantly different among all strain levels. The detachment occurred simply due to a weak adhesive force between the gel and the walls made from silicone; the adhesive force could not withstand the tensile force generated at the gel-wall interface, particularly at high strain magnitudes. Thus, this was not relevant to the Poisson's effect. These findings indicated that, if the gel kept attached to the four walls was started cyclically stretched to 10 or 16%, the gel was detached from the walls at some point during the 24-h stretching period and this resulted in inconsistent cellular deformation during the stretching experiment. To avoid a possible variability in experimental results due to such inconsistent mechanical stimulation, we decided to detach the gel from the transverse walls in advance to the beginning of the stretching experiment and seeded the cells in the area surrounded by the four posts (Figure D). Indeed, it was confirmed that the cell deformation in the gel detached from the transverse wall in advance was consistent at the beginning and the end of the 24 h stretching experiments in all the three strain amplitudes (Fig. 2D). Accordingly, we adopted this experimental setup of detaching the gel from the transverse walls prior to the application of cyclic tensile strain.

(Invitrogen, Life Technologies, CA, USA) according to the manufacturer's protocol. For complementary DNA synthesis, 0.5 mg or 1.0 mg of total RNA was reverse-transcribed using random hexamer primers (Promega, WI, USA) and ImProm II reverse transcriptase (Promega). Real-time quantitative PCR was performed with the Opticon II system (Bio-Rad, CA, USA). Signals were detected using the SYBR Green qPCR Kit (Finnzymes, ThermoFisher Scientific) with the gene-specific primers listed in Table 1. The relative expression of messenger RNA (mRNA) of each targeted gene is expressed as the Ct value of each gene normalized to the Ct value of the GAPDH-encoding gene using the ΔΔCt method (Livak and Schmittgen, 2001).

2.5. Calcium response experiment

To examine the difference in mechanosensitivities of articular chondrocytes between Flox and Ugcg mice, mechanically-induced changes in intracellular calcium ion concentrations were observed. Cultured chondrocytes from these mice at passage 1 were collected with trypsin and seeded in a stretch chamber, with a flat chamber bottom coated with fibronectin at a density of 1.0×10^5 cells/cm^2. The cells in the device were pre-cultured at 37 °C and 5% CO_2 for 24 h prior to setting up for microscopy. Following the pre-culture, the cells in the device were loaded with Fluo4-AM (5 μM, ThermoFisher Scientific) and probenecid (2.5 mM, ThermoFisher Scientific) in culture medium at 37 °C and 5% CO_2 for 30 min. The loading medium was replaced with fresh culture medium supplemented with 1.25 mM of probenecid, and the device was mounted onto the mechanical loading device (Maeda et al., 2013) on the confocal laser scanning microscope (Nikon A1R system). In addition, two cartridge heaters were inserted into the chamber to keep the cells at 37 °C during the confocal imaging. The cells were visualized with a 20× objective lens (Nikon) with the excitation laser wavelength at 488 nm. A field of view (635 μm × 635 μm) was selected as a representative region of fluorescently labeled chondrocytes. First, 5-min imaging, with an imaging frequency of 1 fps, was performed to obtain the pre-strain fluorescence intensity from the labeled cells in the view and was defined as the control. This was followed by a 10-s application of one of 0%, 5%, 10%, or 16% cyclic tensile strain at 1 Hz to the cells. Immediately after the cyclic straining was finished, 5-min imaging of the same cells was repeated to obtain the post-strain fluorescence intensity of the cells. In some cases, this procedure was repeated a few times in the same device. To ensure that the effects of previous straining stimulation were diminished and the fluorescence level of the cells was returned to the initial level, there was a 15-min interval between successive runs. Furthermore, the field of view was changed in each run to avoid the accumulation of possible effects of laser photobleaching in the cells subjected to previous experiments. Three separate sets of the experiments were carried out for each genotype.

The intracellular calcium ion (Ca^{2+}) concentration of individual chondrocytes was determined by obtaining the mean fluorescence intensity within a region of interest outlining each individual chondrocyte (Maeda et al., 2013). The mean intensities were obtained in each frame and plotted against time to examine the temporal profile of intracellular Ca^{2+} concentration. Ca^{2+} transients were manually identified by a single observer, and the number of cells exhibiting Ca^{2+} transients was recorded.

The percentages of the number of chondrocytes exhibiting Ca^{2+} transients within the total number of chondrocytes within the field of view were calculated (Maeda et al., 2013). Using these percentages, the oscillation ratio was defined as the percentage during the post-strain 5-min imaging period divided by the percentage of the

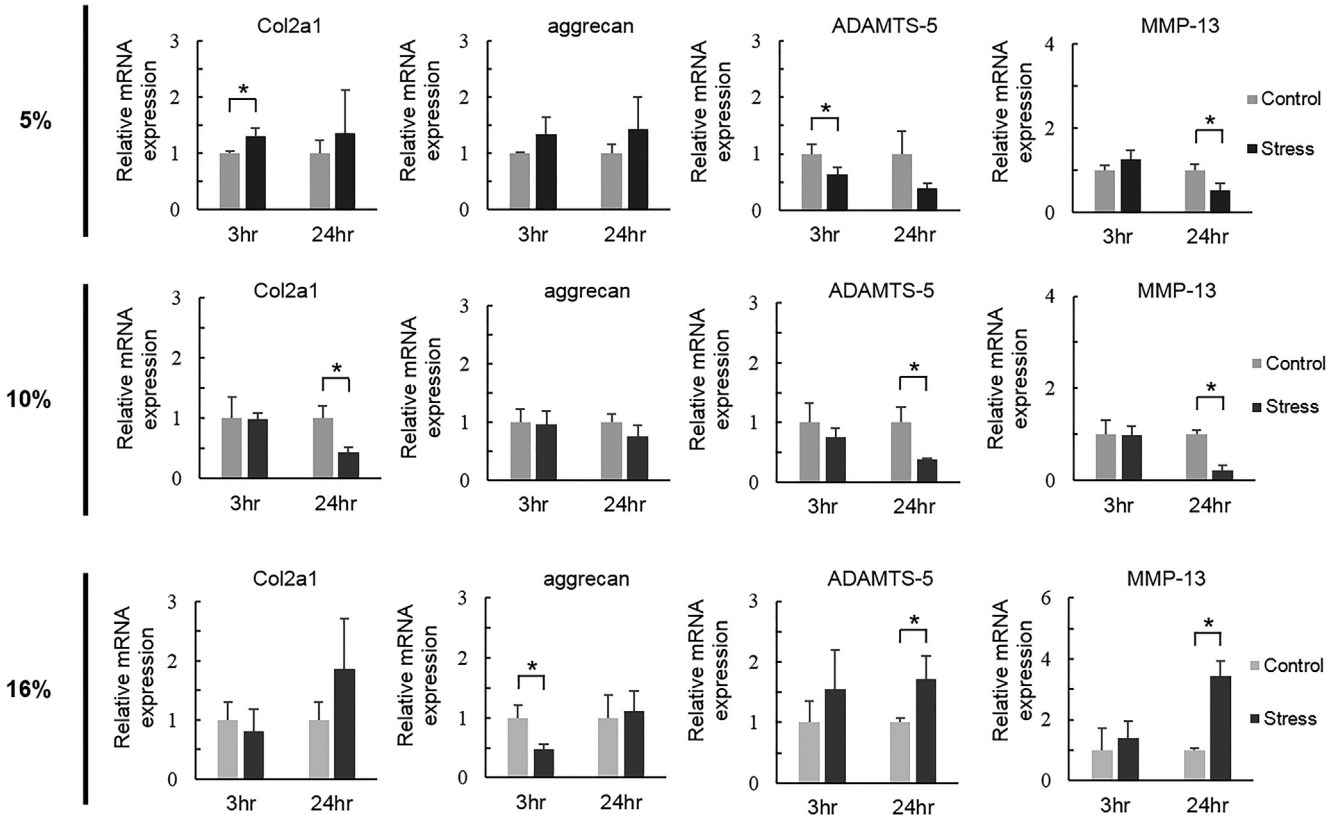

Fig. 3. Quantitative RT-PCR results for anabolic factors (Col2a1 and aggrecan) and catabolic factors (ADAMTS-5 and MMP-13) in chondrocytes from WT mice. Data are presented as relative expression levels to the control group at each time point. Values are the means and 95% CI (n = 3).

pre-strain control (Pingguan-Murphy et al., 2006). The ratio was compared between the Flox and Ugcg groups under the 0%, 5%, 10%, and 16% conditions.

2.6. Statistical analysis

Data are expressed as means and 95% confidence intervals (95% CIs). All data were confirmed for normality and homogeneity using the Shapiro-Wilk test and Bartlett's test, respectively, except for one case. Comparisons of the data were performed using either Student's *t*-test or Welch's *t*-test, depending on the homogeneity of the two groups, and continuous variables were evaluated using one-way ANOVA followed by Tukey-Kramer multiple comparison tests. For the case in which the null hypothesis of normality was rejected (Col2a1 at 3 h in 16% CTS between Flox vs Ugcg, Fig. 4), the Wilcoxon rank-sum test was used. Data analysis was carried out using statistical software JMP Pro 10.0 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Deformation of WT mice chondrocytes within collagen gel

Before CTS culture, the cell aspect ratios under 5%, 10%, and 16% strain were 1.04 ± 0.08, 1.13 ± 0.08, and 1.17 ± 0.08, respectively, and the values were significantly higher under 10% and 16% strain than under 0% strain (1.00 ± 0.10) (P = 0.0028, P = 0.0008, respectively) [Fig. 2(C)]. After CTS culture with 16% strain amplitude at 1 Hz for 24 h, the ratios under 5%, 10%, and 16% strain remained at the same level as those before CTS (1.04 ± 0.09, 1.14 ± 0.11 and 1.22 ± 0.11, respectively), and the values were also significantly higher under 10% and 16% strain than under 0% (P = 0.032, P = 0.002, respectively) [Fig. 2(C)].

3.2. Mechanical stress affects the anabolic and catabolic responses in WT mice chondrocytes

In WT mice chondrocytes, 5% CTS significantly increased the expression of Col2a1 at 3 h (P = 0.029), whereas there was a significant decrease in ADAMTS-5 at 3 h and MMP-13 at 24 h (P = 0.045 and P = 0.016, respectively) [Fig. 3]. In contrast, the application of 10% CTS significantly decreased Col2a1, ADAMTS-5, and MMP-13 at 24 h (P = 0.012, P = 0.014 and P = 0.0005). CTS with 16% strain significantly decreased aggrecan at 3 h (P = 0.016) and significantly increased ADAMTS-5 and MMP-13 at 24 h (P = 0.034 and P = 0.013, respectively).

3.3. Chondrocyte-specific deletion of GSLs enhances catabolism in response to mechanical stress

In a static condition (Non-stress), there were no differences in mRNA expressions between the Flox and Ugcg groups at both 3 h and 24 h [Fig. 4]. With 5% CTS, mRNA expressions of Col2a1 and aggrecan at 3 h were significantly decreased in the Ugcg group compared with the Flox group (P = 0.004 and P = 0.011, respectively). In addition, the expression of MMP-13 at 3 h in the Ugcg group was also significantly decreased compared with the Flox group (P = 0.032). With 10% CTS, the expressions of ADAMTS-5 at 24 h and MMP-13 at 3 h were significantly higher in the Ugcg group compared with the Flox group (P = 0.019 and P = 0.008, respectively). On the other hand, gene expressions in chondrocytes under 16% CTS were generally markedly lower than those under smaller strain amplitudes. No significant differences in the four genes examined were observed between the Ugcg and Flox groups.

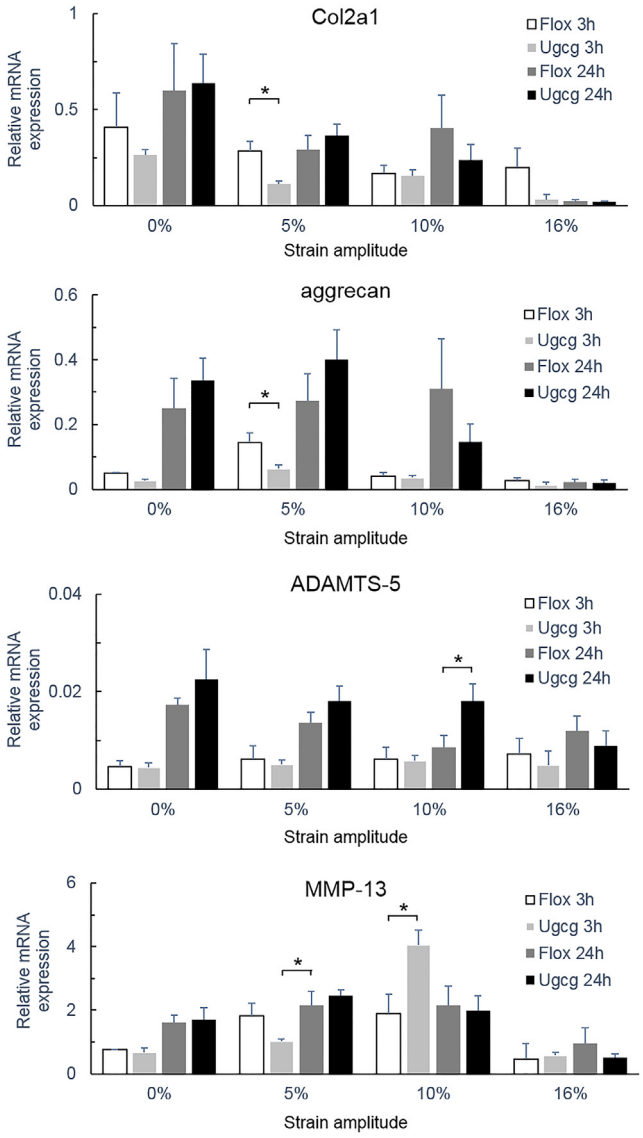

Fig. 4. Quantitative RT-PCR results for anabolic factors (Col2a1 and aggrecan) and catabolic factors (ADAMTS-5 and MMP-13) in chondrocytes from the Flox and Ugcg mice. Data are presented as expression levels relative to the level of GAPDH in each group. Values are the means and 95% CI (n = 3).

Furthermore, the balance between anabolism and catabolism was examined by taking the ratio of Col2a1 expression to MMP-13 expression, as well as the ratio of aggrecan expression to ADAMTS-5 expression [Fig. 5]. There was an overall trend that the Col2a1/MMP13 ratio in CTS samples was lower than the 0% strain samples in both the Flox and Ugcg groups at 3 h and 24 h. On the other hand, 5% CTS showed a high aggrecan/ADAMTS-5 ratio in both the Flox and Ugcg groups. Nonetheless, the ratio of 10% CTS was higher in the Flox group than in the 0% control (anabolic), whereas that in the Ugcg group was lower than in the corresponding controls (catabolic). CTS with 16% strain resulted in a markedly lower ratio in both the Flox and Ugcg groups.

3.4. Chondrocyte-specific deletion of GSLs enhances Ca^{2+} oscillation induced by mechanical stress

Representative patterns of Ca^{2+} oscillations before and after the application of tensile strain are presented in Figs. 5 and 6 for the

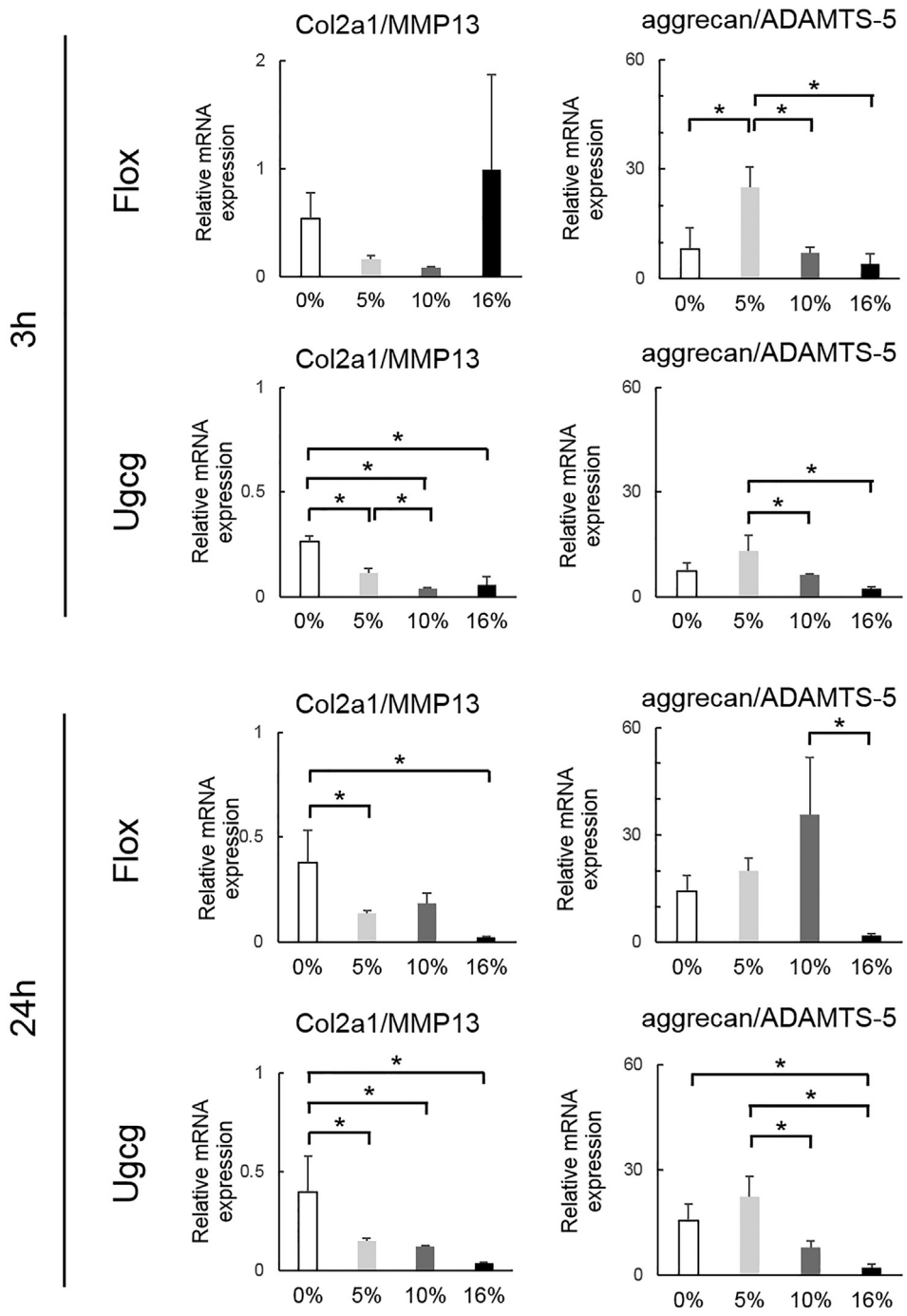

Fig. 5. The balance between anabolism and catabolism was examined by taking the ratio of Col2a1 expression to MMP-13 expression, as well as the ratio of aggrecan expression to ADAMTS-5 expression. Values are the means and 95% CI (n = 3).

Flox and Ugcg groups, respectively. In the static condition (0% strain) and under 5% strain, the Ca^{2+} oscillation ratio was not different between the Flox and Ugcg groups [Figs. 6–8]. Under 10% strain, the Ca^{2+} oscillation ratio was significantly higher in the Ugcg group (1.92 ± 0.47) than in the Flox group (1.45 ± 0.13) (P = 0.005) [Fig. 8]. This was also the case in chondrocytes under 16% strain; the ratio was significantly higher in the Ugcg group (2.15 ± 0.43) than in the Flox group (1.55 ± 0.35) (P = 0.006) [Fig. 8].

4. Discussion

The present results showed that depletion of GSLs induced over-activation of chondrocyte catabolic responses under mechanical stress, indicating that GSLs may be involved in the regulation of the physiological response of articular chondrocytes to mechanical stress.

The characteristics of the raft-like lipid bilayer are known to influence the mechanical properties of the cell membrane (Wang et al., 2014), and GSLs are associated with the cytoskeleton (Jennemann and Grone, 2013). On the other hand, Seito et al. showed that the depletion of GSLs had little effect on the stiffness of chondrocytes measured using an atomic force microscope (Seito et al., 2012). The present result that the elasticity of suspended chondrocytes from the GSL-deficient mice was almost identical to that of control cells was consistent with their results [See Supplementary Fig. 2]. Accordingly, it appears that GSLs have

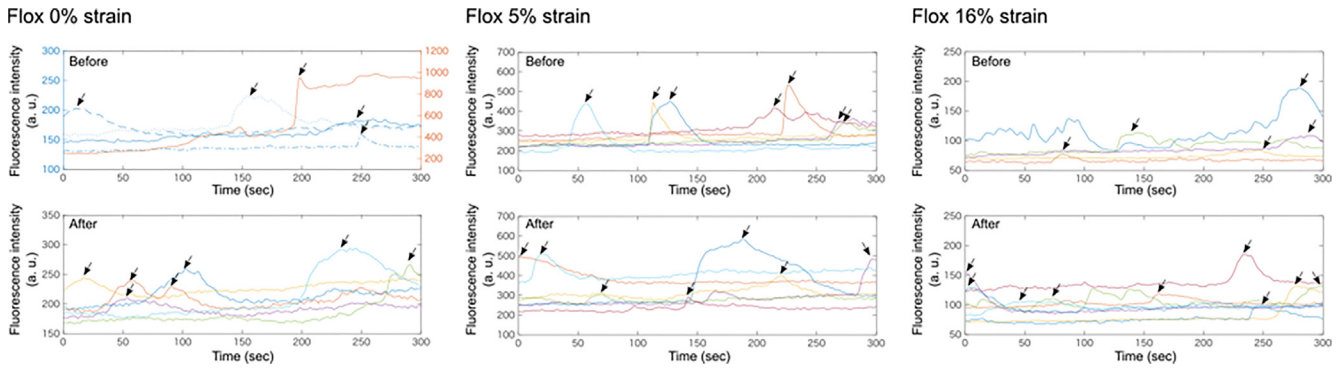

Fig. 6. Representative results of Ca^{2+} imaging of chondrocytes in the Flox group before and after the application of 0%, 5%, or 16% cyclic tensile strain. Data are presented only for those demonstrating elevation of Ca^{2+} during a 5-min imaging period. Black arrows indicate the peak fluorescence intensity of the corresponding cells. The data for the cells before the application of 0% strain are plotted using both left and right y-axes, since one cell exhibited higher baseline and peak fluorescence intensities compared to the rest of the cells in the sample and is thus separately plotted along the right y-axis.

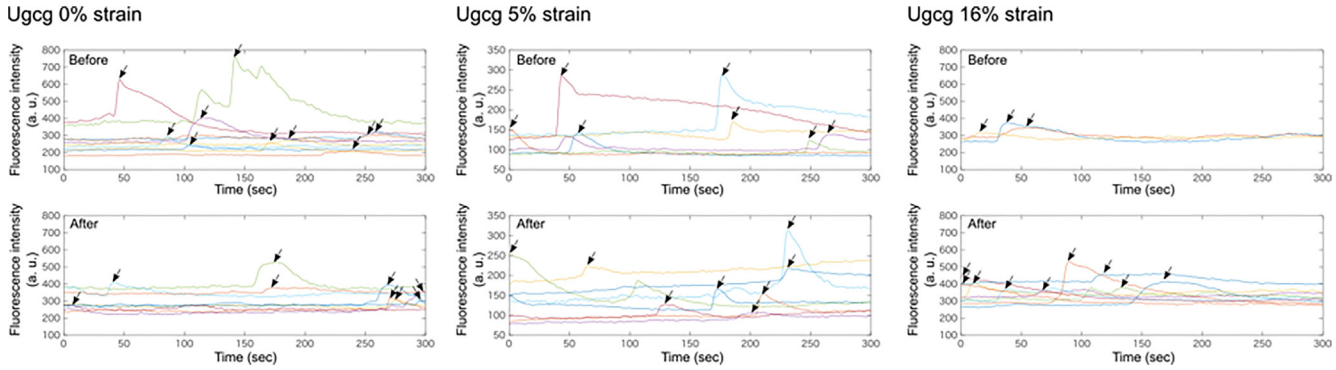

Fig. 7. Representative results Representative results of Ca^{2+} imaging of chondrocytes in the Ugcg group before and after the application of 0%, 5%, or 16% cyclic tensile strain. Data are presented only for those demonstrating elevation of Ca^{2+} during a 5-min imaging period. Black arrows indicate the peak fluorescence intensity of the corresponding cells.

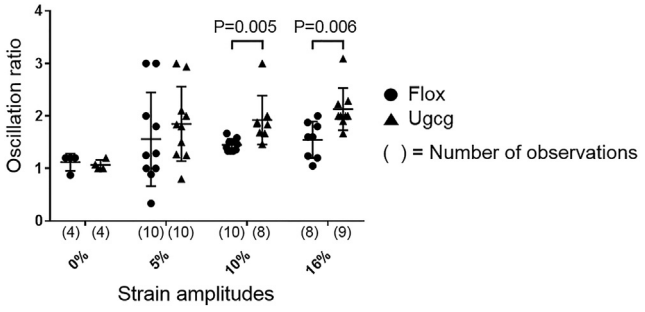

Fig. 8. Ca^{2+} oscillation ratio in chondrocytes from the Flox and Ugcg mice subjected to cyclic tensile strain. The percentages of the number of chondrocytes exhibiting Ca^{2+} transients within the total number of chondrocytes within the field of view were calculated. Using these percentages, the oscillation ratio was defined as the percentage during the post-strain 5-min imaging period divided by the percentage of the pre-strain control. Values are the means and 95% CI.

little influence on the elasticity of articular chondrocytes in our model.

Since GSLs are also known to modulate signal transduction as key mediators in the stress response, including OA (Degroote et al., 2004; Lingwood and Simons, 2010), the expressions of key gene markers of chondrocytes were evaluated under mechanical stress in the present study. Many studies have been performed in 2D culture because of its simplicity and the ease of controlling the culture conditions (Agarwal et al., 2004; Bleuel et al., 2015; Saito et al., 2013). Although 3D culture systems are closer to the in vivo condition than 2D culture systems, it is hard to achieve experimental reproducibility because of spatio-temporal non-uniformity of the mechanical stress due to matrix plasticity and fragility (Homminga et al., 1993; Uchio et al., 2000). To overcome this problem, we have developed a new 3D culture model that can apply a consistent level of mechanical stress to chondrocytes by customizing the fabrication method of the cell-gel constructs. The present results indicated that this model provided a spatio-temporally equal level of mechanical load to the cells [Fig. 2(C)]. Using this model, it was demonstrated with normal chondrocytes that the CTS with 5% amplitude provided an anabolic effect on chondrocytes, the CTS with 10% amplitude had both anti-anabolic and anti-catabolic effects, and 16% amplitude had strong catabolic effects [Fig. 3]. Generally, an appropriate level of mechanical stress has a chondroprotective effect, whereas excessive mechanical stress causes cartilage degeneration in vivo (Bader et al., 2011; Sanchez-Adams et al., 2014). Accordingly, it is thought that our 3D culture model evaluated chondrocyte responses in three situations: appropriate mechanical stress (represented by 5% CTS), moderately high mechanical stress (10% CTS), and excessive mechanical stress (16% CTS).

In the non-stress condition, the depletion of GSLs did not affect mRNA expression of both anabolic and catabolic factors compared with the expressions in their counterparts. However, chondrocytes without GSLs (Ugcg group) were inclined to be catabolic under 10% strain, upregulating ADAMTS-5 expression, while those with GSLs (Flox) took 10% CTS as an anabolic signal, upregulating aggrecan

expression [Figs. 4 and 5]. This suggests that the GSL-depleted chondrocytes have a lower threshold level between anabolic and catabolic mechanical stress and thus take 10% CTS as catabolic stimulation.

To investigate how GSL-depleted chondrocytes perceive mechanical stress, the Ca^{2+} oscillations induced by mechanical stress were examined. In the static condition, GSL depletion had little effect on the Ca^{2+} oscillation ratio, whereas CTS with 10% amplitude or higher increased the Ca^{2+} oscillation ratio in GSL-depleted chondrocytes. The enhanced calcium response in GSL-depleted cells may induce the excessive response of chondrocytes even under a moderately-high level of mechanical stress, which may result in the acceleration of the onset and development of OA.

Several limitations must be considered when interpreting the present results. First, tensile strain was used to investigate the cellular responses, although compressive stress is the predominant type of mechanical loading to chondrocytes in cartilage. It has been established that articular cartilage chondrocytes in vivo are exposed to mechanical loading consisting of compression, shear, hydrostatic pressure, and tension (Bleuel et al., 2015). Although the roles of compressive loading on the regulation of chondrocyte functions have been studied extensively, the effects of tensile strain on chondrocyte functions have also been studied in vitro (Huang et al., 2007; Long et al., 2001). Indeed, it has been demonstrated that articular chondrocytes exhibit responses to tensile strain in a magnitude-dependent manner, upregulating matrix catabolism when subjected to cyclic tensile strain with high amplitudes over 10% (Bleuel et al., 2015), which is essentially similar to the responses to compressive loading. Although the strain amplitude of 16% used in the present study could be a very strong stimulation to chondrocytes, the main purpose of the present study was to determine if the absence of GSLs exacerbates the chondrocyte response to catabolic stimulation. This is an important aspect of the etiology of osteoarthritis, as has been shown by a series of studies by our group (Sasazawa et al., 2014; Seito et al., 2012). Thus, the 16% strain amplitude was adopted as a condition inducing catabolic responses even in normal chondrocytes. Nonetheless, an experiment using more physiological compressive loading in the current 3-D culture model is essential, and we will perform such an experiment as future work. Second, the Ca^{2+} oscillation was examined in a 2D culture environment. Although 3D culture is ideal, there were technical difficulties in observing Ca^{2+} oscillation in 3D culture, especially using collagen type 1 gel where the fluorescent Ca^{2+} indicator did not penetrate into chondrocytes within the gel. Third, although it appeared that GSLs regulated the mechanical response of chondrocytes via Ca^{2+} signaling, how the GSLs regulate the mechanotransduction including the intracellular signaling pathway remains unclear, and this should be clarified in future work. Despite these limitations, the current study provides findings to support our hypothesis that GSLs regulate the responses of articular chondrocytes to mechanical stress, which is relevant to the onset and development of OA.

In conclusion, the present results showed that depletion of GSLs induced overactivation of chondrocyte catabolic responses under mechanical stress, indicating that GSLs may be involved in the regulation of the physiological response of articular chondrocytes to mechanical stress. These regulatory mechanisms are important for maintaining cartilage homeostasis. Therefore, GSLs could be potential target molecules for the treatment of OA.

Declaration of Competing Interest

All authors state that they have no conflict of interest.

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Appendix A. Supplementary material

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