Downregulation of GGPPS in chondrocytes accelerates osteoarthritis progression

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Abstract: Geranylgeranyl pyrophosphate synthase (GGPPS) is a key protein in the mevalonate metabolic pathway, catalyzing the synthesis of geranylgeranyl pyrophosphate (GGPP), which is essential for protein geranylgeranylation. The mevalonate pathway has been reported to participate in the regulation of articular cartilage homeostasis. The role of statins in treating osteoarthritis remains unclear and controversial. Meanwhile, the adverse effects of statins, due to their total inhibition of protein prenylation, are increasingly recognized. We investigated the role of GGPPS in the development of osteoarthritis (OA). We observed downregulated GGPPS protein levels in both mice OA cartilage and human OA samples. GGPPS deficiency aggravated the development of cartilage destruction after surgically induced destabilization of the knee joint, at both 8 and 10 weeks after surgery. In another ageing animal model, the articular cartilage of GGPPS cKO mice exhibited more proteoglycan loss than WT mice at both 4 and 10 months old. We also observed that disruption of GGPPS led to elevated chondrocyte apoptosis at an early stage. Relative to their normal cartilage counterparts, in vitro mRNA levels of ADAMTS5 and MMP-13 were increased 231% and 228%, respectively, in the absence of GGPPS. This correlation between GGPPS expression level and OA progression suggests that GGPPS plays a critical role in maintaining cartilage homeostasis.

Keywords: GGPPS, osteoarthritis, collagen-II cre, ageing, metabolism

Introduction

Osteoarthritis (OA) is the most prevalent chronic joint disorder in the elderly. This disease is characterized by progressive degradation of articular cartilage, which eventually leads to uncontrollable pain and the loss of joint function [1]. Although a variety of risk factors have been identified as contributors to OA pathogenesis, such as heredity, metabolism, and mechanical stress, the exact mechanisms underlying OA remain unclear [2]. No disease-modifying drug efficaciously treats OA.

Several studies have found that statins may have beneficial effects on OA joints [3-6], although the evidence for such effects on human OA remains very limited. Some reports have arrived at the opposite conclusion [7, 8]. Statins have been widely used in clinical practice and sometimes exert severe side effects when treating cardiac hypertrophy and heart failure [9]. We hypothesize that these adverse effects may result from statin suppression of protein geranylgeranylation and protein farnesylation. This hypothesis motivated us to study GGPPS, which is downstream of the mevalonate pathway. We investigated the potential role of GGPPS in osteoarthritis.

Recently, several independent groups have reported that small GTPases have a fundamental role in governing chondrocyte development, hypertrophy, and maturation during endochondral bone formation [10-13]. Rac1, which is one of the most important small GTPases, has been
reported to regulate chondrocyte condensation via N-cadherin, and stimulates chondrogenesis and chondrocyte hypertrophy [13]. Activated Rac1 promoted chondrocyte expression of MMP13, ADAMTS-5, and COLX, partially through the β-catenin pathway [14]. RhoA has been shown to play an opposite role to Rac1 in chondrocyte maturation [11, 15]. These chondrocyte-related G protein functions require post-translational modification for their membrane localization; GGPPS regulates this post-translational modification.

GGPPS is a key protein in the mevalonate metabolic pathway that catalyzes the synthesis of GGPP. Mevalonate-derived prenyl groups (GGPP and FPP) have been shown to play essential roles in many protein prenylation-related cellular functions including cell signaling, cell differentiation, proliferation, and cytoskeleton dynamics [16]. The loss of the isoprenoid intermediate-GGPP due to statin treatment accounted for the inhibition of MMPs expression, and signaling pathway activation [4]. Another in vitro study found that exogenous addition of GGPP completely reversed the statin-mediated decrease in MMP-13 mRNA and protein levels. These results demonstrate that statins modulate chondrocyte metabolism by reducing prenylation of key signaling molecules that control the expression of collagen-degrading enzymes [17]. However, inhibition of protein geranylgeranylation induced more cell apoptosis in synovial fibroblasts, and had a protective role in RA [18]. An in vivo experiment in mice indicated that reduced geranylgeranylation in macrophages can initiate erosive arthritis [19]. Although several lines of evidence support the model of a tight association between GGPPS and articular cartilage metabolism, the role of GGPPS in cartilage homeostasis is unknown.

The role of GGPPS has been revealed in a range of human diseases such as pulmonary inflammation, male infertility, heart failure, and diabetes [9, 20, 21]. Cell type-specific KO experiments have demonstrated clear tissue-specific functions for GGPPS. However, it remains unknown whether and how GGPPS functions in chondrocytes to regulate cartilage tissue homeostasis and integrity.

In the present study, we conditionally deleted GGPPS using Cre transgenic mice (Collagen 2-CreERT). We showed that early postnatal deletion of GGPPS in chondrocytes increases cell death, decreases matrix proteoglycan content, and can cause mild OA-like structural changes both in a DMM OA model and in an ageing mouse model. These findings suggested that GGPPS is essential for chondrocyte survival and structural integrity of the articular cartilage.

Materials and methods

Mice

C57BL6/J mice were used to examine GGPPS expression and location in the development of OA. COL2α1-CreERT2 GGPPSfl/fl mice with GGPPS deficiency were obtained from offspring of GGPPSfl/fl mice bred with COL2α1-CreERT2-transgenic mice. GGPPS cKO mice and their Cre-negative wild-type (WT) control littermates were intraperitoneally injected, at age 2 weeks, with tamoxifen at a dose of 1 mg/10 g body weight, administered daily for 5 days. Genotyping for GGPPS and the Cre transgene was carried out using polymerase chain reactions (PCR). Mice were maintained under pathogen-free conditions and were freely allowed access to food, water, and activity. All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing University Medical School, and were carried out in accordance with Nanjing University Medical School IACUC guidelines.

Human cartilage samples

To obtain human articular cartilage, surgically removed human cartilage tissue was harvested from patients with OA who were undergoing total knee joint replacement, and from patients who were hospitalized at Drum Tower Hospital (Jiangsu, China) because of a traffic accident. We randomly selected four samples from each group. Collection of these samples was approved by the Medical School of Nanjing University ethics committees, and all individuals undergoing surgery provided written informed consent before the procedure.

OA model and ageing model

Experimental OA was induced at the right knee joint of 8-week-old wild-type, GGPPS-cKO, and control mice by transecting the medial meniscotibial ligament under a microscope [22]. Mice
were allowed completely free movement following DMM surgery. The mice were killed, and their knee joints were harvested at eight or ten weeks following surgery. To examine GGPPS expression changes during ageing, we analyzed 4-month-old and 14 month-old wild-type mice. To examine the effect of GGPPS disruption in chondrocytes during ageing, we calculated the degradation of GGPPS-cKO and control mice at 4 months and 14 months of age.

**Histological analysis**

The GGPPS-cKO and control mice (female and male) were killed at 2, 8, and 10 weeks after surgery; at the time of sacrifice each mouse was either 4 months old or 10 months old. The knee joints were fixed in 0.1 M phosphate buffer solution with 4% paraformaldehyde overnight at 4°C, decalcified for one week with 15% EDTA, and embedded in paraffin wax. Each specimen was cut into 5-μm slices along its coronal plane, and stained with safranin O-fast green. Three slices were selected from each medial femoral condyle and medial tibial plateau. The histological OA grade for each field was evaluated (scored 0-6) using the Osteoarthritis Research Society International (OARSI) cartilage OA histopathology grading system. OA grades were assessed by a single observer who was blind to the group membership of each slide.

**Immunohistochemistry**

After epitope retrieval, sections were incubated with primary antibody, followed by secondary antibody. The signal was developed as a brown reaction product, using the peroxidase substrate 3,3′-diaminobenzidine with hematoxylin counterstaining. TUNEL staining was performed with an In Situ Cell Death Detection kit (Promega, USA). All images were obtained under a microscope (Olympus, USA). The following antibodies were used: GGPPS (Proteintech, China), and MMP-13 (Abcam, USA).

**Cell culture and GGPPS knockdown assay**

Sw1353 human chondrosarcoma cells were grown to confluence in Dulbecco's-modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained at 37°C in an atmosphere of 5% CO₂. Knockdown experiments were carried out on SW1353.

Cells were transfected with siRNA for 48 h using the Lipofectamine™ 2000 reagent (Invitrogen), following the manufacturer’s instructions. Next, incubation for 6 h with 10 ng/mL IL-1β was performed before cell collection.

**Quantitative real-time polymerase chain reaction**

Total RNA was isolated from sw1353 cells using TRIzol. First-strand cDNA was synthesized using a Takara System, according to the manufacturer’s protocol (Takara). RNA was quantified spectrophotometrically based on A260 using an ND-1000 spectrophotometer (NanoDrop Technologies, USA). Complementary DNA was produced using a SuperScript First-Strand Synthesis System kit (Invitrogen). Messenger RNA levels of MMP1, MMP3, MMP13, ADAMTS4, ADAMTS5, were measured using SYBR green with an ABI real-time RT-PCR system (Applied Biosystems 7300). The expression levels of genes were defined based on the threshold cycle (Ct), and relative values were calculated using the 2-ΔΔCt method, after normalizing expression to beta-actin. Gene specific primer sequences are shown in Supplementary Table 1.

**Statistical analysis**

All data were expressed as means ± standard deviations. Differences between groups were compared using Student’s t test or the Mann-Whitney U test. Analyses were performed using the GraphPad Prism 5.0 software. A p-value < 0.05 was considered statistically significant.

**Results**

**GGPPS protein was decreased during OA progression in humans and wild-type mice**

We observed GGPPS protein for changes during ageing in the knee joints of wild-type mice. GGPPS protein was mainly observed in the superficial and middle zone in 4-month-old mice knee joints; 14-month-old mice exhibited more severe degeneration of cartilage (Figure 1A). GGPPS-positive cells were significantly decreased in 14-month-old mice compared...
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Figure 1. GGPPS protein is decreased during the progression of osteoarthritis in OA patients and wild-type ageing mice. A. Safranin-O-fast green staining and immunohistochemistry of GGPPS in the medial side of articular surface of 4 month-old and 14 month-old wild-type mice (scale bars are 200 μm). B. Quantitative analysis of GGPPS-positive cells from 4 month-old and 14 month-old mice. *P < 0.05, **P < 0.01, (n=4 per group). C. Safranin-O-fast green staining (scale bars are 500 μm) and immunohistochemistry of MMP-13 and GGPPS in human samples (scale bars are 200 μm). D. Quantitative analysis of MMP-13-positive cells of human OA samples and healthy controls. E. Quantitative analysis of GGPPS-positive cells of human OA samples and healthy controls. Percent GGPPS-positive cells was determined as the ratio of the total number of GGPPS-positive cells to the total number of chondrocytes (n=4 per group).

With 4-month-old mice (Figure 1B). To investigate the role of GGPPS in human OA pathogenesis, we measured GGPPS expression in human OA cartilage. Immunohistochemical study of the OA cartilage revealed significant downregulation of GGPPS expression and upregulation of MMP-13 expression (Figure 1C). Quantitative analysis of MMP-13 and GGPPS expression...
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Figure 1F. Moreover, GGPPS-positive cells had no differences between the two groups in kidney (12-week old; Supplementary Figure 1G).

Disruption of GGPPS in chondrocytes accelerated the development of experimental OA

To further examine the effects of GGPPS loss in chondrocytes during OA development, we created an experimental OA model using GGPPS-cKO mice. To quantify the severity of the joint damage, we analyzed histopathological changes using the OARSI scoring system. WT mice presented maximum scores of 0.90 ± 0.74, for the femur, and 1.00 ± 0.61, for the tibia, at 8 weeks following DMM surgery. In contrast, GGPPS-cKO mice presented significantly higher maximum scores, with 2.00 ± 0.71 for the femur, and 2.60 ± 0.55 for the tibia (*P < 0.05, **P < 0.01, Figure 3A, 3B). Because OA is a progressive disease in old patients, we checked articular change 10 weeks after surgery. WT mice had maximum scores of 1.25 ± 0.61 for the femur, and 2.33 ± 0.82 for the tibia. The maximum scores of GGPPS-cKO mice were also significantly higher (femur 2.83 ± 1.17, tibia 3.83 ± 1.17; *P < 0.05, **P < 0.01, Figure 3C, 3D).

GGPPS protein is decreased in a DMM induced OA model

We examined the protein change of GGPPS in a DMM surgery OA model. We found that WT mice 8 weeks following a DMM surgery exhibited more severe joint degeneration and lower GGPPS expression than control mice (Figure 2). To study the role of GGPPS in OA physiological and pathological processes, we generated cartilage specific GGPPS-cKO mice using the Cre-loxP system (Supplementary Figure 1). The DNA recombination between two loxP sites in the GGPPS allele by Cre recombinase was confirmed by the presence of a recombined allele (Supplementary Figure 1A). The efficiency of the loss of GGPPS expression in chondrocytes was confirmed by immunohistochemistry (IHC). IHC for GGPPS showed remarkably reduced GGPPS protein expression in the femoral epiphyses and tibial plateau of GGPPS-cKO mice (4-week old mice, Supplementary Figure 1B-E). GGPPS-positive cell analysis revealed a nearly 76.6% reduction in GGPPS protein in the knee joints of GGPPS-cKO mice (Supplementary Figure 1F). Moreover, GGPPS-positive cells had no differences between the two groups in kidney (12-week old; Supplementary Figure 1G).

Figure 2. GGPPS protein is decreased in a DMM induced OA model. A. Safranin-O-fast green staining and immunohistochemistry of GGPPS in the medial side of articular surface of OA and control mice (scale bars are 200 μm). B. Quantitative analysis of OARSI score of OA and control mice (n=4 per group). Total OA Score represents the OARSI score of medial femoral condyle and tibial plateau. C. Quantitative analysis of GGPPS-positive cells from OA and control mice (n=4 per group).
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**Figure 3.** Accelerated osteoarthritis (OA) progression in GGPPS-conditional knockout (cKO) mice in a DMM model. A. Safranin-O-fast green staining of the medial femoral condyle (MFC) and the medial tibial plateau (MTP) of WT and GGPPS deletion groups at 8 weeks following DMM surgery (n=8 per group). B. OARSI scores for the medial femoral condyle and tibial condyle in control and GGPPS-cKO mice at 8 weeks following DMM surgery. C. Safranin-O-fast green staining of the medial femoral condyle and tibial plateau of control and GGPPS deletion groups at 10 weeks following DMM surgery (n=8 per group). D. OARSI scores for the medial femoral condyle and tibial condyle in control and GGPPS-cKO mice. Scale bars are 200 μm; *P < 0.05; **P < 0.01.

**Disruption of GGPPS led immediately to an OA phenotype**

To examine the role of GGPPS in articular cartilage homeostasis, safranin-O and toluidine blue staining of the articular cartilage was performed in GGPPS-cKO mice and in control mice (Figure 4A-E). Our results revealed that tibial and femoral proteoglycan loss was significantly higher in GGPPS-cKO mice than in control mice, both at 4 and 10 months. This tibial and femoral proteoglycan loss worsened with time. Although the OARSI scores were low, there were statistically significant differences between the two groups (Figure 4E, 4F). These results indicated that progression of aging-related proteoglycan loss is aggravated by GGPPS deficiency.

**Increased protein levels of chondrocyte apoptotic markers in GGPPS-cKO mice**

Increased cell death was observed in articular cartilage of GGPPS cKO mice. To further examine the mechanism underlying accelerated OA progression in GGPPS-cKO mice, we examined the TUNEL-positive cells, which were increased in GGPPS-cKO mice relative to control mice at 2 weeks after surgery (Figure 5C, 5D). Quantitative analysis of the TUNEL-positive cells indicated a 45.4% increase for the GGPPS cKO group (Figure 5F).

**Relative expression of key genes associated with chondrocyte metabolism**

In a previous study, we showed that GGPPS disruption accelerated OA progression and significantly increased proteoglycan loss in two mouse models of OA. This finding led us to investigate in vitro effects of GGPPS on cartilage metabolism. Treatment of SW1353 chondrocytes with specific siRNAs led to a significant downregulation of GGPPS expression (Figure 6A). Knockdown of GGPPS upregulated the expression of ADAMTS and MMPs, which are involved in osteoarthritis development (Figure 6B-F). Relative to their normal cartilage counterparts, key genes associated with chondrocyte catabolism were upregulated in the absence of GGPPS. The mRNA levels of ADAMTS5 and MMP-13 were increased by 231% and 228%, respectively.
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Type II collagen degradation, and greatly contributes to OA development [26]. Genetically modified mice with constitutively active MMP-13 expression presented early development of OA, and MMP-13 deficiency protected mice against OA cartilage damage [27, 28]. The present study suggests that disruption of GGPPS may facilitate OA development by inducing expression of key genes known to cause cartilage degeneration.

We also found that disruption of GGPPS in articular cartilage induced more apoptosis of chondrocytes. Chondrocytes in OA cartilage demonstrated morphologic changes characteristic of apoptosis [29]. It has been reported that chondrocyte apoptosis is positively associated with degree of cartilage matrix damage, and that the extent of apoptosis varies with cartilage zone and mechanical loading environment of the joint [30]. Apoptotic death of articular chondrocytes has been implicated in the pathogenesis of human osteoarthritis, and several studies

Discussion

To the best of our knowledge, the present study is the first to demonstrate that cartilage samples with OA have decreased GGPPS levels in both human and mice samples (Figure 1). We found that GGPPS cKO mice exhibited more severe joint degeneration than exhibited by WT controls, at 8 and 10 weeks following DMM surgery. We provide evidence, for the first time, indicating that genetic inhibition of GGPPS in mouse articular cartilage aggravates OA development.

In vitro, we found that mRNA levels of ADAMTS5 and MMP-13 were increased 231% and 228%, respectively, in the absence of GGPPS. The proteolytic functions of the ADAMTS and MMPs play important roles in the development of OA. ADAMTS5 is responsible for aggrecan degradation [23, 24], an event occurring early in the development of OA [25]. Furthermore, MMP-13 is considered the most active collagenase for type II collagen degradation, and greatly contributes to OA development [26]. Genetically modified mice with constitutively active MMP-13 expression presented early development of OA, and MMP-13 deficiency protected mice against OA cartilage damage [27, 28]. The present study suggests that disruption of GGPPS may facilitate OA development by inducing expression of key genes known to cause cartilage degeneration.

Figure 4. Histologic changes related to aging in the articular cartilage of GGPPS conditional knockout (cKO) mice compared to wild-type (WT) (Cre-negative) littermate control mice. A-D. Coronal sections of knee joints from mice at age 4 months and 10 months were stained with Safranin-O-fast green to visualize gross morphologic differences in knee joint degeneration and loss of proteoglycan (n=4 per group). E, F. Toluidine blue staining of 10 month old GGPPS cKO and WT mice. G. Scores for proteoglycan loss in the medial femoral condyle (MFC) and medial tibial plateau (MTP) were determined in the knee joints of GGPPS cKO and WT mice at age 4 months. H. Scores for proteoglycan loss in the medial femoral condyle (MFC) and medial tibial plateau (MTP) in the knee joints of GGPPS cKO and WT mice at age 10 months. Scale bars are 200 μm; *P < 0.05; **P < 0.01.
have indicated that caspase inhibitors can reduce the severity of cartilage lesions in experimental OA [31].

These changes may be at least partly responsible for the accelerated progression of cartilage degeneration in GGPPS-deficient mice.

The present study has some limitations. Obtaining age matched controls without OA is challenging for studies of human OA. Therefore, cartilage analyses were performed using “relatively normal” cartilage. We found that the level of GGPPS was decreased in the OA samples when compared with the “relatively normal” samples. A DMM model mouse would further support this concept. At 8 weeks after surgery, the OARSI scores for the GGPPS cKO group were

Figure 5. Increased protein levels of apoptotic markers in GGPPS-cKO mice. A, B. Safranin-O-fast green staining of the medial femoral condyle and medial tibial plateau of WT and GGPPS deletion groups at 2 weeks following DMM surgery (n=4 per group). C, D. TUNEL staining in control and cKO mice (scale bars are 200 μm). E. The histological change between WT and cKO group after 2 weeks post DMM surgery. F. Quantitative analysis of the TUNEL-positive cell counts per field in the joint surfaces. The number of positive cells per field was counted under a microscope at 20× magnification using three sections from three mice.

Figure 6. Relative expression of key genes associated with chondrocyte metabolism when GGPPS was knocked down. Messenger RNA levels were quantified by RT-PCR and normalized to β-actin. The gene expression levels (A-F) were defined from the threshold cycle (Ct), and relative values were calculated by the 2-ΔΔCt method. Data are presented as means ± SD (n=3, in duplicate). *P < 0.05; **P < 0.01.
significantly higher; although degeneration of joints in both of our experimental groups was lower than previous reports had indicated [22]. Because OA was surgically induced by transecting the medial collateral ligament and resecting the medial meniscus, the OA severity varied between subjects. We made observations at two time points to ensure the robustness of this finding.

The present study provides evidence that GGPPS downregulation correlates with OA progression, and can stimulate expression of genes known to cause cartilage degradation in chondrocytes. We observed GGPPS play a critical role in maintaining cartilage homeostasis. Further study is warranted on the adverse effects of statin-induced oversuppression of geranylgeranylation, and GGPPS as a therapeutic strategy.

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Disclosure of conflict of interest

None.

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References


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Supplementary Table 1. Primer sequences for RT-PCR analysis

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Supplementary Figure 1. GGPPS expression in GGPPS-conditional knockout (cKO) mice and control group. A. Genotyping confirmed the presence of the Cre transgene in heterozygote (GGPPS<sup>fl</sup>/<sup>-</sup>) and homozygote (GGPPS<sup>fl/fl</sup>) mice, and its absence in wild-type mice. B-E. Immunohistochemical staining for GGPPS confirmed the absence of GGPPS expression in the articular cartilage of 1 month-old GGPPS<sup>fl/fl</sup> Cre mice treated with tamoxifen compared with control mice (n=3); B and C. Magnification: x200; D and E. Magnification: x400. F. Quantitative analysis of GGPPS-positive cells at each groups (**P < 0.01). G. Immunohistochemistry for GGPPS using the kidney of 16-week-old control and GGPPS-cKO mice (DAB, scale bars =200 μm).