



OPEN An effective approach to cartilage regeneration using antler stem cell-conditioned medium

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Articular cartilage has a low capacity for self-regeneration. Therefore, stem cell therapy has been proposed and is gaining momentum. Antler can self-repair and its cartilaginous tissue can grow at an unprecedented rate (Up to 2 cm/day) and antler regeneration is based on antler stem cells. Therefore, we predicted that antler stem cells or their paracrine factors might be a good source for promoting cartilage repair. In this study, we prepared conditioned medium from antler stem cells (ASC-CM) and evaluated its effect. We implanted the medium into rat cartilage defects and assessed its capacity to promote cartilage repair. The protein composition of ASC-CM was analyzed using via DIA assay. ASC-CM can strongly promote chondrocyte proliferation in vitro; it significantly up-regulates the expression of chondrogenesis-related genes (Aggrecan, Col II, and Sox-9) and promotes glycosaminoglycan formation and type II collagen deposition in cartilaginous tissue; meanwhile up-regulate the apoptosis suppressor gene NAMPT and down-regulate apoptosis gene BAX. In vivo, cartilage defect in rats was significantly repaired using ASC-CM. The compositions of ASC-CM were got and some key proteins such as S100A4 were identified by bioinformatics analysis. The ASC-CM can promote the proliferation of rat-chondrocytes, maintain the chondrocyte phenotype, and inhibit apoptosis of rat-chondrocytes. Therefore, it can promote cartilage repair.

Keywords Antler stem cells, Conditioned medium, Cell proliferation, Cartilage regeneration, Cartilage defect

Abbreviations

ASC-CM	Antler stem cell-conditioned medium
ASC	Antler stem cells
BSA	Bovine serum albumin
hADSC-CM	Human adipose-derived stem cell-conditioned medium
hADSC	Human adipose-derived stem cells
H&E	Hematoxylin and eosin
rat-BMSC	Rat bone marrow mesenchymal stem cells
hBMSC	Human bone marrow mesenchymal stem cells
IHC	Immunohistochemistry
qPCR	Quantitative real-time PCR

Articular cartilage is an avascular connective tissue composed mainly of chondrocytes, proteoglycans (The dominant proteoglycan is *Aggrecan*), and collagen fibrillar^{1–3}. The lack of blood vessels in articular cartilage limits the supply of cellular nutrients, making it difficult to self-regenerate when mechanical damage occurs^{4–6}. The main treatments for cartilage defects are microfracture and artificial joint replacement, but these methods have disadvantages such as susceptibility to infection and short life span of the replacement joint. None of these treatments can regenerate hyaline cartilage^{7–9}. Moreover, the fibrocartilage formed by micro fracture is much inferior to natural hyaline cartilage in biomechanical properties¹⁰. Therefore, new strategies for damaged cartilage regeneration are urgently needed. Mesenchymal stem cell (MSC) therapy has recently been reported as a new

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approach to cartilage regeneration^{11–13}. However, one of the main challenges is identifying and obtaining suitable MSC sources to generate effective bioactive molecules to treat cartilage defects and promote chondrogenesis.

The process of chondrogenesis in deer antlers is very similar to that of articular cartilage formation. Both undergo differentiation of mesenchymal cells into chondrocytes and finally into hypertrophic chondrocytes^{14,15}. Antlers can regenerate and grow rapidly at an impressive rate (Up to 2 cm/day) under natural conditions^{16,17}. To meet the rapid growth of antlers, paracrine factors (Chemokines, cytokines, and growth factors) related to antler tissue development and regeneration play an important role^{18–20}. These paracrine factors regulate their microenvironment. They promote angiogenesis, chondrocyte proliferation, cartilage development, and regulate cartilage phenotype. Compared to stem cells, applying their paracrine factors to treat cartilage defects avoids the problems of immunocompatibility, tumorigenicity, embolism, and infection. The most important thing is that the stem cells cannot be stored for longer time, even cannot be stored for one overnight^{21,22}. However, lyophilized paracrine factors can be stored for few months and does not affect their effectiveness. It is also convenient to produce in large quantities, making it more economical and practical for clinical applications²³. Therefore, this procedure provided a promising therapeutic strategy for the repair of cartilage defects.

Aiming to investigate whether antler stem cell-conditioned medium (ASC-CM) is effective in repairing rat cartilage defects in both vitro and vivo, this study provides a suitable cell source and pathway for cartilage repair. The possible mechanisms of cartilage regeneration using ASC-CM were also discussed.

Materials and methods

The whole experimental procedure in the study is schematically shown in (Fig. 1).

Cell isolation and culture

Antler stem cells (ASC) were obtained following the procedures reported by Wang²⁴. Rat bone marrow mesenchymal stem cells (rat-BMSC; Cat NO.: CP-R131), human adipose-derived stem cells (hADSC; Cat NO.: CP-H202), human bone marrow mesenchymal stem cells (hBMSC; Cat No.: FY200001) and rat chondrocyte (Cat NO.: CP-R087) were purchased from Procell. China.

Stem cell multiplication time

ASC, hBMSC and hADSC were seeded in 6-well plates at a density of 10^5 cells per well; Cells were counted every 3 days until the seventh generation; The Doubling Time (DT) of the cells was calculated from the cumulative cell numbers by the formula $DT = T \times \lg 2 / (\lg N_t - \lg N_0)$. T represents the culture time (h), N_t represents the number of cells at harvesting, and N_0 represents the number of cells at the time of plate inoculation.

Preparation of CM

The method for preparation of CM was described previously²⁵. Briefly, ASC and hADSC were inoculated in DMEM medium (Containing 10% FBS), and when the 5th generation cells grew to 80% confluence, their original medium was replaced with a serum-free medium (Gibco, 31053028, USA). The supernatant liquid was collected after 48 h incubation at 37°C and 5% CO₂ in the incubator. The supernatant was filtered through a 0.2 µm membrane, and then the supernatant was taken after ultrafiltration with a 3 kD ultrafiltration tube for 40 min. They were harvested as CM for use in experiments. The protein concentration of CM was measured by the BCA method and kept at –80 °C.

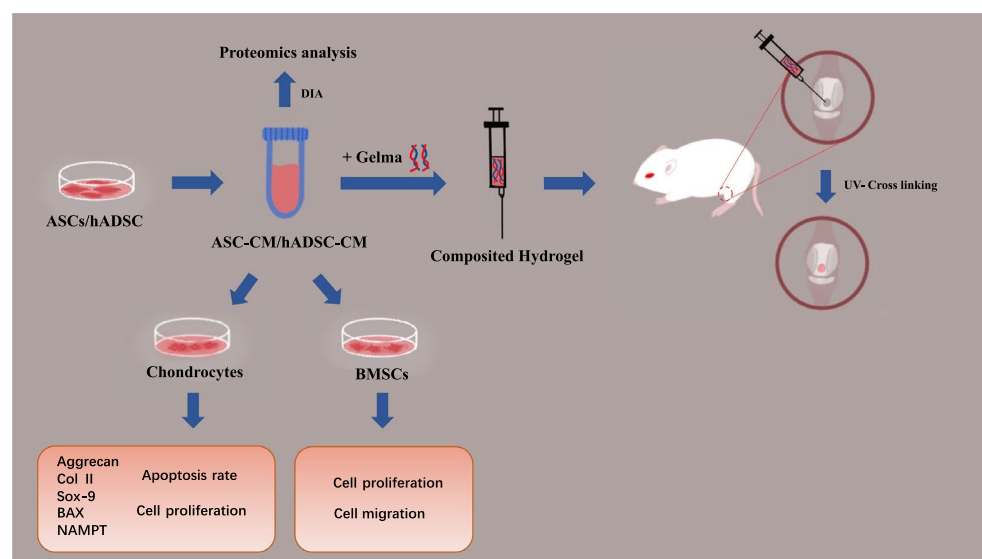


Fig. 1. Schematic drawing of experimental procedure. ASCs Antler stem cells, ASC-CM ASC-conditioned medium, BMSC bone marrow mesenchymal stem cells, Col II collagen type II.

Conditioned medium of antler stem cells (ASC-CM) and human adipose-derived stem cells (hADSC-CM) were prepared as experimental groups and hADSC were used as a positive control. In addition, DMEM was used as a negative control.

Quantitative proteomics analysis

Quantitative proteomics analysis was carried out on the Illumina sequencing platform by Genedenovo Biotechnology Co., Ltd (Guangzhou, China). And the Proteomex change reference number for protein sequences is PXD041259.

Optimization of the CM concentration

Rat-chondrocytes were inoculated in 96-well plates at a density of 2000 cells per well. The ASC-CM group (Initial concentration), DMEM group and diluted ASC-CM with DMEM at 1:2, 1:4, 1:8, 1:16, and 1:32 times were set up. Cell viability was determined using CCK-8 (Beyotime, C0041, China) and the corresponding OD values were measured at 490 nm.

Cell proliferation assay

CCK-8 assay

Rat-chondrocytes and rat-BMSC were seeded in 96-well plates and cultured until the cells reached 80% confluence. The three groups (DMEM, ADSC-CM, and ASC-CM) were maintained at 37 °C in saturated humidity and 5% CO₂ atmosphere for 48 h and 5 days, respectively, and subjected to CCK-8 assay to check the cell viability.

EdU (5-ethynyl-2'-deoxyuridine) assay

Rat-chondrocytes and BMSC were plated in 24-well plates until cells reached 80% confluence. An equal volume of pre-warmed Edu working solution (Epizyme, CX002, China) was added to each well and incubated for 2 h. After removing the original medium, add 100 µl of click additive solution per well for 30 min. And 1000 times diluted Hoechst 33342 (1000×) with PBS was added into each well for 10 min. Positive cells were analyzed in randomly selected optical fields. The proportion of proliferating cells was counted using image j (v.1.6.0).

Migration assay

Culture inserts (Ibidi, Germany) were placed in each well with 70 µl of 3×10^5 cells/ml cell suspension and incubated at 37 °C. Then ASC-CM, ADSC-CM, and DMEM were added respectively for 12 h. The migration results were observed using a microscope (Olympus, Japan). Cell Migration ratio = (Initial Scratch Width—Scratch Width after 12 h.)/Initial Scratch Width × 100%.

RNA extraction and quantitative real-time PCR (qPCR) assay

Total RNA was extracted from rat-chondrocytes using RNA Easy Fast Cell Kit (TIANGEN, DP451, China). RNA was reverse-transcribed into cDNA using Prime Script RT reagent Kit (Takara, RR047A, China). qPCR was performed in 20 µl reaction volumes using TB Green Premix Ex TaqII (Takara, RR820A, Japan) according to the manufacturer's instruction. The primers used in qPCR are shown in (Table 1). The data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Annexin V-FITC apoptosis detection

Cells were stained using Annexin V-FITC Apoptosis Detection Kit (Beyotime, C1062S, China). 100,000 cells were treated with ASC-CM, ADSC-CM and DMEM respectively. 195 µl of the Annexin V-FITC conjugate was added with 5 µl of Annexin V-FITC and 10 µl of propidium iodide staining solution into cells for 20 min. The apoptosis was assayed using flow cytometry.

Gene	Primer
<i>Sox-9-F</i>	GAGTTTGACCAATACCTGCCG
<i>Sox-9-R</i>	GCCTGTTGCTTTGACATCCAC
<i>ColII-F</i>	GACCTGAAACTCTGCCACCC
<i>ColII-R</i>	TGCTCTTGCTGCTCCACCA
<i>Aggrecan-F</i>	CCCCAAATCCCTCATACTCA
<i>Aggrecan-R</i>	AGGTGGCTCCATTCAGACAA
<i>GADPH-F</i>	AAGAAGGTGGTGAAGCAGG
<i>GADPH-R</i>	AAGGTGGAAGAATGGGAGTT
<i>NAMPT-F</i>	AGTTCAACATCCTGCTGGC
<i>NAMPT-R</i>	TTCTCTGTATTTCACCTTCC
<i>BAX-F</i>	CTGTATCCACATCAGCAATCAT
<i>BAX-R</i>	GGACGCATCCACCAAGAA

Table 1. The primers used in qPCR assay.

Repairment of cartilage defects using CM in rats

SD rats (8 weeks old, male, 250 g) were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Shenyang, China). All experiments followed the guidelines and protocols of the Animal Experiment Ethics Committee of the Chinese Academy of Agricultural Sciences (NO. ISAPSAEC-2023-030D). There were five groups in this study, including Control group, DMEM (Negative control) group, ASC-CM (80 mg/ml) group, ADSC-CM (80 mg/ml, positive control) group and Sham group²⁶. Briefly, after rats were anesthetized with sodium pentobarbital (30 mg/kg), a cartilage defect (2 mm in diameter and 1 mm in depth) was caused by an electric drill. CM freeze-dried powder (each 8×10^7 cells yielding about 12 μ g of freeze-dried material) mixed with Gelma (SunPG Bio, China) was implanted directly into the defect sites. Six weeks after surgery, the rats were euthanized by CO₂, and samples were collected.

Histological examination

The embedded samples were cut into 5- μ m-thick sections and analyzed by staining with saffron O solid green and hematoxylin-eosin (H&E). Immunohistochemical study was performed using an immunohistochemical staining kit (MXB, DAB-0031, Beijing). Sections incubated with primary antibodies *collagen II* (Servicebio, China, GB11021, 1:200) at 4 °C overnight. Subsequently, samples were incubated with secondary antibodies, and then stained with hematoxylin.

Statistical analysis

Statistical analysis was performed using Prism 8 (GraphPad software). All data conform to a normal distribution. One-way ANOVA with Dunnett's multiple comparison test was used for more than two groups. In the CCK-8 test, data from different time points were analyzed by repeated-measures ANOVA. The mean $\pm$ SD of at least three independent experiments was used for all quantitative data. Differences were considered significant at $P < 0.05$.

Results

Comparisons of the doubling time of the three stem cells

Compared to hADSC and hBMSC, ASC have the shortest doubling time and the average doubling time of P1-P5 is 20 h (Fig. 2A,B).

Determination of the optimal ratio of ASC-CM in culture medium

The optimal ratio of ASC-CM were determined by proliferation analysis of rat-chondrocytes. Results showed that the group of 1:16 cells have higher proliferation ability. Therefore, a ratio of 1:16 was selected for the subsequent experiments (Fig. 3A).

ASC-CM promoted cells proliferation and migration in vitro

Our results of the CCK8 and Edu assays showed that ASC-CM and hADSC-CM increased the proliferation rate of chondrocytes (Fig. 3B,D,F) and rat-BMSC (Fig. 3C,E,G). However, the ability of ASC-CM to enhance chondrocytes and rat-BMSC proliferation was higher significantly than that of hADSC-CM. The results of migration assay showed that the ASC-CM had the highest migration rates compared to DMEM and hADSC-CM in Rat-BMSC ($P < 0.01$, Fig. 3I,H). ASC-CM significantly increased the migratory capacity of rat-BMSC.

ASC-CM differentially regulated the cartilage mRNA expression

ASC-CM upregulated the mRNA expression of chondrogenesis-related genes significantly. (Fig. 4A-C). It was shown that ASC-CM promoted glycosaminoglycan formation and type II collagen deposition.

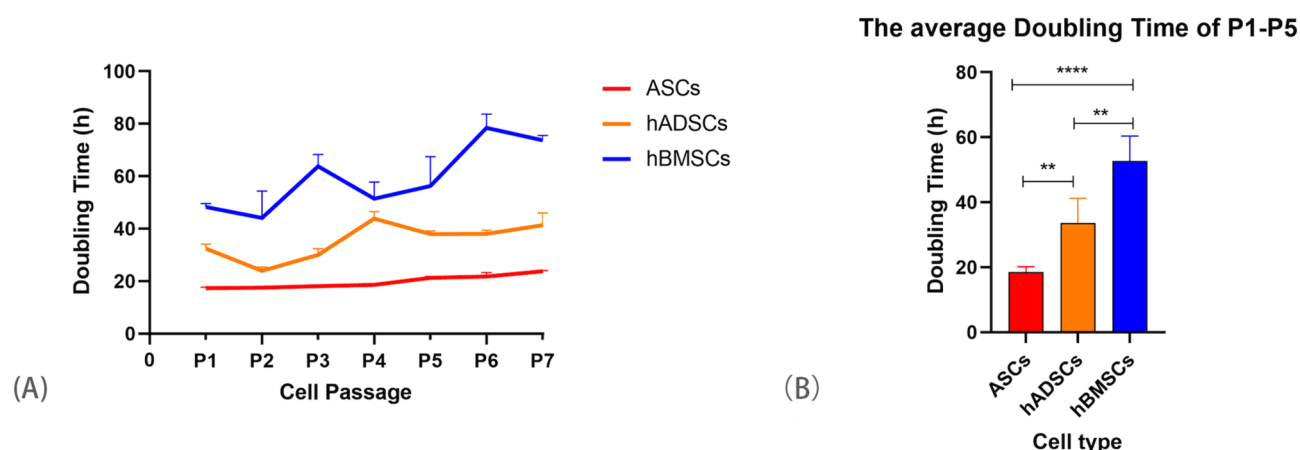


Fig. 2. Stem cell multiplication time. (A)The doubling time of the three stem cells. $n = 3$. (B)The average doubling time of the three stem cells (P1-P5). $n = 3$. ** $P < 0.01$, **** $P < 0.0001$.

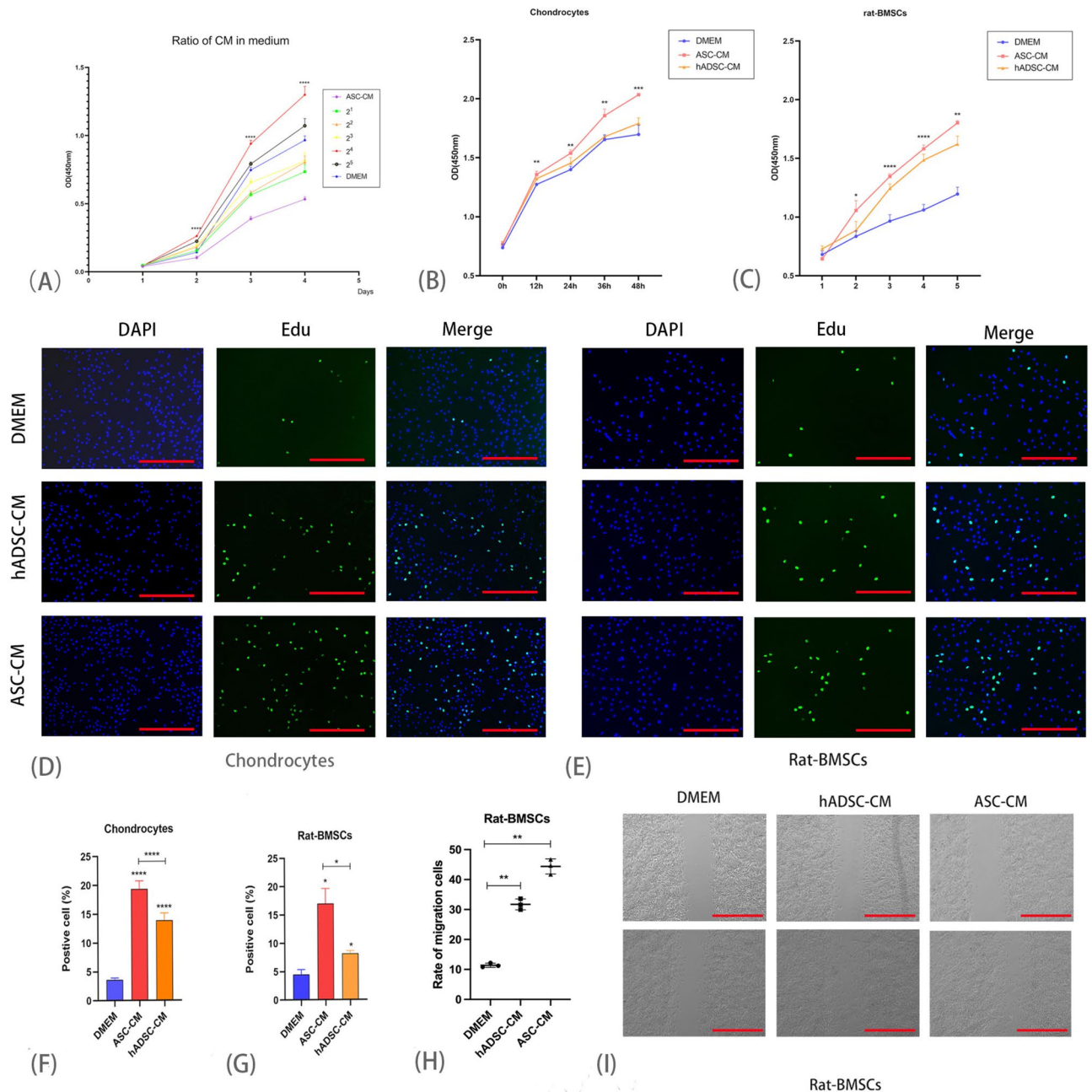


Fig. 3. Effects of the ASC-CM on the proliferation and migration. **(A)** Optimization of the ratio of ASC-CM to medium using rat-chondrocytes, **** $P < 0.0001$. **(B,C)** Effects of ASC-CM (1:16) on the proliferation of rat-chondrocytes and rat-BMSCs, $n = 3$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. **(D,E)** Effects of the ASC-CM on proliferation of rat-chondrocytes and rat-BMSCs by Edu, Scale bar = 400 μm . **(F,G)** Percentages of Edu-positive cells in rat-chondrocytes and rat-BMSCs, $n = 3$, * $P < 0.05$, **** $P < 0.0001$. **(H)** Quantitative analysis of the migrated rat-BMSCs, * $P < 0.05$, ** $P < 0.01$. **(I)** Migration assay of rat-BMSCs, Scale bar = 1000 μm .

ASC-CM suppresses chondrocyte apoptosis

ASC-CM significantly up regulated the mRNA expression levels of the apoptosis suppressor gene *NAMPT* and down regulated apoptosis gene *BAX* (Fig. 4D,E).

Apoptosis detection results showed that compared to hADSC-CM and DMEM, the ASC-CM had the lowest percentage of cells in apoptosis (Fig. 5). Overall, ASC-CM inhibited significantly chondrocyte apoptosis.

ASC-CM repaired the rat knee joints

To achieve sustained release of ASC-CM in vivo, hydrogels containing 80 mg/ml each of ASC-CM, hADSC-CM, and DMEM were fabricated for topical administration to cartilage defects (Fig. 6A).

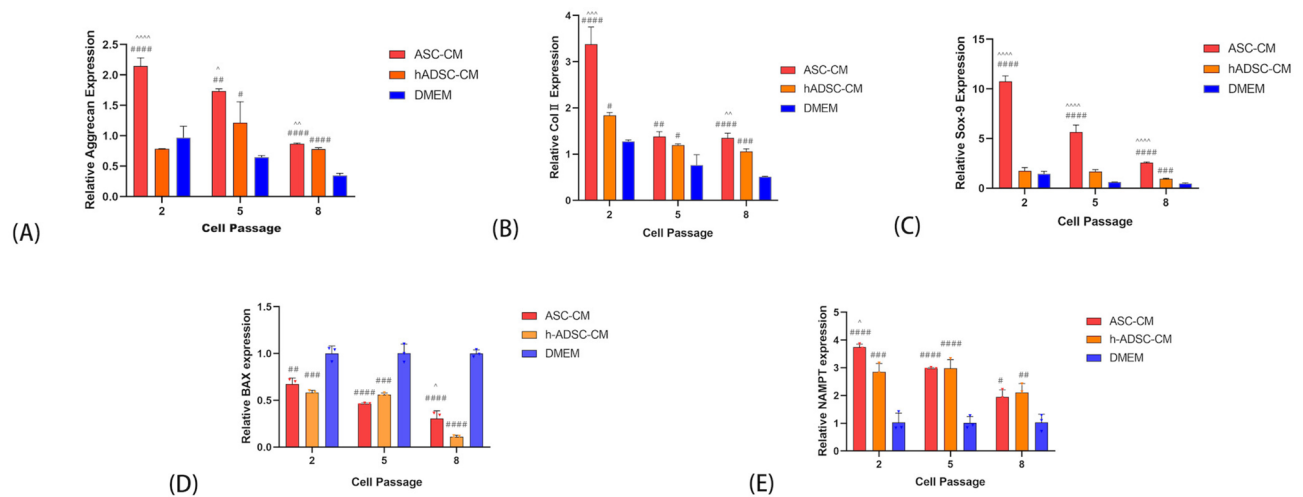


Fig. 4. RNA expression of rat-chondrocytes measured by qPCR. (A–E) qPCR was used to measure the RNA expression levels of aggrecan, collagen II, Sox-9, BAX and NAMPT in chondrocytes. (n = 3), #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001 vs. DMEM group in the same cell passage. ^p < 0.05, ^^p < 0.01, ^^p < 0.0001 vs. hADSC-CM group.

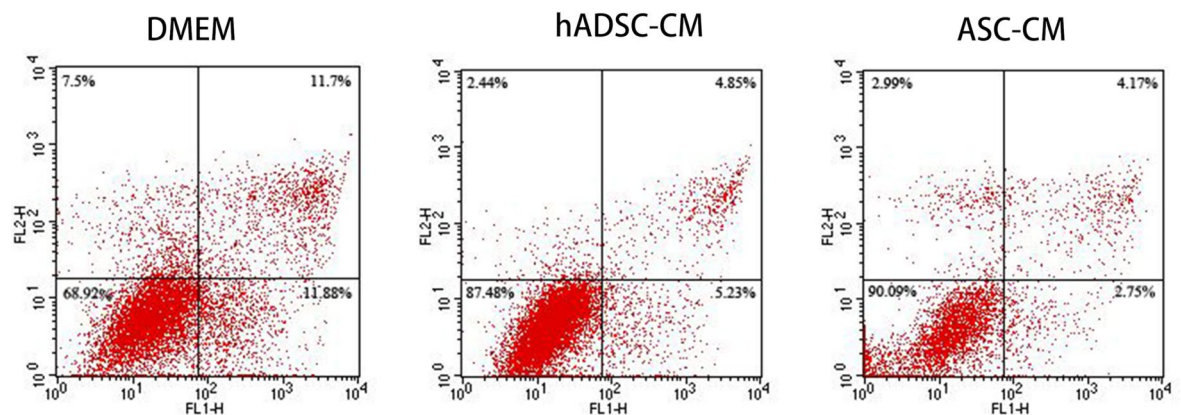


Fig. 5. Effects of the ASC-CM on apoptosis of rat-chondrocytes. The apoptosis rates of ASC-CM, hADSC-CM, and DMEM group are 2.75, 5.23, and 11.88%, respectively.

After 6 weeks, repaired tissue was assessed blindly grossly, using the International Cartilage Repair Society (ICRS) Cartilage Repair Assessment scoring scale. The cartilage in the ASC-CM group was similar to that in the sham group, with intact and smooth surfaces and good integration with the surrounding original cartilage (Fig. 6B).

Concerning histological assessment, Safranin O/fast green and HE staining were performed to assess the regeneration of newly formed cartilage (Fig. 6C). The regenerated cartilage in the ASC-CM group showed intense red staining by Safranin O/fast green staining, similar to the natural cartilage in the sham group. Analysis of the ICRS histological scores (Fig. 6D) showed that the ASC-CM score was higher than the hADSC-CM group, DMEM group and the control group, which were similar to the sham.

Compared to the DMEM and hADSC-CM groups, the ASC-CM group showed higher expression of type II collagen and stronger positive for type II collagen in stained areas, which indicated that ASC-CM facilitated the regeneration of type II collagen and extracellular matrix (Fig. 6E,F).

Quantitative proteomics analysis

The protein components of ASC-CM were analyzed via DIA assay. The datasets generated and/or analysed during the current study are available in the ProteomeXchange repository, (<https://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PX041259>) (Accession number: PX041259). S100A4 and S100B are among the top proteins in relatively high expression, and they regulate the activity of articular rat-chondrocytes and are involved in physiological activities such as cell chemotaxis, tissue development, and repair. The effective promotion of cartilage repair by ASC-CM may be due to the interaction of S100A4 with other cytoskeletal proteins S100B.

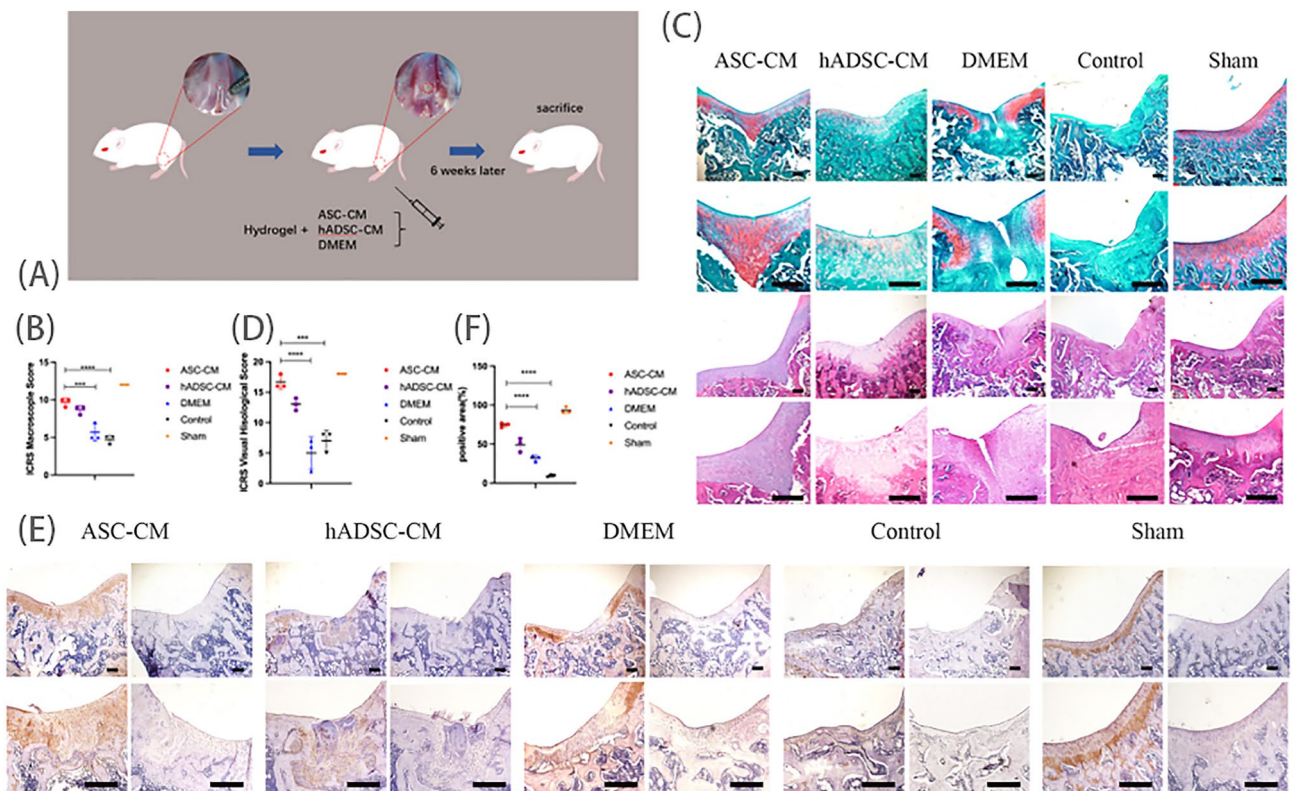


Fig. 6. Histological evaluation of cartilage defect. (A) Experimental procedure. (B) ICRS macroscopic scores of different groups. $n = 3$, $***P < 0.001$, $****P < 0.0001$. (C) HE staining and Safranin O/fast green staining of repaired cartilage. Scale bar = 250 μm . (D) ICRS visual histological scores of different groups. $n = 3$, $***P < 0.001$, $****P < 0.0001$. (E) Immunohistochemical staining of collagen II. Scale bar = 250 μm . (F) Quantitative analysis of immunohistochemical staining of collagen II. $n = 3$, $****P < 0.0001$.

Discussion

Our studies showed that the doubling time of ASC is the shortest among the 3 stem cells (ASC, ADSC, and BMSC), and its conditioned medium can strongly promote chondrocyte proliferation. ASC-CM significantly up-regulated the chondrogenesis-related genes (*Aggrecan*, *Col II*, *Sox-9*) and promoted glycosaminoglycan formation and type II collagen deposition in cartilaginous tissue. Meanwhile, we found that ASC-CM significantly up-regulated the apoptosis suppressor gene *NAMPT* and down-regulated the apoptosis gene *BAX*. In vivo, cartilage defect in rats was significantly repaired using ASC-CM. By analyzing the protein components of ASC-CM, we thought S100A4, S100B might play an important role.

Why antler stem cells (ASC)

Antlers grow rapidly, which is driven by processes such as cell proliferation and differentiation²⁷. ASC are classified as MSC because they express several key embryonic stem cell markers and possess the characteristics of MSC²⁴. ASC initiate the complete regeneration of mammalian accessory organs (Antlers) and have a crucial effect on subsequent antler cartilage tissue formation¹⁶. This experiment confirms that ASC have a faster growth rate and are less susceptible to senescence than hADSC and hBMSC. In addition, ASC stimulated regenerative wound healing in rat skin²⁸, and reduced liver fibrosis²⁹, superior to other tested MSC from human or other animal sources, suggesting no species-specific in the healing ability of ASC.

Why ASC-conditioned medium (ASC-CM)

Current clinical treatment strategies are ineffective in promoting articular cartilage regeneration. And the biochemical and mechanical properties of neocartilage tissue are significantly different from those of normal cartilage. Developing stem cell-based tissue engineering techniques has provided new ideas for cartilage injury repair. Compared with conventional stem cell transplantation, stem cell CM has tremendous advantages: it avoids the difficulty in maintaining cell activity and the risks of cell tumorigenesis, cell dedifferentiation. Moreover, rather than directly differentiating into rat-chondrocytes to repair tissue, MSC regulate local inflammation, apoptosis, and proliferation through paracrine mechanisms. Therefore, ASC-CM might be a good choice to repair cartilage injury.

ASC-CM has been tested in several animal models, demonstrating their beneficial effects^{28–30}. In this study, we found that ASC-CM significantly up-regulated the chondrogenesis-related genes (*Aggrecan*, *Col II*, *Sox-9*) and promoted glycosaminoglycan formation and type II collagen deposition in cartilaginous tissue. Cartilage defect in rats was significantly repaired using ASC-CM in vivo. Moreover, it is also easy to produce in large quantities, making it more economical and practical for clinical applications³¹.

Possible underlying mechanism

By ASC-CM composition analysis, we found that CM contains many cytoskeletal proteins such as S100B, PLS3, Filamin A, and S100A4. It has been shown that S100A4, released from tumor or stromal cells, has the function of downregulating the mRNA expression of the proapoptotic gene *Bax*³². Therefore, it is speculated that S100A4 in CM also downregulated the mRNA expression of the chondrogenic apoptosis gene *Bax*, thus inhibiting chondrocyte apoptosis. S100B is a stimulator of cell proliferation and migration and an inhibitor of apoptosis and differentiation, which may be important for cartilage development, regeneration, and repair³³. In addition, CM also contains TGF- β 1 and TGF- β 2, which have been extensively shown to regulate gene expression in the body to produce matrix components that promote cartilage repair and maintain cartilage phenotype. TGF- β down regulates cartilage-degrading enzymes and stimulates type II collagen synthesis. At the same time, TGF- β has a cartilage-protective effect by eliminating the inhibitory effect of IL-1 on prostaglandins³⁴. TGF- β was found not to induce cartilage differentiation but also to have an inhibitory effect on new cartilage resorption; PDGF was released into the defect site, exerting a chemotactic and mitogenic effect on cells in the surrounding cartilage and infiltrating mesenchymal stem cells, which provided a suitable environment for new tissue formation; IGF plays a key role in cartilage homeostasis, balancing the synthesis and breakdown of proteoglycans³⁵.

Rat-chondrocytes are in a specific “cartilage microenvironment”, and their internal components must be complex and diverse, with more than just a single growth factor present. It is becoming increasingly clear that growth factors can enhance cartilage matrix synthesis synergistically, such as IGF-I and TGF- β , which regulate gene expression in the body to produce matrix components that promote cartilage repair³⁶. Studies have shown that combining multiple growth factors is significantly better than one. In recent years, platelet-rich plasma (PRP) enriched with PDGF, TGF- β , and IGF-1 growth factors have been widely used in treating sports injuries because of their beneficial effects on tissue repair³⁷. Therefore, using ASC-CM for cartilage repair may be more comprehensive and efficient than using a single factor.

Although our in vitro and in vivo data demonstrate superior cartilage-regenerative capacity in S100A4/S100B-high chondroprogenitors compared with h-ADSCs, they do not establish these proteins as the sole drivers of this phenotype. Additional differentially expressed proteins and paracrine cues are expected to contribute. Rigorous proof of causality will demand orthogonal, loss- and gain-of-function strategies—gene knockout or overexpression, neutralizing antibodies, and CRISPR interference—to specifically manipulate S100A4/S100B levels and quantify the resulting functional impact.

Conclusions

Our data demonstrated that the ASC-CM can promote the proliferation, maintain the phenotype, and inhibit apoptosis of rat-chondrocytes. Therefore, it can promote cartilage repair. The proteomics analysis of ASC-CM suggested that these effects might be due to the interaction of S100A4 with other cytoskeletal proteins and the synergistic effects of multiple growth factors.

Data availability

The corresponding author has all the raw data for this study. The datasets generated and/or analyzed during the current study are available in the ProteomeXchange repository at <https://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD041259> (Accession Number: PXD041259).

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Author contributions

HM.S. and J.Z. supervised the design of the experiments; J.Z. and BR.X. participated in animal and cellular experiments; QQ.G., DT.W., ZG.Y., JPL. and XS.L. supervised the experimental procedures; J.Z., JW.Z. and YD.J. participated in cellular and histological experiments.

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Declarations

Competing interests

The authors declare no competing interests.

Institutional review board statement

This study was conducted and reported in strict accordance with the ARRIVE guidelines, including a detailed description of the selection of experimental animals, experimental procedures, data collection, and analysis. We ensured the transparency and reproducibility of the experiments, and all experiments were carried out in compliance with relevant ethical and regulatory standards. The animal study was subject to review and approval by the animal ethics committee of the Institute of Special Economic Animal and Plant Sciences (NO. ISAPSAEC-2023-030D).

Additional information

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