Introduction

Osteoarthritis is the most common joint disease in older adults and is caused by a gradual degradation of articular cartilage leading to reduced mobility, pain, disability and fatigue (Hawker, 2019). No cure has been found to date, but moderate joint loading through physical exercise is known to slow the progression of the disease (Leong and Sun, 2014), indicating the importance of mechanical stimulation on cartilage maintenance and health.

At the cellular level, the disease is characterized by a decreased expression of cartilage matrix proteins (Dunn et al., 2016), an increase in cartilage-degrading enzymes and inflammatory markers, and cell death (Malemud, 2018). Several animal models are routinely used to study osteoarthritis; among those, the destabilization of the medial meniscus (DMM) mouse model, which alters the joint mechanics, is particularly common (Thysen et al., 2015). These offer great insights into the pathogenesis of osteoarthritis but require animal experiments and can be difficult to translate into the human context. In vitro models have also been designed to mimic part of the disease process (Johnson et al., 2016), from monolayer culture to co-cultures and animal explants. Many of these models require explants or primary cells, leading to animal experiments. Furthermore, chondrocyte cell culture on Petri dishes rapidly leads to de-differentiation and loss of the chondrocyte phenotype (Ting et al., 2015).

We previously used the mouse chondrocyte...
progenitor cell line ATDC5 to study the effects of high hydrostatic pressure (HP) on cartilage gene expression (Montagne et al., 2017). High HP was found to decrease chondrocyte marker expression and lead to an increase in stress-, inflammation- and apoptosis-related gene expression. Many of the modulated genes had previously been found modulated in animal models of osteoarthritis. ATDC5 cells are a popular cell line to study chondrogenesis (Yao and Wang, 2013), but they may not be ideal to study chondrocyte physiology: the basal chondrocyte marker expression in ATDC5 cells is relatively low, the cell morphology and behaviour are different from those of mature chondrocytes and the results obtained may therefore be difficult to interpret in the context of osteoarthritis.

Here, we used ATDC5 cells and differentiated them into chondrocytes by seven days of rotation culture. The resulting chondrocyte aggregates were then dissociated by trypsin and monolayers of differentiated ATDC5 cells were formed in Petri dishes. The high chondrocyte marker expression resulting from differentiation, notably that of the aggregan and type II collagen genes Acan and Col2a1, was maintained in Petri dishes for at least three days. Submitting the monolayers to supraphysiological hydrostatic pressure (25 MPa) led to a significant decrease in chondrocyte marker expression (including that of Acan, Col2a1 and the transcription factor gene Sox9), a common feature of osteoarthritic cartilage. High pressure-induced chondrocyte damage was thus reproduced without resorting to primary cells or animal experiments.

Materials and Methods

Reagents
Dulbecco's Modified Eagle Medium (DMEM), trypsin and the antibiotic/antimycotic solution were purchased from Life Technologies Corporation, Grand Island, New York, USA. PBS was from Nissui Pharmaceutical Co., Ltd., Tokyo, Japan. F-12 Nutrient Mixture (Ham) was from MP Biomedicals LLC, Solon, Ohio, USA. Fetal bovine serum (FBS) was from Nichirei Biosciences Inc., Tokyo, Japan. The insulin, transferrin and selenous acid cocktail ITS+ Premix was from BD Biosciences Discovery Labware, Bedford, Massachusetts, USA.

Cell culture
The mouse ATDC5 chondrocyte progenitor cell line was obtained from the Japanese Collection of Research Bioresources Cell Bank, Ibaraki, Japan. Cells were routinely cultured in DMEM/F-12 supplemented with 5% of FBS and 1% of antibiotics in a humidified incubator under a 5% CO2 atmosphere. In order to form cell aggregates, 2.5 x 10^6 ATDC5 cells were seeded in ultra-low attachment 6-well plates (Corning Incorporated, Kennebunk, Maine, USA) in 3.5 mL of DMEM/F-12 medium supplemented with 5% of FBS and 1% of ITS+ Premix, and placed on an orbital shaker rotating at 75 rpm in a humidified incubator.

Cell pressurization
High HP (25 MPa) was applied for up to 24 h using a mechanical system consisting of a pressure chamber placed inside a thermostatic bath set at 37°C, and connected to a high-pressure cylinder operated by a manual pump (Fig. 1a). After 7 days of rotation culture, ATDC5 aggregates were collected, washed in PBS, dissociated in trypsin for 5 minutes, and cells were counted, seeded in 35 mm Petri dishes at 4 x 10^5 cells/dish. After 2 days, the dishes were sealed in polyethylene bags (Seisan Nippon Sha, Ltd., Tokyo, Japan) filled with 15 mL of culture medium and placed in the pressure chamber where a constant 25 MPa pressure was applied for 0, 1 or 24h, as schematized in Fig. 1b. The pressure inside the chamber was continuously monitored by a pressure sensor. As a control, unpressurized cells were placed in a metallic column of the same dimensions as the pressure chamber in the same water bath.

Real-time PCR
Total RNA was extracted using Trizol (Life Technologies Corporation, Carlsbad, California, USA) according to the manufacturer’s instructions. Complementary DNA was then synthesized from 500 ng of RNA using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo Co., Ltd., Osaka, Japan). Real-time PCR was subsequently performed using Thunderbird SYBR qPCR Mix (Toyobo Co., Ltd., Osaka, Japan) in a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, California, USA). All gene expressions were normalized to the expression of the reference gene Rpl13a and the fold changes were calculated using the Delta-Delta Ct method. The primer sequences are listed in Table 1.
Statistics
The graphs show the mean +/- SEM of between 3 and 6 experiments. In order to assess whether the differences observed were statistically significant, a one-way ANOVA was carried out followed by Tukey’s HSD test. P values below 0.05 were considered to show a significant difference.

Results
The ATDC5 cell line is now a well established tool to study chondrogenesis and other aspects of cartilage biology (Yao and Wang, 2013). In order to induce chondrogenesis in ATDC5 cells, we seeded $2.5 \times 10^6$ cells in ultra-low attachment plates on an orbital shaker and observed the formation of aggregates within a few hours, as previously reported with primary articular chon-
drocytes (Furukawa et al., 2003). As shown in Fig. 2a, aggregates slightly less than 100 μm in diameter were observed after 24h; the diameter then slowly increased to around 150 μm after 7 days.

After 7 days, smooth aggregates were formed, which could be easily dissociated in trypsin. The cells obtained from trypsinization adhered to Petri dishes like normal ATDC5 cells but showed a rounder morphology (Fig. 2c), similar to primary, low-passage chondrocytes, whereas non-differentiated ATDC5 cells are more spread out and irregular in shape, with many protruding cellular processes (indicated by white arrowheads, Fig. 2b).

In order to confirm that rotation culture induces chondrogenesis in ATDC5 cell aggregates, PCR was carried out to measure the expression of the chondrocyte markers Acan, Col2a1 and Sox9, the collagenase-3 gene Mmp13 and the Cited2 gene, which produces the chondro-protective transcriptional co-activator CITED2 (Leong et al., 2011; Yokota et al., 2003). Acan and Col2a1 expression was significantly increased in D7 aggregates, and this high expression was maintained in the dissociated ATDC5 cells (Fig. 2). Sox9 expression seemed to be up-regulated but not significantly. Surprisingly, Cited2 expression was significantly reduced in D7 aggregates but returned to its basal level when the dissociated cells were cultured in Petri dishes. Finally, Mmp13 also seemed, surprisingly to be up-regulated by rotation culture, but the differences were not statistically significant (Fig. 3).

In order to check whether the differentiated ATDC5 cells could be used to study load-induced chondrocyte de-differentiation, the trypsinized aggregates were seeded onto Petri dishes and pressurized under 25 MPa for 1 or 24h. PCR analysis showed that Acan, Col2a1 and Sox9 were all significantly down-regulated by high pressure after 24h; Sox9 was even down-regulated after 1h (Fig. 4). As expected, Cited2 was similarly significantly down-regulated after 1 and 24h of pressurization. Surprisingly though, Mmp13 was also down-regulated by pressure after 1 and 24h.
Fig. 3  Chondrocyte gene expression in ATDC5 aggregates and monolayers: mRNA expression of Acan, Col2a1, Sox9, Cited2 and Mmp13 in ATDC5 monolayers before differentiation, aggregates after 7 days of rotation culture and trypsinized D7 aggregates seeded onto Petri dishes, measured by real-time PCR. The bars represent the mean +/- SEM of 3 to 5 experiments. One-way ANOVA followed by Tukey’s HSD were performed to assess whether the differences were statistically significant: *: p<0.05, **: p<0.01.

Fig. 4  Chondrocyte gene expression in dissociated ATDC5 aggregates under high HP: mRNA expression of Acan, Col2a1, Sox9, Cited2 and Mmp13 in trypsinized D7 ATDC5 aggregates pressurized under 25MPa for 0, 1 or 24h, measured by real-time PCR. The bars represent the mean +/- SEM of 4 to 6 experiments. One-way ANOVA followed by Tukey’s HSD were performed to assess whether the differences were statistically significant: *: p<0.05, **: p<0.01.
**Discussion**

During normal joint loading, contact pressures in human articular cartilage have been measured in the range of 0 to 18 MPa, depending on the type of physical activity (Hodge et al., 1986). Due to the high (70–80%) water content of cartilage, over 70% of the compressive load is supported by the cartilage interstitial fluid (Park et al., 2003). Though HP in joints has, to the best of our knowledge, never been measured *in vivo*, one can assume that HP is one of the main physical stimuli sensed by chondrocytes *in vivo* and that the physiological range of pressures varies from 0 to around 20 MPa. The 25 MPa pressure used in the present study is considered slightly above this range and was chosen in order to induce damage without triggering apoptosis; indeed, though high HP seems to slow the proliferation of ATDC5 cells (Montagne et al., 2017), cells continue to proliferate when they are returned to the incubator after pressurization (unpublished observation). Mammalian cell viability becomes affected typically above 100 MPa (Frey et al., 2004).

The down-regulation of Acan, Col2a1 and Sox9 by high HP was therefore expected and has been reported previously in several studies (Elder and Athanasiou, 2009). The down-regulation of Cited2 was also significant under pressure, as previously reported (Montagne et al., 2017), and shows the deleterious effect of excessive HP on chondrocyte physiology. However, CITED2 is believed to protect against cartilage degradation by inhibiting Mmp13 expression (Yokota et al., 2003; He et al., 2019), so the down-regulation of Mmp13 under high HP was unexpected. Interestingly, Ogawa et al. showed that moderate HP promoted chondrogenesis and increased Acan, Col2a1 and Sox9 expression in adipose-derived stem cells while also increasing Mmp13 expression (Ogawa et al., 2015). Mmp13 is also a marker of terminal chondrocyte differentiation and is necessary for bone growth (Dreier, 2010). Its down-regulation under HP may reflect a wide-ranging effect on cartilage differentiation. Alternatively, Mmp13 down-regulation could be an early response mechanism to the sudden down-regulation of extracellular matrix gene expression in order to prevent excessive cartilage degradation, a mechanism, which may be absent in osteoarthritis. It may therefore be important in the future to understand the molecular mechanisms leading to the Mmp13 inhibition under pressure.

ATDC5 cells are a popular cell line to study chondrogenesis. Aggregate formation induces their differentiation by promoting cell-cell contacts (Park et al., 2017). This phenomenon has been used for many years with other cell models in rotation culture (Furukawa et al., 2003) or pellet culture (Tanaka et al., 2004). Consequently, Acan and Col2a1 expression were strongly increased in ATDC5 aggregates. Mmp13 expression seemed to be induced, though not significantly. Over the 7-day culture period, aggregates became gradually larger. This Mmp13 up-regulation may be similar to that observed by Ogawa et al. and indicate that as the aggregates grow, the newly produced extracellular matrix has to be constantly degraded and remodelled.

The up-regulated gene expressions in aggregates were maintained for at least three days after trypsinization and seeding onto Petri dishes, indicating that the chondrocyte phenotype could be maintained for several days. This mature phenotype was also apparent in the cell morphology: differentiated cells seeded onto Petri dishes were reminiscent of primary articular chondrocytes, round, and grew very slowly compared to un-differentiated ATDC5 cells, which grow fast and show many cellular protrusions, indicative of cells with high motility.

Surprisingly, Cited2 expression was significantly inhibited by rotation culture but returned to its basal level after seeding onto Petri dishes. This decrease could account for the increased Mmp13 expression. Furthermore, CITED2 expression knockdown has been shown to inhibit cell proliferation (Kracz et al., 2003); cells in aggregates may decrease their proliferation during differentiation by decreasing Cited2 expression.

In summary, the current protocol yields differentiated cells similar to chondrocytes from a widely used cell line, within a week. Even after aggregate dissociation, the cells maintain their phenotype for several days on Petri dishes and respond to excessive mechanical stimulation in a way similar to primary chondrocytes. This model could be used to investigate the protective effects of drugs and other molecules on mechanically-induced chondrocyte damage.
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References


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