

# The Nicotinamide Adenine Dinucleotide (NAD)-Dependent Deacetylase Sirtuin-1 Regulates Chondrocyte Energy Metabolism through the Modulation of Adenosine Monophosphate-Activated Protein Kinase (AMPK) in Osteoarthritis (OA)

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## Abstract

To clarify how the osteoarthritis (OA)-induced catabolic factor interleukin (IL)-1 $\beta$  affects chondrocyte energy metabolism, and especially to define the downstream pathway linking nicotinamide adenine dinucleotide (NAD)-dependent deacetylase Sirtuin-1 (Sirt-1) to energy metabolism in OA chondrocytes. Human chondrocytes were isolated from articular cartilage samples of patients with OA. The level of energy metabolism of OA chondrocytes was evaluated by monitoring the activity of the energy metabolic sensor, adenosine monophosphate-activated protein kinase (AMPK) and the level of production of adenosine triphosphate (ATP) in chondrocytes in the presence or absence of IL-1 $\beta$  (10 ng/mL). Effects of IL-1 $\beta$  on anabolic and catabolic activities of chondrocytes were analyzed by the levels of production of proteoglycan and matrix metalloproteinase (MMP)-13, respectively. Experiments involving pre-treatment with Sirt-1 inhibitor were also performed to investigate the underlying regulatory mechanism linking Sirt-1 to chondrocyte energy metabolism. IL-1 $\beta$  significantly inhibited the activity of AMPK and production of ATP in OA chondrocytes. The energy metabolism disruption mediated by IL-1 $\beta$  was further decreased by pre-treatment with Sirt-1 inhibitor in OA chondrocytes. Treatment with IL-1 $\beta$  significantly decreased the level of proteoglycan production and significantly increased the level of MMP-13 secretion by chondrocytes. These chondrocyte activities were also reduced by pre-treatment with the Sirt-1 inhibitor in OA chondrocytes. IL-1 $\beta$  inhibits the AMPK - ATP energy metabolic pathway in OA chondrocytes. Our findings also suggest that Sirt-1 activity is involved in anabolic and catabolic cellular activities and that Sirt-1 modulates ATP production through functional regulation of the energy sensor AMPK in chondrocytes.

**Keywords:** Energy metabolism; Adenosine monophosphate-activated protein kinase; Adenosine triphosphate; Chondrocyte; Sirtuin; Osteoarthritis

**Abbreviations:** OA: Osteoarthritis; ATP: Adenosine Triphosphate; AMPK: Adenosine Monophosphate-activated Protein Kinase; Sirt: Sirtuin; Runx2: Runt-related Transcription Factor; MMP: Matrix Metalloprotease; IL: Interleukin; NAD: Nicotinamide Adenine Dinucleotide; NADH: Nicotinamide Adenine Dinucleotide; PBS: Phosphate-buffered Saline; DMEM: Dulbecco's Modified Eagle's Medium; HEPES: 2-[4-(2-hydroxyethyl)-1-piperazinyl] Ethane Sulfonic Acid

## Introduction

Articular cartilage comprises an abundant extracellular matrix containing a sparse population of chondrocytes that are essential for producing, assembling and turning over the articular cartilage matrix components [1,2]. Progressive degeneration of articular cartilage is a common feature of osteoarthritis (OA) [3,4]. OA is the most prevalent joint disease and has a complex pathogenesis and pathophysiology [4-6]. The effects of mechanical force acting on articular cartilage,

obesity, inflammation and aging are among the major catabolic factors in OA, suggesting that both extrinsic and intrinsic stresses acting on articular cartilage matrix and chondrocytes are both causal and contributory factors to the catabolic process in OA [5-8].

Sirtuins are nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylases that control the protein acetylation and cellular metabolism [9-11]. Sirtuins play important roles as key regulators of numerous functions including regulation of the cytoskeleton, cellular differentiation, cell growth, stress tolerance, cellular metabolism, DNA repair, apoptosis, anti-inflammation, as well as control of cellular senescence in a variety of cells [9,12,13]. The sirtuin family members (sirtuin 1-7) execute their functions by deacetylation of target proteins in the different cellular localizations [9,10]. Several reports indicate that sirtuins may have two important roles - "regulation of cellular metabolism" and "response to cellular stresses (stress tolerance)", which are closely involved in the pathogenesis and pathology of a variety of diseases including stress-induced degenerative diseases such as OA [11-13]. Various cellular stresses such as mechanical stress, oxidative stress, inflammation and aging have been recognized as major risk factors for OA, and all sources of stress could affect the regulation of chondrocyte metabolism [5-8,14-17]. Altered

chondrocyte metabolism and changes in their responses to OA-related stresses are implicated in the creation of an imbalance between catabolic and anabolic reactions, leading to osteoarthritic degeneration of articular cartilage [5,8,17]. We postulated that the level of a key regulator of cellular stresses, sirtuin, in chondrocytes could participate in the pathophysiology of OA.

We and other groups have already demonstrated that sirtuin-1 (Sirt-1) has a functional role and is a key regulator of many molecules in both catabolic and anabolic pathways in OA chondrocytes [18-23]. Sirt-1 has been reported to participate in stress responses by regulating cell death and cellular metabolism through the deacetylation of target proteins in chondrocytes [19,20,22]. It has also been shown to promote the expression of cartilage-specific genes in chondrocytes [22,24,25]. In addition, it has been demonstrated that Sirt-1 inhibits chondrocyte apoptosis by suppressing protein tyrosine phosphatase 1B, caspases and mitochondria-related apoptotic signaling proteins or activating the insulin-like growth factor receptor pathway [26,27]. These findings suggest that the downregulation of Sirt-1 in chondrocytes is responsible for their increased apoptosis and the suppression of chondrocyte anabolism in OA. Recent reports reveal that Sirt-1 in chondrocytes may be involved in the pathophysiology of OA.

More recently, we have demonstrated that Sirt-1 regulates the expression of the osteogenic transcription factor Runt-related transcription factor 2 (Runx2) and production of the cartilage matrix-degrading enzyme, matrix metalloproteinase (MMP)-13, in osteoarthritic chondrocytes [18]. It is well known that MMP-13 contributes to OA cartilage degradation [3-5]. The expression level of MMP-13 is significantly higher in the chondrocytes of advanced stage OA cartilage in comparison with early OA or normal knee cartilage [28]. Furthermore, transgenic mice over-expressing MMP-13 in their articular chondrocytes experience joint degeneration similar to human OA, suggesting that recognition of the regulatory mechanism of MMP-13 expression in chondrocytes may contribute to an understanding of the molecular pathophysiology of enzymatic degradation of articular cartilage [29]. There is a general consensus that Runx2 is required for chondrocyte hypertrophy and osteophyte formation in the joint [30,31]. In addition, Runx2 is known to promote activation of the cartilage-degrading enzyme MMP-13 in chondrocytes [32]. Interestingly, our previous study demonstrated that Sirt-1 inactivation inhibited both Runx2 expression and the IL-1 $\beta$ -accelerated production of MMP-13 in chondrocytes [18]. These findings indicate that Sirt-1 activity, which is downregulated by several cellular stresses, may directly influence the expression of Runx2 in chondrocytes, affecting the enzymatic degeneration of articular cartilage and osteophyte formation in OA. We concluded that Sirt-1-regulated-Runx2 expression could control the production of MMP-13 and osteophyte formation by OA chondrocytes [18]. We postulate that Sirt1 activity in chondrocytes could be an important contributing factor in the pathogenesis and pathophysiology of OA. However, it still remains unclear how Sirt-1 regulates chondrocyte anabolic and catabolic metabolic processes during OA progression.

The OA-related catabolic factor IL-1 $\beta$  alters chondrocyte activity and induces chondrocyte apoptosis [4-8]. However, few studies have examined how the catabolic cytokine impacts chondrocyte energy metabolism in articular cartilage. Given the change of chondrocyte energy metabolism during the progression of OA, we hypothesized that the build-up of OA-related catabolic factors may disrupt cellular energy metabolism in cartilage, which then compromises chondrocyte activity, cartilage homeostasis and predisposes cartilage to damage.

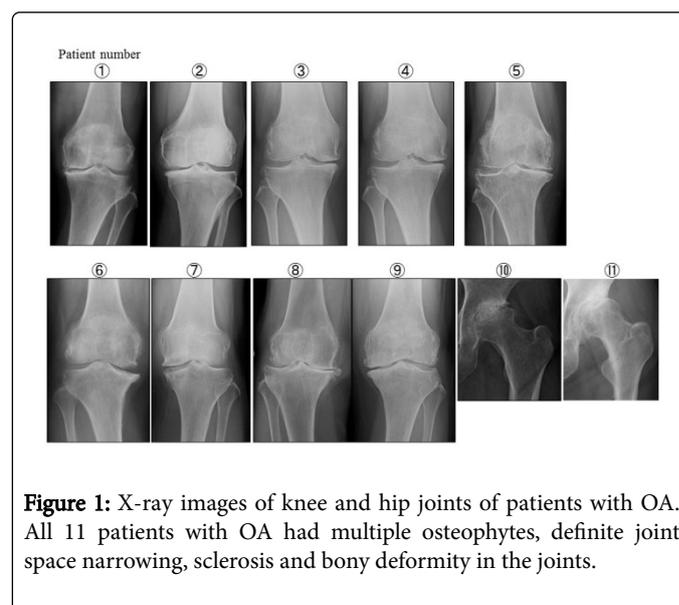
Disturbance in the maintenance of chondrocyte energy metabolism may result in cellular catabolic stress, facilitating the degeneration of articular cartilage.

In this study, we tested our hypothesis using human osteoarthritic chondrocytes stimulated with an OA-related catabolic factor (IL-1 $\beta$ ) and revealed that IL-1 $\beta$  profoundly disrupted chondrocyte energy metabolism. This effect may involve interactions between adenosine triphosphate (ATP) production and the energy metabolic sensor, adenosine monophosphate-activated protein kinase (AMPK). Interestingly, we also found that Sirt-1 regulated both the activity of AMPK and the production of ATP in chondrocytes. AMPK and Sirt-1 are thought to be two critical energy sensors that regulate cellular energy balance. Reduced activities of Sirt-1 and AMPK in chondrocytes may limit energy availability for chondrocyte/cartilage maintenance and homeostasis in OA. We discuss the effect of Sirt-1 on energy metabolism through the regulation of the AMPK - ATP energy metabolic pathway.

## Materials and Methods

### Human chondrocyte cultures

Human articular cartilage tissue samples were obtained from patients' joints during arthroplastic surgery after obtaining informed consent from 11 patients with OA [mean age 72 years (range 58-80 years), 9 knee joints; 2 hip joints]. The protocol of this study was approved by the ethical committee of St. Marianna University School of Medicine (permission number: 1315). The clinical features of patients involved in the current study are summarized in Table 1. Representative pre-operative X-ray features of patients are shown in Figure 1. The severity of knee OA was evaluated by the Kellgren and Lawrence classification [33,34]. The severity of hip OA was also evaluated by the score according to the Kellgren and Lawrence classification for knee OA.



Articular cartilage explants were cut into small pieces, washed with phosphate-buffered saline (PBS) and digested with 1.5 mg/mL collagenase B (Sigma, St. Louis, MO, USA) in Dulbecco's modified Eagle's medium (DMEM) (Sigma) overnight on a shaking platform at

37°C. Isolated chondrocytes were collected following centrifugation, washed three times with PBS, resuspended and cultured in DMEM supplemented with 10% heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine, 25 mM HEPES (2[4-(2-hydroxyethyl)-1-piperazinyl] ethane sulfonic acid), and 100 U/mL penicillin and streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> as previously reported [18].

Patient Number	Age and Gender	Disease	Severity of OA
1	79 years old, male	Lt. knee OA	Grade 3
2	74 years old, male	Lt. knee OA	Grade 3
3	72 years old, female	Rt. knee OA	Grade 3
4	75 years old, female	Rt. knee OA	Grade 3
5	75 years old, female	Rt. knee OA	Grade 3
6	63 years old, female	Rt. knee OA	Grade 3
7	75 years old, female	Lt. knee OA	Grade 3
8	78 years old, female	Rt. knee OA	Grade 4
9	80 years old, female	Lt. knee OA	Grade 3
10	58 years old, female	Lt. hip OA	Grade 4
11	65 years old, male	Lt. hip OA	Grade 4

OA: osteoarthritis; Lt: Left; Rt: Right. Severity of OA: Grade 0 (No feature of osteoarthritis), Grade 1 (doubtful narrowing of joint space and possible osteophytic lipping), Grade 2 (moderate multiple osteophytes, definite narrowing of joint space), Grade 3 (moderate multiple osteophytes, definite narrowing of joint space, and some sclerosis and possible deformity of bones end), Grade 4 (large osteophytes, marked narrowing of joint space, severe sclerosis and definite deformity of bone ends)

**Table 1:** The Clinical characteristics of patients.

### Effects of the OA-related catabolic factor, interleukin (IL)-1 $\beta$ , on Sirt1 expression in chondrocytes

To study the effect of the OA-related catabolic factor, IL-1 $\beta$ , on the expression of Sirt-1 in chondrocytes, chondrocytes were incubated in the presence or absence of IL-1 $\beta$  (10.0 ng/mL) for 24 hours at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. After harvesting the cultured cells, cellular proteins were collected for immunoblotting analyses as previously described [18]. The level of Sirt-1 expression in chondrocytes was analysed by western blotting (Image Quant LAS 4000, GE imagination at work). The antibodies used for western blot analysis were polyclonal against human Sirt-1 (1:1000 dilution; Abcam Inc., Cambridge, UK), and  $\beta$ -tubulin (1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the corresponding secondary antibody conjugated with horseradish peroxidase [Dako, rabbit IgG p04 for anti-Sirt-1 antibody (1:10000 dilution)]. The antibody-bound protein bands were visualized using the extended cavity laser system (GE Healthcare Bio-sciences KK, Tokyo, Japan).

### Effects of Sirt-1 inactivation on energy metabolism in OA chondrocytes

To study the effects of Sirt-1 inactivation on energy metabolism, we examined levels of AMPK and ATP production in the Sirt-1 inhibitor-

treated chondrocytes *in vitro*. Cultured chondrocytes were divided into 4 groups; control (medium only), IL-1 $\beta$ -treated group, Sirt-1 inhibitor-treated group, and IL-1 $\beta$  + Sirt-1 inhibitor-treated group. In the IL-1 $\beta$  + Sirt-1 inhibitor-treated group, chondrocytes were pre-treated with the Sirt-1 inhibitor (S)-35 (sc-204279, 1.0  $\mu$ M, Santa Cruz Biotechnology Inc.) for 6 hours at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. Then, IL-1 $\beta$  (10.0 ng/mL) was added and the cells were returned to the incubator for a further 18 hours under the same conditions. In the IL-1 $\beta$ -treated group or the Sirt-1 inhibitor-treated group, chondrocytes were treated with IL-1 $\beta$  (10.0 ng/mL) or the Sirt-1 inhibitor (S)-35 (1.0  $\mu$ M) for 24 hours at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. At the end of the culture period, the conditioned culture medium was collected and stored at -80°C until analysis. The levels of AMPK in chondrocytes were measured using an AMPK assay kit (Thermo Fisher Scientific K.K., Kanagawa, Japan). The level of ATP production by chondrocytes was examined by the inter Cellular ATP assay kit (Toyo B-NET co., LTD., Tokyo, Japan).

### Effects of Sirt-1 inactivation on proteoglycan and MMP-13 production in OA chondrocytes

To study the effects of Sirt-1 inactivation on chondrocyte activity, we examined levels of production of the articular cartilage matrix component, proteoglycan, and the cartilage-degrading enzyme, matrix metalloproteinase (MMP)-13, in the Sirt-1 inhibitor-treated chondrocytes *in vitro*. As described above, cultured chondrocytes were divided into 4 treatment groups. The levels of MMP-13 produced by chondrocytes were measured using an ELISA kit (MMP-13 assay kit; Amersham Biosciences, Little Chalfont, UK). The level of proteoglycan in chondrocytes was examined by ELISA (DIA source PG-EASIA kit, KAP1461, DIA source Immuno Assays S.A.).

### Statistical analysis

Data were evaluated using Student's t test. Results are presented as mean  $\pm$  95% confidence interval from three independent experiments. Analysis of variance was used for comparisons of more than two groups, and differences between two groups within the set were analysed by a Fisher's protected least-significant difference test. Probability values of <0.05 were considered statistically significant.

## Results

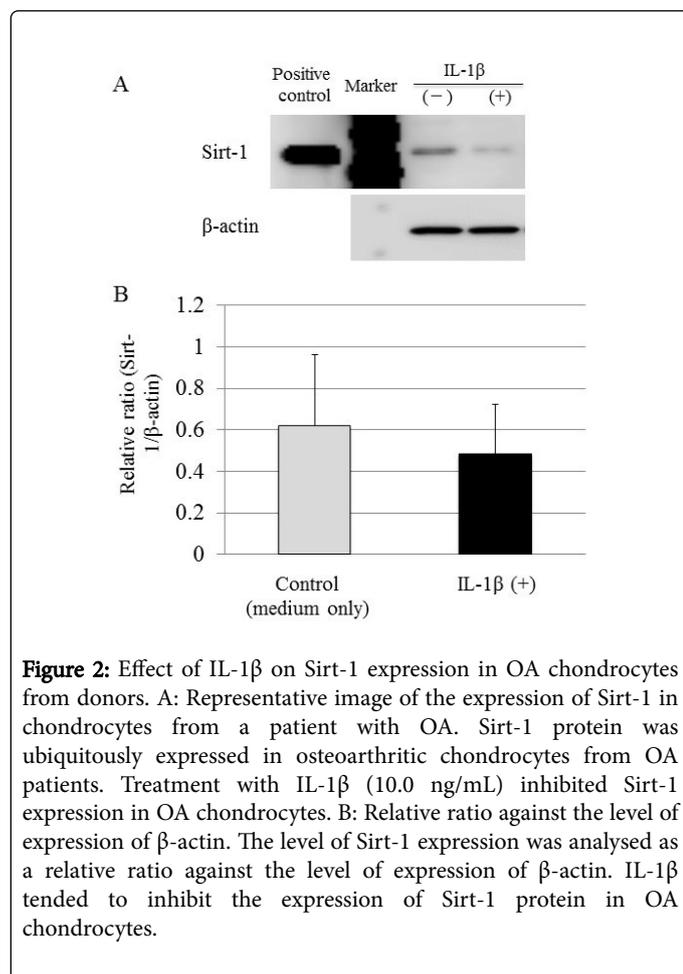
### Severity of knee or hip OA

The clinical features of patients studied are summarized in Table 1. As shown in Figure 1, all 11 patients with OA had multiple osteophytes, definite joint space narrowing, sclerosis and bony deformity in the joints. According to the Kellgren and Lawrence classification, 8 patients with OA were classified as grade 3 and 3 OA patient as grade 4 (Table 1 and Figure 1).

### OA-related catabolic factor IL-1 $\beta$ inhibits the expression of Sirtuin-1 in chondrocytes

Sirt-1 protein was ubiquitously expressed in chondrocytes from patients with OA. As shown in the representative image in Figure 2A, treatment with IL-1 $\beta$  inhibited the expression of Sirt-1 in OA chondrocytes. There was a tendency to inhibit the expression of Sirt-1 in OA chondrocytes which were stimulated by IL-1 $\beta$ , although no

significant difference was observed between control group and IL-1 $\beta$ -treated group (Figure 2B).



**Figure 2:** Effect of IL-1 $\beta$  on Sirt-1 expression in OA chondrocytes from donors. A: Representative image of the expression of Sirt-1 in chondrocytes from a patient with OA. Sirt-1 protein was ubiquitously expressed in osteoarthritic chondrocytes from OA patients. Treatment with IL-1 $\beta$  (10.0 ng/mL) inhibited Sirt-1 expression in OA chondrocytes. B: Relative ratio against the level of expression of  $\beta$ -actin. The level of Sirt-1 expression was analysed as a relative ratio against the level of expression of  $\beta$ -actin. IL-1 $\beta$  tended to inhibit the expression of Sirt-1 protein in OA chondrocytes.

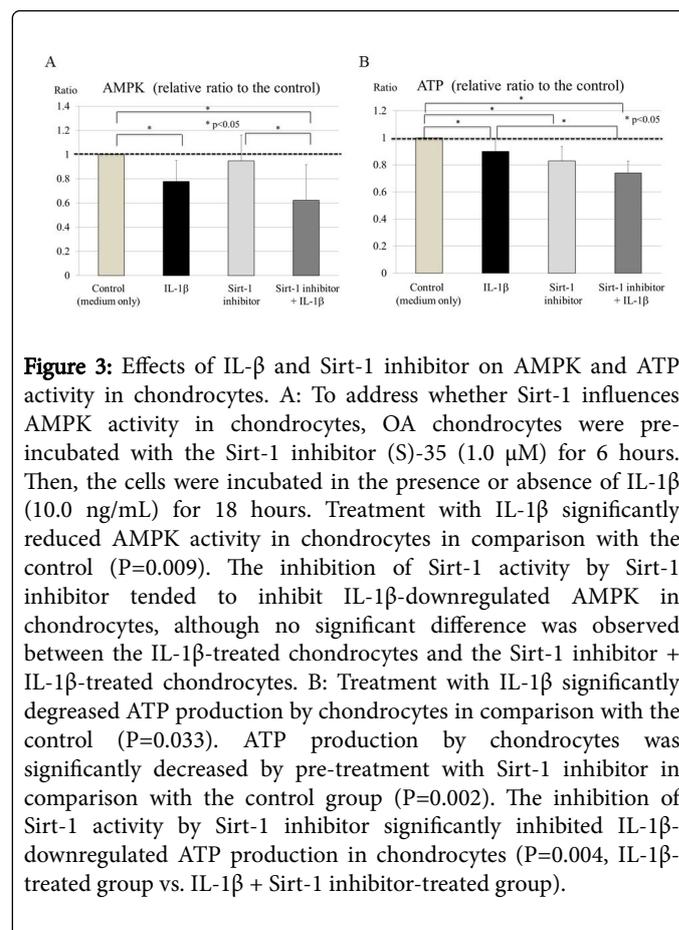
### Sirt-1 regulates activity of the energy metabolic sensor AMPK and the production of ATP in human chondrocytes from patients with OA

As shown in Figure 3A, the level of AMPK in chondrocytes was significantly reduced by IL-1 $\beta$  stimulation in comparison with the control group ( $P=0.009$ ). In addition, treatment with IL-1 $\beta$  significantly reduced the level of ATP production by chondrocytes ( $P=0.033$  compared to control, Figure 3B). These findings indicate that the OA-related catabolic factor IL-1 $\beta$  inhibits levels of the energy sensor AMPK as well as ATP production in chondrocytes, suggesting that energy metabolism is downregulated in osteoarthritic chondrocytes.

To address whether Sirt-1 regulates the levels of AMPK and ATP production in chondrocytes, osteoarthritic chondrocytes from OA patients were pre-treated with Sirt-1 inhibitor and then cellular activities were examined *in vitro*. Pre-treatment with the Sirt-1 inhibitor (S)-35 (1.0  $\mu$ M) showed a tendency to further inhibit the IL-1 $\beta$ -downregulated AMPK in chondrocytes, although no significant difference in the AMPK level was observed between the IL-1 $\beta$ -treated group and the IL-1 $\beta$  + Sirt-1 inhibitor-treated group (Figure 3A). The group treated with Sirt-1 inhibitor alone showed no significant difference in AMPK level in comparison with the control, although

there was a tendency to decrease the mean level of AMPK activity in the Sirt-1-treated group.

ATP production by osteoarthritic chondrocytes significantly decreased following treatment with Sirt-1 inhibitor ( $P=0.002$ ; control group vs. Sirt1 inhibitor-treated group, Figure 3B). Moreover, pre-treatment with Sirt-1 inhibitor further decreased the IL-1 $\beta$ -induced reduction of ATP production in osteoarthritic chondrocytes ( $P=0.004$ ; IL-1 $\beta$ -treated group vs. IL-1 $\beta$  + Sirt-1 inhibitor-treated group, Figure 3B). These data led to our hypothesis that Sirt-1 may modulate the level of the energy sensor AMPK in chondrocytes, resulting in the positive regulation of ATP production in OA.



**Figure 3:** Effects of IL-1 $\beta$  and Sirt-1 inhibitor on AMPK and ATP activity in chondrocytes. A: To address whether Sirt-1 influences AMPK activity in chondrocytes, OA chondrocytes were pre-incubated with the Sirt-1 inhibitor (S)-35 (1.0  $\mu$ M) for 6 hours. Then, the cells were incubated in the presence or absence of IL-1 $\beta$  (10.0 ng/mL) for 18 hours. Treatment with IL-1 $\beta$  significantly reduced AMPK activity in chondrocytes in comparison with the control ( $P=0.009$ ). The inhibition of Sirt-1 activity by Sirt-1 inhibitor tended to inhibit IL-1 $\beta$ -downregulated AMPK in chondrocytes, although no significant difference was observed between the IL-1 $\beta$ -treated chondrocytes and the Sirt-1 inhibitor + IL-1 $\beta$ -treated chondrocytes. B: Treatment with IL-1 $\beta$  significantly decreased ATP production by chondrocytes in comparison with the control ( $P=0.033$ ). ATP production by chondrocytes was significantly decreased by pre-treatment with Sirt-1 inhibitor in comparison with the control group ( $P=0.002$ ). The inhibition of Sirt-1 activity by Sirt-1 inhibitor significantly inhibited IL-1 $\beta$ -downregulated ATP production in chondrocytes ( $P=0.004$ , IL-1 $\beta$ -treated group vs. IL-1 $\beta$  + Sirt-1 inhibitor-treated group).

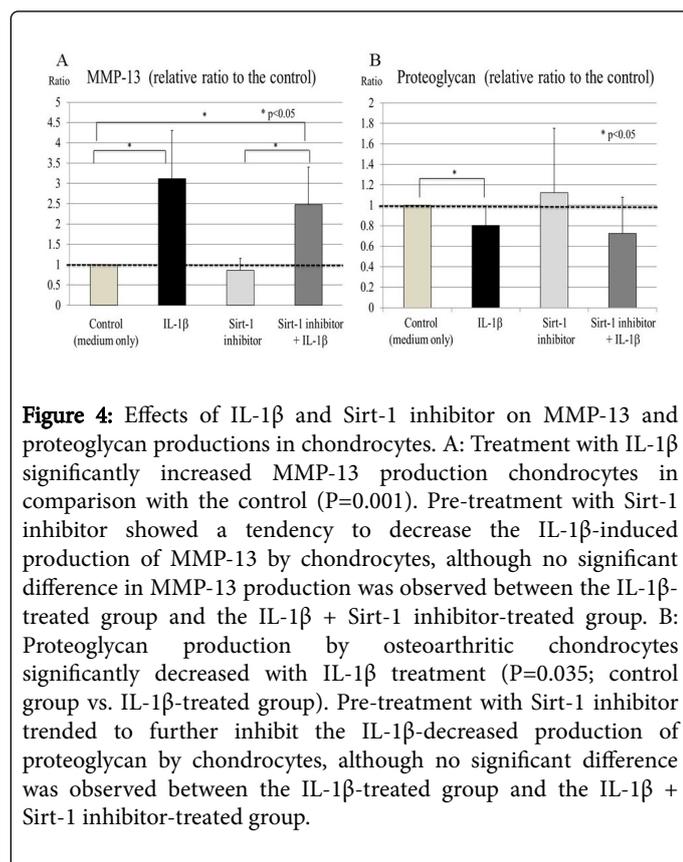
### Effects of IL-1 $\beta$ and Sirt-1 inhibitor on the production of MMP-13 and proteoglycan by human chondrocytes from patients with OA

To address whether Sirt-1 regulates the production of MMP-13 and proteoglycan by chondrocytes, osteoarthritic chondrocytes from OA patients were pre-treated with Sirt-1 inhibitor and then cellular activities were examined *in vitro*.

The production of MMP-13 by chondrocytes was significantly increased by IL-1 $\beta$  stimulation in comparison with the control group (Figure 4A,  $P=0.001$ ). The group treated with Sirt-1 inhibitor alone showed no significant difference in MMP-13 production in comparison with the control. Pre-treatment with Sirt-1 inhibitor showed a tendency to decrease the IL-1 $\beta$ -induced production of MMP-13 by chondrocytes, although no significant difference in

MMP-13 production was observed between the IL-1 $\beta$ -treated group and the IL-1 $\beta$  + Sirt-1 inhibitor-treated group (Figure 4A).

Proteoglycan production by osteoarthritic chondrocytes was significantly decreased by treatment with IL-1 $\beta$  ( $P=0.035$ ; control group vs. IL-1 $\beta$ -treated group, Figure 4B). Pre-treatment with Sirt-1 inhibitor trended to further inhibit the IL-1 $\beta$ -decreased production of proteoglycan by chondrocytes, although no significant difference was observed in proteoglycan production between the IL-1 $\beta$ -treated group and the IL-1 $\beta$  + Sirt-1 inhibitor-treated groups (Figure 4B).



**Figure 4:** Effects of IL-1 $\beta$  and Sirt-1 inhibitor on MMP-13 and proteoglycan productions in chondrocytes. A: Treatment with IL-1 $\beta$  significantly increased MMP-13 production chondrocytes in comparison with the control ( $P=0.001$ ). Pre-treatment with Sirt-1 inhibitor showed a tendency to decrease the IL-1 $\beta$ -induced production of MMP-13 by chondrocytes, although no significant difference in MMP-13 production was observed between the IL-1 $\beta$ -treated group and the IL-1 $\beta$  + Sirt-1 inhibitor-treated group. B: Proteoglycan production by osteoarthritic chondrocytes significantly decreased with IL-1 $\beta$  treatment ( $P=0.035$ ; control group vs. IL-1 $\beta$ -treated group). Pre-treatment with Sirt-1 inhibitor trended to further inhibit the IL-1 $\beta$ -decreased production of proteoglycan by chondrocytes, although no significant difference was observed between the IL-1 $\beta$ -treated group and the IL-1 $\beta$  + Sirt-1 inhibitor-treated group.

## Discussion

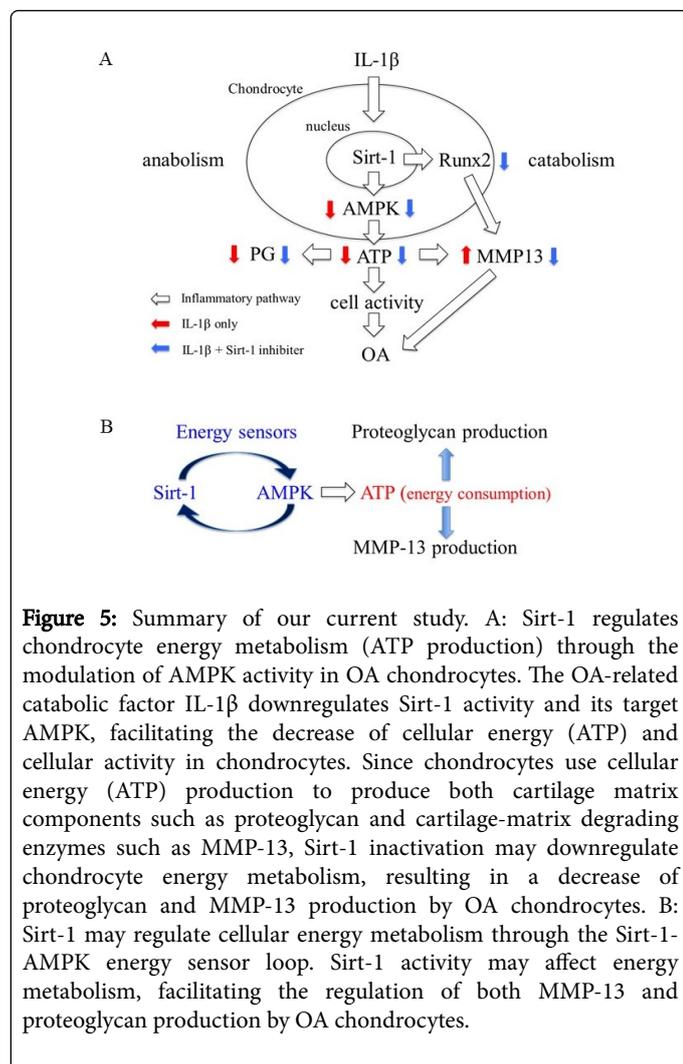
OA is characterized by structural damage and functional deficit of articular cartilage [1,3]. The normal structure and function of articular cartilage is maintained by articular chondrocytes. As an anabolic process, normal chondrocytes produce essential components of articular cartilage, chiefly type II collagen and proteoglycan [1]. In contrast, OA chondrocytes express matrix-degrading enzymes such as matrix metalloproteinase (MMP)-1, -3 and -13, eventually resulting in osteoarthritic cartilage degeneration and destruction. OA chondrocytes are characterized by stimulated catabolic responses as well as reduced anabolic responses to OA-induced stresses [3-6]. In both cases, chondrocytes use cellular energy to produce cartilage matrix components or cartilage-degrading enzymes (MMPs). The status of chondrocyte energy metabolism could influence cartilage homeostasis as well as chondrocyte activity.

To further understand the pathogenesis and pathophysiology of OA, in the current study, we focused on chondrocyte energy metabolism and its related regulatory mechanism. AMPK is known to be the

energy sensor that regulates cellular energy metabolism [35,36]. Once activated, AMPK responds by phosphorylating downstream targets, which allow activation of pathways to produce ATP [36,37]. It has been demonstrated that AMPK is activated by several cellular stresses such as nutrient deprivation, heat shock, hypoxia and exercise [3,35-37]. We postulated that the activity of AMPK and its regulated energy metabolism in chondrocytes may be influenced by OA-related catabolic stresses, such as mechanical and chemical stresses. In the present study, our data reveal that the OA-related catabolic factor IL-1 $\beta$  inhibits both activity of AMPK and ATP production in OA chondrocytes, suggesting that energy metabolism in chondrocytes is downregulated by OA-related factors during the progression of articular cartilage degeneration. Indeed, previous reports demonstrated that the activity of AMPK is decreased in chondrocytes in aged human and mouse OA cartilage [38,39]. Our findings of the downregulation of the AMPK ATP energy metabolic pathway in OA chondrocytes are consistent with their findings. From the results of our present study, we conclude that OA-induced catabolic stresses could lead to the downregulation of AMPK and its related energy production (ATP) in chondrocytes, resulting in an imbalance between anabolic and catabolic responses to OA-related catabolic stresses. AMPK, as an energy sensor, plays a critically-important role in maintaining articular chondrocytes and consequently cartilage.

The NAD-dependent protein deacetylase, Sirt-1, is also recognized as another regulator of energy metabolism [37,40-42]. Sirt-1 has critically-important roles in regulating cellular metabolism and controlling responses to cellular stresses via the regulation of target proteins, and is closely involved in the pathophysiology of stress-induced degenerative diseases including OA [9,13,20,21]. We postulated that cellular stresses could affect cellular energy metabolism through the regulatory mechanism regulated by Sirt-1 as well as AMPK. Several cellular stresses such as mechanical stress, oxidative stress, inflammation and aging, all of which are OA risk factors, induce osteoarthritic cartilage degeneration [5-8]. These OA-related stresses may affect the activity of Sirt-1 as well as AMPK in chondrocytes, consequently facilitating the downregulation of chondrocyte energy metabolism and cartilage matrix homeostasis. Indeed, previous studies demonstrated that decreased expression of Sirt-1 was observed in mouse and human OA cartilages [23,43]. Furthermore, Platou et al. demonstrated that the OA-related catabolic factor, IL-1 $\beta$  reduced the expression of both Sirt-1 mRNA and Sirt-1 protein in chondrocytes [20]. We also found that IL-1 $\beta$  tended to inhibit the expression of Sirt-1 in chondrocytes. Our current study indicates that Sirt-1 insufficiency leads to further reduction in the IL-1 $\beta$ -induced decrease of ATP production by OA chondrocytes. This suggests that Sirt-1, as well as AMPK, is a regulator of energy homeostasis in chondrocytes.

In our current study, Sirt-1 inactivation was shown to decrease both the level of AMPK activity and the production of ATP in OA chondrocytes. This suggests that Sirt-1 activity may positively regulate the activity of AMPK and its resultant ATP production in chondrocytes (Figure 5A). In contrast, previous reports demonstrated that activation of AMPK stimulated Sirt-1 activity in a variety of cell types [44-46]. These findings suggest the existence of a positive feedback loop between Sirt-1 and AMPK in chondrocytes (Figure 5B). This Sirt-1-AMPK feedback loop may control the AMPK-ATP energy metabolic pathway and the energy balance in chondrocytes. In addition, it has been reported that Sirt-1 promotes stress tolerance in chondrocytes [23-27]. Therefore, disruption of the Sirt-1-AMPK feedback loop may not only disrupt chondrocyte energy metabolism but may also decrease chondrocyte stress resistance in OA.



This study indicates that Sirt-1 may regulate the stress response of chondrocytes to OA-related catabolic stresses as well as regulating chondrocyte energy metabolism through the modulation of AMPK activity. Both these changes are implicated in creating an imbalance between catabolic and anabolic pathways, leading to osteoarthritic degeneration of articular cartilage. In the present study, Sirt-1 inactivation induced both a further decrease of IL-1 $\beta$ -reduced production of proteoglycan and a decrease in the IL-1 $\beta$ -accelerated MMP-13 production by OA chondrocytes. Since chondrocytes require cellular energy (ATP) production to produce both cartilage matrix components such as proteoglycan and cartilage matrix degrading enzymes such as MMP-13, Sirt-1 inactivation may downregulate chondrocyte energy metabolism, resulting in decreases of both proteoglycan and MMP-13 productions by OA chondrocytes (Figure 5A). In contrast to our findings, it has been reported that Sirt-1 insufficiency induces further acceleration of IL-1 $\beta$ -induced MMP-13 production by chondrocytes [47]. Regarding the relationship between Sirt-1 and MMP-13, our previous study indicated that Sirt-1 positively regulates the expression of Runx2, positive transcription factor for MMP-13, in OA chondrocytes [18]. We think that Sirt-1 activity may influence MMP-13 expression via Sirt-1-mediated Runx2 expression in OA chondrocytes [18]. Indeed, some studies demonstrated that Sirt-1 activator induces the expression of Runx2 (promotor for MMP-13

expression) in chondrocytes and mesenchymal stem cells [48,49]. Moreover, it has been reported that IL-1 $\beta$  increases MMP-13 expression and requires Runx2 for this effect in chondrocytes [50]. These findings also support our hypothesis that the Sirt-1-Runx2 interaction may affect expression of the cartilage-degrading enzyme MMP-13 in OA chondrocytes. Although further studies are needed to clarify the exact correlation between Sirt-1 and MMP-13 in chondrocytes, we conclude that Sirt-1 insufficiency may induce the downregulation of Runx2 expression, consequently leading to the decrease of Runx2-promoted MMP-13 expression in OA chondrocytes (Figure 5A). In addition, since Sirt-1 may regulate cellular energy metabolism through the Sirt-1-AMPK energy sensor loop, Sirt-1 insufficiency may affect energy metabolism, facilitating the downregulation of MMP-13 and proteoglycan production by OA chondrocytes (Figure 5B).

In conclusion, our present study indicates that the OA-related catabolic factor, IL-1 $\beta$ , inhibits the AMPK/ATP energy metabolic pathway in OA chondrocytes. Our findings also implicate Sirt-1 activity in anabolic and catabolic cellular activities and suggest it may modulate ATP production through functional regulation of the energy sensor AMPK in chondrocytes. Reduced activities of Sirt-1 and AMPK in chondrocytes may limit energy availability for chondrocyte/cartilage maintenance in OA. Disturbance of the Sirt-1-AMPK positive feedback loop in chondrocytes could affect energy balance and homeostasis in chondrocytes/cartilages, resulting in the progression of OA. Although further studies are needed to clarify the regulatory mechanism of chondrocyte energy metabolism, Sirt-1 and AMPK may be therapeutic targets for OA.

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## Competing Interests

The authors declare no conflict of interest.

## Authors' Contributions

All authors read and approved the final version to be published. All authors had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

- The conception and design the study, or acquisition of data, or analysis and interpretation; Hajime Kobayashi, Kazuo Yudoh, Kamada Toshikazu, Koh Terauchi, Naoko Yui, Kanaka Yatabe, Hiroto Fujiya, Hisateru Niki, Haruki Musha.
- Drafting the article or revising it critically for important intellectual content; Hajime Kobayashi, Kazuo Yudoh, Naoko Yui, Koh Terauchi.
- Final approval of the version to be submitted; Hajime Kobayashi, Kazuo Yudoh, Hisateru Niki, Haruki Musha

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