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Impaired glycolytic metabolism causes chondrocyte hypertrophy-like changes *via* promotion of phospho-Smad1/5/8 translocation into nucleus

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SUMMARY

Objective: Hypertrophy-like changes are often observed in chondrocytes during the development of osteoarthritis (OA). These changes play a crucial part in the OA-associated cartilage degradation and osteophyte formation. However, the pathogenesis leading to such changes is still unknown. In this study, we investigated the mechanism by which these hypertrophy-like changes are induced from the viewpoint of impaired glycolytic metabolism.

Methods: The effect of sodium fluoride (NaF) on glycolytic metabolism of cultured chondrocytes was confirmed by measurement of intracellular adenosine triphosphate (ATP) production. Translocation of phosphorylated Smad1/5/8 to the nucleus was evaluated by subcellular fractionation and Western blotting. Chondrocyte hypertrophy-like changes were investigated by real-time RT-PCR and Western blot analysis of differentiation markers.

Results: ATP production was dose-dependently decreased by NaF in the human chondrocytic cell line HCS-2/8. In addition, both chondrocyte proliferation and differentiation were inhibited, whereas cell death was promoted by treatment with NaF. Interestingly, combinational treatment with NaF and lactate enhanced translocation of phospho-Smad1/5/8 to the nucleus, as well as gene expression of *ALP*, *VEGF*, *COL10a1*, and matrix metalloproteinase13 (*MMP13*), which were the markers of late mature and hypertrophic chondrocytes. Furthermore, the production of type X collagen and activation of MMP9 were also promoted under the same conditions.

Conclusions: These findings suggest that decreased ATP production by NaF promotes hypertrophy-like changes *via* activation of phospho-Smad1/5/8 in the presence of lactate. Novel metabolic aspects of OA pathogenesis are indicated herein.

Introduction

Osteoarthritis (OA) is one of the most prevalent joint diseases and is characterized by an age-dependent, slowly progressive degeneration of articular cartilage[1]. In OA, distinct stages can be recognized during disease progression in terms of the degeneration of cartilage[1]. At the initiation stage, the behavior of the chondrocytes becomes altered due to aging or excess mechanical stress, and the cells acquire a hypertrophy-like phenotype, producing type X collagen and matrix metalloproteinase13 (MMP13)[1,2]. Next, during the progression stage, the release and activation of MMP13 lead to degradation of the extracellular matrix (ECM) surrounding the chondrocytes; and the fragmented ECM molecules, forming an altered biomechanical environment, influence these chondrocytes such that the hypertrophy-like changes become accelerated[1,2]. Thereafter, at the late and end stages, chondrocyte clusters, which are formed in an unsuccessful attempt to repair the cartilage tissue, appear in severely damaged cartilage and peri-articular formation of new bone (osteophytosis) occurs[1,2]. Eventually, the cartilage tissue degenerates, thus exposing the underlying bone[1,2]. Under such conditions, this disease clinically leads to loss of joint function[3]. The incidence of OA is thought to be associated with many risk factors, such as metabolic disease, aging, obesity, and mechanical stress[1,2]. However, the pathogenic mechanism, in

Abbreviations: OA, osteoarthritis; NaF, sodium fluoride; MMPs, matrix metalloproteinases; ECM, extracellular matrix; MCTs, monocarboxylate transporters; ATP, adenosine triphosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PCNA, proliferating cell nuclear antigen; GAG, glycosaminoglycan; BMP, bone morphogenetic protein; ACAN, aggrecan.

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particular, the mechanism governing regulation of these hypertrophy-like changes at the initiation stage in OA, is still unclear.

To estimate possible factors involved in the hypertrophy-like changes, we focused on two experimental models[4]. One of the models is an arthritis model induced by the intra-articular injection of monoiodoacetate (MIA), which inhibits the activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), one of the glycolysis enzymes[5]. In this model, widespread chondrocytes are depleted throughout the cartilage tissue, cartilage ECM is also lost in the central part of the articular cartilage, and, furthermore, chondrocytes form clusters at the surface of articular cartilage[4]. The other one is the K/BxN mouse model, in which a spontaneously erosive arthritis is manifested[6,7]. The phenotype is characterized by many features common to rheumatoid arthritis (RA), including leukocyte invasion, synoviocyte proliferation, and erosion of cartilage and bone[6,7]. Recently, it was reported that the arthritis in the K/BxN mouse is caused by autoantibody against glucose-6-phosphate isomerase (GPI), which is another of the glycolysis enzymes[7]. Since these two animal models commonly exhibit severe cartilage destruction, this cartilage destruction may be caused by inhibition of the glycolysis enzymes. Therefore, we hypothesized that a part of cartilage destruction might be triggered by dysregulation of glucose metabolism in chondrocytes. Indeed, it is well known that cartilage is an avascular tissue and that chondrocytes generate energy in the form of adenosine triphosphate (ATP), mostly dependent on anaerobic metabolism of glucose[8]. Therefore, if this metabolism is impaired, intracellular ATP production is profoundly decreased; and the chondrocytes are forced to degenerate, following the pathway to hypertrophy-like changes.

Bone morphogenetic protein (BMP) signaling has been already known to be required for onset of chondrocyte hypertrophy[9]. Since Smad1/5/8 signaling, is a canonical pathway of BMP signaling, involvement of this signaling pathway in the hypertrophy-like changes due to impaired glycolysis is suspected. To examine this hypothesis, we used an *in vitro* culture system employing primary rat chondrocytes isolated from epiphyseal cartilage[10] and two useful immortalized chondrocytic cells lines, human chondrocytic HCS-2/8 cells[11–13] and rat chondrocytic RCS cells[14]. In the present study, we clarified that intracellular ATP production decreased by a few specific inhibitors of glycolysis caused chondrocyte hypertrophy-like changes *via* activation of Smad1/5/8 by lactate, which is an end product of glycolysis.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), α-modification of Eagle's medium (αMEM), and fetal bovine serum (FBS) were purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan), ICN Biomedicals (Aurora, OH), and Nichirei Bioscience Inc. (Tokyo, Japan), respectively. Plastic dishes and multi-well plates were obtained from Greiner Bio-One (Frickenhausen, Germany). Sodium fluoride (NaF) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Sodium lactate, iodoacetate sodium salt, anti-β-actin, anti-proliferating cell nuclear antigen (PCNA), and anti-type X collagen antibodies were obtained from Sigma (St. Louis, MO). Anti-monocarboxylate transporter (MCT)2, anti-MMP13, and anti-SOX9 antibodies were from Millipore (Temecula, CA). Anti-MMP9 was purchased from Triple Point Biologics, Inc. (Forest Grove, OR); and anti-nuclear factor (NF)-κB p65 and anti-MCT1, from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Smad1/5/8 from Cell Signaling Technology (Beverly, MA) and anti-lamin B1 from Life Technologies (Grand Island, NY) were also employed.

Cell culture

HCS-2/8[11–13] cells were inoculated at a density of 4×10^4 cells/cm^2 into culture dishes containing DMEM supplemented with 10% FBS and incubated at 37°C under 5% CO_2. RCS cells[14] were cultured in monolayer, pellet and collagen gel. For monolayer, RCS cells were seeded at a density of 2.5×10^4 cells/cm^2 in DMEM containing 10% FBS and incubated at 37°C under 5% CO_2. For pellet culture, 5×10^6 cells per pellet were centrifuged and then cultured at 37°C under 5% CO_2. For collagen gel 3-dimensional (3-D) culture, the cells were embedded at a density of 2×10^5 cells/dish into collagen scaffold (Cellmatrix; Nitta Gelatin Inc. Osaka, Japan) and cultured under normoxia or hypoxia (5% O_2) by using multigas incubator (BIO-LABO, Tokyo, Japan) at 37°C. Rat chondrocytes were isolated from the epiphyseal cartilage of 5-day-old Wistar rats as described previously[10]. The isolated chondrocytes were seeded at a density of 1×10^4 cells/cm^2 and cultured at 37°C under 5% CO_2 in αMEM containing 10% FBS. The Animal Committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences approved all of the procedures.

Cell proliferation assay

HCS-2/8 cells were inoculated into a 96-well multi-plate at a density of 3×10^4 cells/well; and the next day the medium was replaced with serum-free medium containing NaF at various concentrations (1, 5 and 10 mM). Then the cells were cultured for 16 h. The effect of NaF on cell proliferation was determined by performing a cell proliferation enzyme-linked immunosorbent assay (ELISA), 5-bromo-2′-deoxyuridine (BrdU) colorimetric (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol[15].

Detection of intracellular ATP

For measurement of intracellular ATP, an ATP bioluminescence assay kit was obtained and used according to manufacturer's (Roche Applied Science) recommendations. Briefly, after HCS-2/8 cells had been treated with NaF in the absence or presence of sodium lactate for 16 h, they were harvested. The cells were then boiled for 2 min for the preparation of cell lysates, and 50 μl of samples/standards was transferred to a white plate. Then, the luciferase reagent was added, and the emitted light was measured with a luminometer (Fluoroskan Ascent FL; Thermo Labsystems, Franklin, MA).

Detection of cytotoxicity

For detection and quantification of cell death by NaF, we performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of rat chondrocytes and measurement of the lactate dehydrogenase (LDH) activity in conditioned media of HCS-2/8 cells by using an *in situ* cell death detection kit and cytotoxicity detection kit (Roche Applied Science), respectively.

Luciferase assay

The firefly luciferase reporter construct containing the Smad binding element (SBE) and hypoxia response element (HRE) at -1006/-954 of VEGF promoter[16] and herpes simplex virus thymidine kinase promoter-*Renilla* luciferase reporter plasmid (pRL-TK, internal control: Promega, Madison, WI) were used. HCS-2/8 cells were transfected with 0.9 μg of reporter plasmid in combination with 0.1 μg of pRL-TK using Fugene6 reagent (Roche, Basel, Switzerland). The dual luciferase system (Promega) was used as described previously[16].

Western blot analysis

HCS-2/8 or RCS cells were treated with NaF, sodium lactate or a combination of lactate and NaF. After 18 h, the cell lysates were prepared, and the nuclear and cytoplasmic proteins were separated by using a CelLytic NuCLEAR extraction kit (Sigma) according to the manufacturer's protocol. Western blot analysis was performed as described previously[17].

Quantification of sulfated glycosaminoglycan (GAG) contents

Rat epiphyseal chondrocytes were grown to confluence in 96-well multi-plates containing αMEM supplemented with 10% FBS. Thereafter, the cells were treated with NaF at several concentrations for 4 days. A microassay for sulfated GAG's was performed as described previously[18].

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from HCS-2/8 cells by using ISOGEN reagent (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized with a Takara RNA PCR kit (AMV) Version 3.0 (Takara Shuzo, Tokyo, Japan), and amplification reactions were performed with a SYBR® Green Real-time PCR Master Mix (Toyobo, Tokyo, Japan)[16]. The nucleotide sequences of the primers and expected size of the amplicons are shown in Table I.

Indirect immunofluorescence analysis

RCS cells were cultured on chamber slides (Nunc Inc., Naperville, IL) for 2 days. Then, the cells were treated with NaF combined with lactate. After 22 h, the cultures were washed with phosphate-buffered saline (PBS), fixed with 3.5% paraformaldehyde for 1 h at room temperature, and made permeable with 0.1% NP-40 in PBS. Indirect immunofluorescence analysis was then performed as described previously[19].

Statistical analysis

Statistical analyses were performed by using a software (StatView, version 5.0, SAS Institute Inc., Cary, NC). To assess normality for the observed differences, Kolmogorov–Smirnov test was used and showed no strong departure from normality. Therefore, the statistical analysis was performed using the two-tailed unpaired Student *t* test. Results are presented as mean values with 95% confidence intervals. In the dot plots, exact *P*-values are presented for all data sets with significant differences.

Results

Sugar metabolism impaired by NaF inhibits the proliferation and differentiation and promotes cell death of chondrocytes

Because NaF and iodoacetate are respective inhibitors of enolase and GAPDH, which are critical enzymes of glycolytic pathways, these factors specifically block glycolytic metabolism. We firstly examined whether ATP production in chondrocytic HCS-2/8 cells would be affected or not by NaF or iodoacetate. As shown in Fig. 1(A and B), the content of intracellular ATP in the cells was significantly decreased by NaF and iodoacetate at all doses tested. These results indicate that the decreased intracellular ATP production was due to NaF or iodoacetate, thus suggesting that glycolysis was the principal pathway to supply ATP in the chondrocytes. Next, to investigate the effect of decreased ATP content on chondrocyte proliferation and differentiation, we performed a cell proliferation assay and Western blot analysis of lysates of NaF-treated HCS-2/8 cells by using anti-PCNA antibody. As shown in Fig. 2(A) (upper panel), the signal of PCNA, which is a marker of cell proliferation, was decreased by the treatment with NaF. Similarly, treatment with 5 or 10 mM NaF significantly decreased the incorporation of BrdU into the cells [Fig. 2(A), lower panel]. Subsequently, in order to test the effect of NaF on the differentiation of chondrocytes, we comparatively analyzed sulfated proteoglycan, which is a typical marker of differentiated chondrocytes, accumulated by rat chondrocytes treated with various concentrations of NaF. As a result, the accumulation of sulfated GAG was significantly inhibited by NaF in a dose-dependent manner [Fig. 2(B)]. These findings suggest that both chondrocyte proliferation and differentiation were impaired by decreased intracellular ATP production caused by addition of NaF. Finally, we tested whether treatment with NaF-induced cell death, or not, by TUNEL staining and the measurement of LDH activity released from damaged cells into cultured medium. As shown in Fig. 2(C and D), although 1 mM NaF had no effect, 5 mM NaF remarkably increased TUNEL positive cells and LDH activity in the supernatant. Taken together, these findings show that NaF-elicited blockade of ATP production, a major energy source for chondrocytes, inhibited both cell proliferation and differentiation, while promoting cell death.

No rescue effect of lactate on the ATP production impaired by NaF

We suspected that the impaired chondrocyte proliferation and differentiation by treatment with NaF were due to the reduced ATP

Table I
Sense (S) and antisense (AS) primers used for PCR

Gene	Accession no.	Primer sequence	Expected size (bp)
COL10a1	NM_000493.3	(F) 5′-GAATGCCTGTGTCTGCTT-3′ (R) 5′-TCATAATGCTGTTGCCTGTT-3′	105
MMP13	NM_002427.3	(F) 5′-TGGTGGTGATGAAGATGATTTGTCT-3′ (R) 5′-AGTTACATCGGACCAAACTTTGAAG-3′	375
ALP	NM_001127501.2	(F) 5′-GCACCGCCACCGCCTACC-3′ (R) 5′-CCACAGATTRCCCAGCGTCCTTG-3′	149
VEGF	NM_001171630.1	(F) 5′-CTTGCCTTGCTGCTCTAC-3′ (R) 5′-ACCACTTCGTGATGATTCTG-3′	88
ACAN	NM_013227.3	(F) 5′-GGAGCAGGAGTTTGTCAACA-3′ (R) 5′-CTTCTCGTGCCAGATCATCA-3′	186
β-actin	NM_001101.3	(F) 5′-GATCATTGCTCCTCCTGAGC-3′ (R) 5′-ACTCCTGCTTGCTGATCCAC-3′	100
MCT1	NM_003051.3	(F) 5′-CACCGTACAGCAACTATACG-3′ (R) 5′-CAATGGTCGCCTCTTGTAGA-3′	114
MCT2	NM_004731.3	(F) 5′-CCAAAGCTGTCACCGTATTCTTC-3′ (R) 5′-CTGCCGTATTTATTCACCAAAACA-3′	144

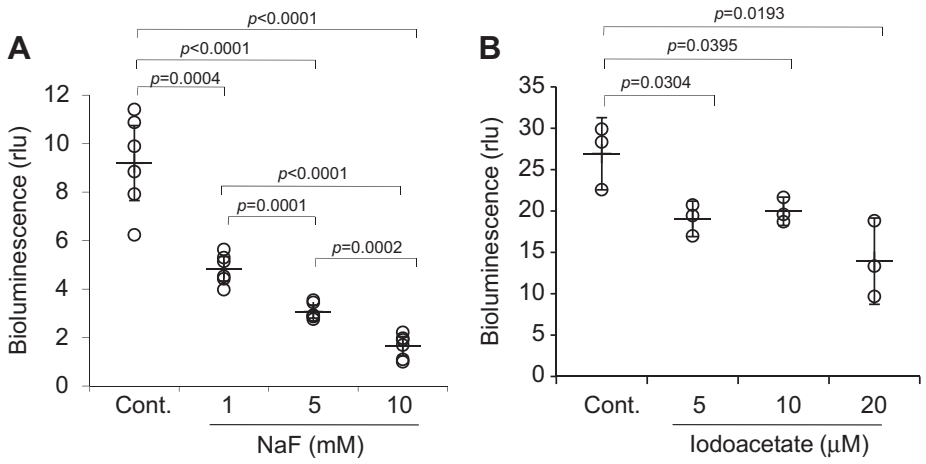

Fig. 1. Metabolic activity of HCS-2/8 cells treated with NaF or iodoacetate as measured by ATP bioluminescence assay. (A) HCS-2/8 cells were cultured until they had reached confluence. Then, they were treated with NaF (1, 5, or 10 mM). Sixteen hours later, cell lysates were prepared and the ATP level was measured with an ATP bioluminescence assay kit. The ordinate shows relative light units (rlu) of luminescence. Each dot represents the value determined for each of the individual samples ($n = 6$), and bars represent means and 95% confidence intervals. Exact *P*-values are indicated between compared groups. (B) HCS-2/8 cells were treated with iodoacetate at 5, 10, or 20 μM for 16 h. The ordinate shows rlu of luminescence. Data show the value of three independent samples, and bars represent means and the 95% confidence intervals. Exact *P*-values are indicated on the graphs.

production. If decreased intracellular ATP production could be rescued by the addition of lactate, which is the end product of the glycolytic pathway, it would indicate that aerobic metabolism after glycolysis could be a major source of ATP. To address this issue, we measured the intracellular ATP content in HCS-2/8 cells treated with NaF or iodoacetate in the presence of sodium lactate. However, there was no recovery of the decreased intracellular ATP production when lactate was present [Fig. 3(A and B)]. Then, we speculated that because MCTs, which mediate lactate transport, might not be expressed in HCS-2/8 cells, intracellular ATP production might not be supported by the addition of lactate. To examine this possibility, we analyzed the gene expression of MCT1 and 2 by performing real-time RT-PCR analysis. As a result, the expression of MCT1 was detected in the untreated HCS-2/8 cells and was decreased in the cells treated with NaF or the NaF combined with lactate [Fig. 4(Aa)]. On the other hand, the gene expression of MCT2 was remarkably increased in HCS-2/8 cells treated with NaF or the NaF combined with lactate in the comparison with that in the untreated ones [Fig. 4(Ab)]. In addition, Western blot analysis revealed that MCT1 protein was decreased in pellet culture of RCS cells treated with NaF, but MCT2 protein was increased [Fig. 4(B)]. Furthermore, immunofluorescence analysis revealed that immunoreactivity for MCT2 was detected in the cytoplasm and on the cell-surface [Fig. 4(C)]. These findings indicate that MCT2 was increased at both mRNA and protein levels by treatment with NaF or NaF combined with lactate, thus suggesting that lactate could have been transported into the NaF-treated chondrocytes *via* MCT2.

Sodium lactate enhances the BMP signaling but not NF-κB signaling under the condition of lowered ATP production in HCS-2/8 cells

Recently, it was reported that MCT1 contributes to cell death through activation of NF-κB in mouse chondrocytic ATDC5 cells[20]. Therefore, to test whether MCT2 induced by NaF could also mediate cell death through the activation of NF-κB, we comparatively analyzed the translocation of NF-κB into the nucleus of HCS-2/8 cells treated with NaF in the presence of lactate. As shown in Fig. 5(A), when lactate was added to HCS-2/8 cells treated with NaF, the translocation of NF-κB into the nucleus showed no change. However, interestingly, more phospho-Smad1/5/8 was translocated to the nucleus of the cells treated with NaF in the presence of lactate than that in its absence [Fig. 5(A)]. As phospho-Smad1/5/8 is involved in BMP signaling[9], these findings suggest that lactate enhanced BMP signaling in HCS-2/8 cells in collaboration with NaF that induces MCT2. To assess this finding, HCS-2/8 cells were transfected with the luciferase reporter plasmid containing HRE and SBE located within VEGF promoter, and reporter gene assay was performed. As shown in Fig. 5(B), luciferase activity was more increased in HCS-2/8 cells treated with NaF in the presence of lactate than in its absence. On the other hand, luciferase activity in the untreated cells did not change with lactate [Fig. 5(B)]. Taken together, these findings suggest that BMP signaling was enhanced by lactate in collaboration with NaF-induced MCT2 that mediated lactate uptake.

Combination of NaF and lactate induces chondrocyte hypertrophy-like changes

Because it has been already reported that BMP signaling promotes chondrocyte hypertrophy[9], we next investigated whether the chondrocyte hypertrophy-like changes were provoked by treatment with the combination of NaF and lactate. As shown in Fig. 6(A), the gene expression of *COL10a1* (a), *ALP* (b), *MMP13* (c), and *VEGF* (d), all of which are markers of hypertrophic chondrocytes, was promoted by treatment with NaF or it in combination with lactate more strongly than by that with PBS or lactate alone. In particular, the gene expressions of *COL10a1* and *ALP* were stimulated to a greater extent by the combination with lactate than by NaF alone. In addition, the gene expression of *AGGRECAN* (*ACAN*, e), which is a marker of mature chondrocytes, was decreased in the cells treated with NaF or NaF combined with lactate compared with its expression in the presence of PBS or lactate alone. It has been generally believed that 3-D culture of chondrocytes under hypoxic condition is suitable for mimicking the cartilage environment. Therefore, RCS cells were embedded into collagen scaffold and were treated with NaF combined with lactate under hypoxia for 24 h. As shown in Fig. 6(B), the gene expression level of *COL10a1* was increased under hypoxia compared with its expression under normoxia. Furthermore, *COL10a1* expression was significantly increased by treatment with NaF combined with lactate under both normoxia and hypoxia. These results suggest that NaF combined with lactate promoted chondrocyte hypertrophy-like changes in both monolayer and 3-D collagen cultures and under both normoxia and hypoxia.

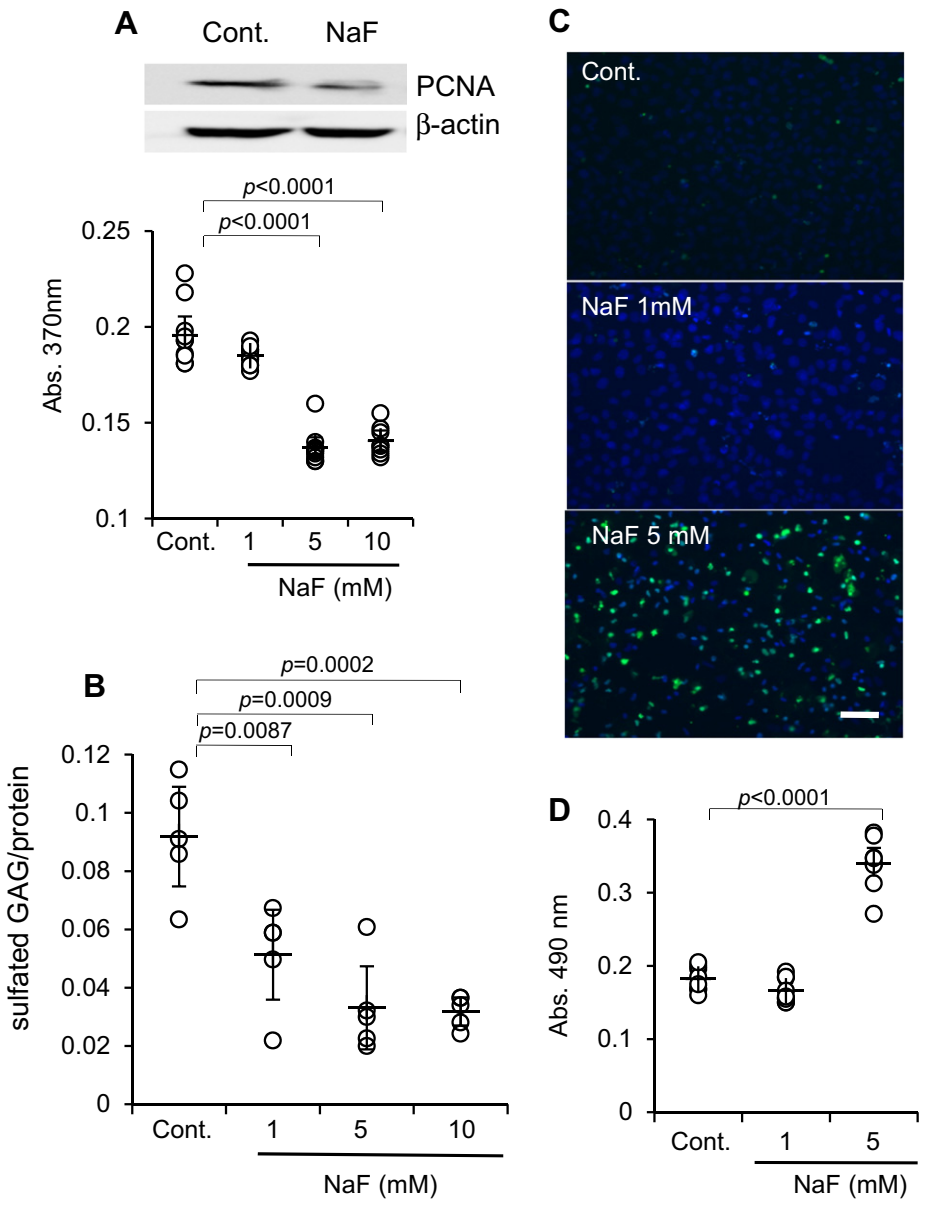

Fig. 2. Effect of NaF on the proliferation, differentiation, and cell death of cultured chondrocytes. (A) Effect of NaF on the proliferation of HCS-2/8 cells. (Upper) HCS-2/8 cells were cultured in 35-mm diameter dishes with DMEM containing 10% FBS. After the cells had reached confluence, they were treated with NaF at 5 mM for 16 h. Then, cell lysates were prepared and Western blot analysis was performed by using anti-PCNA and β-actin antibodies. (Lower) HCS-2/8 cells were inoculated into 96-well multi-plates, and the next day, the medium was replaced with serum-free DMEM containing NaF at 1 ($n = 10$), 5 ($n = 10$) or 10 ($n = 8$) mM. The negative control was added with PBS ($n = 10$). After 16 h, BrdU was added; and the cells were then incubated for an additional 3 h. Measurement of BrdU incorporation was determined as immunoreactivity with anti-BrdU antibody. Each dot shows the value from an independent sample and bars represent mean values and the 95% confidence intervals. Exact *P*-values between the indicated groups, are given on the graph. (B) Effect of NaF on the differentiation of rat chondrocytes. Sulfated GAG microassay using rat epiphyseal chondrocytes was performed as described in Materials and methods. Each dot shows the value from an independent culture ($n = 5$) and bars represent means and the 95% confidence intervals. Exact *P*-values are indicated. (C) Fluorescent TUNEL staining in rat epiphyseal chondrocytes treated with NaF. Rat chondrocytes were cultured in a 24-well multi-plate until they had reached confluence. Then the cells were treated with NaF at 1 or 5 mM. After 16 h, the cells were fixed; and TUNEL staining was then performed. Bar represents 250 μm. (D) Measurement of LDH activity in the conditioned media of HCS-2/8 cells treated with NaF. After the cells were treated with NaF at 1 ($n = 9$) or 5 ($n = 9$) mM for 16 h, cell death was determined by LDH release into the conditioned media. The negative control was added with PBS ($n = 8$). Dots show the values from independent cultures and bars represent means and the 95% confidence intervals. Exact *P*-values are indicated.

Next, to confirm whether NaF combined with lactate also stimulated the production of type X collagen in HCS-2/8 cells, we performed Western blot analysis. As shown in Fig. 7(A), the production of type X collagen was increased by NaF combined with lactate. On the other hand, as it is already known that SOX9 is decreased in hypertrophic chondrocytes[21], we also investigated whether production of SOX9 was decreased by treatment with NaF combined with lactate. As expected, SOX9 production was decreased by NaF, or NaF combined with lactate [Fig. 7(A)]. Additionally, to examine whether or not these results were due to decreased ATP production, we performed similar experiments using iodoacetate. As a result, similar results were obtained (data not shown). Furthermore, the active form of MMP9, which is another marker of hypertrophic chondrocytes, was detected in medium conditioned by HCS-2/8 cells treated with NaF or NaF combined with lactate [Fig. 7(B)]. Taken together, these findings suggest that ATP production deficiency by NaF and lactate accumulation induced the chondrocyte hypertrophy-like changes.

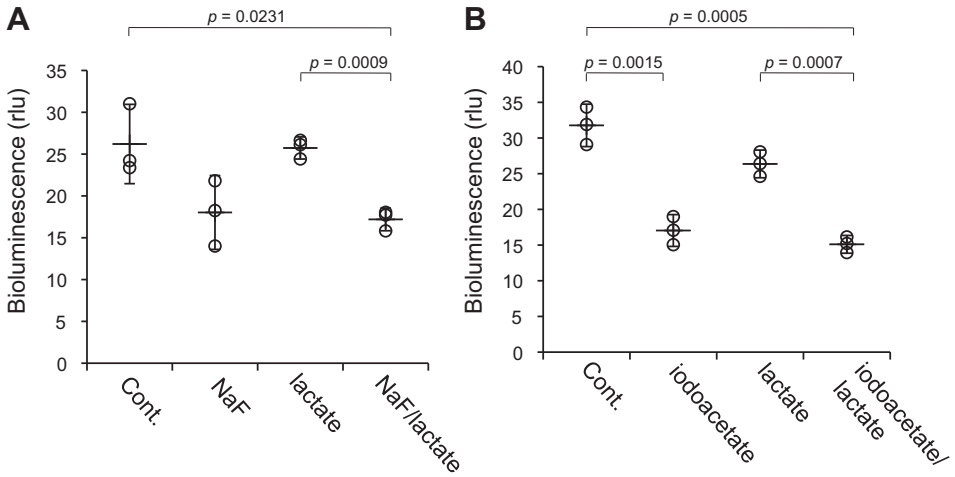

Fig. 3. No rescue by lactate of ATP production decreased due to NaF (A) or iodoacetate (B). HCS-2/8 cells were cultured until they had reached confluence. Then, they were treated with NaF (2 mM) or iodoacetate (10 μM) and/or lactate (1 mM). After 16 h of treatment with NaF, cell lysates were prepared; and the ATP level was subsequently measured with the ATP bioluminescence assay kit. The ordinate shows rlu of luminescence. Data shows all the values from independent samples of $n = 3$, and bars represent means with the 95% confidence intervals. Exact *P*-values are indicated.

Discussion

In this study, we clarified that treatment with NaF impaired chondrocyte proliferation and differentiation, and promoted cell death (Figs. 1 and 2). We also showed that gene expression of MMP13 and activation of MMP9 were induced by treatment with NaF (Figs. 6 and 7). These results suggest that the impaired chondrocyte proliferation and differentiation by NaF is due to the

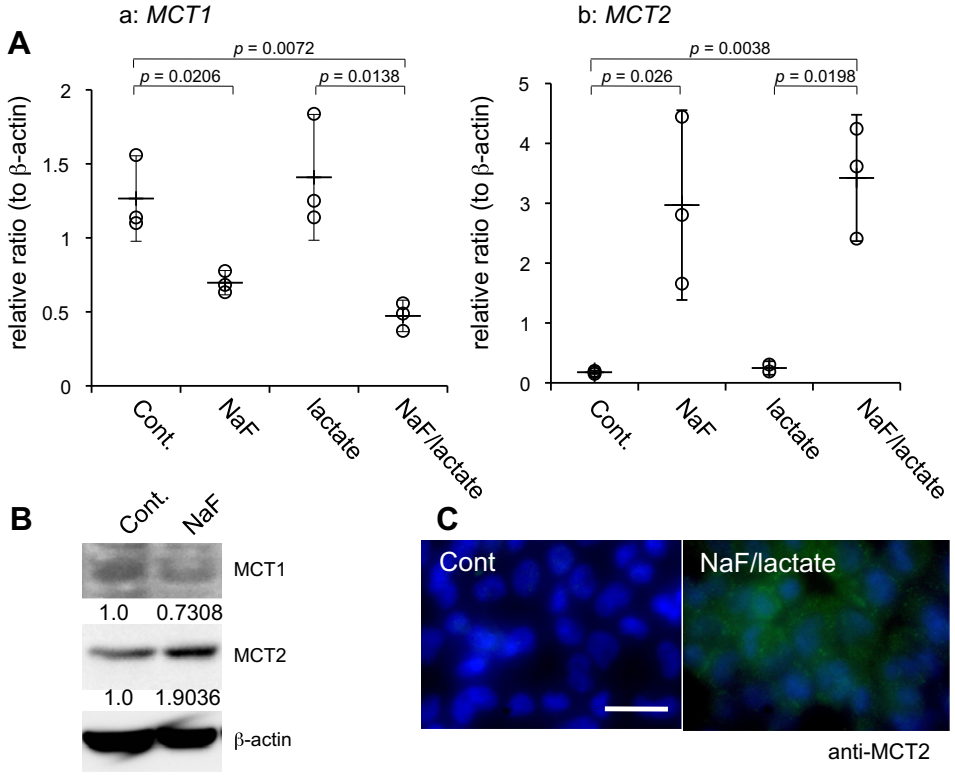

Fig. 4. Promotion of *MCT2* gene expression and production in cultured chondrocytes treated with NaF combined with lactate. (A) Real-time RT-PCR analysis of *MCT1* (a) and *2* (b) mRNAs in HCS-2/8 cells treated with NaF and/or lactate. HCS-2/8 cells were cultured in 35-mm diameter dishes until they had reached confluence, after which they were treated with NaF and lactate. The amounts of *MCT1* and *2* mRNA were normalized to the amount of *β-actin* mRNA. The ordinate shows the relative ratio. Data are presented as the mean values and 95% confidence intervals of three sets of independent cultures. (B) Western blot analysis of MCT1 and MCT2 production in RCS cells treated with 2 mM NaF in pellet culture. RCS cells centrifuged in conical tube were cultured with DMEM containing 10% FBS for 2 days. Thereafter, the cells were treated with NaF for 24 h. Cell lysates were subsequently prepared and Western blot analysis was performed by using anti-MCT1, anti-MCT2 and β-actin antibodies. The amount of MCT1 and 2 was determined densitometrically and these amounts were normalized to the amount of β-actin. Relative ratios (control = 1.0) from independent two cultures are presented. (C) Immunofluorescence analysis of MCT2 expression on/in RCS cells treated with NaF and lactate. RCS cells were cultured in an 8-well chamber slide until they had reached sub-confluence. Then, they were treated with 2 mM NaF combined with 1 mM lactate for 22 h. The cells were visualized under laser-scanning microscopy. MCT2 showed a cell-surface plus cytoplasmic distribution (green), and the nuclei were stained with DAPI. Bar represents 100 μm.

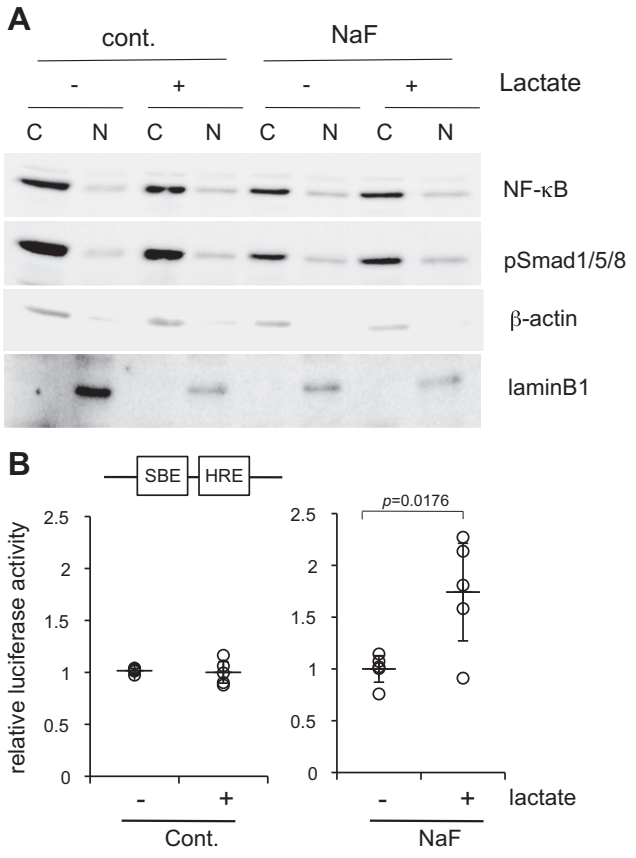

Fig. 5. Promotion of phospho-Smad1/5/8 transport into the nucleus of HCS-2/8 cells by the addition of lactate. (A) HCS-2/8 cells were cultured in 35-mm diameter dishes with DMEM containing 10% FBS until they had become confluent. Then, the medium was replaced with serum-free DMEM containing 2 mM NaF. After 18 h, the cells were incubated for 30 min with lactate at 1 mM; and the nuclear (N) and cytoplasmic (C) proteins were then prepared. Successful fractionation was confirmed by Western blotting of the fraction markers of the nucleus (lamin B1) and cytoplasm (β-actin). Western blotting was performed by using anti-p65 antibody (NF-κB) and anti-phospho-Smad1/5/8 antibody. (B) HCS-2/8 cells were transiently transfected with the firefly luciferase reporter plasmid containing SBE and HRE at -1006/-954 of VEGF promoter together with pRL-TK plasmid (internal control). Next day, the cells were treated with NaF (2 mM) and lactate (1 mM). After 16 h, the cells were treated with 1 mM lactate for 30 min, and the cell lysate was collected. Then, luciferase assay was performed. The data show the means and the 95% confidence intervals of relative values of the measured luminescence of firefly vs *Renilla* luciferase from independent cultures ($n = 5$). Exact *P*-values are indicated where significant differences were observed.

reduced ATP production. Although we expected that ATP production was rescued by the addition of lactate that could be catabolized through pyruvate for aerobic ATP production, it was not rescued against our expectations. This outcome represents anaerobic glycolysis as the major metabolic pathway to supply ATP in chondrocytes. More interestingly, during this degenerative process, lactate stimulated translocation of phospho-Smad1/5/8 into the nucleus (Fig. 5). As a result, the gene expression and protein production level of *COL10a1* were increased, and the production of SOX9, which was decreased in hypertrophic chondrocytes, was decreased (Figs. 6 and 7). These results suggest that decreased ATP production by treatment with NaF promoted chondrocyte hypertrophy-like changes in the presence of lactate.

NaF has been commonly used as an additive to drinking water for the prevention of dental caries. Previously, it was reported that the thickness of the temporomandibular joint cartilage layer in rats is increased when the animals are given 0.1% NaF in their drinking water, an increase attributable to a histologically demonstrated increase in the thickness of the hypertrophic zone[22]. The referenced authors suggested that enlargement of the hypertrophic zone was due to the accumulation of glycogen by inhibition of glycolytic pathways in chondrocytes and to the delayed capillary invasion into cartilage ECM[22]. Based on the above discussion and the results of our experiments, the hypertrophic zone enlarged by fluoride exposure may be attributable to an increase in the number of hypertrophic cells promoted by decreased ATP production, inefficient removal of terminally differentiated hypertrophic chondrocytes caused by delayed vascular invasion, and an increase in cell size caused by glycogen accumulation in the chondrocytes.

Chondrocytes utilize predominantly glycolytic metabolism to produce optimal ATP due to the surrounding avascular microenvironment, yielding lactate as the end product[23,24]. Lactate is released into the surrounding matrix to maintain normal intracellular pH *via* MCTs[23,24]. MCTs are a family of integral membrane proteins that mediate proton-coupled co-transport of monocarboxylic acids across the cell membrane, and nine MCT-related homologs have been identified in mammals thus far[25]. Among them, direct transportation of lactate and pyruvate transport have been demonstrated for mammalian MCT1–MCT4[25]. In chondrocytes, mRNA expression of MCT1, 2, and 4 isoforms, but not the MCT3 isoform, is detectable; and MCT2 expression is the lowest among these isoforms[26]. In this study, we confirmed that MCT2 expression was lower than MCT1 expression in HCS-2/8 cells under normal culture conditions [Fig. 4(A)]. However, treatment with NaF revealed that MCT2 expression was dramatically up-regulated [Fig. 4(A)]. Furthermore, it was previously reported that MCT2 has a tenfold higher affinity for cargo than MCT1 or MCT4[25]. One may expect that pyruvate converted from lactate transported into chondrocytes *via* MCT2 may increase intracellular ATP *via* aerobic catabolism. However, consistent with the fact that chondrocytes predominantly use glycolysis to obtain ATP, lactate had no effect on the production of ATP in HCS-2/8 cells treated with NaF or iodoacetate (Fig. 3). Thus, we hypothesized that lactate might be an activator of cell signaling pathways rather than a pyruvate precursor. A previous study indicates that lactate activates toll-like receptor 4 (TLR4) signaling and NF-κB pathways in macrophages through up-regulation of MCTs[27]. In addition, it was recently revealed that stimulation by lactate leads to activation of signal transducers and activators of transcription 3 (STAT3) and extracellular signal-regulated kinase (ERK)1/2 signaling pathways in human mesenchymal stem cells[28]. In view of these findings, we investigated the effect of lactate on the transport of NF-κB into the nucleus in HCS-2/8 cells treated with NaF but found no promotion of NF-κB transport [Fig. 5(A)]. However, surprisingly, the transport of phospho-Smad1/5/8 into the nucleus was increased by lactate stimulation [Fig. 5(A)]. It is well known that activation of BMP signaling promotes proliferation and differentiation of chondrocytes and furthermore promotes chondrocyte hypertrophy[9]. Our earlier immunohistochemical analysis revealed that BMP-2 is localized in the perichondrium and pre-hypertrophic zone in the growth plate[29]. Furthermore, phosphorylated forms of Smad1/5/8 are immunohistochemically detectable in the pre-hypertrophic zone of the growth plate but not in the perichondrium[30]. These findings suggest that BMP signaling pathways, engaged by phospho-Smad1/5/8, would be activated in the pre-hypertrophic zone. Taken together, our and the referenced data indicate that both initiation of BMP signaling *via* BMP-2 and promotion of phospho-Smad1/5/8 transport into the nucleus by lactate would work cooperatively. As a result of this cooperation between a metabolite and growth factor, pre-hypertrophic chondrocytes may differentiate into hypertrophic chondrocytes efficiently.

Articular cartilage is an avascular connective tissue and has an important role in resistance against mechanical stimulation of

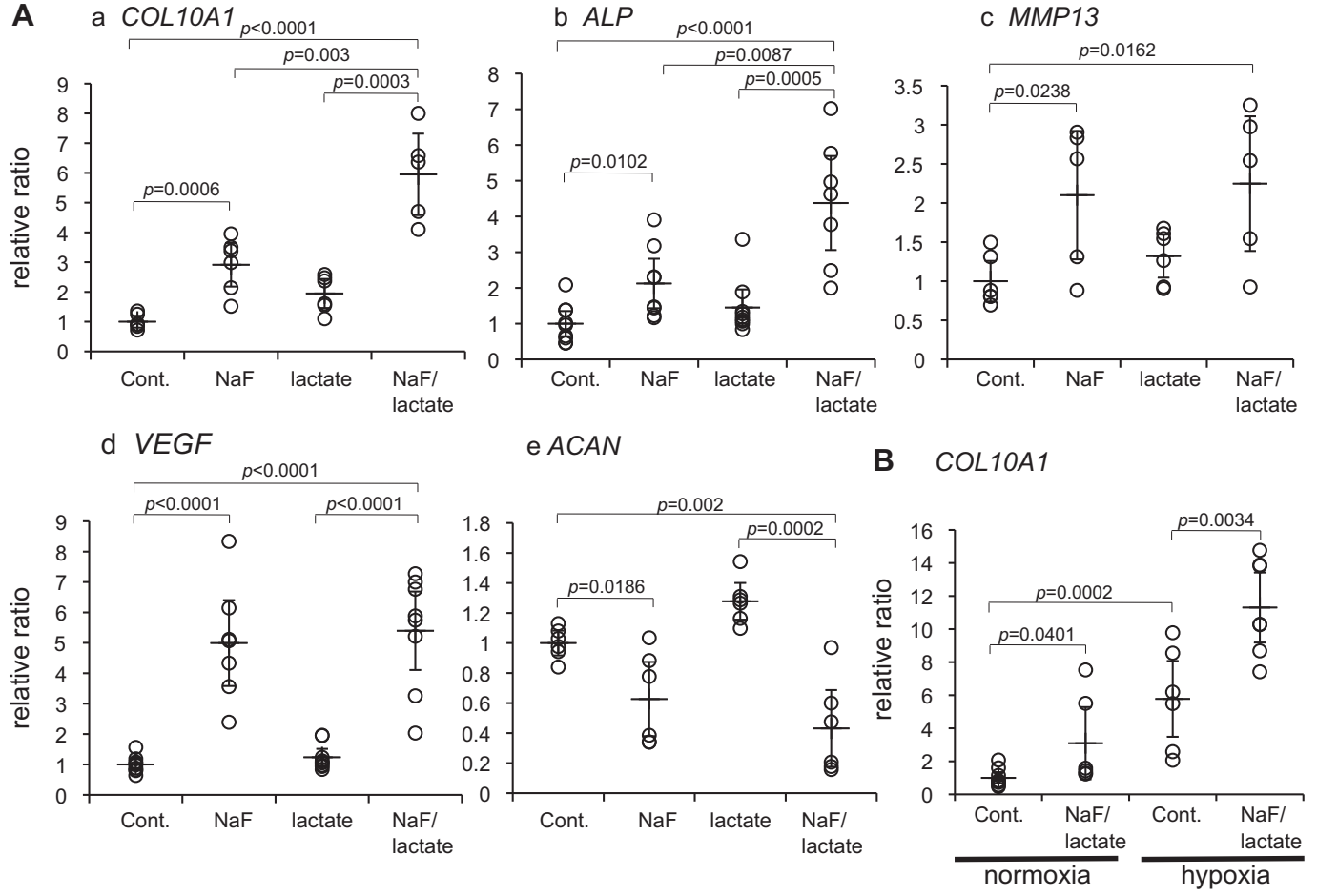

Fig. 6. Real-time RT-PCR analysis of chondrocyte marker gene expression in cultured chondrocytes treated with NaF and/or lactate. (A) HCS-2/8 cells were grown in DMEM containing 10% FBS until they had become confluent. Then, the cells were treated with NaF (2 mM), lactate (1 mM) or NaF combined with lactate; and total RNA was prepared 16 h later. Real-time RT-PCR analysis was performed by using *COL10a1*-, *ALP*-, *MMP13*-, *VEGF*-, *ACAN*- and *β-actin*-specific primers. The amount of expression of each chondrocyte marker genes was normalized to the amount of *β-actin*. (a) Graph shows the expression level of *COL10a1* in HCS-2/8 cells treated with PBS ($n = 6$), NaF ($n = 6$), lactate ($n = 6$), or NaF combined with lactate ($n = 5$). (b) Graph shows the expression level of *ALP* in HCS-2/8 cells treated with PBS ($n = 9$), NaF ($n = 8$), lactate ($n = 9$), or NaF combined with lactate ($n = 7$). (c) Graph shows the expression level of *MMP13* in HCS-2/8 cells treated with PBS ($n = 6$), NaF ($n = 5$), lactate ($n = 6$), or NaF combined with lactate ($n = 5$). (d) Graph shows the expression level of *VEGF* in HCS-2/8 cells treated with PBS ($n = 9$), NaF ($n = 7$), lactate ($n = 9$), or NaF combined with lactate ($n = 8$). (e) Graph shows the expression level of *ACAN* in HCS-2/8 cells treated with PBS ($n = 6$), NaF ($n = 6$), lactate ($n = 6$), or NaF combined with lactate ($n = 6$). In all the graph, the ordinate indicates the relative ratio with respect to control sample (ratio = 1.0), and bars represent means and the 95% confidence intervals. Exact *P*-values are shown between two data sets with significant differences. (B) RCS cells embedded in collagen gel were treated with NaF combined with lactate under the normoxic or hypoxic conditions, and total RNA was prepared 24 h later. Real-time RT-PCR analysis was performed by using *COL10a1* and *β-actin*-specific primers. The amount of expression of *COL10a1* genes was normalized to the amount of *β-actin*. The graph shows the expression of *COL10a1* after the treatment with PBS ($n = 9$) or NaF/lactate ($n = 6$) under normoxia, and that after the treatment with PBS ($n = 7$) or NaF/lactate ($n = 7$) under hypoxia. Bars represent mean values with 95% confidence intervals. The ordinate indicates the relative ratio with respect to control sample under normoxia (ratio = 1.0). Exact *P*-values are shown between compared groups.

joints[31]. Although chondrocytes are the only cells found within articular cartilage, they vary in size and morphology, depending on the zone of articular cartilage above the tidemark, which is divided into three distinct ones, i.e., the superficial, middle, and deep zones[31]. Corresponding to these morphological differences, chondrocyte metabolism reportedly differs among these zones[32]. The zonal difference in chondrocyte metabolism may be important in the differential expression of hypertrophic chondrocyte-related genes. In particular, it was reported that the expression level of *COL10a1* in the deep zone of OA cartilage is greater than that in another zone[32]. In fact, we have preliminary data showing that immunoreactivity of MCT2 was detectable in the deep zone of normal articular cartilage (data not shown). Of note, chondrocytes are recognized to produce a high level of lactate in the internal zones of the avascular articular cartilage[23]. Under such conditions, if metabolic activity in this deep zone of articular cartilage is attenuated by aging or excess mechanical stress, chondrocytes may undergo hypertrophy-like changes leading to the onset and progression of OA. Our preliminary immunohistochemical analysis revealed that the immunoreactivity of GAPDH in the articular cartilage of 18-month-old mice was less than that of the enzyme in 2-month-old mice (data not shown). In addition, we observed that intracellular ATP production was decreased when excess mechanical stress was loaded onto 3-D culture of HCS-2/8 cells (data not shown). These findings indicate that metabolic activity is decreased by aging or excess mechanical stress, which would induce decreased glycolytic metabolic activity, thus accelerating hypertrophy of articular chondrocytes and leading to OA. In addition, it is of particular interest and thus worth investigating whether similar events may occur in the synovial tissue as well.

In conclusion, our study clarified that decreased ATP production in the presence of lactate plays an important role in alteration of chondrocytes to the hypertrophy-like state. Further investigation is needed to verify our hypothesis that the chondrocyte hypertrophy-

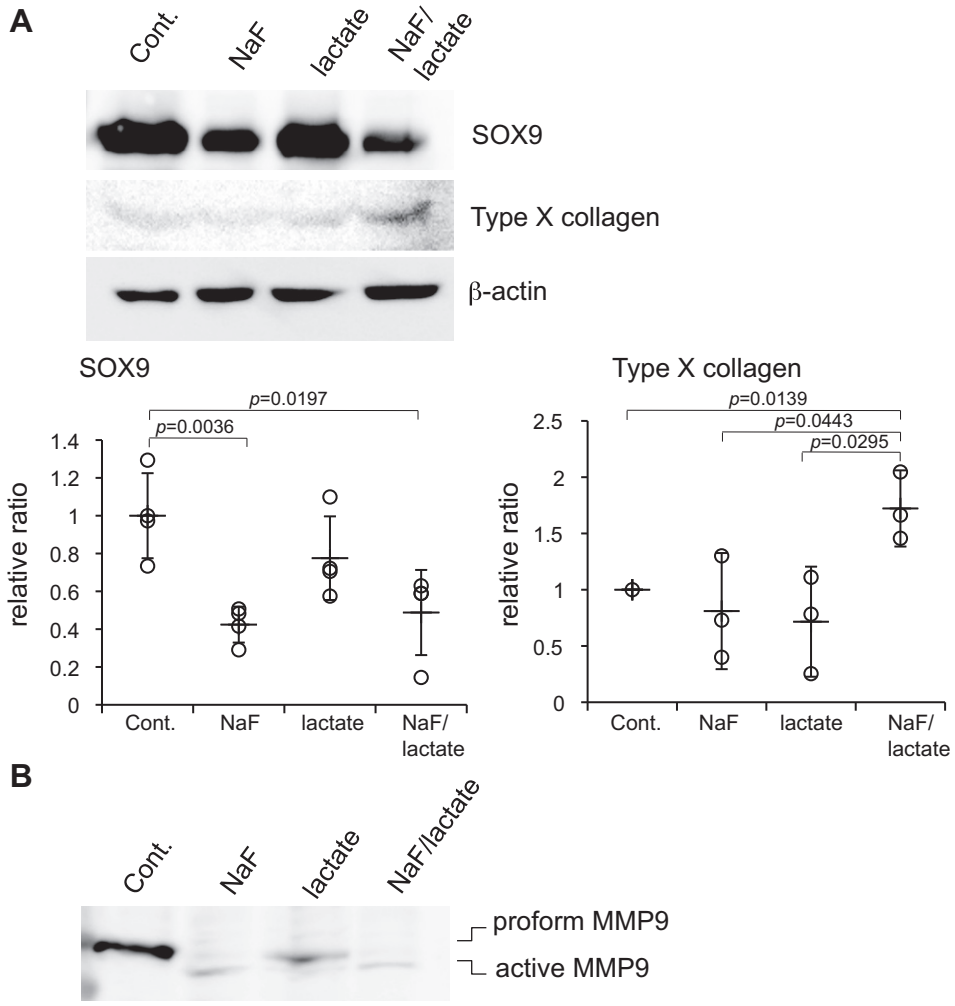

Fig. 7. Western blot analysis of production of SOX9 and type X collagen, and activation of MMP9, in HCS-2/8 cells treated with NaF with or without lactate. (A) HCS-2/8 cells were grown in DMEM containing 10% FBS until they had become confluent. Then, the medium was replaced with serum-free DMEM, and the cells were subsequently treated with NaF (2 mM), lactate (1 mM) or NaF combined with lactate. After 18 h, cell lysates were prepared; and Western blotting was then performed by using anti-SOX9, anti-type X collagen, and anti-β-actin antibodies. The amount of type X collagen and SOX9 was determined densitometrically and normalized to β-actin. Bars presented for type X collagen ($n = 3$) and SOX9 ($n = 4$) are mean values with 95% confidence intervals. Exact *P*-values are shown between compared data sets. (B) After HCS-2/8 cells had reached confluence, the medium was replaced with serum-free medium containing NaF (2 mM), lactate (1 mM) or NaF combined with lactate. Conditioned media were collected 18 h later and concentrated with gelatin Sepharose. Western blot analysis was performed by using anti-MMP9 antibody. The active form of MMP9 was detected in the medium conditioned by cells treated with NaF or NaF combined with lactate but not when the cells had been treated with PBS (cont.) or lactate.

like changes were due to decreased glycolysis and lactate accumulation/uptake.

Authors' contributions

TN performed measurement of intracellular ATP productions, Western blotting, cell proliferation assay, sulfated GAG microassay, immunofluorescence, and real-time PCR experiments using chondrocyte cultures; participated in the study design; and helped to draft the manuscript.

SK designed and coordinated the study and helped to draft the manuscript.

EA isolated chondrocytes and set up primary cultures of them.

MT participated in the study design and helped to draft the manuscript.

Conflict of interest

None to declare.

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