

ARTICLE

Deer antler reserve mesenchyme cells with hyaluronan alleviates cartilage damage in a rat model

Boyin Jia^{1,2} | Xin Li¹ | Xintong Han¹ | Fuquan Ma¹ | Linlin Zhang¹ |
Xue Wang¹ | Xinrui Yan¹ | Yu Zhang¹ | Jianming Li^{2,3} | Pengfei Hu⁴ |
Yusu Wang³ | Naichao Diao^{2,3} | Kun Shi^{2,3} | Ying Zong^{2,3} | Rui Du^{2,3}  |
Chunyi Li⁴

¹College of Animal Medicine/ College of Animal Science and Technology, Jilin Agricultural University, Changchun, China

²Laboratory of Production and Product Application of Sika Deer of Jilin Province, Jilin Agricultural University, Changchun, China

³College of Chinese Medicine Materials, Jilin Agricultural University, Changchun, China

⁴Institute of Antler Science and Product Technology, Changchun Sci-Tech University, Changchun, China

Correspondence

Rui Du, Laboratory of Production and Product Application of Sika Deer of Jilin Province, Jilin Agricultural University, 2888 Xincheng Street, Changchun 130118, China; College of Chinese Medicine Materials, Jilin Agricultural University, 2888 Xincheng Street, Changchun 130118, China.
Email: duyijia@163.com

Chunyi Li, Institute of Antler Science and Product Technology, Changchun Sci-Tech University, 1345 Pudong Road, Changchun 130000, China.
Email: lichunyi1959@163.com

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Abstract

Reserve mesenchyme cells (RMCs) of deer antlers have been considered as the promising cell source for repairing injury-induced articular cartilage or cartilage degeneration. However, systematic investigation of RMC differentiation to repair injured cartilage and its combination with biomaterials has not been reported. The aim of this study was to evaluate the role of RMCs in combination with hyaluronic acid (HA) in promoting chondrogenic differentiation through simulating native environments and their efficacy in articular cartilage repair. The RMCs were cultured in vitro for the characterization of these cells, including morphology, surface marker expression, and multipotent differentiation potential (adipogenesis, chondrogenesis, and osteogenesis). When combined with HA in vitro, RMCs increased expression levels of the chondrogenic marker gene (COL II and COMP) but decreased levels of the hypertrophic marker gene (COL X). Using a rat articular cartilage defect model, we evaluated the effects of RMCs in combination with HA on cartilage defect repair at 4 and 8 weeks through macroscopical, histological, and immunohistochemical examinations. Compared with other groups, treatment with RMCs + HA reduced cartilage loss and degree of cartilage surface worn, whereas cartilage content was significantly increased. These results suggest that the combination of RMCs with HA can effectively repair cartilage defects. We believe that effective cartilage defect repair will benefit from the use of RMCs together with favorable biomaterials, such as HA.

Keywords

cartilage, defects, hyaluronic acid, reserve mesenchyme cells, stem cell therapy

Boyin Jia and Xin Li are equally contributed.

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INTRODUCTION

Articular cartilage is hyaline cartilage, composed mainly of chondrocytes and the extracellular matrix (ECM) [1]. Cartilage tissue lacks blood vessels and obtains nutrients only from the synovial fluid and nearby blood vessels through diffusion [1]. Meanwhile, chondrocytes reside in cartilage lacunae and are not able to migrate to the wound site; thus, the cartilage cannot repair its own defects, and the damage normally gradually deteriorates into osteoarthritis [2]. At present, besides joint replacement, the way of treatments of articular cartilage defects and osteoarthritis mainly includes both pharmacological (chemical, biological, and traditional Chinese medicine) and nonpharmacological methods (patient education programs, weight reduction, coping strategies, and exercise programs) [3, 4]. However, these methods can only temporarily alleviate the symptoms, and cartilage defects will eventually become too big to regenerate or reconstruct.

Mesenchymal stem cells (MSCs) are a type of pluripotent stem cell [5] and can self-renew and differentiate into multilineage cells [6]. These cells can not only directly participate in tissue repair themselves but also can indirectly facilitate repair through immunomodulatory and anti-inflammatory properties [7]. Stem cell therapy has currently been widely used in the treatment of various diseases [8]. In particular, MSCs from different sources were widely used for cartilage defect repair, including bone marrow MSCs (BMSCs), adipose MSCs (AMSCs), synovial MSCs (SMSCs), umbilical cord blood MSCs (UCMSCs), etc. [9–12]. MSCs could migrate from the subchondral bone to the damaged cartilage site and differentiate into cartilage-lineage and bone-lineage cells [13]. The differentiated cells can effectively integrate into the surrounding of newly formed tissues to repair the damaged cartilage [14]. However, some studies recently reported that the directional differentiation of MSCs *in vitro* was not always repeatable, and the phenotype of differentiated chondrocytes may vary [15]. Therefore, the exploration of new MSC sources has never ceased.

Antlers are male secondary sexual characters and organs of bone consisting of cartilage tissue in their growth center, within which mesenchymal stem cells lay, known as reserve mesenchyme cells (RMCs) [16, 17]. It is the proliferation of RMCs that drive antlers to grow so rapidly without known parallel examples [18, 19]. Because RMCs can effectively avoid the tumorigenic tendency, which normally occurs in other MSCs, thereby reducing the risk of RMC use in the treatment of cartilage defects. Meanwhile, RMCs have the potential to differentiate into chondrocytes and other cell types *in vitro* [17, 20]. Therefore, RMCs may provide new cell resources for the treatment of cartilage defects.

Hyaluronic acid (HA) is one of the main components in the joint fluid and cartilage matrix [21]. Direct injection of HA into the joint cavity can effectively improve the viscoelasticity and osmotic pressure of synovial fluid, alleviate joint pain, and increase joint range of motion [22]. However, the effects of HA treatment alone is limited. Previous studies found that intra-articular injection of HA could alleviate pain symptoms in early and moderate osteoarthritis patients, while the level of pain relief in late osteoarthritis patients was relatively low [23, 24]. In addition, high frequency use of HA might cause local adverse reactions [25]. In recent years, use of MSCs in combination with HA for treating cartilage defects has become popular. Zhang et al. (2021) confirmed that HA could enhance the chondrogenic potential of hESC–MSCs *in vitro*. Meanwhile, the composite (MSCs + HA) showed additive effects on the treatment of osteoarthritis *in vivo* in a rabbit model [26]. Chang et al. (2021) found that the expression levels of chondrogenic markers increased in the chondrocytes when treated with HUCMSC + HA *in vitro* [27]. The composite also significantly suppressed the hyaline cartilage destruction in rabbit osteoarthritis [27]. To sum up, MSCs combined with HA both *in vitro* and *in vivo* can effectively repair cartilage defects and osteoarthritis in experimental animal models. Interestingly, antler RMCs were bathed in the ECM richly containing chondroitin sulfate and HA [28–30]. When residing in such an environment, RMCs could proliferate and chondrogenic could differentiate in an unparalleled speed (elongation at 2 cm/day) [19]. Therefore, the addition of HA could facilitate the process when using RMCs to repair cartilage damage.

In this study, we investigated the safety, feasibility, and effectiveness of using the composite RMCs + HA to treat rat articular cartilage defects via injecting directly into the joint cavity. Comparing with other types of MSCs, RMCs may be a better cell source in repair cartilage defects, particularly when combined with HA. Overall, our system may provide a new therapeutic strategy for cartilage defect repair.

MATERIALS AND METHODS

Tissue collection and RMCs culture

The experiments were approved by the Jilin Agricultural University Committee on the use of live animals. Antler tissue samples were collected from three 3-year-old sika deer at 30 days after previous hard antler button casting. The RMCs were isolated from the growing antler tips aseptically as Li described [31]. RMCs culture was performed using the description of Li et al. [32]. Briefly, the samples were cut into small pieces and digested in a DMEM high sugar medium containing

collagenase (150 units/mL). After the removal of collagenase, a digested complex was cultured in the DMEM medium containing 10% FBS in a 5% CO₂ incubator. All RMCs used in the experiments were less than the fourth passage.

Flow cytometry analysis

The immunophenotypes of RMCs cultured at the third passage were confirmed using flow cytometry as Barbier et al. reported [33]. In brief, cultured RMCs were incubated with 5% BSA and a primary antibody, including CD34 (Santa, sc-7324), CD45 (Proteintech, 20103-1-AP), CD73 (Santa; sc-398260), and CD90 (bs-0778R). The cells were washed with PBS and then labeled with the secondary antibody. Flow cytometry analysis was carried out using a flow cytometer (BD biosciences, USA).

Immunofluorescent staining (IF)

Immunofluorescence staining was performed as Javed et al. reported (Javed et al., 2004). Briefly, RMCs were incubated with primary antibodies CD34 (Santa, sc-7324), CD45 (Proteintech, 20103-1-AP), CD73 (Santa; sc-398260), CD90 (bs-0778R), COL II (bs-10589R), COMP (bs-10286R), COL X (bs-0554R), and β -Actin (Proteintech, 60,008-1-I g) for 1 h at room temperature (RT). After washing, RMCs were incubated with the secondary antibodies conjugated with green fluorescence protein (Proteintech, SA00013-1, SA00013-2) for 1 h at RT. Subsequently, the nuclei of the cells were counterstained with DAPI for 5 min at RT. Finally, fluorescent photographs were captured using a fluorescence microscope, and the fluorescent intensity was measured by ImageJ (v1.4.3.67).

RMCs trilineage differentiation

According to the manufacturer's procedures, we used the MesenCult Adipogenic Differentiation kit (Cyagen, GUXMX-90031), Chondrogenic Differentiation kit (Cyagen, HUXMA-90041), and Osteogenic Differentiation kit (Cyagen, GUXMX-90021) to determine the capability of RMCs to differentiate into adipogenic, chondrogenic, and osteogenic lineages. In short, 2×10^5 RMCs were seeded into a 12-well plate or 4×10^5 RMCs were seeded into a 15 mL centrifuge tube. Then, the medium was replaced by a corresponding differentiation medium. The medium was changed every 3 days. After 14 days or 21 days of adipogenic, chondrogenic, and osteogenic differentiation, they were stained with oil red O, Alcian blue, and Alizarin red, respectively.

Real-time reverse-transcriptase polymerase chain reaction (qRT-PCR)

According to the manufacturer's procedures, total RNA was extracted from the cultured cells using the TRIzol reagent (Invitrogen). The reverse transcript reactions were performed using PrimeScript™ RT reagent Kit (Perfect Real Time; Takara, RR037A). qRT-PCR was performed using TB Green®Premix Ex Taq™ II (Tli RNaseH Plus; Takara, Dalian, China, RR820A) in triplicates. The $2^{-\Delta\Delta C_t}$ method was used to calculate the expression levels of mRNAs. The primers were listed in Supplementary Table S1.

Cell viability assay

RMCs were cultured in 0%, 0.05%, 0.5%, 1%, and 4% HA (Macklin, 9004-61-9), and cell viability was measured using the cell counting kit-8 (Biosharp, BS350B). Briefly, a total of 1×10^4 cells were seeded in 12-well plates, and each well contained a 1 mL medium for 1, 3, and 6 days, respectively. CCK-8 reagents were added in a volume of 100 μ L/well and cultivated further for 4 h. The absorbance was measured at 450 nm.

Frozen sections and staining

The third passage RMCs were cultured with 0% or 0.05% HA and induced to subject to chondrogenic differentiation on day 7, 14, and 21, respectively. The samples were embedded in OCT (SAKURA, 4583) at -20°C for 15 min and then cryosectioned at 5 μ m thickness. Sections were stained with Alcian blue (Cyagen, ALCB-10001) and Hematoxylin Eosin (Bioss, C02-04004). The images were photographed using a Leica microscope and analyzed using Image J (1.4.3.67, Bethesda) software.

Western blot

Proteins were isolated from the cartilage-like nodules using the RIPA lysate (Beibokit, BB-3121-2). Protein concentrations were quantified using a BCA protein assay (Beyotime, P0010S). The proteins were subjected to 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, IPVH00010). The membrane was incubated with primary antibodies at 4°C for 12 h. Next, the resultant blots were washed and incubated with secondary antibodies at RT for 60 min. After rinsing with TBST, the bands were visualized using ECL detection reagents (Tanon, 180-5001). Finally, Image J (1.4.3.67, Bethesda) software was used to quantify the protein bands.

Animal experiments and femur gross appearance evaluation

Fifty Sprague Dawley rats (8 weeks old, 250 g) were divided into 5 groups randomly ($n = 10/\text{group}$): PBS, HA, RMCs, RMCs + HA, and Sham control. According to the method described by Yoshida et al., except for the Sham control, a hole with a diameter of 1.5 mm was drilled on the left distal femur, and then, the wound was immediately closed [34]. Subsequently, 100 μL PBS, 100 μL HA (0.05%), RMCs ($1 \times 10^6/\text{knee}$) in 100 μL PBS, or RMCs ($1 \times 10^6/\text{knee}$) in 100 μL HA, respectively, were injected into the articular space of knee joints after surgery. The operated rats were treated 3 times consecutively with an interval of 1 week using the aforementioned solutions. The rats were euthanized, and the femurs were collected at 4 and 8 weeks, respectively. According to the International Cartilage Repair Society (ICRS), the cartilage defects were evaluated macroscopically.

Histological examination

The sampled tissues were histologically processed and stained with Hematoxylin Eosin (HE) staining, Alcian blue (Cyagen, ALCB-10001) staining, Safranin-O-Fast green staining (Phygene, PH1852), and immunohistochemistry using the IHC reagent kit (ZSGB Bio, SP9000). Briefly, the sampled femurs were fixed in 4% paraformaldehyde, after which they were decalcified in 10% ethylenediaminetetraacetic acid decalcifying solution. The specimens were embedded in paraffin and sectioned coronally at 4 μm thickness. Mankin's and OARSI scoring systems were used to determine the degree of cartilage defects. Images were captured with a microscope (Carl Zeiss).

Enzyme-linked immunosorbent assay (ELISA)

The fluid was collected from the knee synovial cavity following injection of 100 μL PBS into the cavity. Based on the instructions of the manufacturers, the levels of inflammation-related factors IL-6 (Sinobestbio, Invitrogen, YX-091260R) and TNF- α (Sinobestbio, Invitrogen, YX-201406R) in the joint synovial fluid collected at 8 weeks were determined using the ELISA kits. Each measurement was repeated three times to obtain the mean value.

Statistical analysis

The statistical analyses were performed using SPSS 20 (IBM). The data were presented as the mean $\pm$ SD of at

least three observations. Statistical differences were validated using a one-way ANOVA. p -value was considered significant when the p value < 0.05 . * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns: not significant.

RESULTS

Characteristics of RMCs

Morphology and status of surface marker expression

After initial isolation and expansion, RMCs (NT-RMCs) displayed fibroblastic morphology (Figure 1A). To characterize the isolated RMCs, the cell-surface antigen profiles were examined via flow cytometry and immunofluorescence. The results of flow cytometry showed that RMCs were more than 98% positive for MSC markers CD73 (98.9%) and CD90 (99.7%), whereas they were less than 0.30% negative for MSC markers CD34 (0.25%) and CD45 (0.28%) (Figure 1B–E). The results of immunofluorescent staining were consistent with the flow cytometry results, showing that CD73 and CD90 were positive for RMCs and CD34 and CD45 were negative (Figure 2). These results indicate that the RMCs have general characteristics of MSCs.

Differentiation ability of RMCs

Adipocytes differentiated from RMCs (Adi-RMCs) contained lipid droplets that were positively stained with oil red O (Figure 3A). Results of qRT-PCR analysis showed that adipogenesis-related genes were highly expressed in the differentiated cells including FABP4 in Adi-RMCs was significantly higher than in NT-RMCs (Figure 3D). Twenty-one days after chondrogenic differentiation, RMCs formed a cartilage-like micromass pellet. The pericellular matrix proteoglycans in the RMCs-differentiated chondrocyte were stained with Alcian blue (Figure 3B). qRT-PCR analysis revealed that the expression of COL II, a chondrogenic marker, significantly increased expression levels after differentiation (Figure 3E). Osteoblasts differentiated from RMCs were positively stained with Alizarin red, mainly on the intracellular mineral deposits (Figure 3C). Results of qRT-PCR analysis showed that the terminal osteogenic marker, COL I, was highly expressed (Figure 3F). The above results indicate that RMCs have the ability of trilineage differentiation in vitro in the appropriate induction culture medium. Taken together, RMCs have the multilineage differentiation ability and thus belong to the category of MSCs.

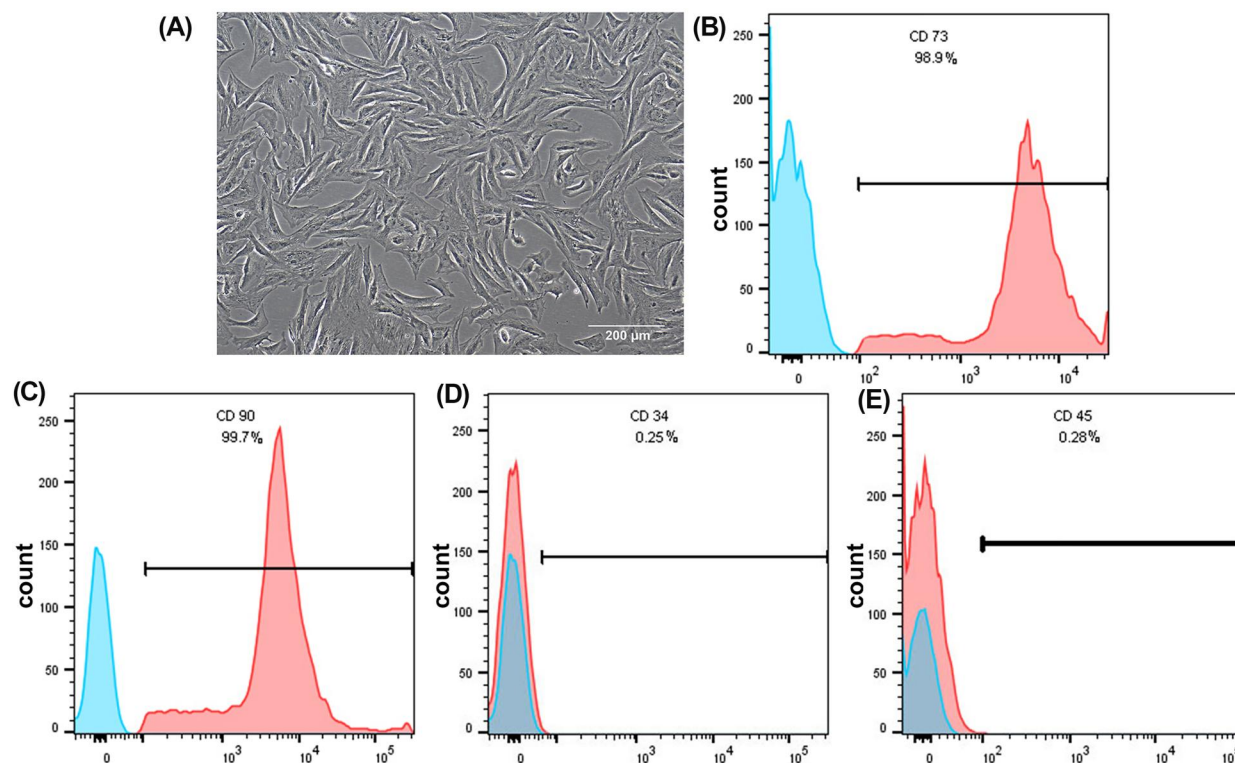


FIGURE 1 Characterization of RMCs. (A) Morphology of cultured RMCs in passage 3. Scale bars: 200 μm. (B–E), Flow cytometry analysis of the passage 3 RMCs positive mark (CD73 and CD90) and negative markers (CD34 and CD45).

Effects of HA on proliferation and chondrogenic differentiation of RMCs in vitro

We used CCK8 to measure the proliferation of RMCs in the different concentrations of HA (0%, 0.05%, 0.5%, 1%, and 4%). The results showed that there was no significant difference in the proliferation rate of RMCs in different concentrations of HA compared to that without HA, but the 4% HA group had the lowest proliferation rate (Figure 4A). This indicates that HA cannot enhance the proliferation ability of RMCs. qRT-PCR analysis showed that HA treatment significantly stimulated the expression of chondrogenic markers COL II and COMP of the RMCs (Figures 4B,C) but inhibited the expression of hypertrophic markers COL X (Figure 4D) compared to the HA-less control. Therefore, HA can enhance chondrogenic differentiation of RMCs, and the effect is most significant at 0.05% concentration.

HA promoted the expression of chondrogenic-related genes in chondrocytes differentiated from RMCs in vitro

In this experiment, RMCs were micromass-cultured in 0.05% HA or HA-less medium and induced to

chondrogenic differentiation for 7, 14, and 21 days, respectively. Cartilage-like nodules were formed with the diameters between 1.0 and 1.5 mm (Figure 5A). The volume of the HA nodules tended to be greater than that of the HA-less control. However, due to a large variation in size in repeats, no significant difference was detected between these two groups. qRT-PCR results showed that compared with the nodules in the HA-less control, HA treatment significantly stimulated the expression of chondrogenic markers COL II and COMP in the nodules, whereas it inhibited the expression of hypertrophic markers COL X (Figure 5B). Histological analysis of the nodules showed that staining of matrix proteoglycans in the HA nodules using alcian blue tended to be stronger than that in the HA-less control (Figure 5C,E). The HE staining results showed that the size of the HA nodules tended to be larger than that in the HA-less control (Figure 5D,F). In the IF staining, compared with the nodules in the HA-less control, HA treatment significantly stimulated the expression of COL II and COMP in the nodules, whereas it significantly inhibited the expression of COL X (Figure 5G,H). The results of western blot were consistent with those of IF staining. Compared to the nodules in the HA-less control, HA treatment significantly stimulated the expression of COL II and COMP, whereas it significantly inhibited the expression of the hypertrophic marker COL X in the nodules (Figure 5I,J).

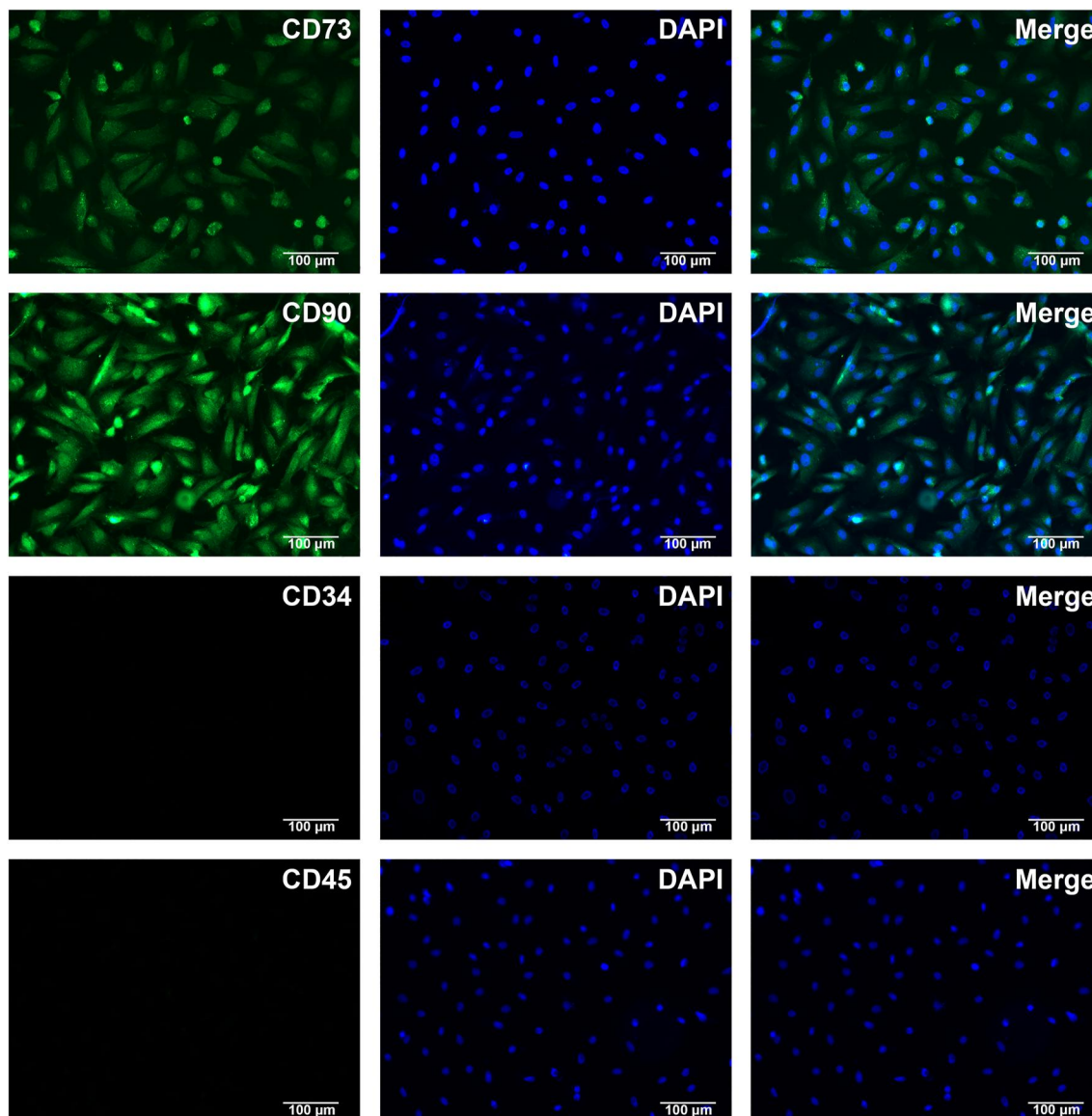


FIGURE 2 Immunofluorescence analysis. The positive markers (CD73 and CD90) and negative markers (CD34 and CD45) of RMCs were detected by immunofluorescence staining (DAPI: blue, the markers of interest: green). Scale bars: 100 μ m.

In conclusion, HA treatment can effectively promote chondrogenesis of RMCs but inhibits the termination of chondrocytes.

Effect of RMCs-HA on repair of cartilage defects in rats

At macroscopic level

Macroscopic evaluation of the effects of femur cartilage defect repair was done. Four weeks after operation, the defects in both the RMCs group and RMCs + HA group were largely filled with newly formed tissue, although the trace of defects was still observable. However, the defects of the PBS group and HA-only group started to

fill with newly formed tissue and they were clearly seen. At the 8th week after operation, the defects in both the RMCs group and RMCs + HA group were completely filled with newly formed tissue, but there was a clear demarcation between the repair defects and the surrounding tissue in the RMCs group. The newly formed tissue in the RMCs + HA group was the one most similar to the adjacent natural cartilage. The defects of the PBS group and HA group were only partially filled with newly formed tissue and they were clearly seen (Figure 6A). According to the ICRS macroscopic assessment, the scores of the RMCs group and RMCs + HA group were significantly higher than those of the PBS group and HA group at the 4th week after operation. It was suggested that using HA alone could not effectively repair cartilage defects. Although there

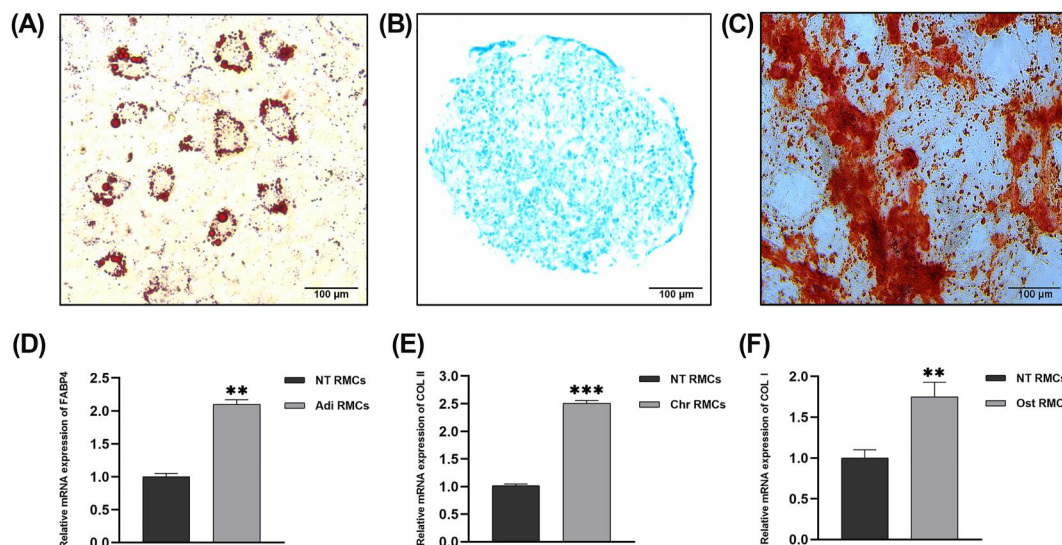


FIGURE 3 Trilineage differentiation capacity of RMCs. (A and D) The capacity of adipogenic differentiation by Oil Red O staining. And FABP4 (adipogenic marker) were detected using qRT-PCR. (B and E) The chondrogenic differentiation capacity by Alcian blue staining. And COL II (chondrogenic marker) were detected using qRT-PCR. (C and F) The capacity of osteogenic differentiation by Allizarin red staining and COL X (osteogenic marker) were also detected. Scale bars: 100 µm.

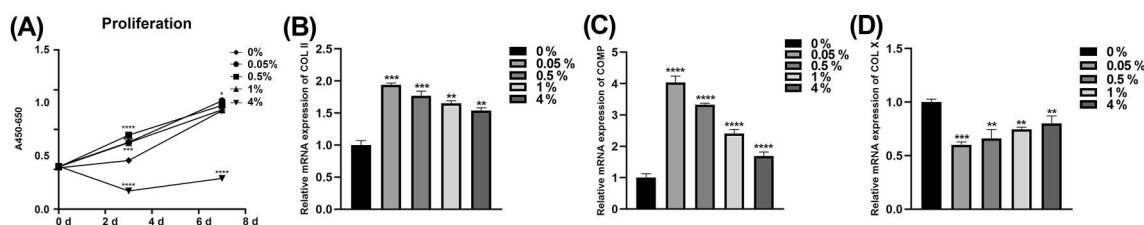


FIGURE 4 Proliferation and chondrogenic differentiation of RMCs in HA. (A): Proliferation of RMCs in different concentrations of HA. (B–E), The expression of chondrogenic genes (COL II, COMP, and COL X) in RMCs treated with different concentrations of HA was analyzed using qRT-PCR.

was no significant difference in the RMCs + HA group and the RMCs group compared to the Sham control at the 8th weeks after operation, the RMCs + HA group still had higher ICRS score than that of the RMCs group (Figure 6B). Therefore, RMCs plus HA were more effective than HA alone or RMCs alone in cartilage defect repair.

At histological level

Histological examination and Mankin's score were used to evaluate the effects of the treatments on rat articular cartilage defect repair at the 4th and 8th week after operation (Figure 7A,C). The results were essentially consistent with macroscopic evaluation (3.4.1). On HE staining, compared to the Sham control, the RMCs + HA group had the best repair results with the top layer of the cartilage well integrated with the adjacent cartilage, and the surface of the top layer cartilage was comparable to that of the sham group (8 weeks

after operation) and had the lowest Mankin's score. The effects of defect repair in the RMCs group were inferior to that in the RMCs + HA group, and although the defects had properly filled with newly formed tissue, the nature of which was not clear; there was a clear demarcation between the newly formed tissue and surrounding original tissue and had a significantly lower Mankin's score. The defects of the HA group were not totally filled at the end of the experiment, and the trace of the defects was still clearly seen; the cartilage layer of the newly formed tissue was relatively thinner than the other groups except for the PBS group. The PBS group showed surface irregularities; large holes in the defects were clearly discernible and had the highest Mankin's score. The results of Safranin-O-Fast green staining provided more information on defect repair in different groups (Figure 7B,D). In the RMCs + HA group, there was no detectable loss of proteoglycan and glycosaminoglycan, representing the lowest OARSI's score. The RMCs group showed a certain degree of proteoglycan and glycosaminoglycan loss and had

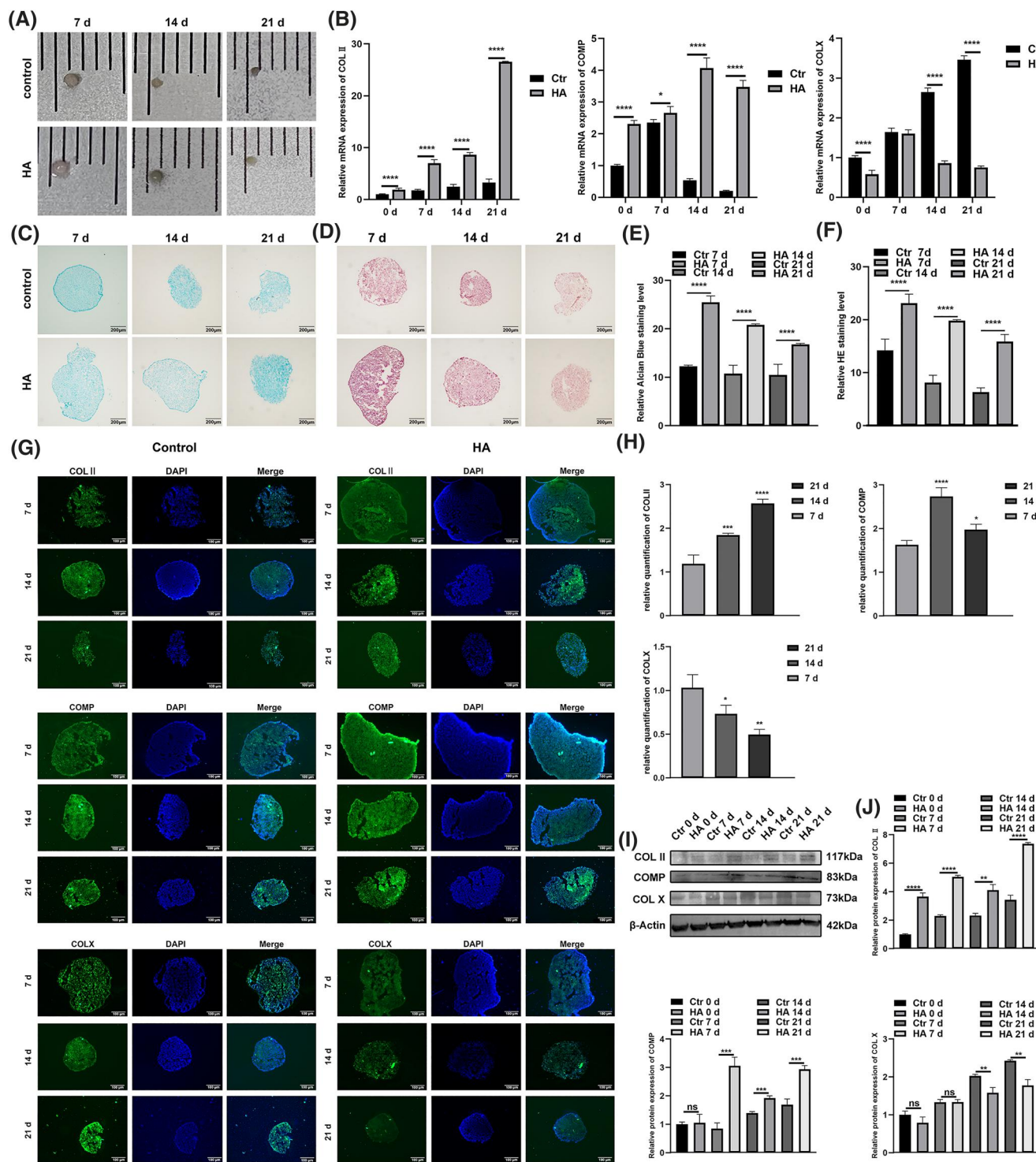


FIGURE 5 Histological analysis of micromass pellets and expression of chondrogenesis related genes. (A): The gross picture of the HA-micromass pellets and control. (B): The expression of chondrogenic genes (COL II, COMP, and COL X) in the micromass pellets and control were analyzed using qRT-PCR. (C): Alcian blue staining of the HA-micromass pellets and control (Scale bars: 200 µm). (D): HE staining of the HA-micromass pellets and control (Scale bars: 200 µm). (E): Quantitative analysis of Alcian blue staining. (F): Quantitative analysis of HE staining. (G): IF of COL II, COMP, and COL X of the HA-micromass pellets and control (Scale bars: 100 µm). (H): Quantitative analysis of IF. (I): The expression of chondrogenic protein (COL II, COMP, and COL X) in the HA-micromass pellets and control were analyzed using western blot. (J): Quantitative analysis of western blot.

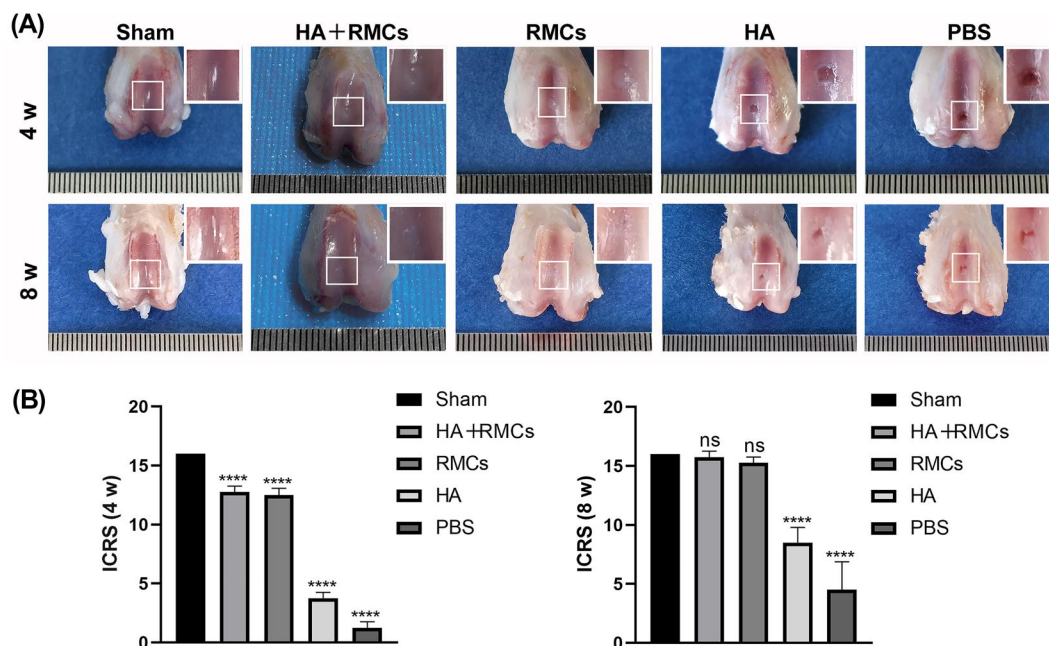


FIGURE 6 Gross appearance evaluation of the femur. (A): Macroscopic examination of the femur at 4 and 8 weeks posttreatment. (B): Macroscopic ICRS scores of the femur at 4 and 8 weeks posttreatment.

higher OARSI's score compared to the RMCs + HA group. However, the PBS group and HA group showed significant loss of proteoglycan and glycosaminoglycan and had the highest OARSI's score. These reductions of the OARSI score were consistent in the articular cartilage at both the 4th and 8th week operation, respectively. Taken together, these data suggest that HA can effectively enhance the role of RMCs in cartilage defect repair.

At molecular level

Immunohistochemical results showed that the PBS group and HA group had the highest level of COL I and COL X and the lowest level of COL II at the 8th week after treatment (Figure 8) compared to the RMCs group and RMCs + HA group. These data indicate that newly formed tissue mainly belongs to fibrocartilage and have hypertrophic tendency. Furthermore, there was no significant difference in expression degree of COL I, COL II, and COL X between the PBS group and HA group (Figure 8). In contrast, the RMCs group and RMCs + HA group had the highest level of COL II and the lowest level of COL I and COL X at the 4th and 8th week after treatment, respectively (Figure 8). These data indicate that the newly formed tissue in these groups belongs to hyaline cartilage and negligible hypertrophy. Moreover, compared with the RMCs group, expression level of COL II in the cartilage of the RMCs + HA group was substantially higher (Figure 8). Together, these data suggest that the newly formed

tissue treated with RMCs + HA was more hyaline cartilage.

The levels of the major inflammatory factors IL-6 and TNF- α in the collected joint synovial fluid were examined using the ELISA method at the 8th week. The levels of IL-6 and TNF- α in the RMCs + HA group were significantly lower than those in other groups except for the Sham control (Figure 9). In general, compared with the Sham control, no significant changes in expression levels of IL-6 and TNF- α in the RMCs + HA group were detected but were detected in the other groups.

DISCUSSION

To the best of our knowledge, this was the first report to use RMCs in combination with HA for the treatment of cartilage defects. At the macroscopic, histological, and molecular levels, both in vitro and in vivo, we confirmed that combined with HA, RMCs highly upregulated the expression of cartilaginous markers, COL II and COMP, and thus differentiated toward chondrogenic lineage; RMCs notably downregulated the expression of the osteogenic marker COL I and the hypertrophic marker COL X and thus inhibited differentiation toward bone replacement. Furthermore, RMCs downregulated the levels of inflammatory factors IL-6 and TNF- α in the joint synovial fluid. Consequently, we believe we have opened up a new avenue by introducing a novel composite of RMCs and HA for the treatment of articular cartilage defects in the clinic setting.

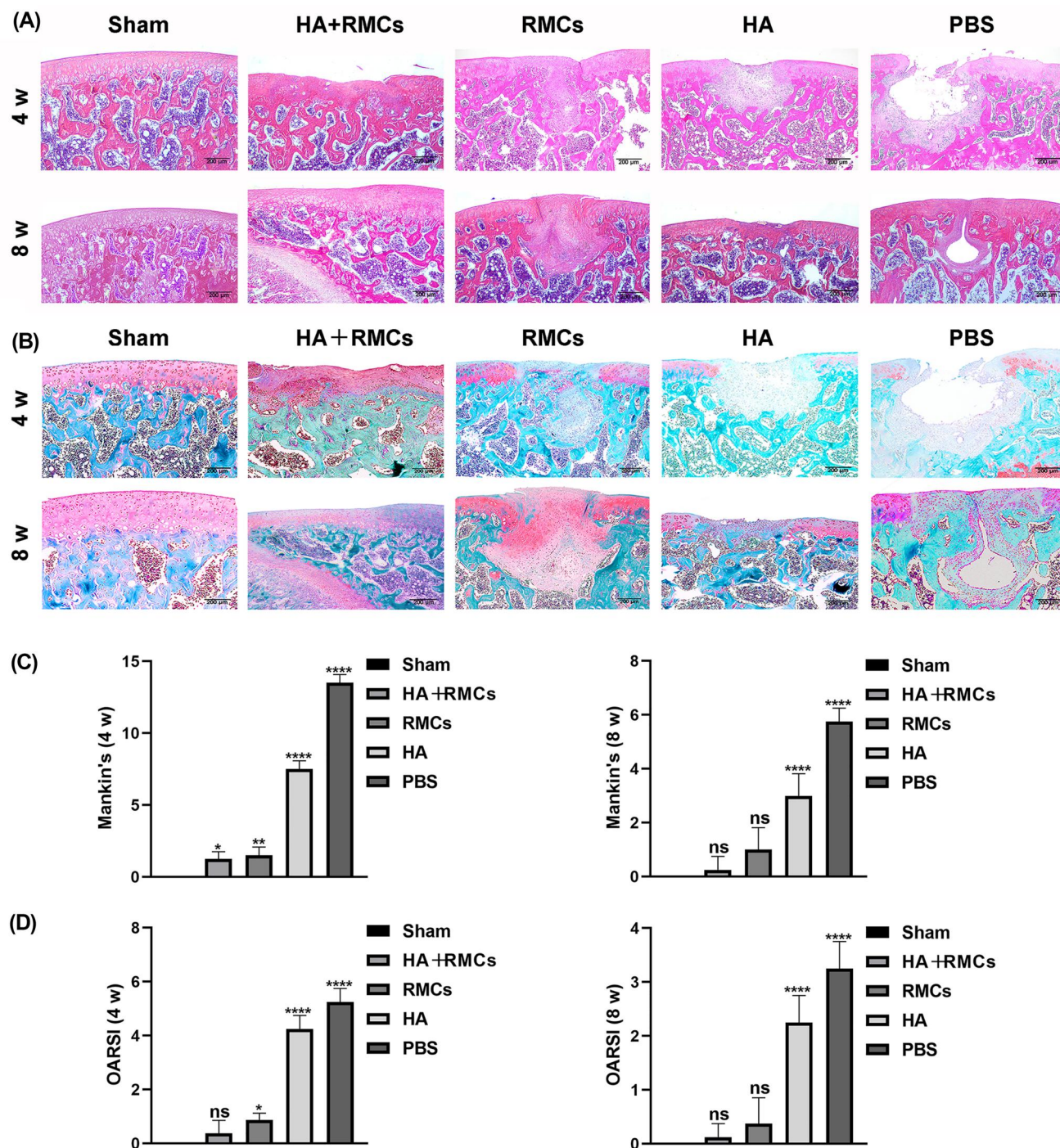


FIGURE 7 Histological analysis of cartilage. (A and C), The cartilage at 4 and 8 weeks posttreatment was evaluated using HE staining and Mankin's scoring. (B and D), The cartilage at 4 and 8 weeks posttreatment was evaluated by Safranin-O-Fast green staining and OARS scoring.

Articular cartilage has limited capacity to repair damage caused by trauma or disease because of its avascularity and low cellular mitotic activity [35]. Untreated joint cartilage defects could cause progressive tissue degeneration, resulting in a 5-fold increase in the risk of osteoarthritis [36]. Although clinical methods such as joint replacement surgery were available, the regeneration of full-thickness cartilage defects was still

a challenge [37]. Among recently investigated approaches, cell-based cartilage regeneration strategies represent a feasible and promising alternative. Although advantages are clear, selecting the optimal cell type for treatment remains a formidable task.

Mesenchymal stem cells have recently attracted much interest for possible clinical use because of their self-renewing potential and multipotency. Thus far,

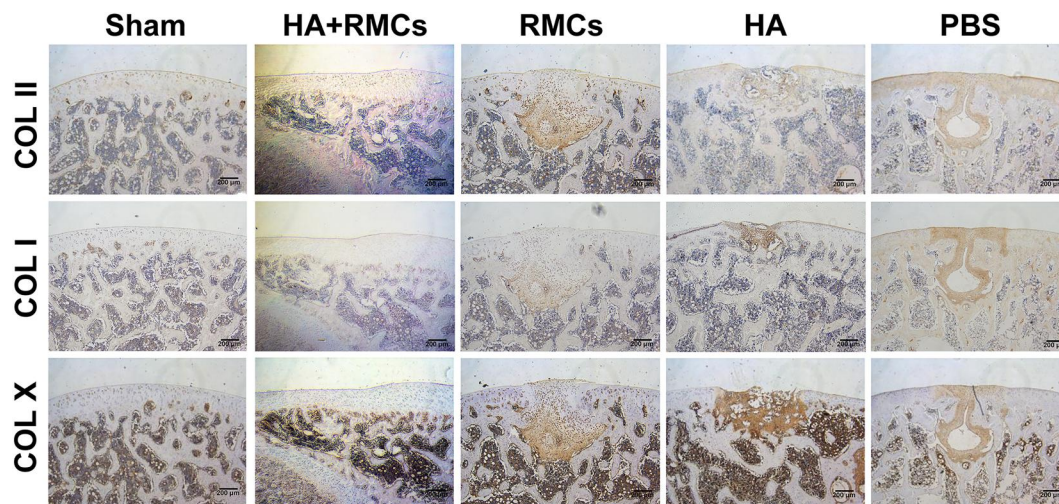


FIGURE 8 Immunohistochemistry analysis of cartilage. (A): The femur condyles were stained with COL II. (B): The femur condyles were stained with COL I. (C): The femur condyles were stained with COL X. Scale bars: 200 μ m.

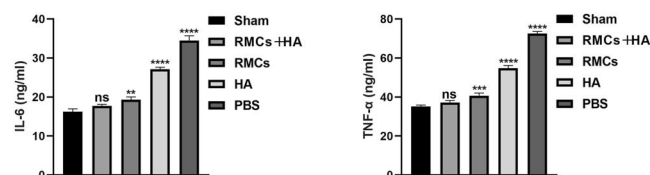


FIGURE 9 Analysis of inflammatory factors in joint synovial fluid. Detection of inflammatory markers IL6 and TNF- α in joint synovial fluid by ELISA 8 weeks after treatment.

MSCs have been identified in a variety of tissues, including the bone marrow, muscle, periosteum, neural tissue, and adipose tissue [38–43]. Among these tissues, the bone marrow has been well established as an MSC source in humans. However, the sample source and cell numbers from bone marrow are relatively limited [44]. In this regard, RMCs are undoubtedly an attractive source because they have unparalleled proliferation potential, bona fide stem cell attributes, and almost unlimited availability, and naturally differentiate into cartilage lineage [17, 32, 45]. However, reports to use RMCs for the treatment of cartilage defects have not been seen thus far.

The extracellular matrices (ECMs) provide a micro-environment for cartilage cells to maintain their homeostasis and differentiation properties [35, 46–48]. Native articular cartilage ECM is a composite material composed primarily of proteoglycans and collagen, and hyaluronan (HA) is the main type of glycosaminoglycan (GAG) during the early stage of chondrogenesis [49–51]. Most importantly, HA is the major physiological component of the articular cartilage matrix and is particularly abundant in the synovial fluid [52]. Proteoglycans bind to an HA backbone to form macromolecules. Thus, HA is a key component in the ECM of articular cartilage. It is known that HA interacts with

cartilage cells through surface receptors such as CD44, enabling the modulation of cell activities such as migration, proliferation, and differentiation, as well as matrix secretion [53, 54]. ECM of deer antler cartilage (growth center) is also made up of collagen and GAG, and the latter consists of approximately 10% and 90% of HA and chondroitin sulfate, respectively [29]. Therefore, HA is also one of the indispensable components of the RMC niche, and this niche sustains antler growth at an unparalleled rate, 2 cm/day [55].

It is reported that HA-based hydrogels have the potential to not only provide structural support during the cartilage repair process but also provide signaling cues that affect the repair process. Besides, exogenous HA is known to be able to directly incorporate into cartilage. Chu et al. reported that the antler decellularized cartilage-derived matrix scaffolds (containing HA) were successfully used to effectively repair cartilage defects in the rabbit model [56].

In recent years, strategies to deliver MSCs together with biomaterials to treat cartilage defects have become popular. Chung et al. (2014) used the composites formed by UCMSCs and different hydrogels to treat cartilage defects and found that the repair effect of UCMSCs with HA hydrogel was the best, and its cellular arrangements and collagen organization pattern were similar to adjacent articular cartilage [57]. The defects filled with HA-based hydrogels seeded with MSCs resulted in a firm, elastic, and translucent cartilage with good integration with the surrounding cartilage [53]. In the present study, we introduced a special type of MSC, that is, RMCs, to treat articular cartilage defects and found that when RMCs bathed in appropriate concentration (approximate to the native antler cartilage) of HA, the repair results were the best compared to the singular use of each component. The composite significantly showed an increased

expression of the chondrogenic marker genes COL II and COMP compared to the control group, whereas a decreased expression of the osteogenic marker gene COL I and the hypertrophic marker COL X. Nenna et al. (2019) reported that HA acts as an autocrine factor for chondrocyte proliferation and differentiation through a variety of downstream effectors as well as an effective inhibitor of chondrocyte hypertrophy and apoptosis [58]. Because COMP, the main component of cartilage noncollagen protein, has obvious tissue specificity, it can serve as a reliable marker of cartilage [59]. COL X is the specific marker of hypertrophic chondrocytes [60]. COL I is one of the most important indicators related to the ECM in early osteogenesis [61]. It is known that during chondrogenic differentiation, COL X plays an important role in matrix organization, calcium binding, and matrix vesicle compartmentalization [62]. Therefore, when RMCs were delivered together with appropriate concentration of HA to the cartilage defect, the RMCs were convincingly coaxed to differentiate into chondrocytes and stabilized at the stage before being subject to hypertrophy. Consequently, we have formulated a totally new and effective composite for treating articular cartilage defects.

Thus far, the mechanism underlying chondrogenic promotion of HA to RMCs without going hypertrophy is not known. Previous studies have found that HA can enhance the synthesis of cartilage ECM in vitro [63, 64]. Similarly, MSCs can also promote chondrogenic differentiation of cells by updating ECM and synthesizing COL II [65]. Amann et al. (2017) reported that in the process of differentiation of AMSCs, HA promoted chondrogenesis by upregulating the expression of GAG and prevented hypertrophy by downregulating the expression of COL X [66]. After intra-articular delivery of HA in the rat OA model, the expression level of the hypertrophic marker COL X was significantly reduced but the expression level of the chondrogenic marker COL II was increased. The results indicate that HA plays an important role in preventing cartilage degradation [67]. Our histological evidence showed that HA effectively promoted the aggregation of RMCs at the transplanted site and prevented cells from spreading. At the same time, HA stimulated the differentiation of RMCs into a stable state of cartilage by upregulating ECM and expression of COL II and COL X.

Besides the behavior of the transplanted cells at the defect site in our study, we also found that the immune system was involved in the process of cartilage repair. The levels of inflammatory factors IL-6 and TNF- α in the joint synovial fluid of the RMCs + HA group were significantly lower than those of the PBS group, HA group, and RMCs group. It is well established that proinflammatory factors, such as IL-6 and TNF- α , participate in the destruction process of joint cartilage. Both TNF- α and IL-6 promote the proliferation of synovial

immune cells, thereby inducing neovascularization and inflammation, as well as the production of matrix metalloproteinases and other cytotoxins, which ultimately lead to cartilage degradation [68, 69]. Therefore, the healing effects of RMCs + HA on cartilage defects may also include the reduction of proinflammatory factors (such as IL-6 and TNF- α). Collectively, effects of the composite of HA and RMCs on cartilage defect repair may be achieved via stimulating synthesis and deposition of cartilage ECM (upregulation of COL II and COMP), inhibiting hypertrophy of cartilage cells (downregulation of COL X), and reducing the inflammatory cascade. If it can be convincingly demonstrated that exosomes or metabolites of RMCs are equally effective to the RMCs themselves at the next step, a new effective composite may be developed and used in the clinical settings.

CONCLUSION

As far as we know, this was the first study to reveal the effect of combined treatment of HA and RMCs on the progress of cartilage defects in vivo and in vitro. We have proved that RMCs conform to the characteristics of MSCs in terms of morphology, expression of surface markers, and trilineage differentiation. HA promoted RMCs to differentiate into chondrocytes. In a rat model of articular cartilage defect, HA was added to provide microenvironment support to promote RMCs to effectively repair cartilage defects in macro and micro histology. In conclusion, findings from this work showed the chondrogenic potential of RMCs in combination with HA, thus supporting their use for the treatment of cartilage defects.

AUTHOR CONTRIBUTIONS

Boyin Jia: Conceptualization (equal); data curation (equal); funding acquisition (equal); investigation (equal); methodology (equal); software (equal); validation (equal); writing – original draft (equal). **Xin Li:** Data curation (equal); methodology (equal); software (equal); validation (equal). **Xintong Han:** Data curation (equal); methodology (equal); software (equal); validation (equal). **Fuquan Ma:** Data curation (equal); methodology (equal); software (equal); validation (equal). **Linlin Zhang:** Conceptualization (equal); data curation (equal). **Xue Wang:** Conceptualization (equal); data curation (equal). **Xinrui Yan:** conceptualization (equal); data curation (equal). **Yu Zhang:** Conceptualization (equal); data curation (equal); methodology (equal). **Jianming Li:** Project administration (equal); resources (equal); supervision (equal). **Pengfei Hu:** Methodology (equal); validation (equal). **Yusu Wang:** methodology (equal); validation (equal). **Naichao Diao:** Resources (equal); supervision (equal). **Kun Shi:** Project administration (equal); resources (equal); supervision

(equal). **Ying Zong**: Funding acquisition (equal); resources (equal); supervision (equal). **Rui Du**: Conceptualization (equal); project administration (equal); supervision (equal); writing – review & editing (equal). **Chunyi Li**: Conceptualization (equal); methodology (equal); writing – review & editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

ETHICS STATEMENT

The animal study was reviewed and approved by Jilin Agricultural University Committee on the use of live animals. Written informed consent was obtained from the owners for the participation of their animals in this study.

ORCID

Rui Du  <https://orcid.org/0000-0001-6818-297X>

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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