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ORIGINAL ARTICLE

Kartogenin Promotes the BMSCs Chondrogenic Differentiation in Osteoarthritis by Down-Regulation of miR-145-5p Targeting Smad4 Pathway

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Abstract

BACKGROUND: Transplantation of mesenchymal stem cells (MSCs) is a potential therapeutic strategy for cartilage degeneration of osteoarthritis (OA). But controlling chondrogenic differentiation of the implanted MSCs in the joints remains a challenge. The role of kartogenin (KGN) for chondrogenesis of MSCs has been widely reported, however, the mechanism of chondrogenesis has not been elucidated in OA.

METHODS: In this study, we investigated the miR-145-5p, TGF-β, Samd4, and p-stat3/stat3 expression in cartilage of OA patients and bone marrow mesenchymal stem cells (BMSCs) treated with KGN or miR-145-5p inhibitor. In addition, the cell proliferation and chondrogenic differentiation *in vitro* and *in vivo* of BMSCs treated with KGN was also detected.

RESULTS: In OA patients, the expression of miR-145-5p was up-regulated, and the expression of TGF-β, Samd4, and p-stat3/stat3 was inhibited. When the BMSCs treated with miR-145-5p inhibitor, the expression of TGF-β, Samd4, and p-stat3/stat3 was also significantly up-regulated. KGN-treated BMSCs had better proliferation and chondrogenic differentiation by up-regulating the expression of Sox 9, Col-2a1, aggrecan *in vitro* and in OA by down-regulation of miR-145-5p targeting Smad4 pathway. Moreover, intra-articular injection of KGN-treated BMSCs had a better pain relief effect in OA.

CONCLUSION: The double effect on cartilage protection and pain relief indicates a great potential of intra-articular injection of KGN-treated BMSCs for the treatment of OA.

Keywords Osteoarthritis · BMSCs · Kartogenin · miR-145-5p · Pain

1 Introduction

Osteoarthritis (OA) is a chronic arthropathy characterized by degeneration, destruction, and hyperosteogeny of articular cartilage. OA affect 303.1 million people worldwide, mainly manifested as joint pain and limited mobility, which seriously affect the limb function and quality of life [1]. Joint replacement is the ultimate and effective treatment for OA, but it often brings heavy economic burden, long recovery time, and catastrophic complications such as infection, prosthesis displacement, and periprosthetic fractures. Therefore, it is of great significance to develop effective early non-surgical treatment of OA [2, 3]. At present, intra-articular (IA) injection is one of the effective treatments for the early stage of OA, which can improve the local concentration of therapeutic agents and reduce the systemic adverse reactions. However, the low delivery efficiency and the momentary retention time of most IA injection agents seriously limit the therapeutic

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effects. In addition, repeated IA administration increases the potential risk of bleeding and infection [4, 5]. Moreover, although IA injection corticoids and hyaluronic acid, and oral non-steroidal anti-inflammatory drugs can alleviate the symptoms of OA, they cannot repair the damaged articular cartilage and reverse the progress of OA [6]. Therefore, the strategy for cartilage regeneration in OA is of great clinical significance.

With the rapid development of regenerative medicine, IA injection of stem cells has become a potential strategy for the treatment of OA. Among many kinds of stem cells, mesenchymal stem cells (MSCs), especially bone marrow mesenchymal stem cells (BMSCs), have become one of the most attractive seed cells for the treatment of OA because of their potential of multi-directional differentiation and the ability to regulate inflammation [7–9]. Previous investigations have demonstrated that IA injection of MSCs was helpful to improve cartilage destruction and relieve pain in OA [10–12]. However, after injection of stem cells into the joint cavity, only a few cells adhere to the cartilage defects, their differentiation ability is not prominent [13]. In addition, the chondrogenic differentiation of MSCs after IA injection is not well controlled, which may limit their clinical applications [14, 15]. Therefore, MSCs with more chondrogenic differentiation potential will contribute to the efficient repair of articular cartilage in OA.

Kartogenin (KGN), a newly small molecule organic compound, has been proved that it can promote chondrogenesis of MSCs, thus inducing articular cartilage regeneration in OA [16, 17]. Previous study has demonstrated that KGN can promote MSCs to differentiate into chondrocytes by activating the core binding factor beta (CBFβ)-Runt-related transcription factor 1 (Runx1) signaling pathway [18], the JNK/Runx1 pathway (suppressing the β-catenin/Runx2 pathway to anti-ossification) [19], and the bone morphogenetic protein 7 (BMP-7)/Smad5 pathway [20]. However, the mechanism of chondrogenic differentiation of BMSCs induced by KGN has not been fully elucidated and needs to be further supplemented, especially the relationship between KGN and microRNA (miRNA). miRNA shows high efficiency to modulate stem cells fate determination and tissue regeneration [21, 22]. Emerging evidence presents the interesting role of some miRNAs in chondrogenesis and OA occurrence [23]. In this study, we focus on whether miR-145-5p and Smad4 pathway play a regulatory role in KGN induced chondrogenesis of BMSCs.

2 Materials and methods

2.1 Materials

Low Glucose Dulbecco's Modified Eagle's Medium (LG-DMEM), streptomycin-penicillin and fetal bovine serum (FBS), bovine serum albumin (BSA), and 0.25% trypsin EDTA were obtained from Gibco (Grand Island, NY, USA). miR-145-5p inhibitor was obtained from Sangon (Shanghai, China). Mesenchymal Stem Cell Chondrogenic Differentiation Medium was obtained from ScienCell (San Diego, CA, USA). Sprague–Dawley rats BMSCs RASMX-01001 was obtained from Cyagen Biosciences (Guangzhou China). Cell Counting Kit-8 (CCK-8) C0039, Calcein acetoxymethyl ester (Calcein-AM)/propidium iodide (PI) C2015M, and Bicinchoninic Acid Protein Assay Kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). miR-145-5p inhibitor was synthesized and provided by GenePharma (Shanghai, China). The 4% paraformaldehyde solution, phosphate buffer saline (PBS), and Triton X-100 were obtained from Beijing Solarbio Science & Technology (Beijing, China). Eastep Super Total RNA Extraction Kit was supplied by Promega Corporation. Perfect Real Time RT reagent kit, Prime Script RT reagent kit, and SYBR Premix Ex Taq II kit were provided by Takara Biotechnology (Dalian, China). TRIzol Reagent was supplied by Invitrogen Life Technology (Carlsbad, CA, USA). Safranine O, alcian blue, and hematoxylin eosin (H&E), stains were obtained from Thermo Fisher Scientific (Shanghai, China). Primary antibodies, TGF-β, Samd4, stat3, p-stat3, Sox 9, Col-2a1, aggrecan, and Col-1 were purchased from Abcam (Cambridge, UK), and secondaries antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

2.2 Tissue samples

This study protocol was approved by the Ethics Committee of Liyuan hospital affiliated to Tongji Medical College of Huazhong University of Science and Technology, and all patients were informed according to the World Medical Association Declaration of Helsinki. A total of 16 cases who underwent joint replacement, including 10 OA patients and 6 non-OA patients were enrolled in this detection (Males: Females = 8:8). All patients were aged 47–77 years (67.32 ± 11.54). During the operation, articular cartilage was collected and cryopreserved at -80 °C for subsequent real-time quantitative PCR (RT-qPCR) and Western blotting detection.

2.3 BMSCs treated with miR-145-5p inhibitor

BMSCs were seeded in 24-well culture plates at a density of 2.0×10^4/well and cultured with LG-DMEM medium containing 10% FBS (v/v), 1% penicillin and streptomycin in a humidified environment with 37 °C and 5% CO_2. In order to study the expression of Samd4, stat3, p-stat3, and TGF-β after the miR-145-5p was inhibited, 50 μM miR-145-5p inhibitor was added into the LG-DMEM for cell culture. The sequence of miR-145-5p inhibitor was 5-AGGGAAAGGAGGGAAAACUGGAC-3', and the sequence of NC inhibitor was 5-UCCCCGGAA-CAAACCCCUUUUGA-3'. BMSCs co-cultured with miR-145-5p inhibitor for 48 h to detect gene expression (*Samd4* and *TGF-β*), and 72 h to investigate protein expression (TGF-β, Samd4, stat3, and p-stat3).

2.4 BMSCs treated with KGN

In order to study the mechanism of KGN inducing chondrogenic differentiation of BMSCs, cells were cultured with LG-DMEM containing 10 μM KGN in 12-well plates at a density of 1×10^5 cells/well. The samples treated with KGN for 48 h to detect gene expression (*miR-145-5p*, *Samd4* and *TGF-β*), and 72 h to investigate protein expression (Samd4, stat3, p-stat3, and TGF-β). To evaluate the chondrogenic differentiation of stem cells induced by KGN, BMSCs were treated with chondrogenic differentiation medium containing 10 μM KGN. The chondrogenic differentiation medium was composed of 1% insulin-transferrin-selenium-sodium pyruvate solution, 25 mg/ml ascorbate-2-posphate, 40 mg/ml L-proline, 100 nM dexamethasone, and 10 ng/ml TGF-β3.

2.5 Cell viability assays

In order to detect the viability of BMSCs treated with LG-DMEM containing 10 μM KGN, CCK-8 assay and Calcein-AM/PI staining were performed. In brief, cells were seeded in 12-well plates at a density of 5×10^4 cells/well. After 1, 3, and 5 days of culture, CCK-8 assay was carried out according to the manufacturer's instructions, and the absorbance was evaluated by a microplate reader (Multiskan EX; Thermo Fisher Scientific, Inc.) at 450 nm. In addition, to observe the cell survival, the cells were stained with the Calcein-AM/PI Double Stain kit according to the manufacturer's protocol, and observed under a FV1000 confocal laser scanning microscope (CLSM, Olympus, Tokyo, Japan) after 3 days of culture. The cell survival rate was quantitatively analyzed using Image J 1.50i (NIH, Bethesda, MD, USA) according to the fluorescent images. In addition, the proliferative ability of BMSCs after intra-articular injection was detected by CCK-8 assay. Briefly, 1×10^7 BMSCs (abbreviated as NC Group) and 1×10^7 BMSCs treated with 10 μM KGN for 2 h (abbreviated as KGN Group) were injected into OA in rat joints. One week later, the articular cavity was harvested for flushing and digestion by 0.25% trypsin EDTA. The harvested cells were cultured in a six well plate, and CCK-8 assays were detected at predetermined time points.

2.6 Cells staining

BMSCs were seeded in 12-well plates at a density of 5×10^4 cells/well, and induced by KGN-containing chondrogenic medium for 14 days, and then stained with safranin O and alcian blue. Briefly, at scheduled time point, the cells were fixed by 4% paraformaldehyde for 5 min and then washed with PBS. Then, according to the manufacturer's instructions, the samples were stained with safranin O for 5 min, and stained with alcian blue for 30 min at room temperature.

2.7 Real-time quantitative PCR (RT-qPCR)

To detect the expression of miR-145-5p, *Smad4*, and *TGF-β*, and chondrogenic differentiation related genes *Sox 9*, *Col-2a1*, *aggrecan*, and *Col-1* in the cells and cartilage tissue, RT-qPCR was conducted. Briefly, total RNA was extracted with TRIzol reagent, and cDNA was synthesized in reverse transcription reaction by 1 ug total RNA using a Prime Script RT reagent kit according to the manufacturer's protocols. The expression of target genes was detected by RT-qPCR using the SYBR Premix Ex Taq II kit according to the manufacturer's protocol. Amplification was performed in 96-well optical reaction plates on the LightCycler 480 (Roche Diagnostics). *GAPDH* and *U6* were used as internal controls for mRNA and miR-145-5p expression, respectively. Primer sequences of genes were

Table 1 Primer sequences of genes

Gene	Oligonucleotide Primers (5'–3')
Sox 9	F: 5'- AGC GAA CGC ACA TCA AGA C-3'
	R: 5'- CTG TAG GCG ATC TGT TGG GG-3'
Col-2a1	F: 5'- TGG ACG ATC AGG CGA AAC C-3'
	R: 5'- GCT GCG GAT GCT CTC AAT CT-3'
Aggrecan	F: 5'- AGA ATC CAC CAC CAC CAG-3'
	R: 5'- ATG CTG GTG CTG ATG ACA-3'
Col-1	F: 5'-TCCGGCTCCTGCTCCTCTTA-3'
	R: 5'-GGCCAGTGTCTCCCTTG-3'
GAPDH	F: 5'- GGA GCG AGA TCC CTC CAA AAT-3'
	R: 5'- GGC TGT TGT CAT ACT TCT CATGG-3'

displayed in Table 1. The relative RNA expression were calculated by the formula of $2^{-\Delta\Delta Ct}$ method [24].

2.8 Western blotting

For *in vitro* studies, total protein was collected from BMSCs cultured with KGN and miR-145-5p inhibitor. For *in vivo* investigations, the cartilage tissues were isolated and washed in PBS twice. The cells or tissues were lysed for 1 h on ice by a Total Protein Extraction Buffer with protease inhibitor according to the manufacturer's instructions and then sonicated. The lysates were collected at 12,000 rpm for 10 min at 4 °C. After the lysates were centrifuged, protein concentrations were determined by a Bicinchoninic Acid Protein Assay Kit. And then the supernatant proteins were collected, denatured, separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with 5% stacking gels and 10% separating gels, and transferred, and then blocked in 5% skim milk for 1 h. The membranes were incubated with primary antibodies, Samd4 (1:200), stat3 (1:300), p-stat3 (1:150), TGF-β (1:200), and β-actin (1:200) overnight at 4 °C after blocking with 5% BSA at room temperature for 1 h. Membranes were then incubated with secondary antibodies for 40 min. The grey bands were calculated using Image J 1.50i software.

2.9 Rat OA model establishment and IA injection of KGN preconditioning BMSCs

A total of 40 Sprague–Dawley rats (male, 8-week-old) were supplied by Medical Laboratory Animal Center of Tongji Medical College of Huazhong University of Science and Technology. All animal procedures were followed and approved by The Ethics Committee of Liyuan hospital affiliated to Tongji Medical College of Huazhong University of Science and Technology (Approve no. LYH 2020–0194). The surgical destabilization of the medial meniscus (DMM) was adopted to induce OA in rat joints as described previously [17]. Briefly, after anesthetizing by intraperitoneal injection of 3% pentobarbital at the dose of 0.2 ml/100 g, the medial parapatella approach was chosen to incise the skin and move the patella to one side to dislocate it. After finding the anterior corner of the medial meniscus, it was removed with microsurgical instruments. After the patella was reset, the surgical wound was sutured in layers. The other hind leg had the same operation. For the Sham group, a same protocol was adopted without DMM operation.

Two weeks after DMM operation, the rats were divided into 4 groups (n = 10) randomly, and were IA injection with the following therapeutic agents once every 2 weeks for 3 times, namely, 100 µl PBS (abbreviated as PBS Group), 1×10^7 BMSCs in 100 µl PBS (abbreviated as BMSC Group), 1×10^7 BMSCs pre-incubated with 10 µM KGN for 2 h then collected and counted in 100 µl PBS for injection (abbreviated as KGN-BMSC Group), the sham group did not receive IA injection of any therapeutic agent (abbreviated as Normal Group). At 8 weeks after IA injection, the rats were sacrificed by overdose of 3% pentobarbita and the articular cartilage was collected for subsequent RT-qPCR, western blotting, and histological examination.

2.9.1 Histological and immunohistochemical evaluation

After scarification, the articular cartilage of knee joints was collected and fixed in 4% paraformaldehyde. The joint samples were decalcified with 0.5 M EDTA solution for about 4 weeks and embedded for paraffin-sectioning. Then, 5 µm thick slices were prepared and stained with H&E and Safranin O-fast green according to the manufacturer's instructions. The Osteoarthritis Research Society International (OARSI) cartilage histopathology assessment system was adopted to assess the destruction of articular cartilage [25]. For immunofluorescence staining in the cartilage, the sections were permeabilized with 0.5% Triton X-100 for 15 min at room temperature and blocked with 1% BSA for 30 min at 37 °C. Subsequently, the sections were incubated with primary antibodies Sox 9 (1:150, anti-mouse polyclonal antibody) plus Col-2a1 (1:150, anti-rabbit monoclonal antibody), and aggrecan (1:50, anti-mouse polyclonal antibody) plus Col-1 (1:150, anti-rabbit polyclonal antibody), respectively, at 4 °C overnight. After washing with PBS for 3 times, the slices were incubated with Cy3-conjugated goat anti-rabbit or goat anti-mouse IgG DyLight 488-conjugated secondary antibodies for 1 h at 37 °C. Finally, the nuclei were stained with DAPI, and the sections were observed under a CLSM.

2.9.2 Behavioral study

In order to research the pain related behavioral changes in OA rats after IA injection, paw withdrawal threshold (PWT) was measured to detect the effects on pain threshold as described previously [26]. In brief, the mechanical allodynia (hypersensitivity), PWT, was evaluated on the basis of the force exerted by von Frey filament (Ugo Basile, Varese, Italy) ranging from 0 to 40 g with a 0.2 g accuracy. The paw sensitivity threshold was regarded as the minimum force for leading to a strong and immediate withdrawal reflex of the paw.

2.9.3 Statistical analyses

All results were calculated as mean ± standard deviation. Comparisons among groups were analyzed with one-way ANOVA followed by Tukey's post hoc test using SPSS 19.0 (SPSS Inc.). $p < 0.05$ was considered as significant difference between groups. All experiments were repeated at least three times independently.

3 Results and discussion

3.1 The expression of miR-145-5p was increased and Samd4 pathway was inhibited in OA patients

To detect the interdependent role of miR-145-5p and Samd4 pathway in OA, we first studied their expression in OA cartilage at gene and protein levels. Consistent with a previous study, the expression of miR-145-5p in OA tissue was enhanced (Fig. 1A) [27]. Meanwhile, the mRNA levels of TGF-β and Samd4, and the transcription levels of TGF-β, Samd4, p-stat3, and p-stat3/stat3 were also found to be decreased (Fig. 1A–C), suggesting that the Smad4 pathway, a critical pathway regulating cartilage differentiation, is inhibited in patients with OA [28]. However, the relationship between miR-145-5p and Smad4 pathway in the progression of OA has not been clarified.

3.2 Samd4 pathway was activated after BMSCs were treated with miR-145-5p inhibitor

miR-145-5p is a target miRNA for Sox 9, and miR-145-5p mimic could inhibit MSCs differentiation into cartilage, and decrease the expression of cartilage-related factors [29]. In addition, Smad4 pathway shows a crucial role in the pathogenesis of OA, and its inhibition will aggravate the disease [30]. Herein, to investigate the relationship between miR-145-5p and Smad4 pathway in the progression of OA, miR-145-5p inhibitor, which predicted by Target. Scan (Fig. 2A), was prepared and cultured with BMSCs. As shown in Fig. 2B, miR-145-5p inhibitor group significantly down-regulated the gene expression of miR-145-5p, as well as the TGF-β and Samd4. Subsequently, western bolting detection further proved that miR-145-5p inhibitor could activate Samd4 pathway by up-regulating the protein expression of TGF-β, Samd4, p-stat3, and p-stat3/stat3 in BMSCs (Fig. 2C, D). These results demonstrated that Samd4 pathway could be activated after BMSCs were treated with miR-145-5p inhibitor.

3.3 The expression of miR-145-5p was decreased and Samd4 pathway was activated in KGN-treated BMSCs

In order to further study the molecular mechanism of KGN promoting BMSCs to differentiate into chondrocytes, we investigate the expression changes of miR-145-5p and

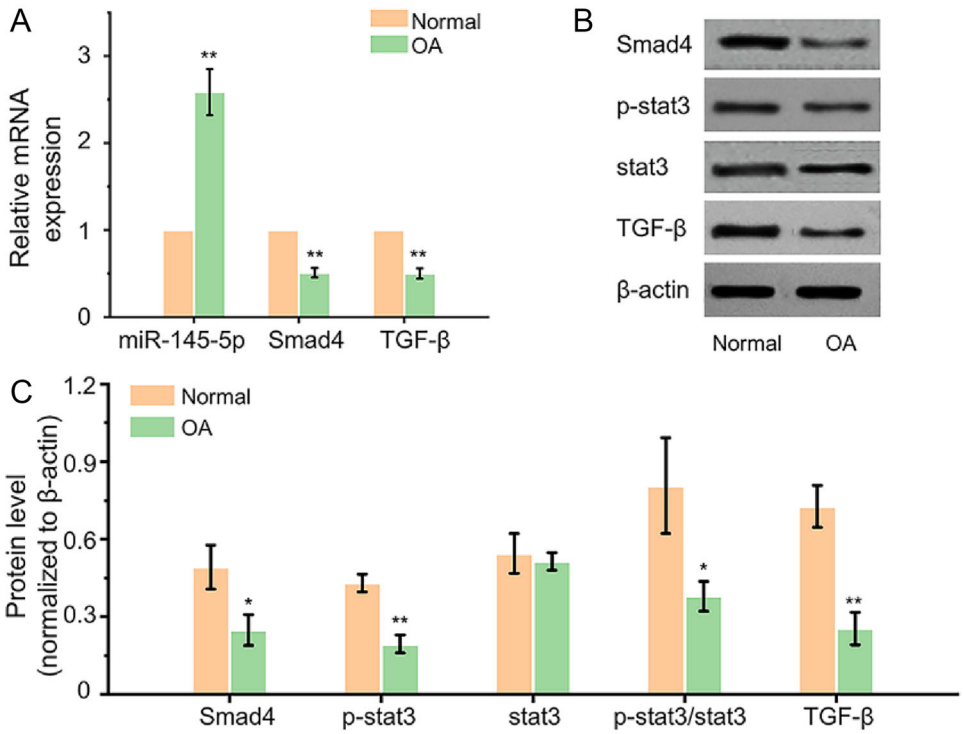

Fig. 1 The expression of miR-145-5p was increased and Samd4 pathway was inhibited in OA patients. **A** The mRNA levels of miR-145-5p, TGF-β, and Samd4 in OA patients. **B**, **C** Protein expression of TGF-β, Samd4, stat3, and p-stat3 in OA patients (*$p < 0.05$ and **$p < 0.01$)

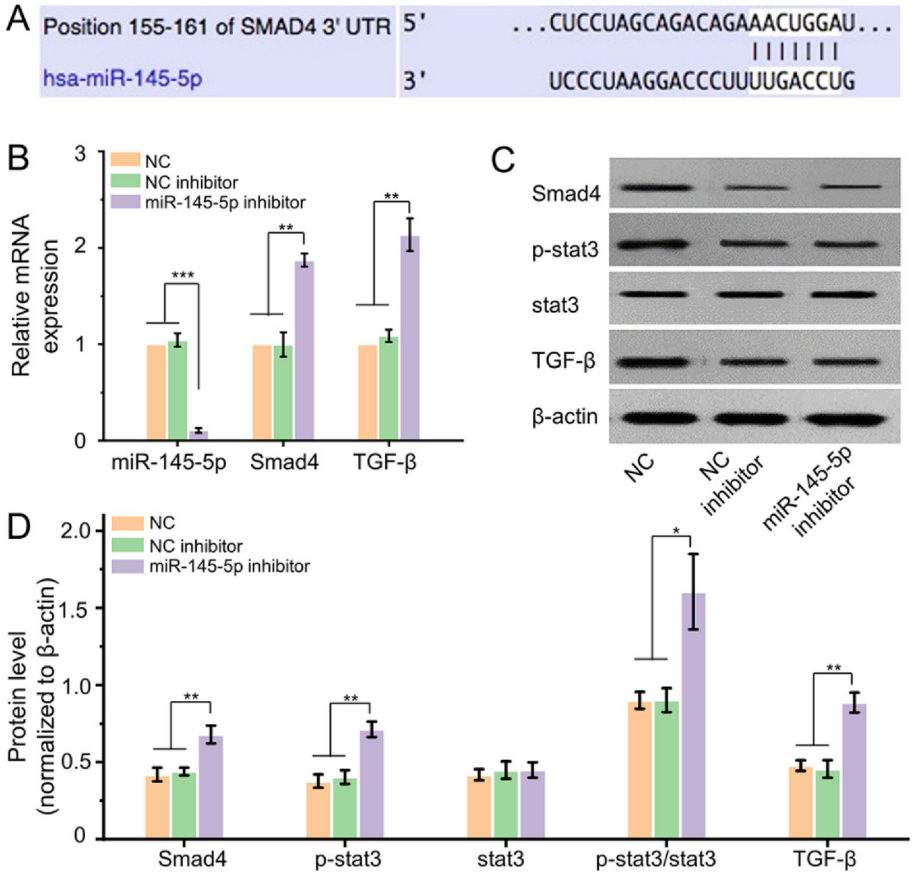

Fig. 2 Samd4 pathway was activated after BMSCs were treated with miR-145-5p inhibitor. **A** Predicted miR-145-5p target sequences. **B** The mRNA levels of miR-145-5p, Samd4, and TGF-β in BMSCs when treated with miR-145-5p inhibitor at 48 h was detected by RT-qPCR. **C**, **D** Protein expression of Samd4, stat3, p-stat3, and TGF-β in BMSCs when treated with miR-145-5p inhibitor at 72 h was detected by western blot (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$)

Smad4 pathway in BMSCs cultured with KGN containing medium. As exhibited in Fig. 3A, compared with NC group, KGN significantly diminished the gene expression of miR-145-5p, and augmented the level of TGF-β and Smad4. In addition, the level of TGF-β, Samd4, p-stat3, and p-stat3/stat3 at transcription level detected by western bolting test was also significantly enhanced (Fig. 3B, C). These results indicated KGN decreased the expression of miR-145-5p and activated the Samd4 pathway in BMSCs.

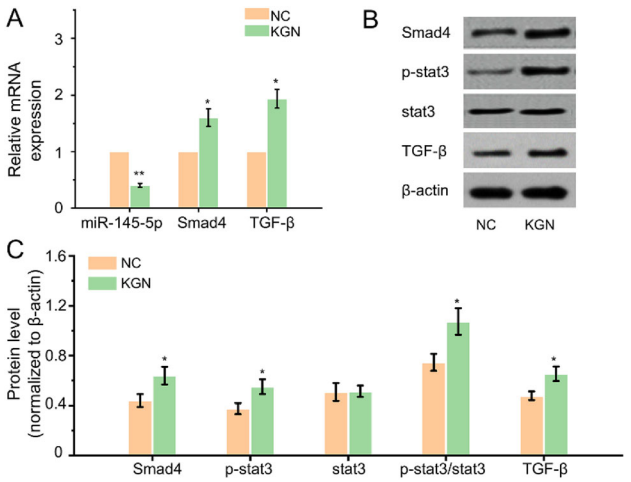

Fig. 3 The expression of miR-145-5p was decreased and Samd4 pathway was activated in KGN-treated BMSCs. **A** The mRNA levels of miR-145-5p, TGF-β, and Samd4 in BMSCs when treated with KGN at 48 h was detected by RT-qPCR. **B**, **C** Protein expression of TGF-β, Samd4, stat3, and p-stat3 in BMSCs when treated with KGN at 72 h was detected by western blot (*$p < 0.05$ and **$p < 0.01$)

3.4 Proliferation and chondrogenic differentiation of BMSCs treated with KGN

For the clinical efficacy of IA injection of stem cells in the treatment of OA, the proliferation and chondrogenic differentiation of injected cells are crucial. Herein, the proliferation of BMSCs treated with KGN was detected by the CCK-8 assay. As displayed in Fig. 4A, with the time extension of incubation, the absorbance of each group increased significantly. What is satisfying is that the cell proliferation in KGN group at 5 days were increased significantly compared with the NC group. Furthermore, Calcein-AM/PI staining was carried to investigate the cell survival, which demonstrated directly that the BMSCs in all group were almost green-stained live cells (Fig. 4B). Then the survival rate in the NC group and KGN group was calculated quantitatively, which was 95.54 ± 2.17% and 94.15 ± 1.30%, respectively, and there was no distinct difference between the groups (Fig. 4C). These results

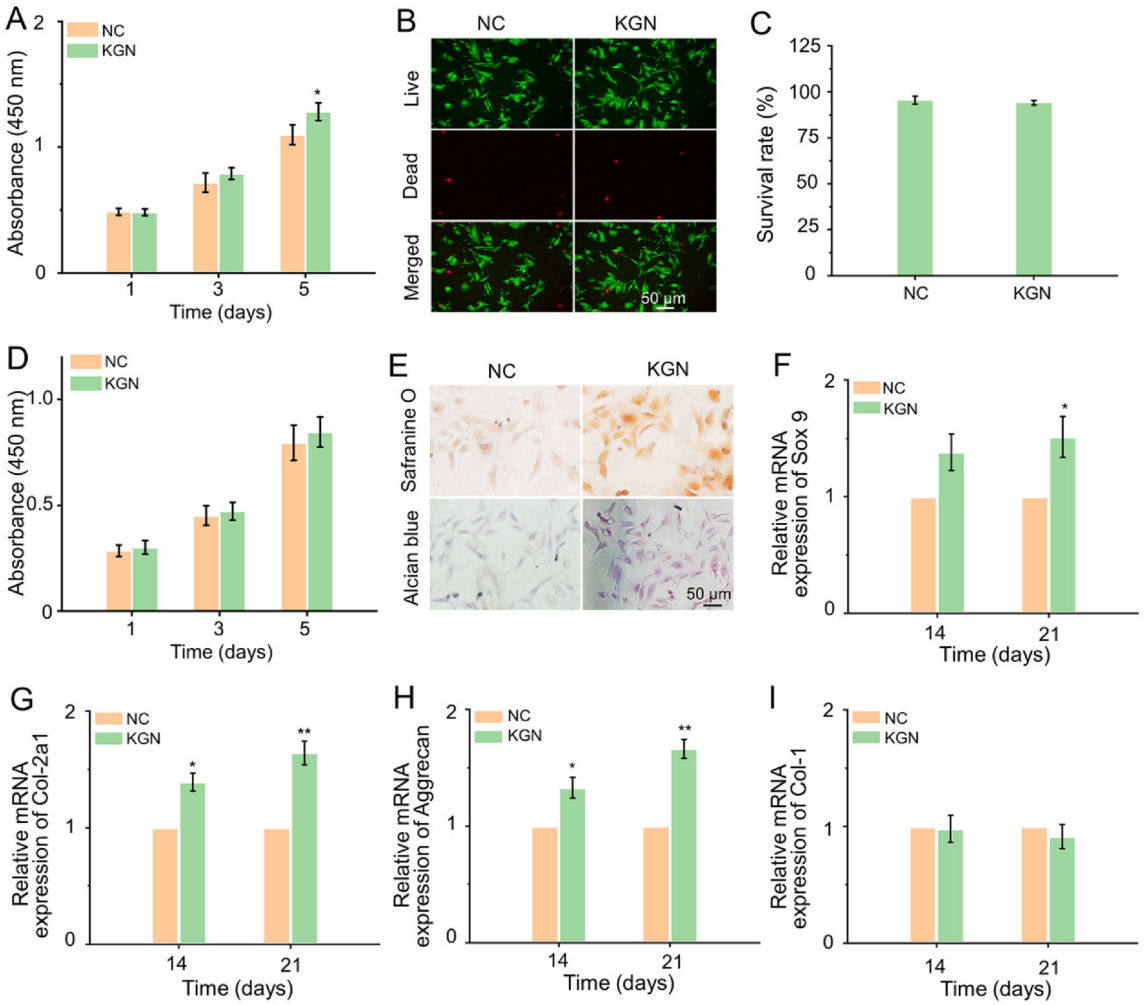

Fig. 4 Proliferation and chondrogenic differentiation of BMSCs treated with KGN. **A** The cell proliferation assay was studied after 1, 3, and 5 days by CCK-8 assay. **B** Representative fluorescence images of Calcein-AM/PI staining incubated for 3 days. **C** The survival rates of BMSCs. **D** The proliferation of BMSCs extracted after intra-articular injection by CCK-8 assay. **E** Safranine O and Alcain blue of BMSCs. **F–I** RT-qPCR analysis of chondrogenic genes *Sox 9*, *Col-2a1*, *aggrecan*, and *Col-1* of BMSCs treated with KGN. (*$p < 0.05$, **$p < 0.01$)

clearly demonstrated that KGN-treated BMSCs had good cell activity and were conducive to cell proliferation *in vitro*, thus taking the advantage of realizing the therapeutic effect in OA. In addition, the proliferative ability of BMSCs after intra-articular injection was detected by CCK-8 assay. BMSCs and KGN-pretreated BMSCs harvested from articular cavity had good proliferation ability. KGN group had a stronger proliferation ability than NC group, but there was no significant difference between the two groups (Fig. 4D).

In addition to cell proliferation and survival, chondrogenic differentiation of stem cells is crucial for the treatment of OA. To detect the function of KGN on the chondrogenic differentiation of BMSCs, cytological staining and RT-qPCR were conducted. Safranine O and Alcain blue staining confirmed that KGN can significantly promote chondrogenic differentiation of BMSCs (Fig. 4E). Furthermore, the results of RT-qPCR revealed that the expression of chondrogenic genes *Sox 9*, *Col-2a1*, and *aggrecan* were up-regulated in KGN group, which were significantly higher than that of NC group (Fig. 4F–H). However, the level of osteogenic related gene *Col-1* was not increased by KGN treatment (Fig. 4I). These results suggested that KGN treatment can promote chondrogenesis rather than osteogenic differentiation of BMSCs.

Chondrogenic differentiation of BMSCs *in vitro* mainly depends on the addition of induction factors. Previous studies have indicated that TGF-β and IGF-1 are the main inducing factors [31–33]. TGF-β is a critical factor in the process of directional differentiation of BMSCs into chondrocytes. The TGF-β can deliver a signal, which is transmitted to the nucleus through Smad pathway, to activate the expression of Sox 9, and then promote the expression of Col-2 [34]. In this study, we applied KGN to

induce chondrogenic differentiation of BMSCs, and explored its potential mechanism. The results indicated that KGN promoted the BMSCs chondrogenic differentiation by down-regulation of miR-145-5p targeting Smad4 pathway, which provides a new strategy for the stem cell therapy of OA.

3.5 IA injection of KGN-pretreated BMSCs reduced cartilage degeneration in OA

The degeneration and destruction of articular cartilage is the basic pathological change and associated with primary clinical symptoms in the OA. In this study, we investigated the potential of KGN-pretreated BMSCs for cartilage regeneration in OA model, which was prepared by DMM surgery.

Eight weeks after IA injection of different therapeutic agents, articular cartilages were collected for histological evaluation. H&E staining indicated that the articular surface in the PBS group was not smooth, cartilages missed and defects formed, subchondral bone exposed, and osteophytes formed, and a large number of cells on the cartilage surface were necrotic. Although the cartilage in the BMSC group maintained a relatively complete gross shape, the cartilage layer became thinner and slightly matte, the chondrocytes on the cartilage surface were disappeared and the deep chondrocytes were arranged loosely and irregularly, and suspicious fiber components were found on the surface of cartilage. However, in the BMSC-KGN Group, the articular cartilage was generally intact, with cells being regularly arranged, which was similar to the cartilage structure in healthy state, namely the Normal group (Fig. 5A). Subsequently, Safranin O-fast green staining was conducted to evaluate the cartilage matrix. The results showed that the cartilage was damaged in the PBS group and BMSC group, resulting in uneven distribution of matrix components and slight or no staining of Safranin O. However, in the BMSC-KGN group and the Normal group, the cartilage indicated a good histological morphology and uniform matrix distribution (Fig. 5B). In addition, the OARSI cartilage histopathology assessment system was applied to estimate cartilage degeneration (Fig. 5C). The score in the BMSC-KGN group was 3.6 ± 1.5, which was significantly lower than PBS group (12.60 ± 2.70) and BMSC group (8.8 ± 2.3), and similar with Normal group (2.0 ± 1.2), respectively.

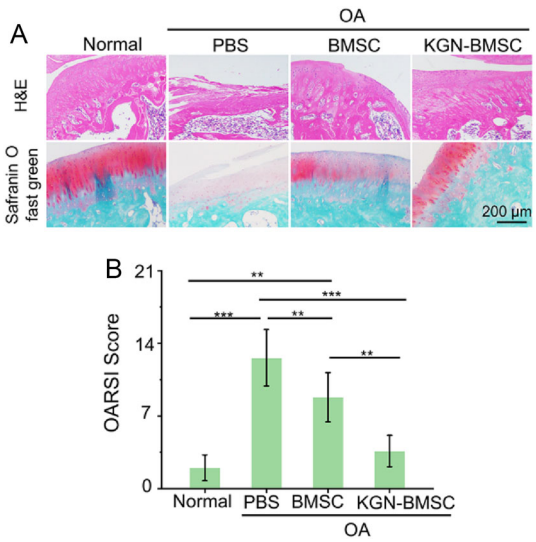

Fig. 5 **A**, **B** H and E and Safranin O staining at 8 weeks after the IA injection. **C** OARSI scores in each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

3.6 IA injection of KGN-pretreated BMSCs induced chondrogenesis in OA

To further investigate the change of chondrogenic differentiation related marks in the cartilage, RT-qPCR and immunofluorescence staining were performed to evaluate the content of Sox 9, Col-2a1, aggrecan, and Col-1 at 8 weeks post-injection. As revealed in Fig. 6A–C, the gene level of *Sox 9*, *Col-2a1*, *aggrecan* in BMSC-KGN group were significantly up-regulated compared with PBS group and BMSC group. Compared with BMSC group and BMSC-KGN group, the expression of *Col-1* in PBS group was significantly higher, indicating the formation of subchondral ossification and osteophyte (Fig. 6D). Subsequently, immunofluorescence double staining was used to further verify the content of Sox 9, Col-2a1, aggrecan, and Col-1 in cartilage, which was roughly the same as the trend of gene expression (Fig. 6E–I).

In general, KGN-pretreated BMSCs have better chondrogenesis capacity in OA than BMSCs injected directly. After IA injection of KGN-pretreated BMSCs, the chondrogenic differentiation related markers in OA cartilage were generally similar to those in healthy non-OA cartilage, although BMSC-KGN group had lower *Sox 9* gene expression and aggrecan protein content compared with Normal group. This phenomenon is attributed to the powerful function of KGN to promote the differentiation of MSCs into chondrocytes, enabling the repair and regeneration of articular cartilage in OA [16, 17].

3.7 IA injection of KGN-pretreated BMSCs relieved pain caused by OA

As a kind of degenerative disease, the pain and dysfunction caused by OA greatly perplex the patients. Peripheral pain mechanisms include the direct activation of nociceptors, as well as sensitization of nociceptors by the inflammation in articular cavity [35, 36]. Local immune cells secrete

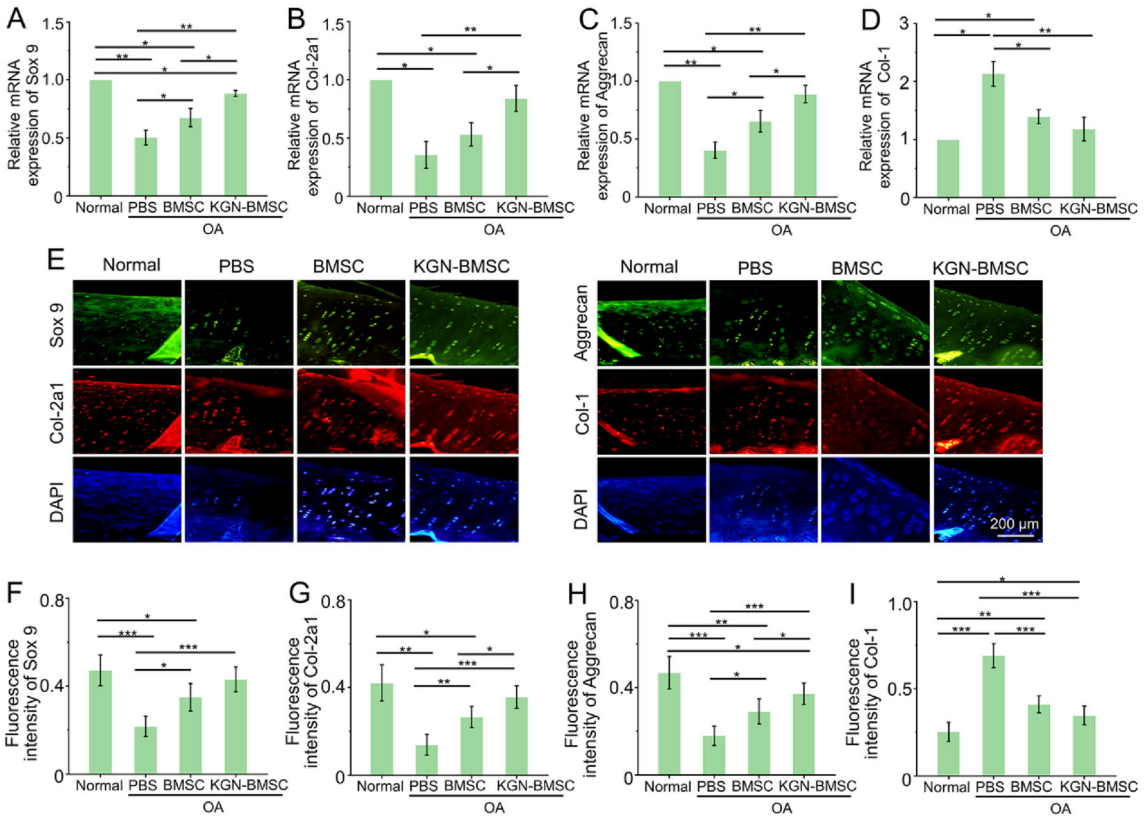

Fig. 6 KGN preconditioning BMSCs promoted cartilage regeneration in OA. **A–D** RT-qPCR analysis of *Sox 9*, *Col-2a1*, *aggrecan*, and *Col-1* in OA rat model at 8 weeks post-injection. **E** Representative immunofluorescence images of Sox 9, Col-2a1, aggrecan, and Col-1 in cartilage at 8 weeks post-injection. (**F–I**) Quantitative statistics of Sox 9, Col-2a1, aggrecan, and Col-1 expression ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$)

inflammatory cytokines and additional molecular mediators that function on the peripheral nerve terminals of nociceptor neurons [37]. In response to the inflammatory mediators, intracellular signaling pathways lead to a phosphorylation cascade, which reduces the threshold for nociceptor neurons to generate action potentials, eventually resulting in heightened pain sensitivity [38]. As displayed in Fig. 7, the PWT values of Normal group, PBS group, BMSC group, BMSC-KGN group were 15.6 ± 2.5, 9.2 ± 2.5, 10.8 ± 2.1, and 13.8 ± 1.7 at 8 weeks after IA injection, respectively. These results revealed that IA injection of KGN-pretreated BMSCs could relieve pain caused by OA.

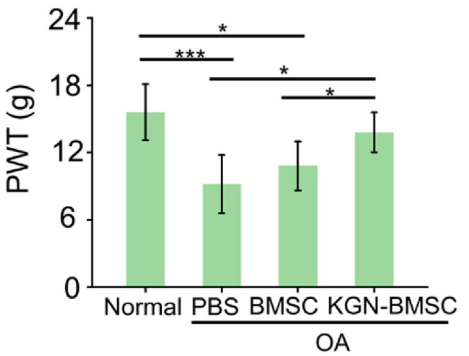

Fig. 7 Paw withdrawal threshold (PWT) was measured to test mechanical allodynia ($*p < 0.05$, $***p < 0.001$)

3.8 IA injection of KGN-pretreated BMSCs reduced cartilage degeneration by decreasing miR-145-5p expression and activating Samd4 pathway in OA

In order to further investigate the molecular mechanism of cartilage protective effect of KGN-pretreated BMSCs *in vivo*, the miR-145-5p expression and Smad4 pathway regulation in the cartilage of OA rats were detected. As exhibited in Fig. 8A–C, compared with PBS group and BMSC group, IA injection of KGN-pretreated BMSCs significantly inhibited the gene expression of miR-145-5p, and augmented the level of TGF-β and Smad4. It is worth mentioning that, IA injection of KGN–pretreated BMSCs could almost restore the expression of miR-145-5p in OA to the level of healthy non-OA state. In addition, the level of TGF-β, Samd4, p-stat3, and p-stat3 at transcription level was detected (Fig. 8D–I). The quantitative analysis of

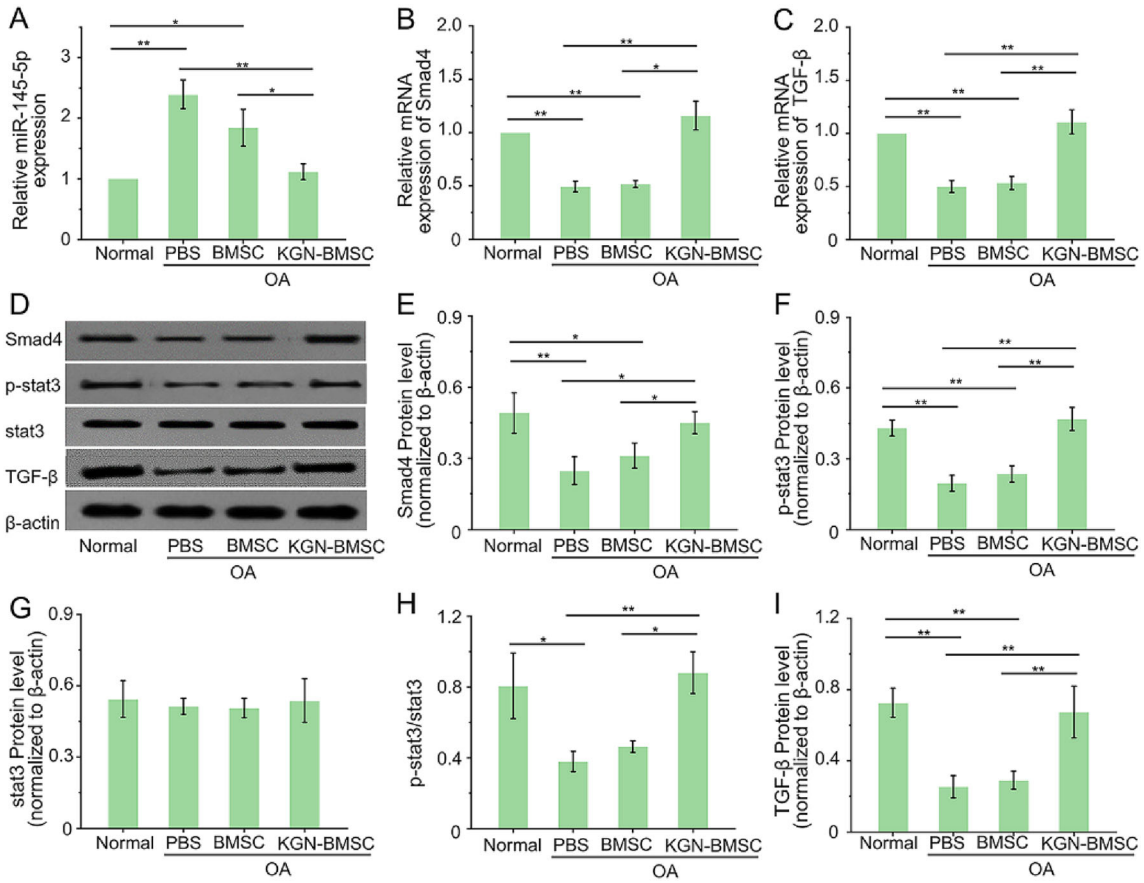

Fig. 8 KGN-pretreated BMSCs decreased miR-145-5p expression and activated Samd4 pathway in OA rats. **A–C** The mRNA expression of miR-145-5p, TGF-β, and Samd4 in cartilage after IA injection. **D–I** Protein expression of TGF-β, Samd4, stat3, and p-stat3 in cartilage after IA injection detected by western blot (*$p < 0.05$ and **$p < 0.01$)

western blotting showed that the content of TGF-β, Samd4, p-stat3, and p-stat3/stat3 in BMSC-KGN group were significantly higher than PBS group and BMSC group, suggesting that the Smad4 pathway was activated in OA. These results further demonstrated that IA injection of KGN-pretreated BMSCs promoted BMSCs chondrogenic differentiation in OA by down-regulation of miR-145-5p targeting Smad4 pathway, thus playing a role in cartilage protection. However, this study lacks a more comprehensive *in vivo* experimental design to fully prove the precise molecular mechanism of KGN-treated BMSCs *in vivo*. As an important part of cell therapy, stem cell therapy plays an important role in organ repair and tissue regeneration. However, understanding the migration, distribution, survival and ultimate fate of stem cells entering the human body is of great significance for basic research and clinical implantation of stem cells. Using BMSCs with fluorescence labeling for transplantation, and check the co-localization of transplanted cell signals with chondrogenic markers in OA cartilage tissue, is of great significance for studying the precise mechanism *in vivo*. However, our *in vivo* observation period was up to 8 weeks after the injection of stem cells into the articular cavity. As the long retention time *in vivo*, the fluorescence will quench due to cell proliferation and differentiation, as well as limited experimental conditions, causing great trouble for the tracing of transplanted BMSCs *in vivo*.

Our results indicated KGN-treated BMSCs could induce BMSCs differentiating into chondrocytes and they filled in the cartilage defects to show the regenerative effect, thus inhibiting cartilage degeneration and reduce OA progression *in vivo*. A previous study has indicated that MSCs have paracrine effects. The growth factors, cytokines, and bioactive substances contained in the microcapsules released from the implanted MSCs can promote the proliferation of chondrocytes and collagen synthesis, which can relieve pain and improve the function of OA patients [39]. At present, the molecular mechanism of BMSCs in cartilage formation is still unclear. In order to avoid fibrocartilage, hypertrophic cartilage and cartilage ossification, it is still necessary to further study the basic research of cartilage-oriented differentiation and expression of MSCs. Herein, the action mechanism of KGN-treated BMSCs to reduce OA progression *in vivo* could be

through chondrogenic differentiation of BMSCs induced by KGN preconditioning to promote the chondrocytes proliferation and collagen synthesis.

4 Conclusion

In conclusion, KGN promotes the BMSCs chondrogenic differentiation by down-regulation of miR-145-5p targeting Smad4 pathway. For intra-articular injection of KGN-pretreated BMSCs, this strategy significantly prevents articular cartilage degeneration and alleviates pain in OA. This suggests intra-articular injection of KGN-pretreated BMSCs is a promising strategy to administer cartilage repairing and pain relief in OA patient in the future.

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Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical statement All animal procedures were performed in accordance with the guidelines for Care and Use of Laboratory Animal Experience and approved by the Liyuan hospital affiliated to Tongji Medical College of Huazhong University of Science and Technology. (Approve no. LYH 2020–0194).

References

1. Peat G, Thomas MJ. Osteoarthritis year in review 2020: epidemiology and therapy. Osteoarthritis Cartilage. 2021;29:180–9.
2. Costello CA, Liu M, Furey A, Rahman P, Randell EW, Zhai G. Association between epidemiological factors and nonresponders to total joint replacement surgery in primary osteoarthritis patients. J Arthroplasty. 2021;36:1502–10.e5.
3. Hailer NP, Kuja-Halkola R, Bruggemann A, Pedersen NL, Michaëlsson K. Body mass index differentially moderates heritability of total joint replacement due to hip and knee osteoarthritis a cohort study of 29,893 Swedish twin pairs. J Bone Joint Surg Am. 2021;103:1319–27.
4. Kon E, Boffa A, Andriolo L, Di Martino A, Di Matteo B, Magarelli N. Subchondral and intra-articular injections of bone marrow concentrate are a safe and effective treatment for knee osteoarthritis: a prospective, multi-center pilot study. Knee Surg Sports Traumatol Arthrosc. 2021. https://doi.org/10.1007/s00167-021-06530-x.
5. Najm A, Alunno A, Gwinnutt JM, Weill C, Berenbaum F. Efficacy of intra-articular corticosteroid injections in knee osteoarthritis: a systematic review and meta-analysis of randomized controlled trials. Joint Bone Spine. 2021;88:105198.
6. Gazendam A, Ekhtiari S, Bozzo A, Phillips M, Bhandari M. Intra-articular saline injection is as effective as corticosteroids, platelet-rich plasma and hyaluronic acid for hip osteoarthritis pain: a systematic review and network meta-analysis of randomised controlled trials. Br J Sports Med. 2021;55:256–61.
7. Barry F, Murphy M. Mesenchymal stem cells in joint disease and repair. Nat Rev Rheumatol. 2013;9:584–94.
8. Bertoni L, Jacquet-Guibon S, Branly T, Desance M, Legendre F, Melin M, et al. Evaluation of allogeneic bone-marrow-derived and umbilical cord blood-derived mesenchymal stem cells to prevent the development of osteoarthritis in an equine model. Int J Mol Sci. 2021;22:2499.
9. Hwang JJ, Rim YA, Nam Y, Ju JH. Recent developments in clinical applications of mesenchymal stem cells in the Treatment of rheumatoid arthritis and osteoarthritis. Front Immunol. 2021;12:631291.
10. Centeno CJ, Busse D, Kisiday J, Keohan C, Freeman M, Karli D. Increased knee cartilage volume in degenerative joint disease using percutaneously implanted autologous mesenchymal stem cells. Pain Physician. 2008;11:343–53.
11. Csaki C, Schneider PR, Shakibaei M. Mesenchymal stem cells as a potential pool for cartilage tissue engineering. Ann Anat. 2008;190:395–412.
12. Maumus M, Guérit D, Toupet K, Jorgensen C, Noël D. Mesenchymal stem cell-based therapies in regenerative medicine: applications in rheumatology. Stem Cell Res Ther. 2011;2:14.
13. Koga H, Shimaya M, Muneta T, Nimura A, Morito T, Hayashi M, et al. Local adherent technique for transplanting mesenchymal stem cells as a potential treatment of cartilage defect. Arthritis Res Ther. 2008;10:R84.
14. Chang YH, Liu HW, Wu KC, Ding DC. Mesenchymal stem cells and their clinical applications in osteoarthritis. Cell Transplant. 2016;25:937–50.
15. Ha CW, Park YB, Kim SH, Lee HJ. Intra-articular mesenchymal stem cells in osteoarthritis of the knee: a systematic review of clinical outcomes and evidence of cartilage repair. Arthroscopy. 2019;35:277–88.e2.
16. Wang SJ, Qin JZ, Zhang TE, Xia C. Intra-articular injection of kartogenin-incorporated thermogel enhancing osteoarthritis treatment. Front Chem. 2019;7:677.
17. Xu X, Liang Y, Li X, Ouyang K, Wang M, Cao T, et al. Exosome-mediated delivery of kartogenin for chondrogenesis of synovial fluid-derived mesenchymal stem cells and cartilage regeneration. Biomaterials. 2021;269:120539.
18. Johnson K, Zhu S, Tremblay MS, Payette JN, Wang J, Bouchez LC, et al. A stem cell-based approach to cartilage repair. Science. 2012;336:717–21.
19. Jing H, Zhang X, Gao M, Luo K, Fu W, Yin M, et al. Kartogenin preconditioning commits mesenchymal stem cells to a precartilaginous stage with enhanced chondrogenic potential by modulating JNK and beta-catenin-related pathways. FASEB J. 2019;33:5641–53.
20. Zhou Q, Zhang JH, Yuan S, Shao JH, Cai ZY, Chen S, et al. A new insight of kartogenin induced the mesenchymal stem cells (MSCs) selectively differentiate into chondrocytes by activating the bone morphogenetic protein 7 (BMP-7)/Smad5 pathway. Med Sci Monitor. 2019;25:4960–7.
21. Kao GS, Tu YK, Sung PH, Wang FS, Lu YD, Wu CT, et al. MicroRNA-mediated interacting circuits predict hypoxia and inhibited osteogenesis of stem cells, and dysregulated angiogenesis are involved in osteonecrosis of the femoral head. Int Orthop. 2018;42:1605–14.
22. Yao C, Wu W, Tang H, Jia X, Tang J, Ruan X, et al. Self-assembly of stem cell membrane-camouflaged nanocomplex for microRNA-mediated repair of myocardial infarction injury. Biomaterials. 2020;257:120256.
23. Geng Y, Chen J, Alahdal M, Chang C, Duan L, Zhu W, et al. Intra-articular injection of hUC-MSCs expressing miR-140-5p induces cartilage self-repairing in the rat osteoarthritis. J Bone Miner Metab. 2020;38:277–88.
24. Bai H, Zhao Y, Wang C, Wang Z, Wang J, Liu H, et al. Enhanced osseointegration of three-dimensional supramolecular bioactive

interface through osteoporotic microenvironment regulation. Theranostics. 2020;10:4779–94.
25. Kang ML, Jeong SY, Im GI. Hyaluronic acid hydrogel functionalized with self-assembled micelles of amphiphilic pegylated kartogenin for the treatment of osteoarthritis. Tissue Eng Part A. 2017;23:630–9.
26. Micheli L, Bozdag M, Akgul O, Carta F, Guccione C, Bergonzi MC, et al. Pain relieving effect of-NSAIDs-CAIs hybrid molecules: systemic and intra-articular treatments against rheumatoid arthritis. Int J Mol Sci. 2019;20:1923.
27. Chen Y, Wang X, Yang M, Ruan W, Wei W, Gu D, et al. miR-145-5p increases osteoclast numbers in vitro and aggravates bone erosion in collagen- induced arthritis by targeting osteoprotegerin. Med Sci Monitor. 2018;24:5292–300.
28. Zhuang X, He L. Effects on osteoarthritis inflammation and apoptosis of chondrocytes under down-regulated smad4 expression by MIR-146A-5P. Acta Medica Mediterranea. 2020;36:1707–13.
29. Zhu J, Fu Q, Shao J, Peng J, Qian Q, Zhou Y, et al. Regulating effect of Circ_ATRNL1 on the promotion of SOX9 expression to promote chondrogenic differentiation of hAMSCs mediated by MiR-145-5p. J Tissue Eng Regen Med. 2021;15:487–502.
30. Hu S, Zhao X, Mao G, Zhang Z, Wen X, Zhang C, et al. MicroRNA-455-3p promotes TGF-beta signaling and inhibits osteoarthritis development by directly targeting PAK2. Exp Mol Med. 2019;51:1–13.
31. Peng XB, Zhang Y, Wang YQ, He Q, Yu Q. IGF-1 and BMP-7 synergistically stimulate articular cartilage repairing in the rabbit knees by improving chondrogenic differentiation of bone-marrow mesenchymal stem cells. J Cell Biochem. 2019;120:5570–82.
32. Qasim M, Le NXT, Nguyen TPT, Chae DS, Park SJ, Lee NY. Nanohybrid biodegradable scaffolds for TGF-β3 release for the chondrogenic differentiation of human mesenchymal stem cells. Int J Pharm. 2020;581:119248.
33. Taghavi M, Parham A, Raji A. The combination of TGF-beta 3 and BMP-6 synergistically promotes the chondrogenic differentiation of equine bone marrow-derived mesenchymal stem cells. Int J Pept Res Ther. 2020;26:727–35.
34. Ruiz M, Maumus M, Fonteneau G, Pers YM, Ferreira R, Dagneaux L, et al. TGF beta i is involved in the chondrogenic differentiation of mesenchymal stem cells and is dysregulated in osteoarthritis. Osteoarthritis Cartilage. 2019;27:493–503.
35. Boyden SD, Hossain IN, Wohlfahrt A, Lee YC. Non-inflammatory causes of pain in patients with rheumatoid arthritis. Curr Rheumatol Rep. 2016;18:30.
36. Meeus M, Vervisch S, De Clerck LS, Moorkens G, Hans G, Nijs J. Central sensitization in patients with rheumatoid arthritis: a systematic literature review. Semin Arthritis Rheum. 2012;41:556–67.
37. Zhang A, Lee YC. Mechanisms for joint pain in rheumatoid arthritis (RA): from cytokines to central sensitization. Curr Osteoporos Rep. 2018;16:603–10.
38. Pinho-Ribeiro FA, Verri WA Jr, Chiu IM. Nociceptor sensory neuron-immune interactions in pain and inflammation. Trends Immunol. 2017;38:5–19.
39. Nazempour A, Van Wie BJ. Chondrocytes, mesenchymal stem cells, and their combination in articular cartilage regenerative medicine. Ann Biomed Eng. 2016;44:1325–54.