Running Head and First Author:

SHORT COMMUNICATION: MILK BASIC PROTEIN AFFECTS CHONDROGENIC CELLS

NAKATANI ET AL.

Title:

Short communication: **Milk basic protein promotes proliferation and inhibits differentiation of mouse chondrogenic ATDC5 cells**

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Interpretive Summary:

Short communication: **Milk basic protein promotes proliferation and inhibits differentiation of mouse chondrogenic ATDC5 cells.** *By Nakatani et al.*, page xx. It has been reported that the intake of milk basic protein (**MBP**) increases bone density in clinical trials. However, there are few studies of MBP in cartilage, the tissue adjacent to bone. We therefore investigated the effect of MBP on a chondrocyte cell line, ATDC5. Addition of MBP to ATDC5 cells promoted their proliferation and suppressed their differentiation to calcified chondrocytes. Thus, MBP could contribute to

maintaining the cartilage tissue by promoting the proliferation of chondrocytes with suppressing their differentiation toward calcification.

ABSTRACT

It has been reported that the intake of milk basic protein (**MBP**) increases bone density by promoting bone formation and suppressing bone resorption. However, there are few studies of MBP in cartilage, the tissue adjacent to bone. We therefore investigated the effect of MBP on a chondrocyte cell line, ATDC5. In the proliferative assay using the WST-1 method, the addition of 10, 100, and 1000 μg/mL of MBP to ATDC5 cells significantly increased the cell number by about 1.2-, 1.5-, and 1.7-fold, respectively, compared to the control cells. The cell cycle analysis using flow-cytometry revealed that the proportion of S- and G2/M-phase cells was increased but that of G0/G1 phase was decreased in a dose-dependent manner of MBP addition. We measured the alkaline phosphatase (**ALP**) activity of MBP-treated ATDC5 cells to examine the differentiation stage of the cells. The ALP activity was suppressed in a dose-dependent manner of MBP addition, especially drastically in higher dose of MBP (100 and 1000 μg/mL). The Alizarin Red S staining intensity, the indicator for calcification of cells was lower in the MBP-treated (100 μg/mL) cells than in non-treated control cells. In the reverse transcription-polymerase chain reaction (**RT-PCR**) experiment, the mRNA level of *sex determination region Y box 9* (***Sox9***) and *type II collagen* (***Col2***) were significantly increased in the MBP-treated cells compared to the control cells. Significant decrease of the mRNA level of *Runt-related transcription factor 2* (***Runx2***) and *type X collagen* (***Col10***) was also observed in the MBP-treated cells. These results suggested that MBP promoted the proliferation of chondrocytes with suppressing their differentiation toward calcification.

Key words

milk basic protein, chondrocyte, proliferation, differentiation genes

SHORT COMMUNICATION

Milk basic protein (**MBP**), a basic protein fraction in milk, is contained in whey protein (Kruger et al., 2007). *In vitro* experiments have reported that basic protein fraction of whey protein promotes the proliferation of osteoblasts and suppresses the differentiation of osteoclasts (Takada et al., 1996, 1997). *In vivo* experiments have shown that MBP prevents bone loss in postmenopausal osteoporosis as a result in suppressing the osteoclast mediated bone resorption. (Toba et al., 2000). Some clinical studies showed MBP supplementation prevented bone loss also in healthy women (Aoe et al., 2001 and 2005). Another study showed MBP supplementation enhanced fracture healing in mice (Yoneme et al., 2015). It is well known that chondrocytes aggregate and differentiate in such as fracture site before replacement of bone. However, few studies have evaluated the effect of MBP on cartilage tissues.

Joint epiphyses are covered with cartilage that possesses high shock absorbability, elasticity, and lubricity for the smooth movement of the joints (Scott, 2003). Chondrocytes, present in cartilage tissues, provide articular cartilage tissues with glycosaminoglycan (**GAG**), hyaluronic acid, and collagen (Blair et al., 2001). Chondrocytes differentiate from undifferentiated mesenchymal cells into proliferating chondrocytes, mature chondrocytes, hypertrophic chondrocytes, calcified chondrocytes, and finally into bone cells. This process of differentiation is called intra-cartilage ossification (Cancedda et al., 2000).

In this study, we investigated the effect of MBP on the proliferation and differentiation of chondrocytes, as well as the differentiation-related genes in chondrocytes. A murine chondrocyte progenitor cell line, ATDC5, was used as the model reproducing intra-cartilage ossification such as differentiation from proliferative chondrocyte to calcified chondrocyte (Atsumi et al., 1990).

First, we investigated the proliferative effect of MBP at the proliferative stage of ATDC5 using the WST-1 method. MBP was obtained from MEGMILK SNOW BRAND Co., Ltd. (Tokyo, Japan). The major components of MBP were lactoferrin (53.6%), lactoperoxidase (36.1%), angiogenin (3.6%) and cystatin C (0.05%) (Ono-Ohmachi et al., 2018). ATDC5 cells (RIKEN Cell Bank, Ibaraki, Japan) were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12

medium (**DMEM/F12**), supplemented with 5% fetal bovine serum (Nichirei, Tokyo, Japan), penicillin (50 IU/mL), streptomycin (50 μg/mL), and kanamycin (50 μL/mL), and maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded at 1.5×10^3 cells/well in a 96-well micro-plate, and after 18 h, MBP (1, 10, 100, and 1000 μg/mL) was added to these wells. Forty-eight hours after the addition of MBP, the culture medium was exchanged with a medium containing 10% WST-1 reagent (Roche Diagnostics, Basel, Switzerland). After incubation for 5 h, the absorbance of the medium at 440 nm was measured using the Spectra Max M2e (Nippon Molecular Devices, Tokyo, Japan). We found that the addition of 10, 100, and 1000 μg/mL of MBP significantly increased the cell number by about 1.2-, 1.5-, and 1.7-fold, respectively, compared to the control group (Figure 1A). Thus, the addition of MBP could significantly increase the proliferation of chondrocytes.

Next, we investigated the mechanism behind this effect by performing cell cycle analysis using flow-cytometry. Eighteen hours after seeding ATDC5 cells at 1.3×10^5 cells/dish in a 6-cm dish, MBP (100 and 1000 μg/mL) was added. After 24 h of incubation, a cell suspension was prepared using phosphate buffered-saline (-) and 0.25% trypsin-ethylenediamine tetraacetic acid solution. After removing the supernatant by centrifugation, 70% ethanol was added, and the resultant suspension was stored at -30 °C. The stored suspension was again centrifuged to remove the supernatant. The residual cells were prepared using the Muse Cell Cycle Kit (Merck Millipore, Billerica, MA, USA) and 5,000 of these cells were analyzed using the Muse Cell Analyzer (Merck Millipore). The effect of MBP on the cell cycle on proliferative chondrocyte stage of ATDC5 cells is shown in Figures 1B and C. In the control group, 62% of the cells was in G0/G1 phase, 16% in S phase, and 22% in G2/M phase. In the group with 100-μg/mL MBP, the percentage of cells in the G0/G1 phase was decreased by about 3%, while those in S phase and G2/M phase were increased by about 3% and 1%, respectively, compared to the control group. In the group with 1000 μg/mL MBP, percentage of cells in G0/G1 phase was significantly decreased by 14%, while those in S phase and G2/M phase increased by about 6% and 8%, respectively.

The alkaline phosphatase (**ALP**) activity is known to increase before the calcification of chondrocytes (Balcerzak et al., 2003; Fedde et al., 1999). Therefore, it could be used as an index of

differentiation into hypertrophied chondrocytes. We measured the ALP activity of MBP-treated ATDC5 cells to examine the early differentiation stage of the cells. Twenty-four hours after seeding the cells at 3×10^3 cells/well in a 96-well micro-plate, MBP (1, 10, 100, and 1000 μg/mL) was added. Four days after the addition of MBP, the ALP activity staining was performed. An ALP staining solution was prepared by dissolving naphthol AS-BI-phosphate (Sigma Aldrich, St. Louis, MO, USA) and Fast Red Violet LB salt (Sigma Aldrich) in a 0.05-M solution of 2-amino-2-methyl-1,3-propanediol (pH 9.8). After fixing the cells with a 20%-formalin solution for 20 min and rinsing with water, the ALP staining solution was added, and this solution was incubated for 20 min at 37°C. Dye photographs were captured using a scanner and quantified with the image processing software, ImageJ (NIH, Bethesda, MD, USA). The ALP activity of the chondrocytes was found to be suppressed by the addition of MBP in a concentration-dependent manner (Figure 2A). With higher concentrations of MBP (100 and 1000 μg/mL), the ALP activity was drastically decreased to about one-tenth or less of the control group.

In hypertrophic chondrocytes, the production of extracellular matrix-degrading enzymes is increased, and type X collagen (**Col10**) induces the differentiation of the cells into calcified chondrocytes through apoptosis and calcium deposition (Cancedda et al., 2000). We prepared a staining solution by adjusting the concentration of Alizarin Red S (Sigma Aldrich) to 1% (w/v) using aqueous ammonia and pure water. ATDC5 cells, which were cultured with MBP (100 μg/mL) for 13 days in an ITS (5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL sodium selenite)-added medium as inducer agents, was fixed with 20% formalin for 10 min and washed thrice with water. The staining solution was added to the cells and incubated overnight at room temperature. Dye photographs were captured with a scanner and quantified using ImageJ. We found that addition of MBP tended to suppress the mineralization of the ATDC5 cells (Figure 2B).

Transcription factors such as the sex determination region Y box 9 (**Sox9**), Sox5, Sox6, and runt-related transcription factor 2 (**Runx2**) are known to be involved in the regulation of differentiation of chondrocytes. Sox9 promotes the early stage and suppresses the late stage of the differentiation (Ikeda et al., 2005). Runx2 promotes the late stage of the differentiation, transforming the cells into hypertrophic chondrocytes and calcified chondrocytes (Zhang et al., 2015). In this study, we

performed reverse transcription-polymerase chain reaction (**RT-PCR**) to examine the changes in the expression of differentiation-related genes in MBP-treated ATDC5 cells. For extracting sufficient amount of RNA, ATDC5 cells were seeded at a density of 2.0×10^5 cells/dish on 60-mm dishes. The day when confluence was achieved was considered day 0, and the RNA was extracted using Maxwell RSC and LEV simplyRNA Cells Kit (Promega Corporation, Madison, WI, USA) on days 0, 3, 7, 14, and 21. cDNA was prepared from 1 μg of the total RNA of each sample by adding Oligo (dt) and reverse transcriptase (SuperScript III, Thermo Fisher Scientific, Waltham, MA, USA), and then using the Veriti 96 well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The PCR primers were as follows: for GAPDH, 5'-TTGACCTCAACTACATGG-3' (forward) and 5'-ATGAGGTCCACCACCCTG-3' (reverse); for Sox9, 5'-TGCAGCACAAGAAAGACCAC-3' (forward) and 5'-CCCTCTCGCTTCAGATCAAC-3' (reverse); for Runx2, 5'-GCACTACCCAGCCACCTTTACC-3' (forward) and 5'-GTCAGCGTCAACACCATCATTC-3' (reverse); for type II collagen (**Col2**), 5'-GTGGAGCAGCAAGAGCAAGGA-3' (forward) and 5'-CTTGCCCCACTTACCAGTGTG -3' (reverse); for Col10, 5'-CCACCTGGGTTAGATGGAAAA -3' (forward) and 5'- AATCTCATCAAATGGGATGGG -3' (reverse). The PCR product was electrophoresed on a 2.0%-agarose gel at 50 V for 10 min, switched to 100 V for 20 min, and then stained with 5% ethidium bromide solution. The products of the electrophoresis were visualized under ultraviolet irradiation using Gel Doc ™ EZ Imager (Applied Biosystems) and analyzed with the Image Lab software (Bio Rad Laboratories, Hercules, CA, USA). The expression levels of *Sox9, Runx2, Col2* and *Col10* mRNA were compared to that of *glyceraldehyde-3-phosphate dehydrogenase* (***GAPDH***). As shown in Figure 3, the mRNA level of *Sox9* was significantly increased on days 7 in the MBP-added group, compared to the control group. The mRNA level of *Col2* as early marker of differentiation of cartilage also increased remarkably in the MBP-added group compared to the control group on day 3, 7, 14 and 21. On the other hands, the mRNA level of *Runx2* in the MBP-added group was lower than that of control on day 7, 14 and 21, especially on day 3. The mRNA level of *Col10*, a calcified chondrocyte marker, also decreased significantly in the MBP-added group compared to the control group on day 3.

The results of our study shed light on the effects of MBP on chondrocytes. We found that the

proliferation of chondrocytes was promoted by MBP-treatment. The cell cycle was likely accelerated by MBP, so more cells were probably arrested at the S and G2/M phases. Although MBP promoted the proliferation of chondrocytes, the mRNA levels of *Sox9* and *Col2* were increased by MBP. These results mean that MBP promotes the proliferation of proliferative chondrocyte and induces the differentiation from the proliferative chondrocytes to mature ones. Furthermore, we found that the addition of MBP caused the suppressing mRNA expression of *Runx2* and *Col10* and decreased the ALP activity and mineralization. The differentiation of chondrocytes is known to progress initially with an increase in the ALP activity until the mineralization stage is reached (Balcerzak et al., 2003). We speculated that the differentiation was delayed because it was indirectly affected by a prolonged growth phase (Figure 4). Thus, we showed that MBP promoted proliferation by affecting the cell cycle of chondrocytes. Further studies are necessary to clarify the mechanism and component behind this action of MBP on chondrocytes and to investigate the effect of MBP on chondrocytes *in vivo*.

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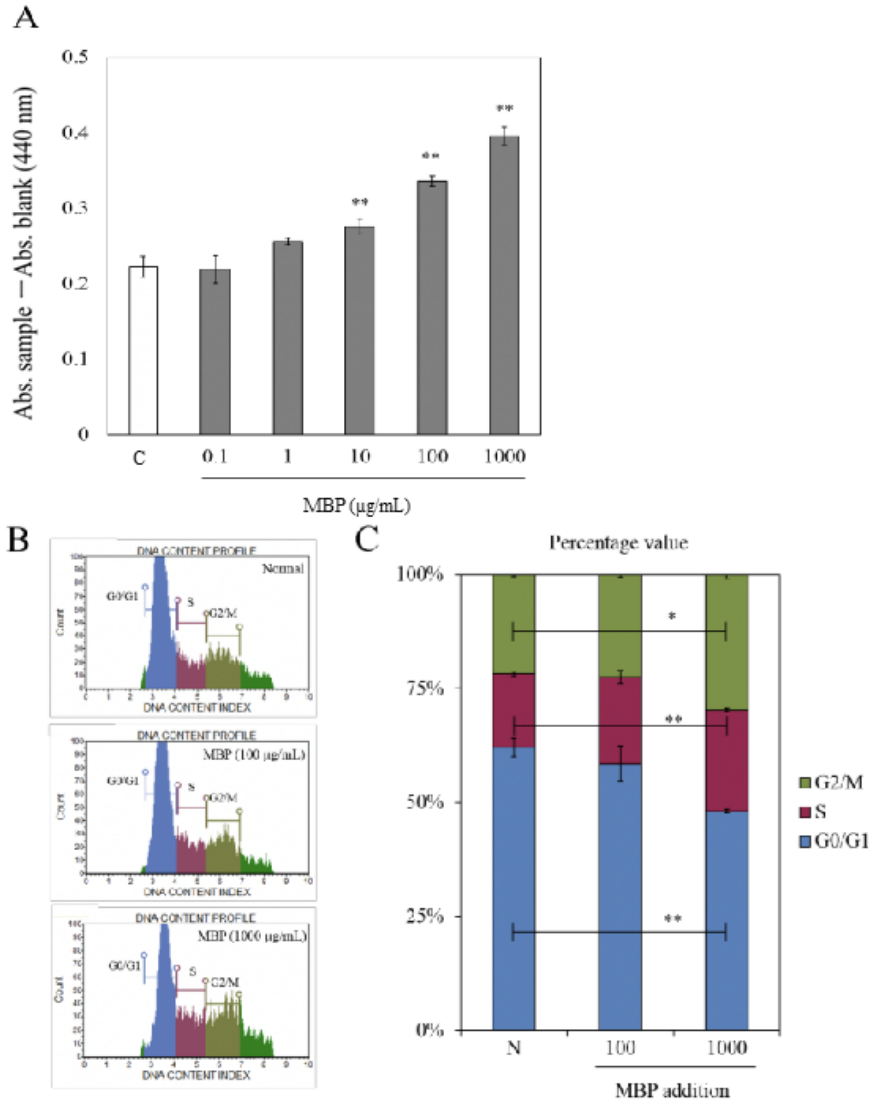
Figure legends

Figure 1. Effect of addition of milk basic protein (MBP) on the proliferation and cell cycle ratio of ATDC5 cells. (A) Cell proliferation, (B) DNA content index histogram and (C) percentage value. Data are presented as the mean ± S.E. Dunnett's test vs. control (C) * $p < 0.05$ ** $p < 0.01$ (n = 3~5).

Figure 2. Effect of milk basic protein (MBP) on (A) alkaline phosphatase (ALP) activity and (B) mineralization of ATDC5 cells. Data are presented as the mean ± S.E. Dunnett's test or student's *t* test *vs*. control (C) ** $p < 0.01$ (n = 4).

Figure 3. Effect of milk basic protein (MBP) on the expression of genes related to differentiation of ATDC5 cells. Expression levels of *Sox 9*, *Runx2*, *Col2* and *Col10* are indicated relative to that of *GAPDH*. Data are presented as the mean ± S.E. student's *t* test vs. control (C) *$p < 0.05$ **$p < 0.01$ (n = 3).

Figure 4. Schematic diagram of the effect of milk basic protein (MBP) on chondrocytes.



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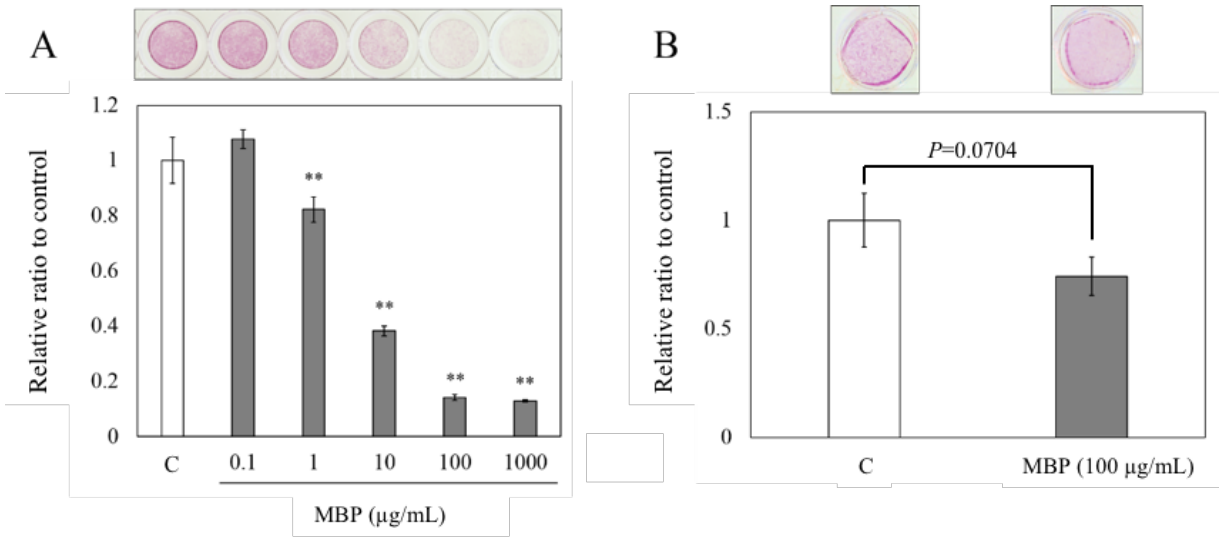

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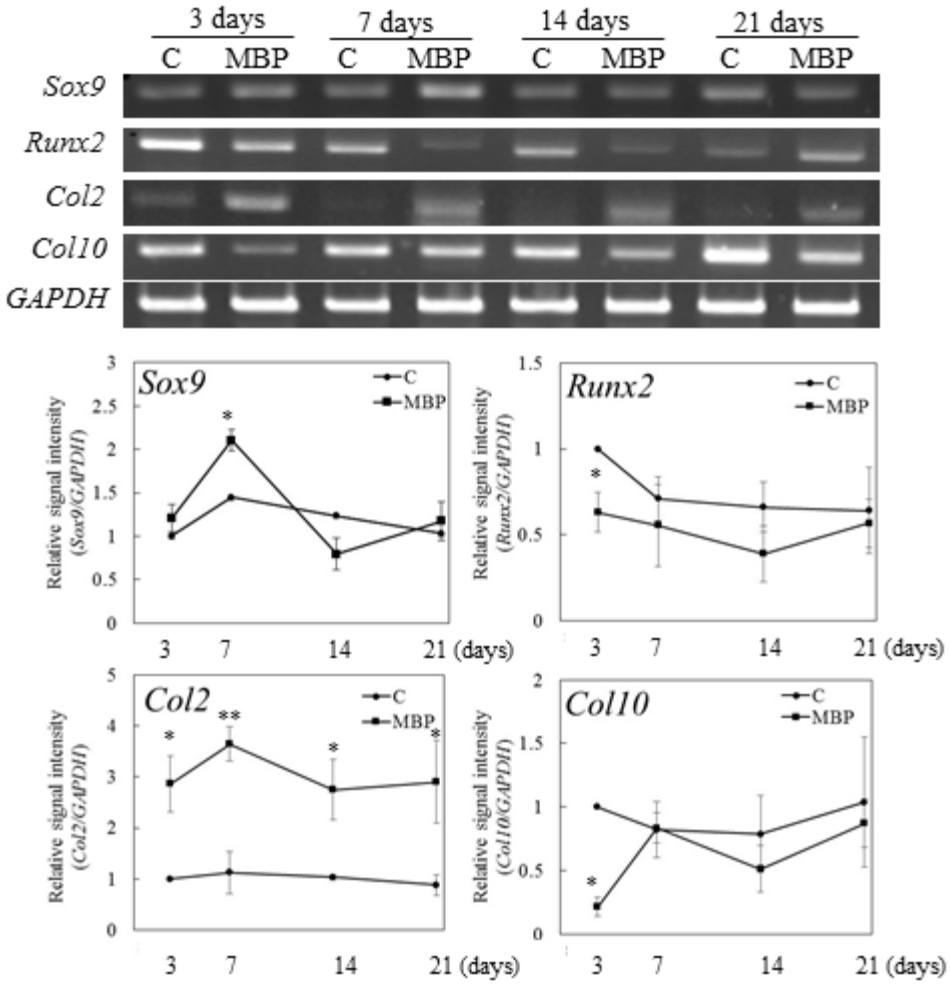
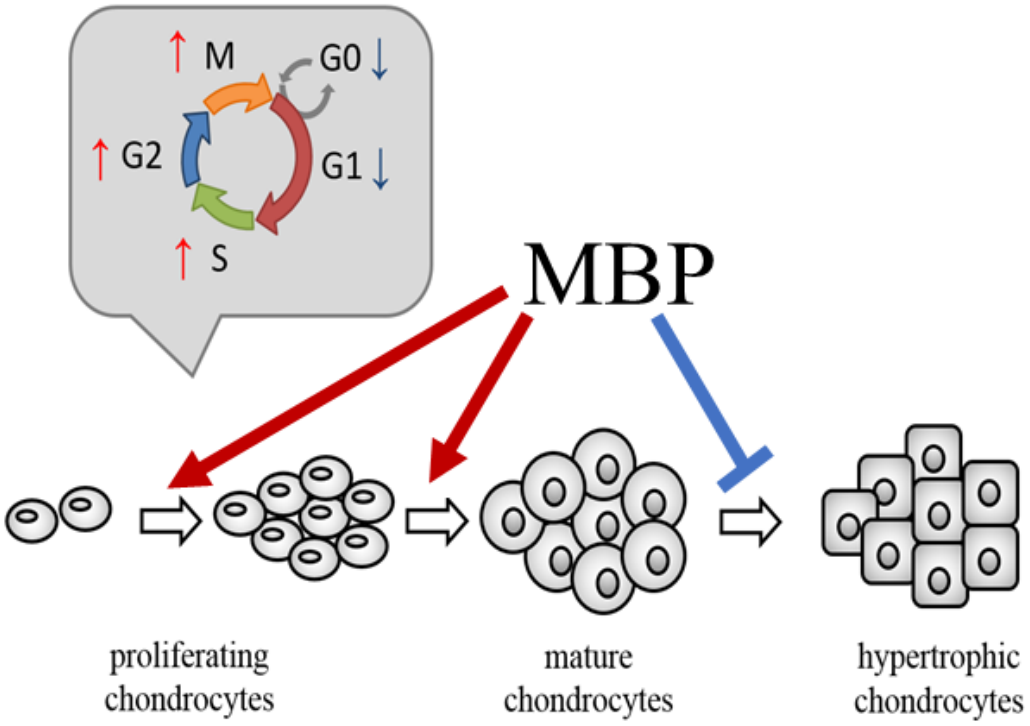


JDS-18-15656, Figure 3



JDS-18-15656, Figure 4