Intra-articular Injection of SHP2 Inhibitor SHP099 Promotes the Repair of Rabbit Full-thickness Cartilage Defect

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Abstract

Background

Cartilage repair has been a challenge in the field of orthopedics for decades, highlighting the great significance of investigating potential therapeutic drugs. In this study, we explored the effect of SHP2 inhibitor, SHP099, as a small molecule drug on cartilage repair.

Methods

Human synovial mesenchymal stem cells (SMSCs) were isolated and their three-way differentiation potential was examined. After treated with chondrogenic medium, the chondrogenic effect of SHP099 on SMSCs was examined by Western blot, qPCR, and immunofluorescence (IF). To explore chondrogenic effects of SHP099 in vivo, full-thickness cartilage defects with microfracture were constructed in the right femoral trochlear of New Zealand White rabbits. Intra-articular injection of SHP099 or normal saline were performed twice a week for 6 weeks. Cartilage repair were evaluated by hematoxylin and eosin (H&E) staining, Safranin O/Fast Green staining. Immunohistochemistry (IHC) for collagen II (COL2) was also conducted to verify the abundance of cartilage extracellular matrix after SHP099 treatment. The mechanism involving yes associated protein (YAP) and WNT signaling was investigated in vitro.

Result

The SMSCs isolated from human synovium represented optimal multi-differentiation potential. SHP099 increased chondrogenic markers (SOX9, COL2) expression and decreased hypertrophic markers (COL10, RUNX2) in SMSCs. The inhibition of YAP and WNT signaling was also observed. Moreover, compared with the normal saline group at 6 weeks, intra-articular injection of SHP099 resulted in better defect filling which formed more hyaline cartilage-like tissue with more glycosaminoglycan (GAG) and COL2.

Conclusion

SHP099 promotes the repair of rabbit full-thickness cartilage defect, representing a potential therapeutic drug for cartilage repair.

Introduction

Articular cartilage is a layer of hyaline cartilage that covers the end of the bone, which is rich in collagen II (COL2) and glycosaminoglycan (GAG). The components of articular cartilage ensures its role in maintaining the abrasion resistance and stretching properties(1-3). Cartilage can be injured by mechanical, chemical and miceobiological reasons, usually accompanied by disability induced by arthritis in the terminal stage(4). Considering that cartilage has no regenerative capacity, finding a rational and effective treatment for cartilage repair is of great significance(5).
Several growth factors, such as transforming growth factor β (TGFβ), can promote the homing and chondrogenesis of mesenchymal stem cells in cartilage defects(6). Compared to growth factors, small-molecule drugs produce less immune response because of their small molecular weight and relatively low production cost, these characteristics provides new insights for the treatment of cartilage repair(7). It is meaningful to develop or find a small molecule drug that can promote cartilage repair.

Src-homology 2-containing protein tyrosine phosphatase 2 (SHP2) is encoded by the PTPN11 gene(8), which plays various roles in organism development in response to stimulating factors and growth factors(9, 10). A previous study found that the cartilage mass and chondrogenic-related proteins, such as SOX9 and COL2, were increased in the epiphyseal area of SHP2-deficient mice which conditionally deleted PTPN11 in ephiphyseal cells(11). This indicates that SHP2 might play an important role in chondrogenic differentiation and cartilage formation. Meanwhile, SHP2 interacted with yes associated protein (YAP), a target of Hippo signaling pathway, which inhibited the chondrogenic process(12). Furthermore, SHP2 activated WNT signaling through parafibromin/YAP during the development of tumor(13). Whether SHP2 can be considered as a therapeutic target for cartilage repair are not investigated yet. SHP099 is a newly discovered SHP2 inhibitor that stabilize SHP2 in an auto-inhibited conformation(14), SHP099 was hypothesized as a potential small molecule drug used for cartilage repair in this study.

Synovial mesenchymal stem cells (SMSCs) showed optimal chondrogenic ability which can be harvested by a less invasive technique(15). In this study, SMSCs were isolated and their stemness was examined. We demonstrated that SHP099 promoted the chondrogenic differentiation of human SMSCs partially via the inhibition of YAP/WNT signaling in vitro. Moreover, intra-articular injection of SHP099 promoted cartilage defects repair in vivo. Our results suggest that SHP099 is a potential therapeutic drug for cartilage repair.

**Method And Materials**

**Clinical specimen**

The research was approved by the Ethical Committee of the Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School (2009022). The synovium tissues were obtained from OA patients undergoing a total knee replacement.

**Animals**

All animal experiments have complied with the approval of the Animal Committee of Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University (2019AE002). We have complied with all rules and regulations.

Adult female New Zealand rabbit (2.5kg~3kg, n=10) were purchased from the Animal Center of the Nanjing Medical University (Jiangsu, China). After one-week adaptive breeding, 10 rabbits received a
cartilage defect surgery in their right femoral pulley, and they were divided into treatment group and sham group randomly. Intra-articular injection of 3mL of 20µM SHP099 (MCE) twice a week was performed in the treatment group (n=5), and 3mL saline was injected into the articular cavity of the rabbits in the control group (n=5). Samples were collected at 6 weeks after surgery.

**Cartilage defect model**

The rabbit cartilage defect model is widely used in the research of cartilage repair because of their larger joints and greater endogenous healing potential(16, 17). The full-thickness cartilage defect with microfracture is considered to have more stem cell infiltration to more comprehensively evaluate the effect of external stimulatory factors(16). Each rabbit was anesthetized using intramuscular injection of 5 mg droperidol. The rabbits were placed in a supine position with a medial patella approach to expose the femur pulley, where cartilage defect with a diameter of 2.7mm and a depth of 3mm was constructed using an osteoarticular transplantation system.

**Cell culture condition and treatment**

SMSCs were isolated from synovium of OA patients after type I collagenase treatment for one night (Gibco) and cultured using Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin and streptomycin (Gibco) in cell incubator at 37 °C.

To induce a chondrogenic environment, we constructed a chondrogenic medium with 10ng/mL TGF-β3 (PeproTech), 10^{-7} M dexamethasone (Sigma), 50µg/mL L-Ascorbic acid (Sigma), 40µg/mL L-proline (Sigma), 1% FBS (Gibco), and 1% penicillin and streptomycin (Gibco) as previous studies(18). To study the impact of SHP099 on the chondrogenic process, SMSCs were induced with SHP099 for 1 week to detect mRNA level and protein expression.

**Cell viability assay**

Cell viability of SHP099 on synovial stem cells was assessed by CCK-8 assay kit (ThermoFisher Scientific) according to the instructions. Cells were cultured in 96-well plates and incubated with different concentrations of SHP099 for 1 week. CCK-8 reagents were added to every well for 3 hours at 37 °C. At least 5 measurements each group were carried out by detecting the absorbance at 450nm.

**Western blotting**

The total protein was extracted from SMSCs by RIPA lysis buffer (Solarbio) adding 1% phosphatase inhibitor cocktail (Bimake), and 1% phenylmethylsulfonyl fluoride (Sigma). The protein concentration was detected by BCA protein assay kit (Thermo scientific). According to the standard procedures, proteins were separated on 10% SDS gels (EpiZyme) and transferred on the polyvinylidene fluoride membranes (Bio-Rad). After blocked with 5% milk (Bio-Rad) for 1 hour at 37°C, the primary antibodies of SOX9 (1:1000, CST), COL2 (1:2000, abcam), COL1 (1:2000, Abcam), COL10 (1:2000, Abcam), β-catenin (1:1000, Santa Cruz), YAP (1:2000, CST), parafibromin (1:1000, Santa Cruz), p-GSK-3β (1:1000, CST), GSK-3β
(1:1000, CST), p-ERK (1:1000, CST), ERK (1:1000, CST), SHP2 (1:1000, CST), GAPDH (1:2000, CST) were incubated on the membrane. After rinsed with TBST for 3 times, horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG (1:5000, Biosharp) were incubated on the membrane to conjugate primary antibody. All images were detected by the Imaging System (Tanon). Finally quantitative analysis of protein grayscale value was performed using Image J (NIH).

**Quantitative real-time polymerase chain reaction (qPCR)**

Total RNA was extracted using RNA-Quick Purification Kit (ES Science) from SMSCs cultured in 6-well plates. After synthesizing cDNA using HiScriptIIQ RT SuperMix for qPCR (Vazyme), the qPCR was constructed on a light cycler instrument (LightCycler 480-II, Roche) in a 20µL SYBR Green qPCR kit (Vazyme) according to instructions. The primers were used as follows: **COL10** forward 5′-ATGCTGCCACAATACCCTTT-3′ and reverse 5′-GGTAGTGGGCCCTTTATGCT-3; **COL2** forward 5′-TGGACGATCGGCAGGACC3′ and reverse 5′-GCTGCGGATGCTCTCAATCT-3; **SOX9** forward 5′-GCTCTGGAGACTTCTGAACGA-3′ and reverse 5′-CCGTTCTTCACCGACTTCCT-3; **RUNX2** forward 5′-TGTTTACTGCTATGGCGGGTA-3′ and reverse 5′-TCTCAGATCGTTGAACCTTGCTA-3′.

**Cellular immunofluorescence (IF)**

The sterilized square slides are placed in a six-well plate to inoculate cells, which was taken out after stimulation and fixed with paraformaldehyde for 15 minutes. After treatment of 0.3% Triton-100 (Biosharp) for 15 minutes, the slides were blocked by 5% bovine serum albumin (BSA, Biofroxx) for 1 hour at 37 °C. And then the slide was incubated by the primary antibody of **COL2** (1:200, abcam) overnight. After incubated by the FITC or TRITC conjugated second antibody (Biosharp) for 1 hour at room temperature, the fluoroshield mounting medium with DAPI (abcam) were used to stain the nucleus. The fluorescence images were obtained with a fluorescence microscope (Zeiss).

**Histologic staining**

After the sample has been fixed in 10% formalin for 7 days and decalcified in 15% ethylenediaminetetraacetate (EDTA)-buffered saline solution (Sunshine) for 2 months. The samples were dehydrated, transparent and embedded in paraffin tissue, and cut into sections with a thickness of 3µm. The sections were stained with H&E staining (Beyotime Biotechnology), Safranin O/Fast Green staining (Solarbio). The images were obtained under a light microscope (Olympus).

**Immunohistochemistry (IHC) staining**

After hydration by gradient ethanol from 100% to 50%, the sections were incubated by 3% H₂O₂ for 15 min. After antigen retrieval by pepsin (sigma) and blocked by goat serum (Sigma), the primary antibody of **COL2** (abcam) was incubated on the sections overnight at 4 °C. The second antibody and ultra-sensitive DAB kit (Typng) were incubated on the section the next day. The images were obtained by a light microscope (Olympus).
Statistical analyses

Students’ t-test and one-way ANOVA were used to analyze the results. GraphPad Prism software was used to make graphics, and the data were expressed as Mean ± SD or SEM, P<0.05 was considered significant.

Results

Isolation and identification of SMSCs

SMSCs were isolated and represented a spindle-like morphology at passage 3 (P3) (figure 1A). Following the identification criteria for stem cells, surface markers of SMSCs were analysed, flow cytometry analysis demonstrated that SMSCs were positive for CD90 and CD105, and negative for CD34 and CD45 (figure 1B).

Round morphology was observed in SMSCs cultured in the chondrogenic medium, the chondrogenic differentiation potential of SMSCs was evaluated by Safranin O staining for GAGs (figure 1C, left panel). The osteogenic ability of SMSCs was confirmed by alizarin red staining for deposited calcium mineral (figure 1C, middle panel). While the adipogenic potential was observed by Oil red staining for small cytoplasmic lipid droplets (figure 1C, right panel). These results demonstrated that SMSCs represented optical differentiation capacity.

SHP099 promotes the expression of cartilage-related markers

To verify the chondrogenic effect of SHP099, we investigated the expression of cartilage-related markers in SMSCs after SHP099 treatment on the basis of the chondrogenic medium as previous studies(18). Less than 20µM SHP099 did not influence cell viability (figure 2A). After one-week culture, SHP099 promoted the expression of SOX9 and COL2, and decreased the expression of hypertrophic marker COL10 in a dose-dependent manner. However, the expression of COL1 was not significantly affected by SHP099 treatment (figure 2B). Consistent with the protein level, SHP099 increased the mRNA expression of SOX9 and COL2, and decreased COL10 and RUNX2 (figure 2C). The IF results revealed that COL2 expression was upregulated after SHP099 treatment (figure 2D).

Intra-articular injection of SHP099 promotes the cartilage defects repair in vivo

To evaluate the chondrogenic effect of SHP099 in vitro, the cartilage defect model was constructed (figure 3A). At 6th week after surgery, we observed better repair of cartilage defects in SHP099 group (figure 3B), and the SHP099 group obtained higher International Cartilage Repair Society (ICRS) score (figure 3C). The cartilage surface was smoother in the SHP099 group (figure 3D). Additionally, the Safranin O/Fast Green staining showed that the repaired tissue in SHP099 treatment group contained more GAGs (figure 3E). The repaired cartilage in the defects contained more COL2 after SHP099 treatment (figure 3F).
SHP099 promotes the chondrogenesis via inhibiting YAP signaling pathway

To further explore the mechanism of SHP099 promoting the cartilage repair, we found the protein level of YAP, parafibromin and β-catenin was decreased after SHP099 treatment (figure 4A), suggesting the inhibition of YAP/WNT pathway by SHP099. The decreases of p-GSK3β and p-ERK were also observed after SHP099 treatment (figure 4B). To determine whether YAP was a key protein of this process, we knocked down YAP by Small interfering RNA (siRNA) and found that the expression levels of β-catenin, SOX9 and COL2 were increased, which was consistent with the results of SHP099 treatment (figure 4C).

Discussion

Stem cell-based treatments play an important role in skeleton tissue repair and regeneration, but the natural chondrogenic ability of MSCs is still unsatisfactory(19). It is necessary to promote the chondrogenic process through modulating molecular mechanisms. In our study, SHP099 increased cartilage-related genes (SOX9, COL2) expression in SMSCs. Meanwhile, intra-articular injection of SHP099 resulted in significant improvement in the cartilage repair. Mechanically, SHP099 probably promoted chondrogenesis by YAP/WNT signaling pathway.

MSCs from different tissues, including bone marrow, synovium, and adipose tissues, can be recruited into cartilage defects to promote cartilage repair(16), and SMSCs have better chondrogenic properties(20). In our study, SMSCs showed good proliferation capacity and three-way differentiation capability when they were cultured to the third generation, we therefore believe that SMSCs could be used as an ideal object for assessment of the chondrogenic process.

Small molecule drugs can be applied in many forms, such as intra-articular injection or in combination with growth factors or scaffolds. Intra-articular injection is a simple and efficient therapeutic choice in the cartilage repair(21). In this study, after intra-articular injection of SHP099, not only the macroscopic observation of the repaired cartilage showed satisfied results, but also the Safarnin O/Fast Green staining and COL2 expression were elevated. Similar to our results, Kartogenin (KGN) was discovered as a small molecule drug, which could protect cartilage in the destabilization of the medial meniscus (DMM) mice model(22). More small molecule compounds and underlying mechanism still need to be investigated for potential clinical application of cartilage repair.

In a previous study focusing on cholangiocarcinoma, SHP2 mediated its chemosensitivity via upregulating YAP activity(23), which could decrease the degradation of β-catenin by regulating parafibromin(24). Excessive activation of YAP inhibits the differentiation and maturation of cartilage(12, 25). In our study, SHP099 decreased the expression of YAP during chondrogenesis. And after YAP was knocked down using siRNA, the up-regulation of cartilage-related proteins was observed, which is consistent with the results using SHP099, this revealed that SHP099 might promote chondrogenesis via YAP. A previous study showed that a WNT inhibitor PKF118-310 could promote the cartilage-phenotype(26). Our results showed that SHP099 and YAP knock down could both decrease WNT-
indicative protein β-catenin, indicating that SHP099 probably inhibited WNT signaling via YAP, resulting
in the promotion of chondrogenesis (Figure 5).

MAPK (mitogen-activated protein kinase) pathway plays a key role in a variety of cellular responses,
including proliferation, differentiation, and apoptosis. The inhibition of extracellular signal-regulated
kinase (ERK)-1 and ERK-2, two MAPK subtypes, enhanced chondrogenesis by up to 1.7-fold in micromass
cultures of chick embryo mesenchyme(27). Our results showed that SHP099 inhibited the expression of
p-ERK, which might be part of the reason why SHP099 promoted the chondrogenesis of SMSCs.

Conclusion

SHP099, a SHP2 allosteric inhibitor, enhances the repair of full-thickness cartilage defect, indicating a
new therapeutic approach for cartilage repair.

Abbreviations

SMSC: synovial mesenchymal stem cell
IF: immunofluorescence
H&E: hematoxylin and eosin
IHC: immunohistochemistry
YAP: yes associated protein
COL2: collagen II
GAG: glycosaminoglycan
TGFβ: transforming growth factor β
SHP2: Src-homology 2-containing protein tyrosine phosphatase 2
EDTA: ethylenediaminetetraacetate
ICRS: International Cartilage Repair Society
DMM: the destabilization of the medial meniscus
MAPK: mitogen-activated protein kinase
ERK: extracellular signal-regulated kinase

Declarations
Acknowledgements

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Ethics declarations

Ethics approval and consent to participate

The research was approved by the Ethical Committee of the Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School (2009022). The synovium tissues were obtained from OA patients undergoing a total knee replacement.

All animal experiments have complied with the approval of the Animal Committee of Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University (2019AE002). We have complied with all rules and regulations.

Consent for publication

Not applicable.

Competing Interests

Nothing to disclose

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All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Conception and design: Ziying Sun, Xingquan Xu, Zhongyang Lv, Dongquan Shi. Experiment execution: Ziying Sun, Xingquan Xu, Zhongyang Lv, Jiawei Li, Heng Sun, Kuoyang Sun, Rui Wu, Jia Xu, Guihua, Wenqiang Yan. Result analysis: Ziying Sun, Xingquan Xu, Zhongyang Lv, Jiawei Li, Yannick Xiaofan Yang. Drafting of the article: Ziying Sun, Xingquan Xu, Dongquan Shi. Critical revision of the article for important intellectual content: Qing Jiang, Dongquan Shi. Final approval of the article: Ziying Sun, Zhongyang Lv, Dongquan Shi. Figure design: Ziying Sun, Xingquan Xu, Zhongyang Lv, Jiawei Li.

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Figures
Figure 1

The isolation and identification of synovial mesenchymal stem cells (SMSCs). (A) SMSCs represented a spindle-like shape (scale bar: 50 µm). (B) Representative flow cytometry results of the representative surface markers of MSCs which included CD90, CD105, CD45 and CD34. (C) Representative images of SMSCs cultured in chondrogenic, osteogenic, and adipogenic medium and their staining by Safranin O, Alizarin Red, and Oil red staining respectively (scale bar: 50 µm).
Figure 2

SHP099 promoted the chondrogenic process of SMSCs in vitro. (A) The results of cell viability of SMSCs after different concentrations of SHP099 treatment for 1 week. *P < 0.05. (B) Representative images of western blot of Collagen I (COL1), Collagen X (COL10), Collagen II (COL2), SOX9, and GAPDH after treatment of chondrogenic medium (CM) and SHP099 or not. The experiment was repeated independently three times. (C) The mRNA expression of SOX9, COL2, COLX, RUNX2 after stimulation with
Intra-articular injection of SHP099 promoted the cartilage repair of the New Zealand Rabbit. (A) Representative images of the cartilage defect with a diameter of 2.7mm and a depth of 3mm in the rabbit femur pulley. (B) Representative general view of cartilage defect repair effect between control group and SHP099. (C) Graph showing the degree of defect repair and integration to border zone for control and SHP099 groups. (D-F) Representative images of different concentrations of SHP099. *P < 0.05. (D) Representative images of immunofluorescence (IF) of COL2 of SMSCs with different concentrations of SHP099 are shown. (scale bar: 50 µm)
SHP099 intraarticular injection group. (C) The ICRS scores of control group (Control) and intraarticular injection group (SHP099), which assessed degree of defect repair, intergration to border zone, macroscopic appearance, and overall repair assessment. *P < 0.05 (D) The H&E staining of sections of control group (Control) and intra-articular injection of SHP099 group (SHP099). (scale bar: 200 µm). (E) The Safranin O/Fast Green staining of control group (control) and intraarticular injection of SHP099 group (SHP099). (scale bar: 200 µm). (F) Representative image of immunohistochemistry (IHC) of COL2 between control group (control) and intraarticular injection of SHP099 group (SHP099). (scale bar: 200 µm).

Figure 4
SHP099 promoted chondrogenic process via yes-associated protein (YAP) and WNT pathway. (A) Protein expression of SOX9, β-catenin, YAP, parafibromin, and GAPDH was analyzed using western blotting after stimulation of chondrogenic medium (CM) and SHP099. The statistical graphs of gray value were shown. *P < 0.05. (B) The protein expression of WNT-related proteins and p-ERK after stimulation was detected by western blotting, including β-catenin, p-GSKβ, GSKβ, p-ERK, ERK and GAPDH. (C) The protein expression levels of COL2, β-catenin, SHP2, YAP, SOX9 and GAPDH after interfering with YAP were obtained by western blotting.
Diagram illustrating the proposed mechanism of SHP2 in cartilage repair.