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Title Page

Enhanced chondrogenesis of iPS cells from neonatal-onset multisystem inflammatory disease occurs via the caspase-1-independent cAMP/PKA/CREB pathway

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Running header: Dysregulated SOX9 upregulation in NOMID chondrocytes independent of the NLRP3 inflammasome

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Abstract

Objective. Neonatal-onset multisystem inflammatory disease (NOMID) is a dominantly inherited autoinflammatory disease caused by *NLRP3* mutations. NOMID pathophysiology is explained by the NLRP3 inflammasome, which produces interleukin (IL)-1β. However, epiphyseal overgrowth in NOMID is resistant to anti-IL-1 therapy and may therefore occur independently of the NLRP3 inflammasome. This study investigated the effect of mutated NLRP3 on chondrocytes using NOMID patient-derived induced pluripotent stem (iPS) cells.

Methods. We established isogenic iPS cells with wild-type (WT) or mutant NLRP3 from two NOMID patients with *NLRP3* somatic mosaicism. The iPS cells were differentiated into chondrocytes *in vitro* and *in vivo*. The phenotypes of chondrocytes with WT and mutant NLRP3 were compared, particularly the size of the chondrocyte tissue produced.

Results. Mutant iPS cells produced larger chondrocyte masses than WT iPS cells owing to glycosaminoglycan overproduction, which correlated with increased expression of the chondrocyte master regulator SOX9. In addition, *in vivo* transplantation of mutant cartilaginous pellets into immunodeficient mice caused disorganized endochondral ossification. Enhanced chondrogenesis was independent of caspase-1 and IL-1, and thus the NLRP3 inflammasome. Investigation of the human *SOX9* promoter in chondroprogenitor cells revealed that the CREB/ATF-binding site was critical for *SOX9*

overexpression caused by mutated *NLRP3*. This was supported by increased levels of cAMP and phosphorylated CREB in mutant chondroprogenitor cells.

Conclusion. We demonstrated that the intrinsic hyperplastic capacity of NOMID chondrocytes is dependent on the cAMP/PKA/CREB pathway, independent of the NLRP3 inflammasome.

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Systemic autoinflammatory syndromes are caused by defects in the innate immune system, especially pattern recognition receptors, which result in uncontrolled inflammatory responses (1). Neonatal-onset multisystem inflammatory disease (NOMID) is a systemic autoinflammatory disease caused by *NLRP3* mutation (2). The clinical features of NOMID include neonatal-onset persistent inflammation, urticarial rash, chronic aseptic meningitis, and arthropathy characterized by tumor-like expansive lesions in epiphyseal portions of long bones (3). *NLRP3* is mainly expressed in hematopoietic cells, especially monocytes/macrophages, and in chondrocytes (4). In monocytes/macrophages, once NLRP3 is activated by its ligand, a multi-protein complex called the NLRP3 inflammasome forms, resulting in the activation of caspase-1, which cleaves pro-interleukin (IL)-1β into active IL-1β (5-8). The molecular mechanism by which NOMID-associated *NLRP3* mutations lead to the activation of the NLRP3 inflammasome has not been fully elucidated. However, it is hypothesized that mutated NLRP3 can trigger the formation of the NLRP3 inflammasome independently of ligand binding, which causes dysregulated IL-1β secretion and uncontrolled multisystem inflammation. This hypothesis is supported by the fact that a targeted therapy against IL-1β effectively controls systemic inflammation in NOMID (9-11). However, epiphyseal overgrowth in NOMID is resistant to anti-IL-1 therapy (12).

Sequential radiological imaging and histological analyses of tissue biopsies suggest that the main pathophysiology of NOMID arthropathy is not inflammation but disorganization of cartilage cell columns that leads to tumor-like expansive lesions (13). These clinical and pathological findings suggest

that mutant *NLRP3* induces epiphyseal overgrowth in NOMID via mechanisms unrelated to the NLRP3 inflammasome. However, the function of NLRP3 in chondrocytes has not been elucidated, let alone the mechanism underlying epiphyseal overgrowth in NOMID.

Cartilage is a flexible connective tissue in the skeletal system and consists of chondrocytes and extracellular matrix (ECM). The growth plate consists of a column of chondrocytes that separate the epiphysis and metaphysis of a long bone. The primary function of these chondrocytes is to provide a cartilage template on which bone can form through endochondral ossification. In the growth plate, maturing chondrocytes are organized into resting, proliferating, prehypertrophic, and hypertrophic zones. Growth factor signaling stimulates mesenchymal progenitor cells in the resting zone to proliferate, upon which they move to the proliferating zone. These cells subsequently produce cartilage-specific ECM consisting of type II (collagen 2 [COL2]) and type XI collagens, and proteoglycans, such as aggrecan (ACAN) and cartilage oligomeric matrix protein (COMP). These cells then exit the cell cycle, differentiate, become hypertrophic, produce type X collagen and matrix metalloproteinases, and finally undergo apoptosis. The remaining cartilaginous matrix is mineralized and provides a scaffold on which bone can form (14, 15).

It is difficult to obtain bone tissues from NOMID patients owing to ethical reasons. Moreover, osteochondrogenic progenitor cells often cannot be obtained from postnatal human tissues in sufficient quantities, while acquiring such cells in sufficient quantities from human fetuses or embryos is ethically

challenging. Furthermore, although a mass-like lesion called a "spike" is observed in mouse models of NOMID arthropathy (16), these models do not recapitulate the epiphyseal overgrowth observed in NOMID. These issues have prevented elucidation of the pathophysiological mechanism underlying epiphyseal overgrowth in NOMID. In this study, we applied a newly developed chondrocyte differentiation system to induced pluripotent stem (iPS) cells derived from NOMID patients. This system allowed chondrocytes to be obtained in sufficient quantities to directly study the effect of mutated NLRP3 on chondrocyte phenotypes, focusing on the involvement of the NLRP3 inflammasome and the master regulator of chondrocyte differentiation, SOX9.

Materials and Methods

Cell culture

Undifferentiated iPS cells from two NOMID patients with NLRP3 somatic mosaicism (p.Tyr570Cys and p.Gly307Ser) were established as previously described (17). From each patient, at least three clones of iPS cells with mutant or wild-type (WT) *NLRP3* were established. In all experiments, mutant and WT isogenic cells were compared (3). Culture of undifferentiated iPS cells and chondrogenic differentiation was performed as previously described (Umeda et al., manuscript attached) with some modifications. iPS cells were seeded onto a Matrigel (BD)-coated dish, cultured in mTeSR medium (STEMCELL) for 9 days, and then transferred to initial differentiation medium. This medium was changed once on Day 3. On Day

6, a single cell suspension was prepared with 0.05% trypsin-EDTA. These cells were plated onto fibronectin-coated dishes, cultured in chondroprogenitor medium, and passaged every 3 days. We called these cells 'chondroprogenitor cells'. For chondrogenesis, chondroprogenitor cells that had been passaged 3–5 times were used (Fig. 1A).

Chondrogenesis assay

Serum-free chondrogenic medium was described previously (18). Two-dimensional (2D) micromass culture was performed by spotting a 5 μl droplet of chondroprogenitor cells (1.5×10^5) onto the well of a fibronectin-coated 24-well plate in serum-free chondrogenic medium supplemented with 40 ng/ml platelet-derived growth factor-BB (PDGF-BB) (R&D) and 1% fetal calf serum. The medium was changed every 3 days. From Day 21, 10 ng/ml transforming growth factor (TGF) β3 (R&D) was added, and from Day 25, 50 ng/ml bone morphogenetic protein 4 (BMP4) (WAKO) was replaced with 40 ng/ml PDGF-BB. For three-dimensional (3D) pellet cultures, chondroprogenitor cells were passaged once in chondroprogenitor media containing 5 ng/ml basic fibroblast growth factor and 10 ng/ml TGFβ3, and then cultured for 3 days. Aliquots of 2.5×10^5 cells were centrifuged to form pellets, which were cultured in 0.5 ml of serum-free chondrogenic media supplemented with specific factors as outlined above. Fixation and staining of the 2D micromass and 3D pellet cultures were performed as previously described (18). Glycosaminoglycan (GAG) and sulfated proteoglycan levels and DNA content were quantified as

previously described (19).

Chondrogenesis in vivo

Cartilaginous pellets formed by 3D cell pellet cultures over 20 days were wrapped in a 0.5 cm × 1 cm Gelfoam (Phizer) and transplanted beneath the dorsal skin of immunodeficient NOD/Shi-scid, IL-2Rγ(null) (NOG) mice. Four weeks later, cartilage and bone particles were harvested, fixed with paraformaldehyde for 24 hours, embedded in plastic, sectioned, and stained with hematoxylin and eosin (HE), von Kossa, or Alcian blue, as previously reported (18).

Enzyme-linked immunosorbent assay (ELISA) and Western blotting

The concentration of cAMP was measured using an ELISA (Cell Signaling). Antibodies against CREB, phosphorylated CREB (Cell Signaling), and β-actin (Santa Cruz) were used for Western blotting, as previously described (20).

Gene expression profiling

Total RNA was extracted and reverse-transcribed to cDNA using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. RT-qPCR was performed as previously described (19). The expression levels of each gene from duplicate or triplicate reactions were normalized against the

level of the *BACT* transcript and are shown relative to their expression in the osteosarcoma cell line ANOS (21) or a human articular cartilage sample.

Reporter assay for the human SOX9 promoter

To measure the activity of the human *SOX9* promoter in chondroprogenitor cells, the 5' untranslated region (UTR) of the human *SOX9* gene (−927 to +84 bp) was inserted into a pGL3-luciferase reporter plasmid (Promega), as previously described (22). Site-directed mutagenesis of the known transcription factor-binding sites of the human *SOX9* promoter was performed as previously described (23). The residue was mutated to the nucleotide that was least likely to be at this position, based on consensus sequences in the JASPAR transcriptional database (24). Sequence information is provided in the Supplementary data. Chondroprogenitor cells were plated at a density of 50,000 cells/well in 6-well plates, transfected with 2 μg DNA/well using the FuGENE 6 transfection reagent, and harvested at 24 hours after transfection. Luciferase activity was measured as previously described (23). Additionally, 10 μM forskolin (Sigma) and 10 μM SQ22536 (Sigma) was used to activate and inhibit adenylate cyclase, respectively.

Statistical analysis

Data were analyzed using the Student's t-test. P-values less than 0.05 were considered to be

statistically significant.

Ethical issues

This study was approved by the ethics committee of Kyoto University and was performed in accordance with the Declaration of Helsinki. A sample of human articular cartilage was obtained from a patient who underwent knee surgery after obtaining informed consent that the sample could be used for research purposes.

Results

Chondrocytes differentiated from iPS cells with mutated NLRP3 produce a large cartilaginous mass

To investigate the pathophysiology of NOMID arthropathy, we attempted to recapitulate this phenotype by using chondrocytes that were differentiated from iPS cells derived from patients with NOMID. We obtained iPS cells from two NOMID patients with arthropathy who had *NLRP3* somatic mosaicism, as previously described (17). We established isogenic iPS cell clones that had mutated or WT NLRP3, which allowed us to examine the effects of NLRP3 mutations in the same genetic background (25, 26).

To produce chondrocytes from these iPS cells, we utilized a protocol in which chondrocytes are obtained from cells of neural crest character (Umeda et al., manuscript attached) (Fig. 1A). We performed

two chondrogenic assays, a 2D micromass culture assay and a 3D pellet assay. The former is suitable for experiments with exogenous inhibitors or activators, whereas the latter generates more mature chondrocytes for *in vivo* transplantation assay. First, we confirmed the phenotype of cartilage samples by performing Alcian blue staining, which labels ECM excreted by chondrocytes, and immunostaining for COL2, which is specifically expressed in chondrocytes (Fig. 1B). Following culture in chondrogenic medium, cells derived from WT and mutant iPS cells were positive for Alcian blue and COL2, which confirmed that the iPS cells had successfully differentiated into chondrocytes. Importantly, the 2D micromass and 3D pellet samples derived from mutant iPS cells were significantly larger than those derived from WT iPS cells, up to Day 74 of culture (Fig. 1B and C).

Next, we examined mRNA expression of cartilage-related genes expressed in proliferating chondrocytes (early markers; *COL2A1*, *ACAN*, *COMP*, and *SOX9*) and those expressed in hypertrophic chondrocytes (late markers; *IHH*, *COL10A1*, *VEGFA*, and *MMP13*) in samples obtained by the aforementioned method (Fig. 1D). These genes were expressed in 3D pellet samples obtained from mutant and WT iPS cells, further indicating that chondrocyte differentiation was successful and that 3D pellets contained chondrocytes at various differentiation stages. The expression levels of both early and late chondrogenic markers were significantly higher in mutant samples than in WT samples (Fig. 1D). Taken together, these data show that chondrocytes were successfully differentiated *in vitro* from iPS cells derived from NOMID patients, and that chondrocytes differentiated from iPS cells with mutant *NLRP3*

produce large cartilaginous masses *in vitro*. They also demonstrate that the entire chondrocyte differentiation process, from precursors to late chondrocytes, is enhanced in mutant cells compared to WT cells.

The production of large cartilaginous masses by mutant iPS cells is owing to ECM overproduction, not to increased cell proliferation

We next sought to determine what causes the chondrocyte masses derived from mutant iPS cells to be larger than those derived from WT iPS cells. First, we analyzed the proliferation potential of chondroprogenitor cells. Population doubling time did not significantly differ between mutant and WT chondroprogenitor cells up to 15 passages, after which the cells stopped proliferating (Fig. 2A). Next, we determined the number of differentiated chondrocytes by analyzing DNA content and GAG production, which is a major cartilaginous ECM component. In 2D micromass and 3D pellet cultures, the DNA content in differentiated chondrocyte tissue derived from mutant and WT iPS cells did not significantly differ. This suggests that a similar number of chondrocytes were produced from mutant and WT iPS cells. By contrast, chondrocytes derived from mutant iPS cells produced more GAG than those derived from WT iPS cells in 2D micromass (Fig. 2B) and 3D pellet (Fig. 2C) cultures. These data indicate that the larger amount of chondrocyte tissue produced from mutant iPS cells is not due to an increased number of chondrocytes, but to an increased amount of cartilaginous ECM produced per cell.

In vivo differentiation of chondrocytes from mutant iPS cells reveals dysregulated endochondral ossification

Radiological examination of affected long bones in NOMID patients shows enlargement of the epiphysis with abnormal ossification. The *in vitro* differentiation system did not induce chondrocyte calcification, probably due to the lack of cell components or factors necessary for the final differentiation step. Therefore, we used an *in vivo* differentiation system as a model for endochondral ossification, in which immature 3D pellet samples were transplanted into NOG mice. The transplanted cartilage mass was vascularized *in vivo* (Fig. 3A). Mutant pellets were larger than WT pellets, both at transplantation and harvesting, and this size difference increased during *in vivo* differentiation (Fig. 3B). Following von Kossa staining, which detects calcium deposits, calcification was detected in both WT and mutant pellets (Fig. 3A). Interestingly, Alcian blue staining revealed that mutant pellets contained more residual cartilage components than WT pellets. In addition, calcified areas were scattered throughout mutant pellets, whereas they were localized in specific regions and were clearly separated from Alcian blue-positive areas in WT pellets. Taken together, these data indicate that in our *in vivo* model, chondrocyte tissue differentiated from mutant iPS cells grows larger and exhibits disorganized ossification compared to chondrocyte tissue differentiated from WT iPS cells.

Enhanced chondrogenesis of mutant iPS cells is independent of the NLRP3 inflammasome

The inflammatory phenotype of NOMID is caused by gain-of-function *NLRP3* mutations, leading to activation of the NLRP3 inflammasome (27). Therefore, we examined the involvement of the NLRP3 inflammasome in the formation of cartilaginous masses. First, we analyzed the expression of the NLRP3 inflammasome components in 2D cartilage samples. Mutant and WT cartilage samples both expressed NLRP3, but did not express ASC, pro-caspase-1, or pro IL-1β by Western blotting (data not shown). This suggests that the formation of large cartilaginous masses by mutant chondrocytes occurs independently of the NLRP3 inflammasome.

To confirm that chondrogenesis of mutant iPS cells is enhanced independently of the NLRP3 inflammasome, we used inhibitors of components of the NLRP3 inflammasome, namely, Ac-YVAD-CHO, which inhibits caspase-1, and recombinant human IL-1Ra, which antagonizes IL-1. Ac-YVAD-CHO (10 μM) treatment, as well as recombinant human IL-1Ra (1 μg/ml) treatment, during 2D micromass culture did not prevent the formation of large cartilaginous masses (Fig. 4A, B), *SOX9* upregulation (Fig. 4C), or overproduction of GAG (Fig. 4D) by chondrocytes derived from mutant iPS cells. The same was true when samples were treated with higher concentrations of these inhibitors (up to 1000-fold higher, data not shown). Taken together, these data strongly indicate that the enhanced chondrogenesis of mutant iPS cells is independent of caspase-1 and IL-1, and thus the NLRP3 inflammasome.

Upregulation of NLRP3 correlates with the upregulation of SOX9 in chondroprogenitor cells

To dissect the mechanism underlying the enhanced chondrogenesis of mutant iPS cells, we analyzed the time-course of chondrocyte-specific gene expression in chondroprogenitor cells (Fig. 5). Expression of *SOX9*, *COL2A1*, and *NLRP3* started to be upregulated in chondroprogenitor cells at Day 6. Importantly, at Day 15, *SOX9* and *COL2A1* were upregulated more in mutant chondroprogenitor cells than in WT chondroprogenitor cells, whereas *NLRP3* was upregulated similarly in both types of cells (Fig. 5). By contrast, at this time-point, the other chondrocyte-specific markers *ACAN* and *COMP* were not expressed in either type of cells (Fig. 5). Thus, differential upregulation of *SOX9* in chondroprogenitor cells correlated with the upregulation of *NLRP3* and preceded the upregulation of *COMP* and *ACAN*.

The CREB/ATF-binding site of the SOX9 promoter is critical for mutated NLRP3-dependent enhancement of SOX9 expression

Next, we focused on *SOX9* because it was upregulated together with *NLRP3*, and this preceded the upregulation of other chondrocyte-specific markers. We analyzed the activity of the human *SOX9* promoter in chondroprogenitor cells in which the level of *SOX9* mRNA was increased. We created a luciferase reporter construct containing the 5' UTR of human *SOX9*, which encompasses −927 to +84 bp of the transcription start site. This fragment has basal promoter activity and putative binding sites for five

transcription factors, namely, NFAT, AP-1, NF-κB, Sp1, and CREB/ATF (Supplementary data). This fragment showed no promoter activity in the monocytic cell line THP-1 or the erythroleukemic cell line K562, which do not express endogenous *SOX9* (data not shown). Importantly, human *SOX9* promoter activity was higher in mutant chondroprogenitor cells than in WT chondroprogenitor cells (Fig. 6A). To identify the element of the human *SOX9* promoter region that responds in a mutated NLRP3-dependent manner, we performed site-directed mutagenesis of the sites of this promoter that bind the transcription factors NFAT, AP-1, NF-κB, Sp1, and CREB/ATF (Supplementary data). Among the reporters with these mutations, the reporter that harbored a mutation in the CREB/ATF-binding site showed the least upregulation of *SOX9* promoter activity in mutant cells (Fig. 6A, Supplementary data). Thus, we speculate that the CREB/ATF-binding site is critical for activation of the human *SOX9* promoter in a mutated NLRP3-dependent manner.

The cAMP/PKA/CREB pathway is critical for SOX9 upregulation caused by mutated NLRP3

To further explore the association between mutated NLRP3 and the cAMP/PKA/CREB pathway, we examined the effect of an adenylate cyclase activator and inhibitor (forskolin and SQ22536, respectively) on the activity of the human *SOX9* promoter and *SOX9* mRNA expression (Fig. 6B). Among mutant chondroprogenitor cells, *SOX9* promoter activity was 2.3-fold higher in forskolin-treated cells than in vehicle-treated cells, whereas *SOX9* promoter activity was 2-fold lower in SQ22536-treated cells

than in vehicle-treated cells (Fig. 6B). Similar effects were observed in WT chondroprogenitor cells, although they were less pronounced. These data correlated well with the effects of forskolin and SQ22536 on *SOX9* mRNA expression. We also examined the effects of forskolin and SQ22536 on 3D chondrocyte pellet formation (Fig. 6C). In comparison to pellets of vehicle-treated mutant cells, pellets of mutant cells treated with forskolin and SQ22536 were 2.0-fold larger and 2.1-fold smaller, respectively. Similar effects were observed in WT cells, although they were less pronounced. These data clearly indicate that upregulation of *SOX9* following activation of adenylate cyclase is involved in the enhanced chondrogenesis of mutant iPS cells.

We next measured the cAMP concentration to demonstrate that the activity of adenylate cyclase is increased in mutant chondroprogenitor cells. The concentration of cAMP was 4-fold higher in mutant chondroprogenitor cells than in WT chondroprogenitor cells at Day 15 and 36 (Fig. 6D). By contrast, the concentration of cAMP was similar in mutant and WT iPS cells, in which *NLRP3* expression was low.

Finally, we examined the level of phosphorylated CREB in chondroprogenitor cells. CREB is phosphorylated by cAMP-activated PKA. According to Western blot analysis, the level of phosphorylated CREB was higher in mutant chondroprogenitor cells than in WT chondroprogenitor cells at Day 15 and 36 (Fig. 6D). Taken together, these data indicate that the cAMP/PKA/CREB pathway plays an important role in the upregulation of *SOX9*, and therefore enhanced chondrogenesis, in chondroprogenitor cells with

mutant NLRP3 (Fig. 6E).

Discussion

Disease-specific iPS cells have been extensively used to investigate the pathogenesis of diseases and to discover novel drugs. This approach is particularly useful to study rare diseases because tissues are often difficult to obtain from patients with such diseases. In this study, we used disease-specific iPS cells to study NOMID. Using this approach, we produced chondrocyte tissues with mutant and WT NLRP3, and revealed a previously unidentified connection between the inflammasome-associated molecule NLRP3 and the master regulator of chondrocyte differentiation SOX9.

SOX9 was upregulated during the differentiation of iPS cells into chondrocytes, and this was particularly pronounced in mutant iPS cells. During cartilage development, *SOX9* is highly expressed in immature chondrocytes and is required for the condensation and differentiation of mesenchymal cells. During the early stages of chondrogenesis, SOX9 activates the transcription of many cartilage-specific ECM genes, including *COL2A*1, *ACAN*, and *COMP*, by directly interacting with SOX5 and SOX6 (28, 29). Overexpression of *SOX9* in chondrocytes using a recombinant adeno-associated virus significantly increases the synthesis of major ECM component in chondrocytes, without affecting their proliferation, *in vivo* and *in vitro* (30, 31). In addition, retroviral transduction of *SOX9* increases ECM production in

human chondrocytes *in vitro* (32). These data correlate well with our observation that *SOX9* overexpression driven by mutated NLRP3 caused overproduction of ECM, but did not increase chondrocyte proliferation.

It remains to be determined how enhanced expression of *SOX9* in chondrocytes leads to epiphyseal overgrowth in NOMID patients. Conditional transgenic mice have been used to show that overexpression of *SOX9* in *COL2A1*-positive cells inhibits terminal differentiation of hypertrophic chondrocytes and endochondral bone formation (29). Although we have not directly confirmed the expression level of SOX9 in samples derived from NOMID patients, this previous study might help to link the findings of the current study with the clinical phenotype of NOMID patients.

We identified the cAMP/PKA/CREB pathway as being critical for the upregulation of SOX9 mRNA in a mutant NLRP3-dependent manner. cAMP is an intracellular second messenger that is involved in a variety of cellular processes (33). cAMP/PKA/CREB signaling is crucial in chondrogenesis, and synergism between cAMP and SOX9 is particularly important (34-36). Co-transfection of CREB-binding protein (CBP) and p300 increases SOX9 activity (35). PKA phosphorylates SOX9 and thereby increases SOX9 activity, which results in the upregulation of the *COL2A1* promoter through the interaction between CBP and SOX9 (34). In addition, the PKA inhibitor H89 blocks chondrogenesis in the chick limb bud (36). These data support the idea that cAMP/PKA/CREB signaling upregulates *SOX9* to enhance chondrogenesis.