



Changes in acetyl-CoA mediate Sik3-induced maturation of chondrocytes in endochondral bone formation

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ABSTRACT

The maturation of chondrocytes is strictly regulated for proper endochondral bone formation. Although recent studies have revealed that intracellular metabolic processes regulate the proliferation and differentiation of cells, little is known about how changes in metabolite levels regulate chondrocyte maturation. To identify the metabolites which regulate chondrocyte maturation, we performed a metabolome analysis on chondrocytes of Sik3 knockout mice, in which chondrocyte maturation is delayed. Among the metabolites, acetyl-CoA was decreased in this model. Immunohistochemical analysis of the Sik3 knockout chondrocytes indicated that the expression levels of phospho-pyruvate dehydrogenase (phospho-Pdh), an inactivated form of Pdh, which is an enzyme that converts pyruvate to acetyl-CoA, and of Pdh kinase 4 (Pdk4), which phosphorylates Pdh, were increased. Inhibition of Pdh by treatment with CPI613 delayed chondrocyte maturation in metatarsal primordial cartilage in organ culture. These results collectively suggest that decreasing the acetyl-CoA level is a cause and not result of the delayed chondrocyte maturation. Sik3 appears to increase the acetyl-CoA level by decreasing the expression level of Pdk4. Blocking ATP synthesis in the TCA cycle by treatment with rotenone also delayed chondrocyte maturation in metatarsal primordial cartilage in organ culture, suggesting the possibility that depriving acetyl-CoA as a substrate for the TCA cycle is responsible for the delayed maturation. Our finding of acetyl-CoA as a regulator of chondrocyte maturation could contribute to understanding the regulatory mechanisms controlling endochondral bone formation by metabolites.

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1. Introduction

Most bones except for part of the cranial bones and part of the clavicle are formed during development through endochondral bone formation [1,2]. Endochondral bone formation starts with the condensation of undifferentiated mesenchymal cells, which subsequently differentiate into chondrocytes. The chondrocytes proliferate and produce cartilage extracellular matrix (ECM) to form primordial cartilage, at which point they stop proliferating and undergo hypertrophy at the center of the primordial cartilage. Cartilage ECM is composed of collagen fibrils and proteoglycan. Proliferating chondrocytes produce type II collagen, whereas hypertrophic chondrocytes produce type X collagen, and ECM around

the hypertrophic chondrocytes gradually calcifies. The course of these temporal alterations in the chondrocyte maturation process is translated into spatial changes in primordial cartilage: a zone of proliferating chondrocytes, a zone of hypertrophic chondrocytes and a zone of calcified hypertrophic chondrocytes are stacked from the end to the center of the primordial cartilage. The zone of calcified hypertrophic chondrocytes is finally degraded and replaced by bone. Thus, strict regulation of the chondrocyte maturation process is important for proper bone formation and growth. A network of signaling molecules including salt inducible kinase 3 (Sik3) is responsible for the regulation [3]. Sik3 is one of three isoforms, each of which regulates distinct tissues and organs. Sik3 induces chondrocyte maturation by converting proliferative chondrocytes into hypertrophic chondrocytes. This function was demonstrated by the cartilage phenotype of Sik3 knockout (KO) mice, in which the width of the zone of proliferative chondrocytes is longer than that in wild-type mice [4]. While this cartilage phenotype is attributed primarily to the lack of Sik3 in

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Abbreviations

cKO mice	conditional knockout mice
Col11a2	type XI collagen $\alpha 2$ chain gene
d.p.c.	days postcoitum
Hdac4	histone deacetylase 4
KO mice	knockout mice
Pdh	pyruvate dehydrogenase
Pdk4	pyruvate dehydrogenase kinase 4
Sik3	Salt inducible kinase 3
Tfam	mitochondrial transcription factor A

chondrocytes, systemic effects expressed as abnormalities in other organs or tissues could also affect the cartilage homeostasis.

Recent studies have revealed that intracellular metabolic processes regulate the proliferation and differentiation of cells. Indeed, metabolites are involved in regulating gene expressions, signal transductions, and various protein activities in cellular decision-making processes [5,6]. Growth cartilage is avascular and hypoxic [7]. This condition should make the metabolism of cartilage unique compared with other tissues, but little is known about how metabolites regulate the differentiation of chondrocytes. In the present study, we sought metabolites that regulate chondrocyte maturation during endochondral bone formation using *Sik3*-deficient chondrocytes and found the amount of acetyl-CoA was decreased. We then examined possible roles of acetyl-CoA in the regulation of chondrocyte maturation.

2. Materials and methods

2.1. *Sik3* knockout and conditional knockout mice

Sik3 KO mice were reported previously [4]. To prepare *Sik3* conditional knockout (cKO) mice which lack *Sik3* in a chondrocyte-specific manner, we crossed *Sik3^{flox/flox}* mice [8] with *11Enh-Cre* mice [9,10]. *11Enh-Cre* mice express *Cre* under the control of the promoter and enhancer sequences of type XI collagen $\alpha 2$ chain gene (*Col11a2*). For genotyping, genomic DNA was isolated from tail tips and subjected to PCR analysis according to methods previously described for the *Cre* transgene [10] and *Sik3* allele [4,8]. We designated the *11Enh-Cre; Sik3^{flox/flox}* mice as *Sik3* cKO mice and *11Enh-Cre; Sik3^{flox/+}* mice as control mice. Mice used in this study except for Fig. 1A were younger than 0-days old. The sex of the animals younger than 0-days old was not determined, because sex does not appear to affect the results at these ages. All experiments were approved by our institutional animal committee and institutional biosafety committee.

2.2. Metabolome analysis

Eleven *Sik3* cKO mice and 12 control mice were used. The epiphyseal cartilage of the proximal and distal humerus, proximal and distal femur and proximal tibia were harvested from the mice at birth. Samples from 3, 4 and 4 cKO mice were respectively dedicated to 1 batch ($n = 3$ batches). Samples from 4 control mice were respectively dedicated to 3 batches ($n = 3$ batches). Each batch consisted of 41–47 mg of frozen cartilage and was plunged into 750 μ L of 50% acetonitrile/Milli-Q water containing internal standards (H3304-1002, Human Metabolome Technologies, Inc., Tsuruoka, Japan) at 0 °C in order to inactivate enzymes. The tissue was homogenized ten times at 3,500 rpm for 60 s using a tissue homogenizer (Micro Smash MS100R, Tomy Digital Biology Co., Ltd.,

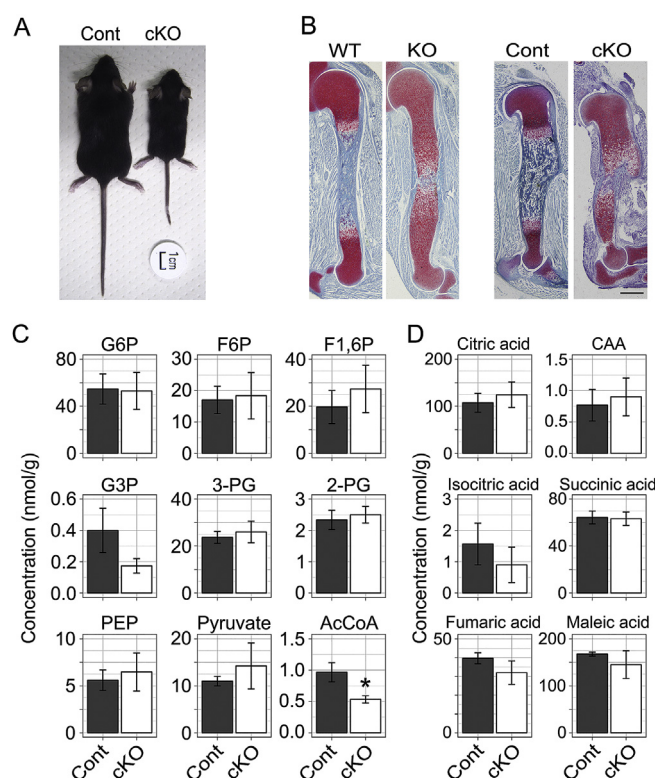


Fig. 1. Phenotype of chondrocyte-specific *Sik3* cKO mice and metabolome analysis. (A) Appearance of female *Sik3* conditional knockout mice at 4 weeks old. Bar, 1 cm. (B) Histological frozen sections of humeri from *Sik3* KO at around 18.5 day postcoitum (d.p.c.) and *Sik3* cKO mice at around 18.0 d.p.c. Safranin O-fast green-iron hematoxylin staining. Bar, 500 μ m. (C and D) Results of the metabolome analysis of epiphyseal cartilage from the humerus, femur and tibia of *Sik3* cKO mice and control mice at birth. Concentrations of metabolites in glycolysis (C) and the TCA cycle (D) are shown. Data are shown as means $\pm$ s.d. * $P = 0.016$ compared to control by Welch's *t*-test ($n = 3$). Cont, *11Enh-Cre; Sik3^{flox/flox}*; cKO, *11Enh-Cre; Sik3^{flox/flox}*; WT, *Sik3^{+/+}*; KO, *Sik3^{-/-}*; G6P, Glucose 6-phosphate; F6P, Fructose 6-phosphate; F1,6P, Fructose 1,6-diphosphate; G3P, Glyceraldehyde 3-phosphate; 3-PG, 3-Phosphoglyceric acid; 2-PG, 2-Phosphoglyceric acid; PEP, Phosphoenolpyruvic acid; AcCoA, Acetyl CoA; CAA, cis-Aconitic acid.

Tokyo, Japan), and then the homogenate was centrifuged at $2,300 \times g$ and 4 °C for 5 min. Subsequently, 400 μ L of upper aqueous layer was centrifugally filtered through a Millipore 5-kDa cutoff filter at $9,100 \times g$ and 4 °C for 120 min to remove the proteins. The filtrate was centrifugally concentrated and re-suspended in 50 μ L of Milli-Q water for CE-MS analysis. Metabolome measurements were carried out through a facility service at Human Metabolome Technologies, Inc.

2.3. Metatarsal organ culture

Second, third and fourth metatarsal primordial cartilage of the forelimb bud was cultured as described previously [11]. Metatarsal cartilage was dissected from mouse embryos at 14.5 days postcoitum (d.p.c.) and cultured in 500 μ L α -modified essential medium without nucleosides (Invitrogen) supplemented with 0.05 mg/mL ascorbic acid (Sigma-Aldrich), 0.3 mg/mL L-glutamine (Merck), 0.05 mg/mL gentamicin (Invitrogen), 0.25 mg/mL Fungizone (Invitrogen), 1 mM β -glycerophosphate (Merck), and 0.2% FBS (Gibco) in 24-well plates in a humidified atmosphere of 5% CO_2 in air at 37 °C (day 0). We collected six metatarsals from both forepaws of each mouse embryo. One metatarsal was cultured in each well. The number of metatarsals used in each experiment is indicated in the figures or figure legends.

2.4. Preparation of reagents

CPI613 (Tocris, Minneapolis) was dissolved in DMSO (Nakalai-tesque, Kyoto) at concentrations of 10 and 1 mM to prepare stock solutions. Rotenone (TCI, Tokyo) was dissolved in DMSO at concentrations of 1 μ M and 0.1 μ M to prepare stock solutions. The stock solutions were diluted with culture medium.

2.5. Histological analysis

For frozen sections, the samples were harvested, fixed with 4% paraformaldehyde, and embedded in SCHEM compound (SECTION-LAB). The sections were prepared at 8- μ m thickness with Cryofilm type 2c(9) (SECTION-LAB) using a CM3050S cryomicrotome (Leica) according to the method described by Kawamoto [12].

For paraffin-embedded sections, samples were dissected, fixed in 4% paraformaldehyde, processed and embedded in paraffin. Sections were prepared at 4.5- μ m thickness and subjected to safranin O-fast green-iron hematoxylin staining or von Kossa staining.

2.6. Immunofluorescent staining of histological sections

Paraffin-embedded sections were deparaffinized and incubated in 20 mM sodium citrate, 10 mM EDTA and 0.05% Tween-20 buffer (pH 6.2) at 98 °C for 30 min to retrieve the antigen. Then, sections were treated with 10 mg/mL bovine testis hyaluronidase (Sigma-Aldrich, St. Louis) in tris-buffered saline (pH 6.2) at 37 °C for 30 min. After blocking with Blocking One Histo (Nakalai-tesque, Kyoto), the sections were incubated with primary antibodies overnight at 4 °C.

The primary antibodies used were as follows: rabbit anti-type II collagen (Abcam, ab34712, 1: 500), mouse anti-type X collagen (Quartett, 1-COQ97-02, 1: 500), rabbit anti-pyruvate dehydrogenase kinase 4 (Thermo Fisher Scientific, PA5-13776, 1: 500), rabbit anti-pyruvate dehydrogenase (Cell Signaling Technologies, C54G1, 1: 500), rabbit anti-phosphorylated pyruvate dehydrogenase (Abcam, ab92696, 1: 500), and rabbit anti-acetylated histone H3 lysine 27 (Abcam, ab4729, 1: 500). Immune complexes were detected using secondary antibodies conjugated to Alexa Fluor 546 and 647 (Thermo Fisher Scientific, 1: 2000). Prolong gold with DAPI (Thermo Fisher Scientific, 1: 1000) was used.

2.7. Microscope

Culture bright field images were acquired on an inverted microscope (Eclipse Ti; Nikon) equipped with a camera (C4742-80-12AG; Hamamatsu Photonics) and NIS Elements software program (Nikon). Histological brightfield images were acquired on an upright microscope (Eclipse Ni-U; Nikon) equipped with a camera (DS-Fi3; Nikon). Immunofluorescence images were captured on an inverted confocal laser scanning microscope (LSM700; Carl-Zeiss) and ZEN 2012 software program (Carl-Zeiss). Image processing was done on open source software imageJ/Fiji.

2.8. Statistical analysis

The methods of statistical analysis are described in the figure legends. P values < 0.05 were considered to be statistically significant.

3. Results

3.1. Chondrocyte-specific *Sik3* cKO mice showed similar cartilage phenotype with *Sik3* KO mice

Although conventional *Sik3* KO mice show major abnormalities in cartilage [4], *Sik3* is known to regulate glucose and lipid metabolism in liver [13,14]. To exclude the possibility that systemic changes due to abnormal metabolism in other organs may be responsible for the cartilage phenotype in *Sik3* knockout mice, we prepared *11Enh-Cre; Sik3^{fllox/fllox}* cKO mice, in which *Sik3* was deleted specifically in chondrocytes. The *Sik3* cKO mice showed chondrodysplasia phenotype such as dwarfism (Fig. 1A). Histological analysis of the humerus indicated that *Sik3* cKO mice have similar abnormalities in endochondral bone formation as *Sik3* KO mice (Fig. 1B). Both mice showed an elongated zone of proliferating chondrocytes, indicating delayed chondrocyte hypertrophy. These results suggest that the delayed chondrocyte hypertrophy is caused by the absence of *Sik3* in chondrocytes.

3.2. Metabolome analysis of *Sik3*-deficient chondrocytes revealed decreased acetyl-CoA concentration

To search for metabolites that might be involved in the regulation of chondrocyte maturation during endochondral bone formation, we used *Sik3* KO cartilage as material for abnormally matured chondrocytes. We subjected the epiphyseal cartilage of the humerus, femur and tibia from 5-day-old *Sik3* cKO mice and control mice to metabolome analysis. Notably, the concentration of acetyl-CoA was significantly less in *Sik3*-deficient chondrocytes than in control chondrocytes (Fig. 1C and D).

Acetyl-CoA is derived from glucose, fatty acid, and amino acid. In the process of glycolysis, glucose is broken into pyruvate. Then, pyruvate dehydrogenase (Pdh) catalyzes the oxidative decarboxylation of pyruvate to produce acetyl-CoA, a two-carbon acetyl unit that is ligated to the acyl-group carrier, CoA [15], in mitochondria. Pdh is inactivated by phosphorylation by pyruvate dehydrogenase kinase (Pdk). Therefore, we analyzed the expression of these enzymes in *Sik3* KO mice. Histological analysis revealed that chondrocytes in the center of metatarsal primordial cartilage at 16.5 d.p.c. are hypertrophic in control mice but not in *Sik3* KO mice, as indicated by the morphology in sections stained with safranin O and immunoreactivity against anti-types II and X collagen antibodies (Fig. 2). As proliferative chondrocytes mature into hypertrophic chondrocytes during endochondral bone formation, collagenous elements in ECM changes from type II collagen to type X collagen. These results indicated that chondrocytes in the center of the metatarsals are prevented from undergoing hypertrophy in *Sik3* KO mice. Immunohistochemical analysis revealed that the chondrocytes in the center of primordial cartilage expressed more Pdk4 and more phosphorylated Pdh in *Sik3* KO mice than in wild-type mice (Fig. 2), which explains the reduced concentration of acetyl-CoA in *Sik3* KO chondrocytes.

Acetyl-CoA reacts with oxaloacetate to yield citrate, which proceeds to be oxidized through the TCA cycle. Citrate is transported to the cytosol as a substrate for ATP-citrate lyase to regenerate acetyl-CoA and oxaloacetate [16]. Acetyl-CoA in the cytosol is used to synthesize fatty acid and sterol [17] and is a source for histone acetylation [18]. Immunohistochemical analysis indicated that the level of histone 3 acetylation in chondrocytes at the center of primordial cartilage in KO mice did not differ from that in control mice (Fig. 2). Because it was reported that the activity of ATP-citrate lyase is important for the regulation of histone acetylation in articular chondrocytes in adult human [19], changes in acetyl-CoA may not immediately affect the histone acetylation level.

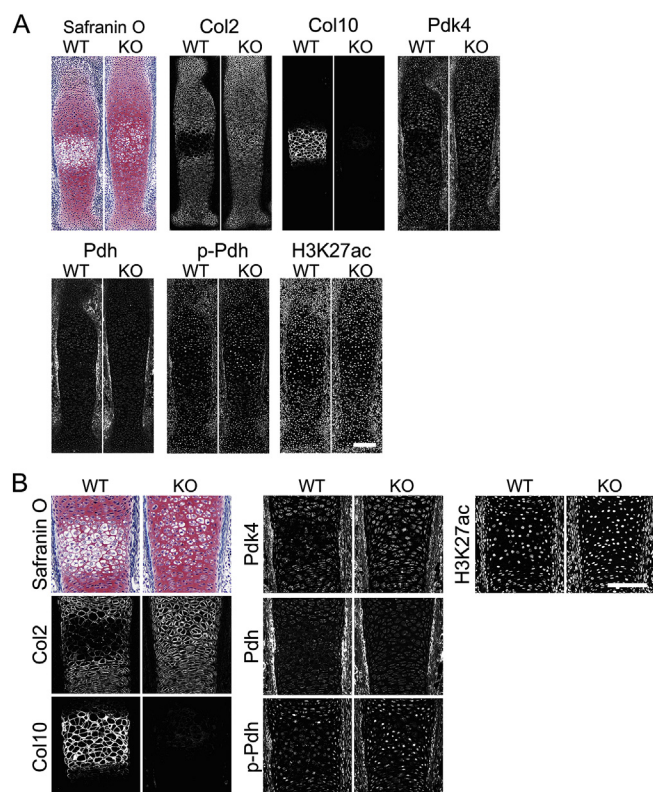


Fig. 2. Immunohistochemical expression analysis of proteins related to chondrocyte differentiation and acetyl-CoA synthesis in the third metatarsals in the forelimbs of *Sik3* KO and WT mice at 16.5 d.p.c. Semi-serial paraffin-embedded sections were stained with Safranin O-fast green-iron hematoxylin and immunostained with antibodies that recognize the proteins indicated at the top (A) or the left (B) of each pair of panels. The data are representative of three mice. (A) Entire primordial cartilage of metatarsals are shown. (B) Magnifications of the central region of each primordial cartilage of metatarsals are shown. WT, *Sik3*^{+/+}; KO, *Sik3*^{-/-}; Col2, type II collagen; Col10, type X collagen; Pdk4, pyruvate dehydrogenase kinase 4; Pdh, pyruvate dehydrogenase; p-Pdh, phosphorylated pyruvate dehydrogenase; H3K27ac, acetylated histone H3 lysine 27. Scale bars, 100 μ m.

3.3. Metatarsal organ culture experiments

To examine whether the decreased concentration of acetyl-CoA is the cause or result of the reduced chondrocyte hypertrophy, we employed an organ culture of metatarsal primordial cartilage from wild-type mice. We harvested metatarsals from mice at 14.5 d.p.c., at which point chondrocyte hypertrophy had not occurred yet, and subjected them to organ culture in the presence or absence of CPI613, an inhibitor of Pdh. Seven days later, the center of the metatarsals turned opaque in the absence of CPI613 (Fig. 3A, top). Histological analysis indicated that cells at the center are hypertrophic, as evident in the section stained with safranin O, and that ECM at the center is calcified, as indicated by positive von Kossa staining (Fig. 3A, top). The opaque region corresponds to calcified hypertrophic cartilage. The addition of 10 μ M CPI613 reduced the extent of the opaque area in the metatarsals (Fig. 3A and B). Histological analysis confirmed the reduced width of the zone of hypertrophic chondrocytes and the reduced area of calcification (Fig. 3A). These results suggest that the inhibition of Pdh delays the maturation process of chondrocytes, including hypertrophy and ECM calcification. Together with the fact that delayed chondrocyte hypertrophy is associated with the reduced concentration of acetyl-CoA in *Sik3*-deficient cartilage, this finding indicates that the reduced concentration of acetyl-CoA is the cause of delayed chondrocyte maturation during endochondral bone formation.

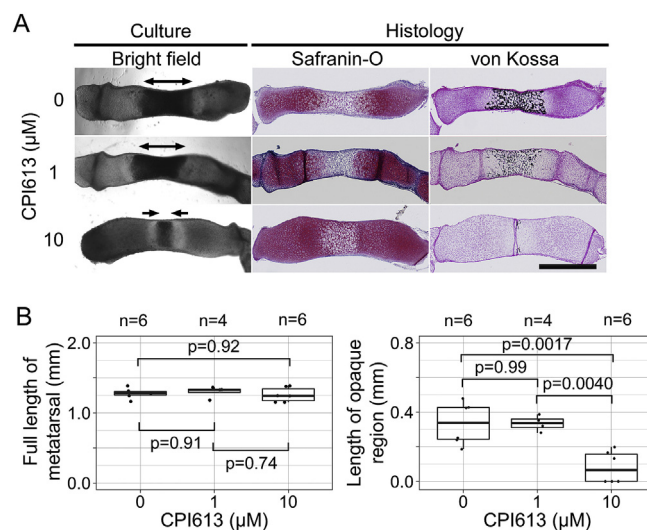


Fig. 3. Metatarsal primordial cartilage were organ cultured in the absence or presence of 1 or 10 μ M CPI613, a Pdh inhibitor, for 7 days. (A) Left, samples were subjected to observation under bright field. Arrows indicate the length of the opaque region. Middle and right, samples were subsequently subjected to histological analysis. Semi-serial sections were stained with Safranin O-fast green-iron hematoxylin (middle) or von Kossa (right). Scale bar, 500 μ m. (B) Boxplots of the lengths of the whole metatarsals (left graph) and opaque regions (right graph). Median, 25% and 75% percentiles are shown. Points correspond to individual results per metatarsal. The numbers above the boxplots indicate the number of metatarsals. P values by one-way ANOVA with Tukey's Kramer test are indicated.

To get insight into the mechanisms by which the reduced concentration of acetyl-CoA delays chondrocyte maturation, we examined the TCA cycle. Rotenone is an inhibitor of complex I (NADH-CoQ reductase) of the respiratory chain, blocking the transfer of electrons from iron-sulfur centers in complex I to ubiquinone and thus the creation of ATP in mitochondria. Chondrocytes were viable in metatarsals for four days in the presence of 1 nM rotenone, as indicated by the increased total length of the metatarsals (Fig. 4A,C). On the other hand, the size of the opaque region at the center of the metatarsals decreased in the presence of rotenone in a dose dependent manner (Fig. 4A–C). These results suggest that disruption of the TCA cycle delays the maturation of chondrocytes.

4. Discussion

Regarding the molecular mechanisms by which *Sik3* induces chondrocyte hypertrophy, we previously reported that *Sik3* anchors Hdac4 in the cytoplasm, thereby releasing Mef2C, a crucial facilitator of chondrocyte hypertrophy, from Hdac4 suppression in nuclei [4]. Another group demonstrated that SIK3 induces the phosphorylation and subsequent proteosomal degradation of DEPTOR, an inhibitor of mTOR, thereby activating mTOR signaling [20]. In addition to these mechanisms, our findings that a reduced concentration of acetyl-CoA is the cause of delayed chondrocyte maturation in *Sik3* KO cartilage suggest a new mechanism in which *Sik3* increases acetyl-CoA to induce chondrocyte hypertrophy.

How changes in acetyl-CoA affect chondrocyte maturation remain to be analyzed. Acetyl-CoA acts in various cellular metabolism including as a substrate for the TCA cycle. Our data indicated that disruption of the respiratory chain with rotenone results in the delay of chondrocyte maturation in a metatarsal organ culture system. These data are consistent with the recent finding that the impairment of mitochondrial respiration by inactivation of mitochondrial transcription factor A (Tfam) delays chondrocyte

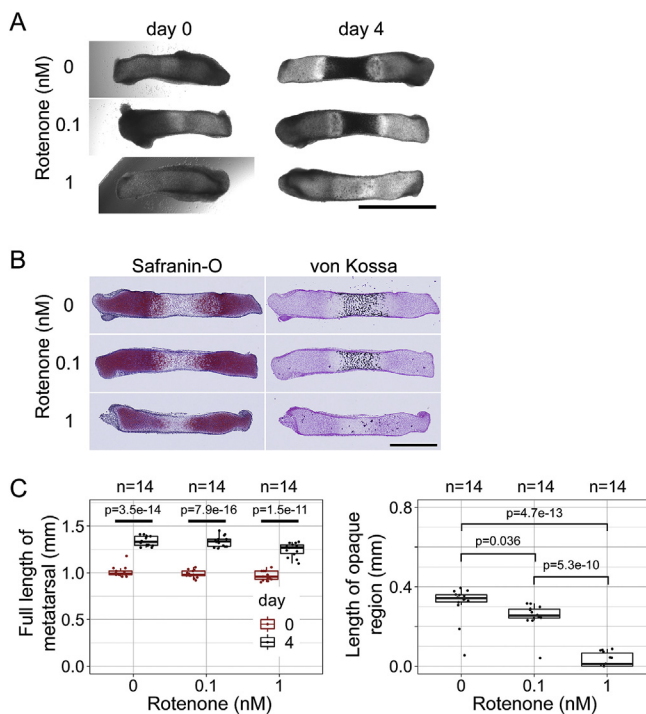


Fig. 4. Metatarsal primordial cartilage were organ cultured in the absence or presence of 0.1 or 1 nM rotenone for 4 days. (A) Samples in culture were subjected to observation under bright field. Samples at the start (left, day 0) and end (right, day 4) of the organ culture. Scale bar, 500 μ m. (B) Samples at the end of the organ culture were subjected to histological analysis. Semi-serial sections were stained with Safranin O-fast green-iron hematoxylin (left) or von Kossa (right). Scale bar, 500 μ m. (C) Boxplot of metatarsal lengths. Median, 25% and 75% percentiles are shown. The points correspond to the individual results per metatarsal. The numbers above the boxplots indicate the number of metatarsals. Left, full lengths of the metatarsals were measured. Red denotes measurements at the start of the organ culture and black denotes measurements at the end of the organ culture. p values were calculated by two-sided Student t-test. Right, lengths of the opaque regions of metatarsals at the end of the organ culture. p values were calculated by one-way ANOVA with Tukey's Kramer test.

hypertrophy in mice [21]. Thus, it is possible that acetyl-CoA induces chondrocyte maturation by acting as a substrate for the TCA cycle. However, this hypothesis appears inconsistent with the hypertrophic chondrocytes depending on oxidative phosphorylation for ATP production less than proliferative chondrocytes [22]. Further experiments are needed.

Another interesting point of our study is that metatarsal primordial cartilage survived in the presence of 1 nM rotenone, a concentration under which other type of cells such as neuroblastoma cells may die [23]. This result is consistent with the recent finding that chondrocytes lacking Tfam survive in mice [21], supporting the notion that mitochondrial respiration is not required for chondrocyte survival.

Our results also suggest that a decrease of acetyl-CoA in Sik3-deficient chondrocytes was caused by an increased expression of Pdk4. It remains to be analyzed how the Sik3 signal regulates the expression of Pdk4 in chondrocytes. It has been reported that the transcription of *Pdk4* is activated by forkhead factor FOXO [24,25]. In addition, it has been reported that Sik phosphorylates and sequesters HDAC4 into the cytoplasm, which in turn increases the acetylation levels of FOXO in the nucleus and decreases FOXO transcriptional activities in the liver [26] and drosophila energy balance [27]. The possibility that Sik3 decreases the expression of Pdk4 through HDAC4 phosphorylation and FOXO acetylation in chondrocytes needs to be tested.

The maturation of chondrocytes should be regulated strictly for

appropriate bone formation. Our results collectively suggest that changes in acetyl-CoA play a role in the regulation of chondrocyte maturation. Clarifying the effects of metabolites on chondrocyte differentiation would contribute to understanding the mechanisms regulating endochondral bone formation.

Conflicts of interest

The authors have no conflicts of interest to declare.

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