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Intraarticular 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 orthopaedics for decades, highlighting the significance of investigating potential therapeutic drugs. In this study, we explored the effect of the SHP2 inhibitor SHP099, 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 treatment with chondrogenic medium, the chondrogenic effect of SHP099 on SMSCs was examined by western blot, qPCR, and immunofluorescence (IF). Micro-mass culture was also used to detect the effect of SHP099. To explore the chondrogenic effects of SHP099 *in vivo*, full-thickness cartilage defects with microfractures were constructed in the right femoral trochlea of New Zealand White rabbits. Intraarticular injection of SHP099 or normal saline was performed twice a week for 6 weeks. Cartilage repair was evaluated by haematoxylin and eosin (HE) staining and 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 signalling was investigated *in vitro*.

Results: SMSCs isolated from human synovium have optimal multi-differentiation potential. SHP099 increased chondrogenic marker (SOX9, COL2) expression and decreased hypertrophic marker (COL10, RUNX2) expression in SMSCs. In micro-mass culture, the SHP099-induced cartilage tissues had a better result of Safranin O and Toluidine blue staining and are enriched in cartilage-specific collagen II. Inhibition of YAP and WNT signalling was also observed. Moreover, compared to the normal saline group at 6 weeks, intraarticular injection of SHP099 resulted in better defect filling, forming increased hyaline cartilage-like tissue with higher levels of glycosaminoglycan (GAG) and COL2.

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

The Translational potential of this article: This study provides evidence that SHP2 inhibition promotes chondrogenesis and the repair of cartilage in defect area, which could be a novel therapeutic approach for cartilage repair.

1. 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 ensure its role in maintaining abrasion resistance and stretching properties [1–3]. Cartilage can be injured by mechanical, chemical and microbiological reasons, usually accompanied by disability induced by arthritis in the terminal stage [4]. Considering that cartilage has limited regenerative capacity, finding a rational and effective treatment for cartilage repair is of great significance [5].

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Studies have revealed that joint tissue contains several types of stem cells and chondrocyte progenitors that can differentiate into chondrocytes for cartilage repair under specific conditions [6]. Compounds that promote the chondrogenic differentiation of mesenchymal stem cells (MSCs) may enhance the repair of damaged cartilage [7]. Several growth factors, such as transforming growth factor β (TGFβ), promote the homing and chondrogenesis of mesenchymal stem cells in cartilage defects [8]. Compared to growth factors, small-molecule drugs induce less of an immune response, and their relatively low production cost provides new insights for cartilage repair [9]. The discovery of kartogenin as a small-molecule drug for cartilage regeneration led to numerous novel applications in cartilage repair [10–13]. Identifying a new small-molecule drug with chondrogenic effects will provide new perspectives for cartilage repair.

Src-homology 2-containing protein tyrosine phosphatase 2 (SHP2) is encoded by the PTPN11 gene [14], playing various roles in organism development in response to stimulating factors and growth factors [15,16]. A previous study found that cartilage mass and chondrogenic-related proteins, such as SOX9 and COL2, were increased in the epiphyseal area in SHP2-deficient mice with conditional deletion of PTPN11 in epiphyseal cells [17]. This finding indicates that SHP2 might play an important role in chondrogenic differentiation and cartilage formation. Meanwhile, SHP2 interacts with yes-associated protein (YAP), a target of the Hippo signalling pathway, which inhibited the chondrogenic process [18]. Furthermore, SHP2 activates WNT signalling through parafibromin/YAP during tumour development [19]. However, whether SHP2 can be considered a therapeutic target for cartilage repair has not yet been investigated. SHP099 is a newly discovered SHP2 inhibitor that stabilizes SHP2 in an autoinhibited conformation [20] and can bind to the interface of the N-terminal SH2, C-terminal SH2 and phosphatase domains, inhibiting SHP2 activity through an allosteric mechanism [21]. Whether SHP099 can be applied to diseases of the skeletal system has not yet been explored.

Considering that SHP2 participates in cartilage development, to further explore the role of SHP2 in cartilage regeneration, in this study SHP099 was tested for its potential as a small-molecule drug for cartilage repair. We examined the effect of SHP099 on chondrogenesis of synovial mesenchymal stem cells (SMSCs) and performed articular injection of SHP099 to detect its effect on the repair of rabbit full-thickness cartilage defects.

2. Method and materials

2.1. Clinical specimens

This research was approved by the Ethical Committee of the Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School (2020-156-01). Synovium tissues were obtained from five patients undergoing total knee replacement.

2.2. Animals

All animal experiments were performed in compliance with the approval of the Animal Committee of Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University (2019AE002).

Adult female New Zealand rabbits (2.5 kg–3 kg, n = 8) were purchased from the Animal Centre of Nanjing Medical University (Jiangsu, China). After one week of adaptive breeding, 8 rabbits received cartilage defect surgery in their right femoral pulley and were subsequently randomly divided into treatment and control groups. Intraarticular injection of 3 mL of 20 μM SHP099 (MCE) was performed twice a week in the treatment group (n = 4), while 3 mL saline was injected into the articular cavity of rabbits in the control group (n = 4). Samples were collected 6 weeks after surgery.

2.3. Cartilage defect model

Cartilage defect models using rabbits are widely used in cartilage repair research due to their larger joints and increased endogenous healing potential [22,23]. Full-thickness cartilage defects with microfractures are considered to experience increased stem cell infiltration, allowing for a more comprehensive evaluation of the effects of external stimulatory factors [22]. Each rabbit was anaesthetized 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 defects with a diameter of 2.7 mm and a depth of 3 mm were created using osteochondral transplantation instrumentation.

2.4. Cell culture conditions and treatments

SMSCs were isolated from the 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% foetal bovine serum (FBS) (Gibco) and 1% penicillin and streptomycin (Gibco) in an incubator at 37 °C. After culturing to the third generation, cell surface markers were analysed using a flow cytometer (BD Accuri C6 Plus). For micro-mass culture, 5×10^5 cells were seeded at the center of the 24-well plate and cultured for two weeks. Lentivirus was purchased from Genechem (Shanghai) and cells were transfected according to the manufacturer's requirements.

Osteogenic medium containing 1 nmol/L dexamethasone (Sigma), 20 mmol/L sodium glycerophosphate (Sigma) and 50 mg/L ascorbic acid was used for osteogenic differentiation of SMSCs, and adipogenic medium containing 10 μg/mL insulin (Sigma), 1 μM dexamethasone (Sigma), and 0.5 mM isobutylmethylxanthine (Sigma) was used for adipogenic differentiation.

To induce a chondrogenic environment, we utilized chondrogenic medium containing 10 ng/mL TGF-β3 (PeproTech), 10^{-7} M dexamethasone (Sigma), 50 μg/mL L-ascorbic acid (Sigma), 40 μg/mL L-proline (Sigma), 1% FBS (Gibco), and 1% penicillin and streptomycin (Gibco) as described in previous studies [24]. To determine the impact of SHP099 on the chondrogenic process, SMSCs were induced with SHP099 for one week followed by detection of mRNA levels and two weeks followed by detection of protein expression.

2.5. Cell viability assay

The cell viability of SHP099 on SMSCs was assessed using a CCK-8 assay kit (Thermo Fisher 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 each well for 3 h at 37 °C. At least 5 measurements in each group were performed by detecting the absorbance at 450 nm. And crystal violet staining (Beyotime Biotechnology) was performed according to the instructions.

2.6. Western blotting

Total protein was extracted from SMSCs in RIPA lysis buffer (Solarbio) supplemented with 1% phosphatase inhibitor cocktail (Bimake) and 1% phenylmethylsulfonyl fluoride (Sigma). The protein concentration was determined using a BCA protein assay kit (Thermo Scientific). According to standard procedures, proteins were separated on 10% SDS gels (EpiZyme) and transferred to polyvinylidene fluoride membranes (Bio–Rad). After blocking with 5% milk (Bio–Rad) for 1 h at 37 °C, primary antibodies against 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), and GAPDH (1:2000, CST) were incubated on the membrane. After rinsing with TBST 3 times,

horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG (1:5000, Biosharp) was incubated on the membrane to conjugate the primary antibody. All images were detected using the Chemiluminescent Imaging System (Tanon). Finally, quantitative analysis of protein greyscale values was performed using ImageJ (NIH).

2.7. Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted using an RNA-Quick Purification Kit (ES Science) from SMSCs cultured in 6-well plates. After synthesizing cDNA using HiScriptIIQ RT SuperMix for qPCR (Vazyme), qPCR was performed on a light cycler instrument (LightCycler 480-II, Roche) in a 20 μL SYBR Green qPCR kit (Vazyme) according to the instructions. The primers used were as follows: *COL10* forwards 5′-ATGCTGCCACAAATACCCTTT-3′ and reverse 5′-GGTAGTGGGCCTTTTATGCCT-3; *COL2* forwards 5′-TGGACGATCAGGCGAAACC-3′ and reverse 5′-GCTGCGGATGCTCT-CAATCT-3; *SOX9* forwards 5′-GCTCTGGAGACTTCTGAACGA-3′ and reverse 5′-CCGTTCTTCACCGACTTCCT-3; *RUNX2* forwards 5′-TGG TTACTGTCATGGCGGGTA-3′ and reverse 5′-TCTCAGATCGTTGAACCT TGCTA-3′.

2.8. Cellular immunofluorescence (IF)

Sterilized square slides were placed in six-well plates to inoculate cells, which were removed after stimulation and fixed in para-formaldehyde for 15 min. After treatment with 0.3% Triton-100 (Bio-sharp) for 15 min, the slides were blocked in 5% bovine serum albumin (BSA, Biofroxx) for 1 h at 37 °C. Then, the slides were incubated with primary antibody against COL2 (1:200, Abcam) overnight. After incubation with FITC- or TRITC-conjugated secondary antibody (Biosharp) for 1 h at room temperature, fluoroshield mounting medium containing DAPI (Abcam) was applied to stain the nucleus. Fluorescence images were obtained using a fluorescence microscope (Zeiss).

2.9. Histological staining

After the samples were fixed in 10% formalin for 7 days, they were decalcified in 15% ethylenediaminetetraacetate (EDTA)-buffered saline solution (Sunshine) for 2 months. The samples were subsequently dehydrated, made transparent, embedded in paraffin tissue, and cut into sections with a thickness of 3 μm. The sections were stained with HE

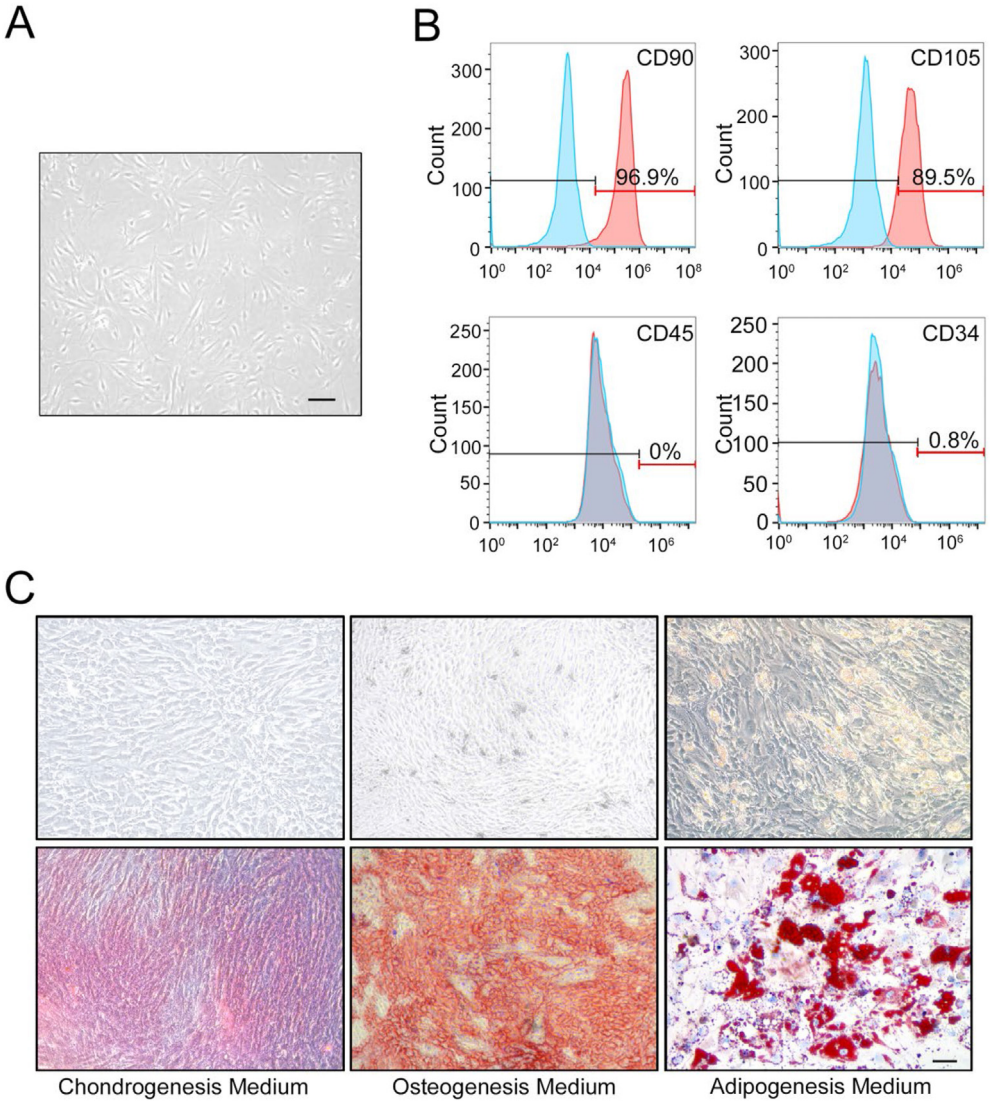

Fig. 1. The isolation and identification of synovial mesenchymal stem cells (SMSCs) (A) SMSCs represented a spindle-like shape (scale bar: 50 μm) **(B)** Representative flow cytometry results of the representative surface markers of MSCs which included CD90, CD105, CD45 and CD34 **(C)** Representative 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: 100 μm).

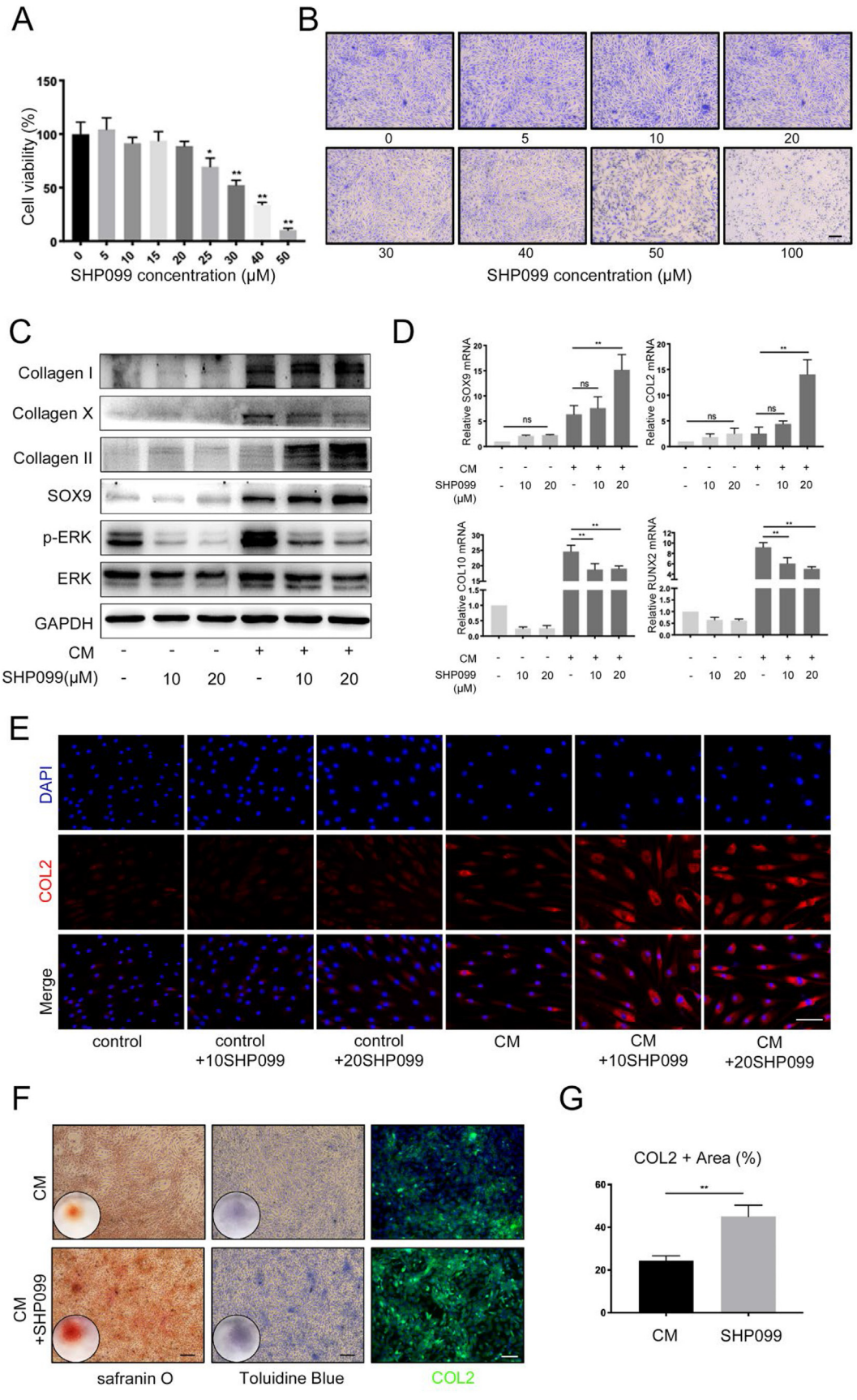

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Fig. 2. SHP099 promoted the chondrogenic process of SMSCs *in vitro* (A) The results of cell viability of SMSCs after different concentrations of SHP099 treatment for 1 week. *P < 0.05 (**B**) Representative images of crystal violet staining of SMSCs after different concentrations of SHP099 treatment for 1 week **(C)** Representative images of western blot of Collagen I (COL1), Collagen X (COL10), Collagen II (COL2), SOX9, p-ERK, ERK and GAPDH after treatment of chondrogenic medium (CM) and SHP099 or not for two weeks. The experiment was repeated independently three times **(D)** The mRNA expression of *SOX9*, *COL2*, *COLX*, *RUNX2* after stimulation with different concentrations of SHP099 for one week. The experiment was repeated independently three times. *P < 0.05 **(E)** Representative images of immunofluorescence (IF) of COL2 of SMSCs with different concentrations of SHP099 for two weeks are shown. The experiment was repeated independently three times (scale bar: 100 μm) (**F**) Representative images of Safranin staining, Toluidine blue staining and IF of COL2 of micro-mass results with different concentrations of SHP099 for two weeks (scale bar: 100 μm) (G) COL2 positive area of micro-mass results with SHP099 or not. *P < 0.05.

(Beyotime Biotechnology) and safranin O/fast green (Solarbio). Images were obtained under a light microscope (Olympus).

2.10. Immunohistochemistry (IHC) staining

After hydration in gradient ethanol from 100% to 50%, the sections were incubated in 3% H_2O_2 for 15 min. After antigen retrieval by pepsin (Sigma) and blocking with goat serum (Sigma), the sections were incubated with primary antibody against COL2 (Abcam) overnight at 4 °C. The sections were incubated with secondary antibody and an ultrasensitive DAB kit (Typng) the next day. Images were obtained using a light microscope (Olympus).

2.11. Statistical analyses

Student's t-test and one-way ANOVA were used to analyse the results. GraphPad Prism software was used to make the graphics, and data are expressed as the mean ± SD or SEM. $P < 0.05$ was considered significant.

3. Results

3.1. Isolation and identification of SMSCs

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

Round morphology was observed in SMSCs cultured in chondrogenic medium, and the chondrogenic differentiation potential of SMSCs was evaluated using safranin O staining for GAGs (Fig. 1C, left panel). The osteogenic ability of SMSCs was confirmed by alizarin red staining for deposited calcium minerals (Fig. 1C, middle panel). Adipogenic potential was observed using oil red staining for small cytoplasmic lipid droplets (Fig. 1C, right panel). These results demonstrated that the SMSCs had achieved optical differentiation capacity.

3.2. SHP099 promotes expression of cartilage-related markers

To verify the chondrogenic effect of SHP099, we investigated the expression of cartilage-related markers in SMSCs in response to SHP099 treatment in chondrogenic medium as described in previous studies [24]. Less than 20 μM SHP099 did not influence cell viability (Fig. 2A and B). Studies have shown that the main indicator for observing whether the SHP2 protein is inhibited is to detect whether the phosphorylation of ERK protein is inhibited [25,26]. SHP099 could significantly inhibit phosphorylation of ERK protein in SMSCs, and after two weeks of culture, SHP099 promoted expression of SOX9 and COL2 and decreased expression of the hypertrophic marker COL10 in a dose-dependent manner. However, expression of COL1 was not significantly affected by SHP099 treatment (Fig. 2C). Consistent with the observed protein levels, SHP099 treatment increased the mRNA expression of *SOX9* and *COL2* and decreased *COL10* and *RUNX2* after stimulation of one week (Fig. 2D). The IF results revealed that COL2 expression was upregulated after SHP099 treatment for two weeks (Fig. 2E). The results of micro-mass culture staining with Safranin O and Toluidine Blue also showed SHP099 promoted chondrogenic process, and the rate of COL2-positive area significantly increased after stimulation for two weeks (Fig. 2F and G).

3.3. Intraarticular injection of SHP099 promotes repair of cartilage defects in vivo

To evaluate the chondrogenic effect of SHP099 *in vitro*, a cartilage defect model was constructed (Fig. 3A). Six weeks after surgery, we observed better repair of cartilage defects in the SHP099 group (Fig. 3B), which also exhibited a higher International Cartilage Repair Society (ICRS) macroscopic evaluation score (Fig. 3C). The cartilage surface was also smoother in the SHP099 group (Fig. 3D). Additionally, safranin O/fast green staining revealed that the repaired tissue in the SHP099 treatment group contained more GAGs (Fig. 3E). Furthermore, the repaired cartilage in the defects contained higher levels of COL2 in response to SHP099 treatment (Fig. 3F). Meanwhile, the ICRS histological evaluation score increased after SHP099 treatment (Fig. 3G).

3.4. SHP099 promotes chondrogenesis by inhibiting the YAP signalling pathway

Concerning the mechanism by which SHP099 promotes cartilage repair, we found that protein levels of YAP, parafibromin and β-catenin were all decreased in response to SHP099 treatment (Fig. 4A), suggesting that SHP099 inhibits the YAP/WNT pathway. The Decreased p-GSK3β levels were also observed after SHP099 treatment (Supplementary Figure). To determine whether YAP was a key protein in this process, we knocked down YAP using small interfering RNA (siRNA) and found that expression levels of β-catenin was decreased, SOX9 and COL2 were increased, consistent with the results of SHP099 treatment (Fig. 4B). And after over-expressing YAP protein, we found that the elevation of COL2 and SOX9 induced by SHP099 was suppressed, and β-catenin was elevated (Fig. 4C).

4. Discussion

Stem cell-based treatments play an important role in skeletal tissue repair and regeneration, but the natural chondrogenic ability of MSCs is still unsatisfactory from a therapeutic perspective [27]. SMSCs exhibit optimal chondrogenic ability and can be harvested using a less invasive technique [28]. In this study, SMSCs were isolated, and their stemness was examined. We demonstrated that SHP099 promoted chondrogenic differentiation of human SMSCs partially through inhibition of YAP/WNT signalling *in vitro*. Moreover, intraarticular injection of SHP099 promoted repair of cartilage defects *in vivo*. Our results suggested that SHP099 is a potential therapeutic drug for cartilage repair.

MSCs from different tissues, including bone marrow, synovium, and adipose tissues, can be recruited into cartilage defects to promote cartilage repair [22], and SMSCs have superior chondrogenic properties [29]. Previous studies have primarily derived synovial tissue from arthroscopic surgery, but such samples are more difficult to obtain [30]. In our study,

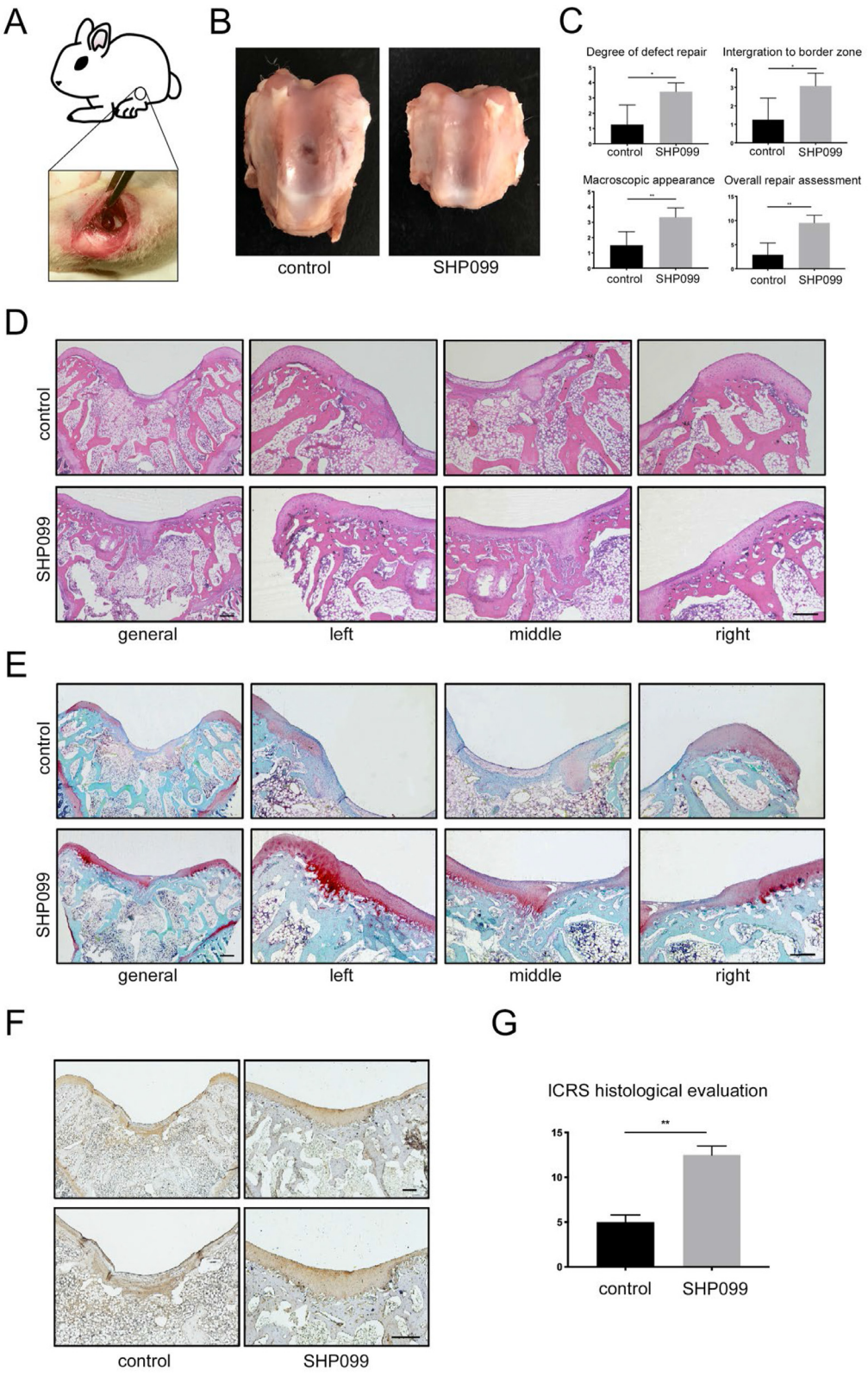

Fig. 3. Intra-articular injection of SHP099 promoted the cartilage repair of the New Zealand Rabbit (A) Representative images of the cartilage defect with a diameter of 2.7 mm and a depth of 3 mm in the rabbit femur pulley **(B)** Representative general view of cartilage defect repair effect between control group and SHP099 intraarticular injection group **(C)** The ICRS macroscopic evaluation 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. There are four samples in each group. *P $<$ 0.05 **(D)** The H&E staining of sections of control group (Control) and intra-articular injection of SHP099 group (SHP099) (scale bar: 500 μm) **(E)** The Safranin O/Fast Green staining of control group (control) and intraarticular injection of SHP099 group (SHP099) (scale bar: 500 μm) (**F**) Representative image of immunohistochemistry (IHC) of COL2 between control group (control) and intraarticular injection of SHP099 group (SHP099) (scale bar: 500 μm) (**G**) The ICRS visual histological evaluation scores of control group (Control) and intraarticular injection group (SHP099). *P $<$ 0.05.

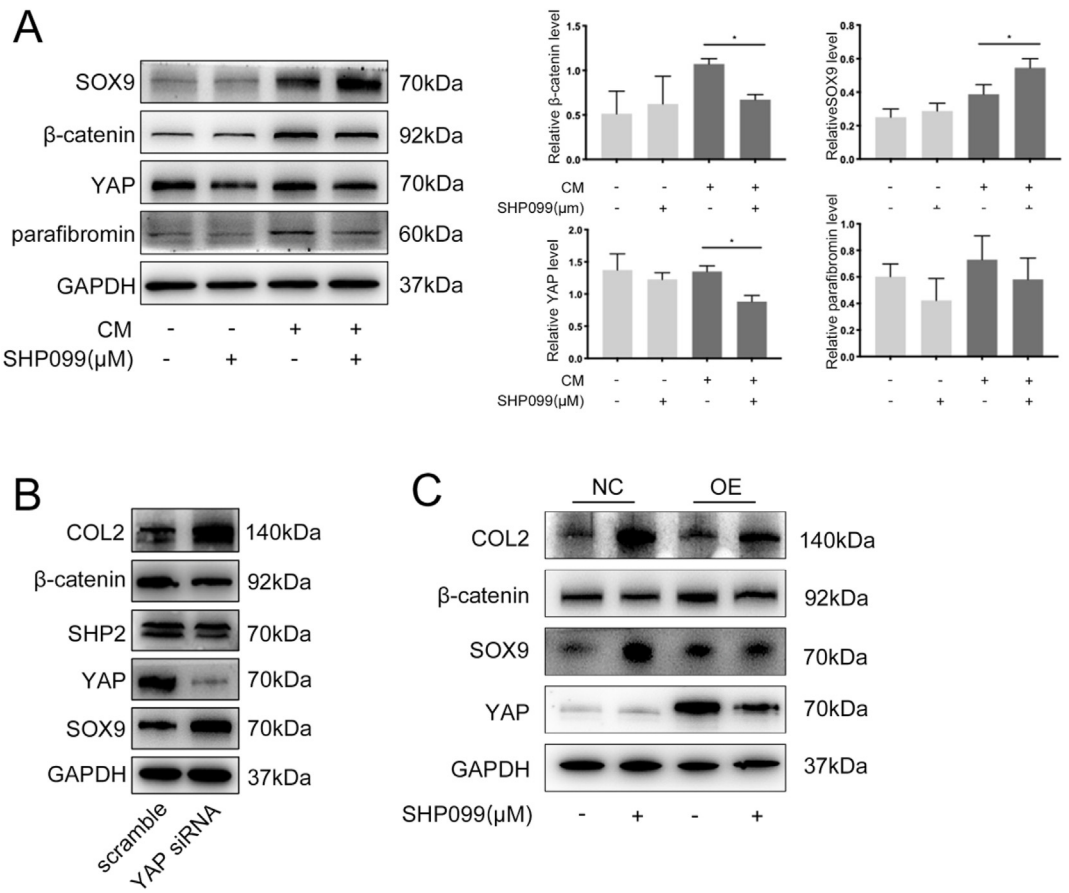

Fig. 4. SHP099 promoted chondrogenic process via yes-associated protein (YAP) and WNT pathway (A) Protein expression of SOX9, β-catenin, YAP, parafibromin, and GAPDH was analyzed using western blotting after stimulation of chondrogenic medium (CM) and SHP099. The statistical graphs of gray value were shown. The experiment was repeated independently three times. *P $<$ 0.05 **(B)** The protein expression levels of COL2, β-catenin, SHP2, YAP, SOX9 and GAPDH after interfering with YAP were obtained by western blotting (**C**) The protein expression levels of COL2, β-catenin, YAP, SOX9 and GAPDH after overexpression of YAP with SHP099 or not were obtained by western blotting. NC: negative control OE: overexpression.

SMSCs from TKR patients also exhibited good proliferation capacity and three-way differentiation capability when cultured to the third generation. Therefore, we believe that SMSCs represent an ideal source for assessment of the chondrogenic process.

Small molecule drugs can be applied in many forms, such as through intraarticular injection or in combination with growth factors or scaffolds. Intraarticular injection is a simple and efficient therapeutic choice in cartilage repair [31]. In this study, in response to intraarticular injection of SHP099, not only did macroscopic observation of the repaired cartilage display satisfactory results but also safranin O/fast green staining and COL2 expression were elevated. Similar to our results, kartogenin (KGN) was discovered as a small-molecule drug that protects cartilage in destabilization of the medial meniscus (DMM) in a mouse model [32]. Additional small molecule compounds and the underlying mechanism still need to be investigated for potential clinical application in cartilage repair.

In a previous study focusing on cholangiocarcinoma, SHP2 mediated chemosensitivity by upregulating YAP activity [33], which decreased the degradation of β-catenin by regulating parafibromin [34]. Excessive activation of YAP inhibits the differentiation and maturation of cartilage [18,35]. In our study, SHP099 decreased YAP expression during chondrogenesis. After YAP was knocked down using siRNA, the upregulation of cartilage-related proteins was observed, consistent with the results using SHP099, revealing that SHP099 might promote chondrogenesis *via* YAP. A previous study showed that a WNT inhibitor, PKF118-310, promoted the cartilage phenotype [36]. Our results showed that SHP099 and YAP knockdown both decreased the WNT-indicative protein β-catenin, indicating that SHP099 likely inhibits WNT signalling *via* YAP, resulting in the promotion of chondrogenesis (Fig. 5).

Studies showed that SHP099 could suppress RAS–ERK signalling to inhibit the proliferation of receptor-tyrosine-kinase-driven human cancer cells [37]. The MAPK (mitogen-activated protein kinase) pathway plays a key role in a variety of cellular responses, including proliferation, differentiation, and apoptosis. MAPKs participated in the regulation of Wnt pathway activities both *in vitro* and *in vivo*, expression of active MKK6, an upstream activator of p38 protein was found to increase the expression of β-catenin through phosphorylation of GSK3β protein [38]. There are few studies on the correlation between ERK and Wnt in the field of chondrogenesis. Inhibition of extracellular signal-regulated kinase (ERK)-1 and ERK-2, two MAPK subtypes, enhanced chondrogenesis by up to 1.7-fold in micro-mass cultures of chick embryo mesenchyme [39]. Our results demonstrated that SHP099 inhibited the phosphorylation of ERK in SMSCs, which might be part of the reason that SHP099 promotes the chondrogenesis of SMSCs. Compared to only intervening in the Wnt pathway, SHP099 can simultaneously inhibit the phosphorylation of ERK protein, which may have a certain strengthening effect on cartilage formation.

Meanwhile, there are several limitations in this study. Firstly, there is no evaluation of the mechanical properties of formed cartilage, which could provide a comprehensive understanding of cartilage repair. And

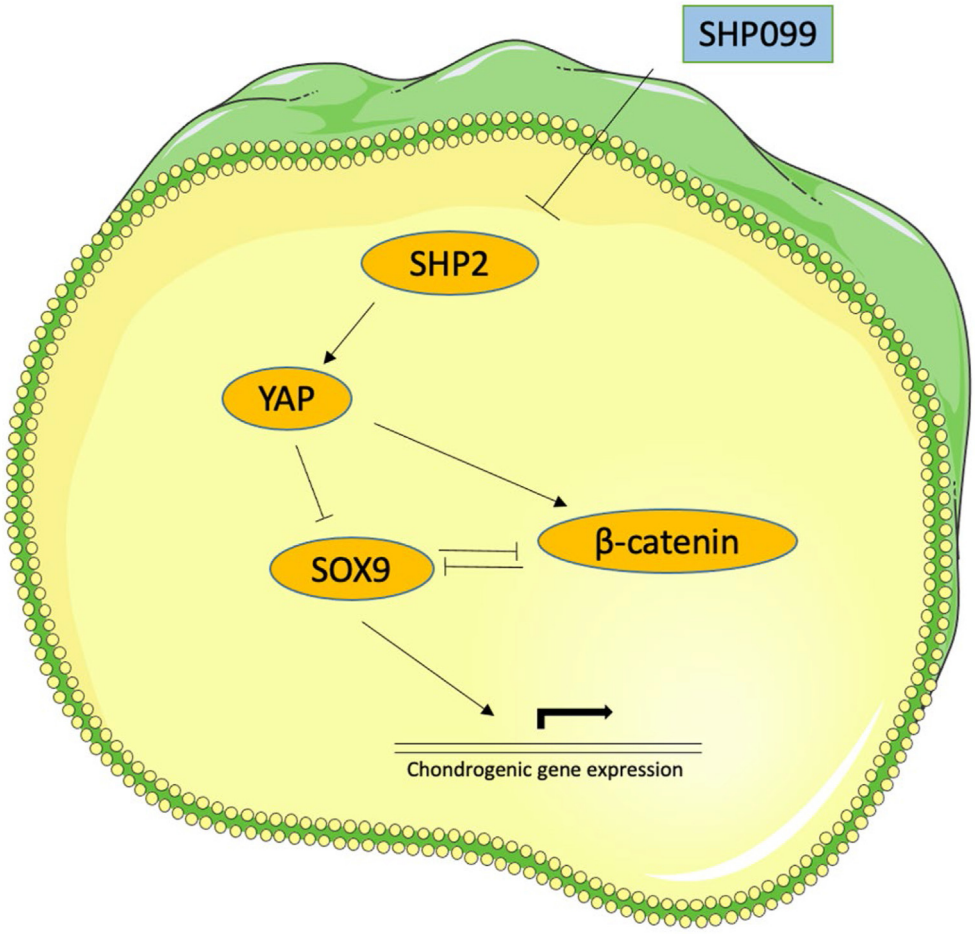

Fig. 5. Diagram illustrating the proposed mechanism of SHP2 in cartilage repair.

this study did not explore the chondrogenic effect of SHP099 in bone marrow-derived mesenchymal stem cells (BMSCs). At the same time, how the SHP2 protein acts on the YAP protein during chondrogenesis still needs to be further explored.

5. Conclusion

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

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Declaration of competing interest

Nothing to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2022.01.001.

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