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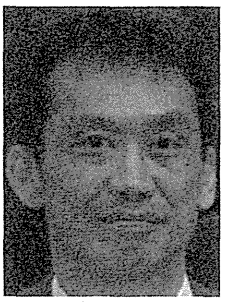
Profile



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Review Article

Genetics of Ossification of the Posterior Longitudinal Ligament of the Spine: A Mini Review

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Ossification of the posterior longitudinal ligament of the spine (OPLL) is a common disease in aging populations and sometimes results in serious neurological problems due to compression of the spinal cord and nerve roots. OPLL is a multi-factorial (polygenic) disease controlled by genetic and environmental factors. Studies searching for the genetic component of OPLL, using linkage and association analyses, are in progress and several susceptibility genes have been reported. This paper reviews the recent progress in the genetic study of OPLL and comments on its future task.

Key Words: Genetic association studies, Ossification of posterior longitudinal ligament, Polymorphism

Epidemiology

The posterior longitudinal ligament of the spine (PLL) is a ligament that runs behind the spinal column (vertebral bodies and intervertebral discs). PLL is situated anterior to the spinal cord within the spinal canal. Ossification of the PLL (OPLL; MIM 602475) is a disease state caused by ectopic ossification. OPLL is a common disease. The incidence of OPLL is 1.9-4.3% in Japan.[1,2] Comparable incidence has been reported in other countries, especially in East Asia.[3] The average age of onset is over 50 years with male predominance.[4] OPLL presents with neurological symptoms due to compression of spinal cord and nerve roots as well as neuropathic pain and stiffness of the neck and trunk. These symptoms affect motility and quality of life of the patients.

Etiology

From the etiological point of view, OPLL is divided into 2 categories; primary (idiopathic) and secondary (syndromic). The latter includes OPLL associated with monogenic diseases like hypophosphatemic rickets/osteomalacia. Several forms of hypophosphatemic rickets are known, including an X-linked form (MIM 307800) caused by phosphate regulating endopeptidase homolog, X-linked (*PHEX*) mutations (MIM 300550), an autosomal dominant form (MIM 193100) caused by fibroblast growth factor 23 (*FGF23*) mutations (MIM 605380), an X-linked recessive form (MIM 300554) caused by chloride channel, voltage-sensitive 5 (*CLCN5*) mutations (MIM 300008), and autosomal recessive forms caused by dentin matrix acidic

phosphoprotein 1 (*DMP1*) (MIM 600980), hypophosphatemic rickets, autosomal recessive 2 (*ARHR2*) (MIM 613312) or ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*) (MIM 173335) mutations. 'tiptoe walking' (*TTW*) mouse, which has a spontaneous nonsense mutation in *ENPP1* is a good model for OPLL.[5] Also, OPPL is a frequent complication in patients with endocrine disorders including hypoparathyroidism[6] and acromegaly/gigantism.[7] However, most cases of OPLL are idiopathic. Therefore, I refer to idiopathic OPLL hereafter.

Many reports on the underlying mechanisms of OPLL have suggested that OPLL is a multi-factorial (polygenic) disease influenced by genetic and environmental (non-genetic) factors. Several clinical factors including age,[8] diabetes mellitus (DM)[9] and obesity[10] have been reported as risk factors for OPLL. In addition, vitamin A-rich diet, exercise and abnormal mechanical stress to the head have been considered as environmental factors for OPLL.[7] On the other hand, OPLL has a strong genetic preposition. A study using 347 OPLL families reported a prevalence of OPPL of 26% in the parents of the probands and 29% in the sibs.[11] Matsunaga et al.[12] studied the association between OPLL and human leukocyte antigen (HLA) haplotypes in families of 24 patients with OPLL and found higher prevalence of OPLL in the siblings showing a higher share of identical HLA haplotypes. As in other multi-factorial diseases, genome studies are revealing the genetic factors of OPLL. A lot of linkage and association studies have been conducted and many genes/loci that link to OPLL susceptibility have been reported (Table 1).

Table 1. Previously reported ossification of the posterior longitudinal ligament of the spine susceptibility genes

Gene	Chromosome	Literature (1st author, journal, year)
TLR5	1q41	Chung, J Korean Neurosurg Soc, 2011 [24]
HLA haplotype	6p21	Matsunaga S, Spine, 1999 [12]
RXRB	6p21	Numasawa T, J Bone Miner Res, 1999 [14]
COL11A2	6p21	Koga H, Am J Hum Genet, 1998 [13]
RUNX2	6p21	Liu Y, Clin Orthop Relat Res, 2010 [23]
IL-1β	6q13	Ogata N, Spine, 2002 [20]
ENPP1 (NPPS)	6q22-q23	Nakamura I, Hum Genet, 1999 [18]
ESR1	6q25	Ogata N, Spine, 2002 [20]
IL-15RA	10p15	Kim DH, Cytokine, 2011 [25]
BMP9	10q11.22	Ren Y, PLoS One, 2012 [26]
VDR	12q13	Kobashi G, Spine, 2008 [21]
BMP4	14q22-q23	Furushima K, J Bone Miner Res, 2002 [16]
TGFB3	14q24	Horikoshi T, Hum Genet, 2006 [27]
TGFB1	19q13	Kamiya M, Spine, 2001 [19]
BMP2	20p12.3	Wang H, Eur Spine J, 2008 [22]
COL6A1	21q22	Tanaka T, Am J Hum Genet, 2003 [15]

TLR, toll-like receptor; RXRB, retinoic X receptor β; COL, collagen; RUNX, runt-related transcription factor; IL, interleukin; ENPP, ectonucleotide pyrophosphatase/phosphodiesterase; NPPS, nucleotide pyrophosphatase; ESR, estrogen receptor; VDR, vitamin D (1,25-dihydroxyvitamin D3) receptor; BMP, bone morphogenetic protein; TGFB, transforming growth factor-beta.

Linkage study

The first one was a sib-pair linkage analysis conducted by a Utah group,[13] which examined 53 families by a non-parametric linkage analysis focusing on the HLA region and found a significant linkage on D6S276 ($P=6\times10^{-6}$). Subsequently, by a candidate gene approach using in 280 patients and 210 controls for positional candidates around the marker, they found an association with collagen, type XI, alpha 2 (*COL11A2*) ($P=4\times10^{-4}$). *COL11A2* (MIM 120290) encodes one of the 3 α-chains of type XI collagen, a cartilage-specific collagen. The group also reported association ($P=0.0028$) with retinoid X receptor, beta (*RXRB*) (MIM 180246) adjacent to *COL11A2*.[14]

A group led by Inoue expanded on the study by increasing the number of sibs and found a significant linkage at D21S1903 on 21q by a genome-wide linkage study.[15] They conducted an association study of 150 candidate genes in a 20-Mb region around the marker using 280 OPLL patients and 210 controls, and found association with collagen, type VI, alpha 1 (*COL6A1*) ($P=3\times10^{-6}$). COL6A1 (MIM 120290) encodes one of the 3 α-chains of type VI collagen. Furushima et al.[16] performed a linkage study for candidate genes selected from expression profiles during osteoblastic differentiation of human mesenchymal stem cells and found suggestive evidence of linkage with bone morphogenetic protein 4 (*BMP4*) (MIM 112262).

Those studies are interesting but were dependent on small number of samples (172 at the most), and most of the subjects were collected in very limited areas. Karasugi et al.[17] performed a large-scale genome-wide linkage study using 410 Japanese OPLL individuals (214 affected sib-pairs); however, they could not replicate the previous linkage results nor find any new loci. In stratification analyses for definite cervical OPLL that included subjects with more than 2 ossified vertebrae only, they found loci with

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suggestive linkage on 1p, 2p, 7q, 16q, and 20p. Fine mapping using additional markers detected the highest nonparametric lod (NPL) score (3.43, $P=0.00027$) at D20S894 on chromosome 20p12 in a subgroup that had no complication of DM.

Association study

Several groups worked on candidate gene association studies. A number of genes/loci associated with the OPLL susceptibility have been reported, including genes for nucleotide pyrophosphatase/phosphodiesterases (NPPS)/ENNP1[18], transforming growth factor (TGF)-β1[19], estrogen receptor (ESR),[20] interleukin 1, beta (IL-1β),[20] vitamin D receptor (VDR),[21] bone morphogenetic protein 2 (BMP2),[22] runt-related transcription factor 2 (RUNX2),[23] toll-like receptor 5 (TLR5),[24] interleukin 15 receptor, alpha (IL-15RA),[25] and BMP9 [26] (Table 1). However, the results of these studies are not sufficiently convincing because of their small sample sizes, small number of sequence variants examined and lack of functional proof of the variants and/or genes. Few variants per gene (usually only one single nucleotide polymorphism [SNP]) were examined; the statistical significance of their association is not sufficient judging by current standards.

At present, the largest study is the case-control association study that examined 109 sequence polymorphisms in 35 candidate genes using a ~1,600 case-control cohort and found the association of TGF beta 3 (*TGFB3*) ($P=0.00040$). [27] *TGFB3* (MIM190230) is a well-known gene related to osteogenesis and located in the weak linkage region identified by the previous linkage study;[15] however, the association has not been replicated in other studies to my knowledge. Like other susceptibility genes so far reported, replication studies with decent scale are necessary for the association.

Future directions

The results of Karasugi et al.[17] indicate that OPLL is genetically heterogeneous, which is consistent with the vast diversity of its clinical features, including sex predominance, age at onset and prognosis by location of the lesion (*i.e.*, cervical, thoracic, lumbar) and type of ossification (*i.e.*, continuous, segmental, mixed). By stratification, *i.e.*, subgroup analysis based on clinical and demographic parameters, we can reduce the heterogeneity of the cases and hence expect to increase the power of detection in association studies. However, stratification is a trade-off with a decrease of the sample number. Larger scale studies enrolling thousands of subjects will be necessary. As linkage studies have a theoretical limitation in pinpointing the location of the susceptibility gene, association studies with high-density SNPs should be the future strategy. Like in other common bone and joint diseases,[28-31] genome-wide association study (GWAS) is awaited. Whole exome and whole genome sequencing are also promising approaches.

Since OPLL is a multi-factorial disease, both genetic and environmental factors must be clarified for better understanding of its etiology and pathology as well as for correct diagnosis, prediction of prognosis and effective treatment of the patients. One of the important future tasks is a longitudinal study of cohorts with detailed clinical information that could evaluate environmental factors based on the adjustment of genetic factors by genotyping results. In this point, larger scale studies will also be necessary. To accomplish such tasks within a certain period of time, international collaboration is the only way to go. I am optimistic because international collaborations have succeeded in many association studies of bone and joint diseases.[32-35]

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骨関節疾患におけるゲノム医学研究の現状と展望

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骨・関節疾患における
ゲノム医学の進歩

骨関節疾患におけるゲノム医学研究の現状と展望

池 川 志 郎*

要旨：ヒトゲノムプロジェクトを出発点とするゲノム研究の急速な進歩は，ゲノムワイド相関解析や全ゲノムシーケンスの成功となって結実した。多くの疾患で遺伝的要因が明らかになり，その成果が，整形外科領域を含む臨床医学を大きく変えようとしている。医療におけるパーソナルゲノム時代の到来が間近に迫っている。わが国での研究を中心に，整形外科疾患のゲノム医学研究の歴史，現状，そして展望について述べた。人類の共通の課題である骨関節の common disease/ありふれた疾患の解明，制圧に向けて，ゲノム疫学研究を効率的に進めていく必要がある。

はじめに
―ゲノム研究の進歩―

ヒトゲノムプロジェクト［http://www.nhgri.nih.gov/HGP］により，ドラフトシーケンス，すなわち，約30億のDNAの塩基からなるヒトゲノムの塩基配列の概略が決定されてから[1]，10年が経った。以来，ゲノム解析研究は，大方の科学者の予想をはるかに超えた速度で進展し続けている（表1）。

一時は，実現不可能とも言われていたヒトゲノムプロジェクトは，遺伝子特許などゲノムビジネスを目指す企業の参入とそれによって駆動されたシーケンス解析技術の革新的進歩により，2003年に予定を早めて終了した[2]。公表されたヒトゲノムのシーケンスを基に，一塩基多型（SNP；single nucleotide polymorphism）をはじめとするヒトの遺伝子多型（genetic polymorphism）のカタログ化が始まり，2002年には早くも，全ゲノム相関解析（GWAS；genome-wide association study）の最初の成功例が報告されている[3]。それは，日本人による心筋梗塞の疾患感受性遺伝子についての報告であった。さらに，HapMap project[4]の成功に伴い，SNPをはじめとする遺伝子多型を用いた相関解析が爆発的に発展・普及し，医学，社会に大きな影響を与えるようになった。2007年にはScience誌のbreak-through of the year，すなわちその年のNo. 1 scientific eventとして遺伝子多型研究とそれに基づくGWASが選ばれている（ちなみに，第2位はiPS/reprogramming）[5]。

2010年代に入ってからは，超高速シーケンスが，これも爆発的に発展・普及し，今や，1,000ドルも出せば，誰でも自分のゲノムの塩基配列，すなわち，生物としての自分のプログラムを知ることができる時代になろうとしている[6]。そして，その医療への適用として，ゲノム情報に基づいた，論理的で戦略的な医療体系が構築されようとして

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Genomic medicine of bone and joint diseases；present and future

Key words：Bone and joint disease, Genomic medicine, GWAS

表 1　ゲノム研究上の主な出来事（わが国に関係することを中心に）

1990	ヒトゲノムプロジェクト（Human Genome Project）の開始 Huntington 病の遺伝子の発見（ポジショナルクローニングの最初の成功例）
1999	ミレニアムプロジェクトの開始
2002	ヒトゲノムの標準塩基配列の概略（draft sequence）の決定 世界最初の人種特異的な SNP のデータベース（JSNP）の公開 世界最初の全ゲノム相関解析（GWAS；Genome-wide association study）の成功
2003	ヒトゲノムの標準塩基配列の決定 「個人の遺伝情報に応じた医療の実現プロジェクト（第 1 期）」が開始 バイオバンクジャパンの創設
2005	HapMap project による染色体地図，common SNP データベースの完成
2007	"Human genetic variation" が Science 誌の break-through of the year に 個人のゲノム配列の公開
2010	日本人 1 人の全ゲノム配列の決定
2011	東北メディカル・メガバンク 難病の次世代シーケンス拠点の開始

いる。臨床の現場で全ゲノムシーケンスが行われる時代，パーソナルゲノム時代の到来が間近に迫っている。先制医学，オーダーメイド医療，ゲノム創薬…，数年前には一部の医科学研究者，医師の夢だったことが，達成可能な現実の目標となってきた。ゲノム医学研究の成果が，臨床医学を変えようとしている。整形外科領域も例外ではない。

本稿では，わが国で行われている研究を中心に骨関節疾患のゲノム医学研究の現状と展望について述べる。

Ⅰ．ゲノム医科学の進歩

ゲノム研究の進歩は，多くの疾患の遺伝的要因を明らかにした。遺伝病には大別して，単一遺伝子病（monogenic disease）と多因子遺伝病（polygenic disease）がある。単一遺伝子病，すなわちメンデル式の遺伝をする狭義の遺伝病においては，患者さんの家系を用いた連鎖解析や候補遺伝子アプローチにより，多くの原因遺伝子が発見された。近年の超高速シーケンス技術の爆発的な進歩は，発見の速度をさらに加速させるだろう。多因子遺伝病とは，疾患感受性遺伝子と呼ばれる複数の原因遺伝子と環境要因の影響によって起こる疾患である。糖尿病，高血圧，骨粗鬆症といった"ありふれた"疾患（common disease），一般集団で非常に頻度の高い疾患の多くが，多因子遺伝病である。日本で始まった GWAS[3] により，一時代前には夢物語であった，ゲノムの中に隠れているこの多因子遺伝病の原因，疾患感受性遺伝子の同定が可能となった。

Ⅱ．骨関節疾患のゲノム医科学

骨関節疾患におけるゲノム医科学研究の対象も，単一遺伝子病と多因子遺伝病の 2 つカテゴリーに分けられる。骨関節の単一遺伝子病としては，骨系統疾患（skeletal dysplasia），結合組織疾患（connective tissue disease）などがある。いずれもまれな難病の集合である。代表的な骨系統疾患としては骨形成不全症，軟骨無形成症がある。代表的な結合組織疾患としては Marfan 症候群，Ehlers-Danlos 症候群がある。骨関節の多因子遺伝病としては，関節リウマチ，骨粗鬆症，変形性関節症，強直性脊椎炎，椎間板ヘルニア，大腿骨頭壊死，側弯症，股関節脱臼など多くの疾患が挙げられる。整形外科領域は，多因子遺伝病の宝庫である。

Ⅲ．骨関節疾患のゲノム医科学研究における日本の貢献

骨関節疾患のゲノム医科学研究に対して，わが国は大きな貢献をしてきた。単一遺伝子病については，骨系統疾患コンソーシウム［http://www.riken.jp/lab-www/OA-team/JSDC/］を中心とする日本の研究者により，短体幹症，脊椎骨幹端異形成症など数多くの原因遺伝子が同定されている。嚆矢となったのが，長崎大学の新川ら[7] のグループの Camurati-Engelmann 病（MIM 131300）の原因遺伝子の発見である。彼らは，骨形成の亢

進を主徴とするこの疾患の原因遺伝子 *TGFB1* を，日本人の大家系を用いた連鎖解析とそれに続く候補遺伝子アプローチにより同定した。*TGFB1* 遺伝子は，成長因子の TGF-β1（transforming growth factor β1）をコードする。TGF-β は，多様な機能を持つ大きな成長因子ファミリーで，骨・軟骨の発生・分化に深く関与する。Camurati-Engelmann 病での原因遺伝子の発見は，TGF-β ファミリーに属する遺伝子が原因遺伝子とわかった骨関節疾患の最初の例である。以来，多くの TGF-β/BMP ファミリーの遺伝子が骨関節疾患の原因遺伝子として同定された[8]。

多因子遺伝病については，鹿児島大のグループが，1998 年にユタ大学の Leppert の率いるグループが日本人の OPLL サンプルを用いて罹患同胞対法による連鎖解析（sib-pair linkage analysis）を行い，21 番染色体上に OPLL の感受性遺伝子座位を同定するのに大きな貢献をした[9]。これは，罹患同胞対法による連鎖解析の，骨関節疾患における最初の成功例である。

相関解析については，理化学研究所（理研）SNP 研究センター（現，統合生命医科学研究センター）/東京大学リウマチ膠原病内科の山本のグループが，関節リウマチに対して大規模相関解析を行い，世界に先駆けて，疾患感受性遺伝子 *PADI4*（peptidylarginine deiminase 4）を発見している[10]。われわれのグループは，2005 年に世界に先駆けて，変形性関節症（変形性股関節症）の GWAS を行った[11]。以来，変形性膝関節症[12]，骨粗鬆症[13]，特発性側弯症[14][15]，椎間板ヘルニア[16]で GWAS を行い，疾患感受性遺伝子を同定している。

Ⅳ．ゲノム解析研究における日本のアドバンテージ

ゲノム解析研究，特に相関解析を行うのに日本にはいくつかの有利な点がある。

1．人種的な均一性が高い

症例（case）-対照（control）間の疾患以外の属性の差は，当然，相関解析において擬陽性をもたらす確率が高くなる。ロシア人を対照として，ナイジェリア人の変形性関節症の患者を用いて相関解析をする－といった研究を考えれば，自明であろう。とはいえ，日本人も単一の民族ではなく，本土クラスター，沖縄クラスターの 2 つの遺伝的なパターンに分かれる。前者が 90% である[17]。しかし，『イギリス人』や『アメリカ人』に比べて，はるかに均一性が高いことは自明であろう。集団の均一性が高いことは，集団・人種特異的な低頻度の多型が解析上の問題となる次世代シーケンス解析でも非常に有利である。

2．医療の水準が高い

遺伝学は結局のところ，表現型と遺伝子型の対応関係を調べていく学問である。出発点の表現型，すなわち診断が間違っていては当然，良い結果は得られない。臨床情報がきちんと把握・理解されていなければ，仕事にならない。例えば，単一遺伝子病の変異の解析において，誤った診断のもとに，見当違いの遺伝子をシーケンスしても，決して遺伝子変異はみつからないだろう。多因子遺伝病の相関解析で患者群に違う疾患が混入すれば，正しい結果は出ないであろう。

3．人口が多い

相関の検出力，精度はサンプルの数に依存する。母集団の人口が少ないと，発生率，有病率の低い疾患では，必要な数の患者サンプルを集めることが不可能になる。骨系統疾患，結合組織疾患の多くは，発生率 1/100,000 以下の希少疾患である。母集団の大きさは，相関解析でも問題となる。有病率 1% の疾患の患者を 1,000 人集めるには，最低 10 万人の母集団が必要である。よって common disease でも有病率があまり高くない疾患では，地域コホートによる研究は難しい。

Ⅴ．ゲノム疫学

今後の医学研究において，国際的な競争に勝つには，これらのアドバンテージを生かして戦略的に研究を進めていく必要がある。その際の鍵となるのがゲノム疫学研究である。Common disease は遺伝的要因と環境要因の総合的な作用，ないし相互作用によって発症するわけだから，両方の要因を現実の患者さんから直接的に探るゲノム疫学

の手法は有効である。

ゲノム疫学には，出発点となるコホートを整備する必要がある。ゲノム疫学のためのコホートは，疾患コホートと住民コホートに大別される。各コホートには，費用，検体の収集の容易さ，環境因子の均一化など，研究上の一長一短がある。例えば，疾患コホートは，患者群を集めるのが容易で，日常の臨床がそのまま患者検体の収集につながる。一方，対照群については，診断にコストがかかる疾患においては収集が難しくなることが多い。住民コホートの利点は，コホートのサイズや地理的条件にもよるが，環境因子が比較的均一化されることである。一番の欠点は，有病率が高くない疾患では，患者群の数が十分でなくなることである。

Ⅵ．ゲノム解析を念頭に置いたコホート

わが国でも，ゲノム解析を念頭に置いたいくつかのコホートが形成されている。以下，主なものを挙げる。

1．ゲノム解析を念頭に置いた疾患コホート

1）思春期特発性側弯症

側弯症臨床学術研究グループは，慶応義塾大学の松本守雄准教授を中心とする側弯症の専門医集団で構成されたグループ（北海道大学，獨協医科大学，東京都済生会中央病院，聖隷佐倉市民病院，名城病院，神戸医療センター，福岡市立こども病院，北里研究所病院，順天堂大学）である。このグループは，3,000例近い特発性側弯症患者の疾患情報とDNAを収集した。この世界最大規模の側弯症の疾患コホートを用いて，理研・統合生命医科学研究センターとの共同研究で，2011年に世界に先駆け特発性側弯症の発症に関連する遺伝子*LBX1*を[14]，2013年には*GPR126*を発見している[15]。

2）椎間板ヘルニア

慶応義塾大学（千葉一裕准教授），富山大学（川口善治准教授），京都府立大学（三上靖夫准教授），函館中央病院（金山雅弘部長）の4施設からなるグループは，1,500例近い腰椎椎間板ヘルニア患者の疾患情報とDNAとを収集し，理研・統合生命医科学研究センター，香港大学ほかとの国際共同研究で，椎間板ヘルニア/椎間板変性症に関連する遺伝子*CHST3* (chondroitin sulfate sulfotransferase 3) を発見している[16]。

3）厚生労働省の難治性疾患等克服研究事業

特発性大腿骨頭壊死班（班長：九州大学岩本幸英教授），脊椎靱帯骨化症班（班長：慶応義塾大学戸山芳昭教授）の2つの研究班で，疾患コホートを収集している。All Japanの体制で，患者情報とDNAとを収集し，GWASを行おうとしている。

2．住民（地域）コホート

1）三重大学コホート

三重大学の内田淳正教授，須藤啓広教授らのグループは，1997年より，三重県で住民検診を利用した地域コホートにより，骨粗鬆症，変形性関節症の疫学研究を行っている[18][19]。異なる地域特性を持つ住民コホート，すなわち，山間部に位置し，農業，林業を中心とする宮川村と，海に面して，漁業，農業を中心とする南勢町の2つのコホートを設定しているのが特徴である。これらのコホートのゲノム解析が，理研との共同研究で行われており，既に，いくつかの疾患感受性遺伝子の発見につながっている[20]~[23]。

2）ROAD

東京大学整形外科のグループ（川口浩准教授，中村耕三教授）は，2005年から，住民コホートによる変形性関節症の疫学研究，ROAD(Research on Osteoarthritis Against Disability）に取り組んでいる。異なる地域特性を持つ住民コホートを設立したのが特徴で，都市部として東京都板橋区，山村部として和歌山県日高川町，漁村部として和歌山県日高川町太地町を選び，都市型コホート1,350人，山村型コホート864人，漁村型コホート826人のデータを収集している[24]。この3つのコホートの約3,000人のベースライン調査を出発点にゲノム解析と追跡調査により，変形性関節症の危険因子の同定，およびその経過に及ぼす因子を解明することを目指している。同じコホートを用いて，中途から骨粗鬆症に対する同様の疫学研究も行われている[25]。

また，和歌山県立医科大学整形外科のグループ

は，ROADの内の和歌山県の2コホートを用いて，椎間板ヘルニア，椎間板変性症のゲノム疫学研究を計画している。日本，中国，香港，フィンランド，英国などが参加する国際コンソーシアムInternational Spine and Pain Consortium（ISPC）が始まっており，世界規模のゲノム疫学研究が行われる予定である。

おわりに

骨関節の遺伝病は，人類の共通の課題である。特に骨関節のcommon disease/ありふれた疾患は現在，および将来の世界中の，医学的，社会的，経済的な深刻な問題である。ゲノム研究，特にゲノム疫学研究は，common diseaseに目の前の現実の患者さんからアプローチすることのできる有効な方法である。研究上，一番の問題である研究の規模，サンプルサイズの問題を克服するには，コホート間の協力，データの統合が必要である。

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整形外科用語の散歩道

国分正一

462. Soleus muscle ヒラメ筋

下腿三頭筋 triceps sulae におけるヒラメ筋の特長を考えてみた。1関節筋が上腕三頭筋で2つであるのに対して，下腿では本筋のみである。腓腹筋の2頭に覆われるが，アキレス腱の両脇に一部顕われ，触診が可能である。遅筋線維の割合が優位の由で，myogelosis になりにくい筋と読める。

Johann Vesling（1598-1649）が本筋を，Dover に限らず広く大西洋・北海・地中海で獲れるヒラメ Solea solea に似るとして，soleus と名付けた。確かに左側の筋の後姿が似る。彼はドイツの Minden に生まれ，イタリアは Venezia の西 40 km の Padova で解剖学・外科学教授となった。1632年に著した解剖書 "Syntagma Anatomicum" は1759年の山脇東洋の『蔵志』発刊に影響を与えた。

「左ヒラメに右カレイ」は日本での見分け方である。ところが Dover sole は右向き right-eyed である。替って，「大口ヒラメに口細カレイ」，あるいは歯の大と小が万国共通の決め手らしい。

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Enhanced Chondrogenesis of Induced Pluripotent Stem Cells From Patients With Neonatal-Onset Multisystem Inflammatory Disease Occurs via the Caspase 1–Independent cAMP/Protein Kinase A/CREB Pathway

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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-1β (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 was undertaken to investigate the effect of mutated NLRP3 on chondrocytes using induced pluripotent stem cells (iPSCs) from patients with NOMID.**

Methods. **We established isogenic iPSCs with wild-type or mutant NLRP3 from 2 NOMID patients with NLRP3 somatic mosaicism. The iPSCs were differentiated into chondrocytes in vitro and in vivo. The phenotypes of chondrocytes with wild-type and mutant NLRP3 were compared, particularly the size of the chondrocyte tissue produced.**

Results. **Mutant iPSCs produced larger chondrocyte masses than wild-type iPSCs 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. **Our findings indicate 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 monocyte/macrophages, and in chondrocytes (4). In monocyte/macrophages, once NLRP3 is activated by its ligand, a multiprotein complex called the NLRP3 inflammasome forms, resulting in the activation of caspase 1, which cleaves pro–interleukin-1β (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 radiologic imaging and histologic analyses of tissue biopsy specimens 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 pathologic 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 and type XI collagens, and proteoglycans, such as aggrecan and cartilage oligomeric matrix protein. 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 due 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 pathophysiologic mechanism underlying epiphyseal overgrowth in NOMID. In this study, we applied a newly developed chondrocyte differentiation system to induced pluripotent stem cells (iPSCs) 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 iPSCs from 2 NOMID patients with NLRP3 somatic mosaicism (p.Tyr570Cys and p.Gly307Ser) were established as previously described (17). From each patient, at least 3 clones of iPSCs with mutant or wild-type NLRP3 were established. In all experiments, mutant and wild-type isogenic cells were compared (3). Details of the procedure for culture of undifferentiated iPSCs and chondrogenic differentiation are available from the author upon request. The iPSCs were seeded onto a Matrigel (Becton Dickinson)–coated dish, cultured in mTeSR medium (StemCell Technologies) 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 (Figure 1A).

Chondrogenesis assay. Serum-free chondrogenic medium has been described previously (18). Two-dimensional

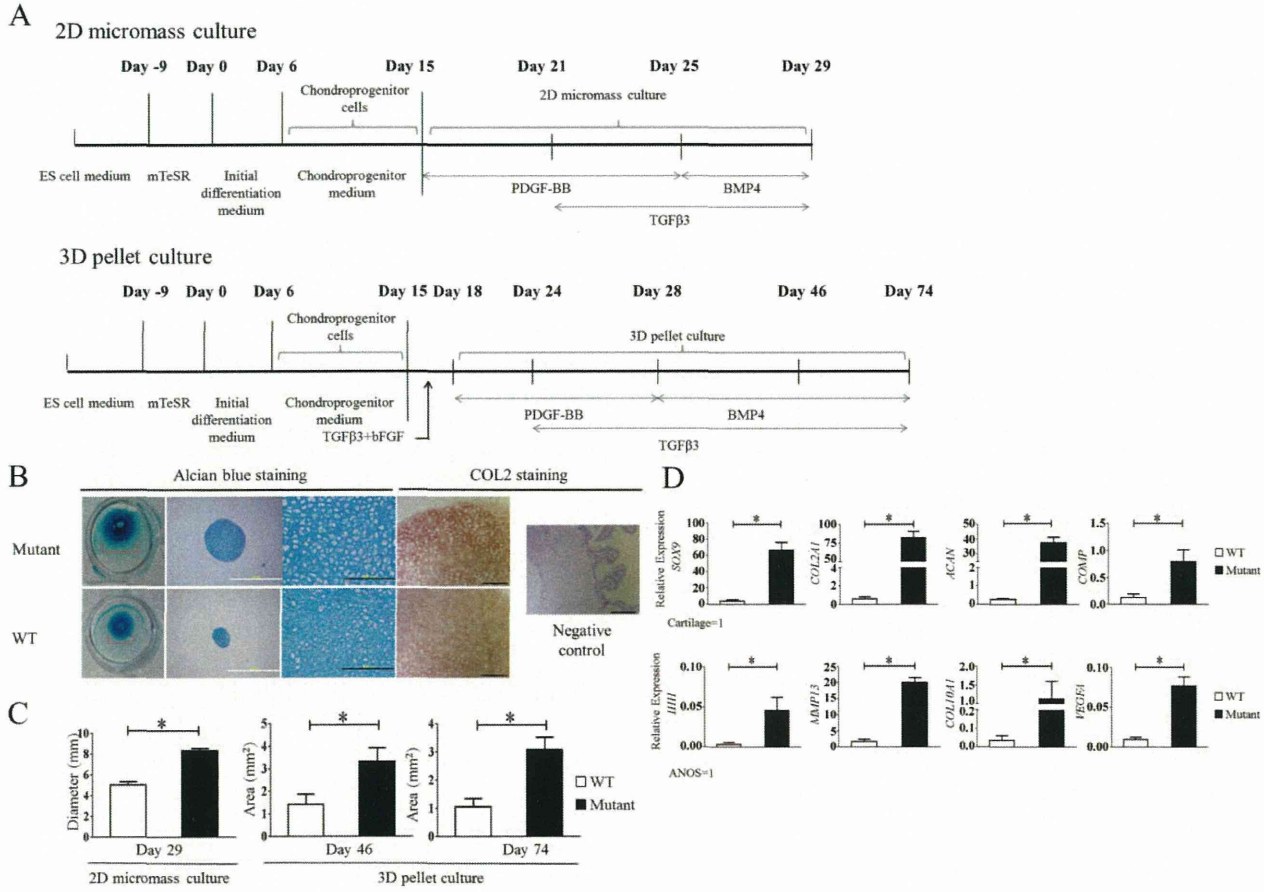

Figure 1. Successful differentiation of chondrocytes from induced pluripotent stem cells (iPSCs) with wild-type (WT) or mutant NLRP3 obtained from a patient with neonatal-onset multisystem inflammatory disease. **A,** Schematic representation of the culture conditions used to differentiate chondrocytes from iPSCs. **B,** Immunohistochemical staining of chondrocytes differentiated from iPSCs. Alcian blue staining of the 2-dimensional (2-D) micromass culture, Alcian blue staining of the 3-dimensional (3-D) pellet culture, higher-magnification images of Alcian blue staining of the 3-D pellet culture, anti–type II collagen (anti-COL2) antibody staining of the 3-D pellet culture, and anti–type II collagen antibody staining of mouse bladder (negative control) are shown. White bars = 2.0 mm; black bars = 0.2 mm. **C,** Quantitative analysis of the sizes of chondrocyte tissue masses in 2-D micromass cultures and 3-D pellet cultures. **D,** Cartilage-specific gene expression in 3-D pellet cultures. Expression of mRNA for each gene is shown relative to that in human cartilage (for *SOX9*, *COL2A1*, *ACAN*, and *COMP*) or the osteosarcoma cell line ANOS (for *IHH*, *MMP13*, *COL10A1*, and *VEGFA*), which were both set at 1. Bars in **C** and **D** show the mean ± SEM of 3 independent clones, from which duplicate measurements (**C**) or triplicate measurements (**D**) were obtained. Data are representative of 3 independent experiments and were obtained using iPSCs from patient 1 (p.Tyr570Cys); similar data were obtained using iPSCs from patient 2 (p.Gly307Ser). * = $P < 0.05$. ES = embryonic stem; PDGF-BB = platelet-derived growth factor BB; BMP-4 = bone morphogenetic protein 4; TGFβ3 = transforming growth factor β3; bFGF = basic fibroblast growth factor.

(2-D) 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 of platelet-derived growth factor BB (PDGF-BB; R&D Systems) and 1% fetal calf serum. The medium was changed every 3 days. Beginning on day 21, 10 ng/ml of transforming growth factor β3 (TGFβ3; R&D Systems) was added, and beginning on day 25, 40 ng/ml of PDGF-BB was replaced with 50 ng/ml of bone morphogenetic protein 4 (Wako). For 3-dimensional (3-D) pellet cultures, chondroprogenitor cells were passaged once in chondroprogenitor medium containing 5 ng/ml of basic fibroblast growth factor and 10 ng/ml of 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 medium supplemented with specific factors as outlined

above. Fixation and staining of the 2-D micromass and 3-D 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 3-D cell pellet cultures over 20 days were wrapped in a 0.5 cm × 1 cm Gelfoam (Pfizer) 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 (H&E), 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 Technology). Antibodies against CREB, phosphorylated CREB (Cell Signaling), and β-actin (Santa Cruz Biotechnology) 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. Quantitative reverse transcriptase–polymerase chain reaction 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 (5′-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 transcription database (24). Sequence information is provided in Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38912/abstract. Chondroprogenitor cells were plated at a density of 50,000 cells/well in 6-well plates, transfected with 2 μg of DNA/well using the FuGene 6 transfection reagent, and harvested 24 hours after transfection. Luciferase activity was measured as previously described (23). Additionally, 10 μ*M* forskolin (Sigma) and 10 μ*M* SQ22536 (Sigma) was used to activate and inhibit adenylate cyclase, respectively.

Ethics approval. 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 and provided informed consent that the sample could be used for research purposes.

Statistical analysis. Data were analyzed using Student's *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

Production of a large cartilaginous mass by chondrocytes differentiated from iPSCs with mutated NLRP3. To investigate the pathophysiology of NOMID arthropathy, we attempted to recapitulate this phenotype by using chondrocytes that were differentiated from iPSCs derived from patients with NOMID. We obtained iPSCs from 2 NOMID patients with arthropathy who had *NLRP3* somatic mosaicism, as previously described (17). We established isogenic iPSC clones that had mutated or wild-type NLRP3, which allowed us to examine the effects of NLRP3 mutations in the same genetic background (25,26).

To produce chondrocytes from these iPSCs, we used a protocol in which chondrocytes are obtained from cells of neural crest character (details are available from the author upon request) (Figure 1A). We performed 2 chondrogenic assays, a 2-D micromass culture assay and a 3-D 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 type II collagen, which is specifically expressed in chondrocytes (Figure 1B). After culture in chondrogenic medium, cells derived from wild-type and mutant iPSCs were positive for Alcian blue and type II collagen, which confirmed that the iPSCs had successfully differentiated into chondrocytes. Importantly, the 2-D micromass and 3-D pellet samples derived from mutant iPSCs were significantly larger than those derived from wild-type iPSCs, up to day 74 of culture (Figures 1B and C).

Next, we examined the expression of mRNA for cartilage-related genes expressed in proliferating chondrocytes (early markers; *SOX9*, *COL2A1*, *ACAN*, and *COMP*) and those expressed in hypertrophic chondrocytes (late markers; *IHH*, *MMP13*, *COL10A1*, and *VEGFA*) in samples obtained by the method described above (Figure 1D). These genes were expressed in 3-D pellet samples obtained from mutant and wild-type iPSCs, further indicating that chondrocyte differentiation was successful and that 3-D 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 wild-type samples (Figure 1D). Taken together, these data show that chondrocytes were successfully differentiated in

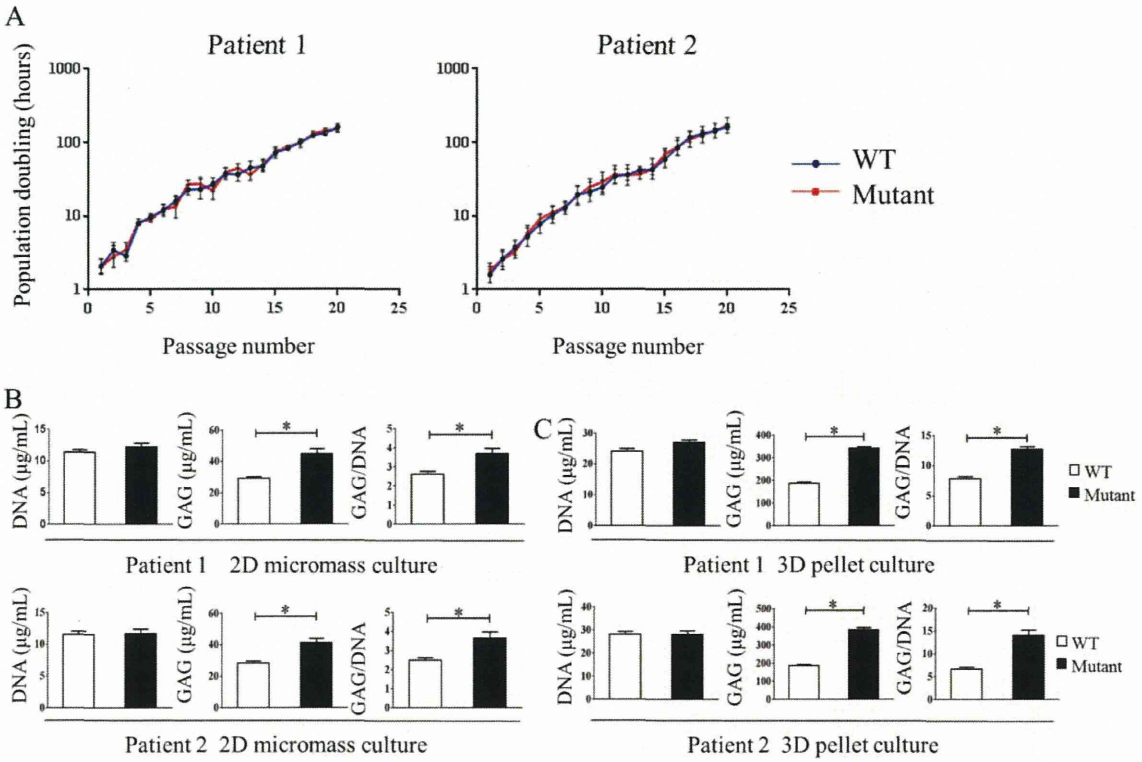

Figure 2. Formation of large cartilaginous masses by mutant iPSCs from patients with neonatal-onset multisystem inflammatory disease is due to the overproduction of extracellular matrix and not due to increased cell proliferation. **A,** Growth curves of chondroprogenitor cells differentiated from mutant and wild-type iPSCs. Values are the mean ± SEM of 3 independent clones, from which duplicate measurements were obtained. **B** and **C,** DNA concentration, glycosaminoglycan (GAG) concentration, and the ratio of GAG concentration to DNA concentration in 2-D micromass (**B**) and 3-D pellet (**C**) cultures. Bars show the mean ± SEM of 3 independent clones, from which triplicate (**B**) or duplicate (**C**) measurements were obtained. Data are representative of 3 independent experiments with consistent results. * = $P < 0.05$. See Figure 1 for other definitions.

vitro from iPSCs derived from NOMID patients, and that chondrocytes differentiated from iPSCs 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 wild-type cells.

The production of large cartilaginous masses by mutant iPSCs is due to ECM overproduction, not to increased cell proliferation. We next sought to determine what causes the chondrocyte masses derived from mutant iPSCs to be larger than those derived from wild-type iPSCs. First, we analyzed the proliferation potential of chondroprogenitor cells. Population doubling time did not significantly differ between mutant and wild-type chondroprogenitor cells up to 15 passages, after which the cells stopped proliferating (Figure 2A). Next, we determined the number of differentiated chondrocytes by analyzing DNA content and GAG production, which is a major cartilaginous ECM component. In 2-D micromass and 3-D pellet cultures, the DNA content in differentiated chondrocyte tissue derived from mutant and wild-type iPSCs did not significantly differ. This suggests that a similar number of chondrocytes were produced from mutant and wild-type iPSCs. In contrast, chondrocytes derived from mutant iPSCs produced more GAG than those derived from wild-type iPSCs in 2-D micromass culture (Figure 2B) and 3-D pellet culture (Figure 2C). These data indicate that the larger amount of chondrocyte tissue produced from mutant iPSCs 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 iPSCs reveals dysregulated endochondral ossification. Radiologic examination of affected long bones in NOMID patients shows enlargement of the epiphysis

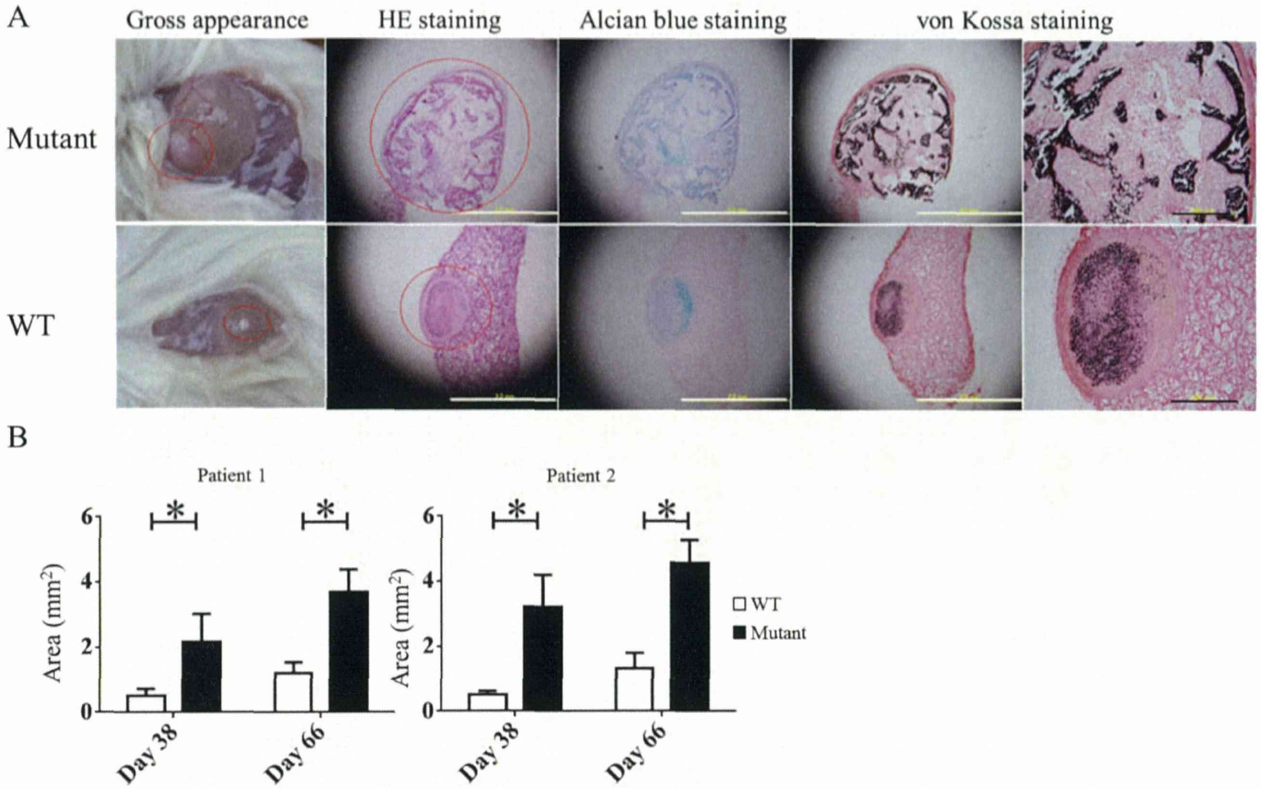

Figure 3. In vivo maturation of 3-D cell pellets derived from mutant or wild-type iPSCs from patients with neonatal-onset multisystem inflammatory disease. **A,** Images of 3-D cell pellets derived from mutant or wild-type iPSCs following transplantation into immunodeficient mice. Gross appearance, hematoxylin and eosin (H&E) staining, Alcian blue staining, von Kossa staining, and higher-magnification images of von Kossa staining are shown. Red circles indicate bone or cartilage pellets in gel form. White bars = 2.0 mm; black bars = 0.2 mm. Results shown were obtained using iPSCs from patient 1; similar results were obtained using iPSCs from patient 2. **B,** Quantitative analysis of the size of pellets when they were transplanted (day 38) and harvested (day 66). Bars show the mean ± SEM of 3 independent clones, from which duplicate measurements were obtained. Data are representative of 3 independent experiments. * = $P < 0.05$. See Figure 1 for other definitions.

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 3-D pellet samples were transplanted into NOG mice. The transplanted cartilage mass was vascularized in vivo (Figure 3A). Mutant pellets were larger than wild-type pellets, both at transplantation and harvesting, and this size difference increased during in vivo differentiation (Figure 3B). Following von Kossa staining, which detects calcium deposits, calcification was detected in both wild-type and mutant pellets (Figure 3A). Interestingly, Alcian blue staining revealed that mutant pellets contained more residual cartilage components than wild-type 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 wild-type pellets. Taken together, these data indicate that in our in vivo model, chondrocyte tissue differentiated from mutant iPSCs grows larger and exhibits disorganized ossification compared to chondrocyte tissue differentiated from wild-type iPSCs.

The enhanced chondrogenesis of mutant iPSCs 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