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Original Article

The in vivo chondrogenesis of cartilage stem/progenitor cells from auricular cartilage and the perichondrium

Xiaodie Zhang[1*], Lin Qi[2*], Yahong Chen[1], Zhezheng Xiong[1], Junjie Li[1], Peng Xu[1], Zongqin Pan[1], Huizhong Zhang[1], Zhuxin Chen[1], Ke Xue[1], Kai Liu[1]

[1]*Department of Plastic and Reconstructive Surgery, Shanghai 9th People's Hospital, Shanghai Jiao Tong University School of Medicine, 639 Zhi Zao Ju Road, Shanghai 200011, China;* [2]*Department of Radiology, Huadong Hospital, Fudan University, 221 West Yan-An Road, Shanghai 200040, China.* [*]*Co-first authors.*

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Abstract: Bone marrow-derived stem cells are commonly studied for cartilage tissue engineering and regeneration medicine applications, but their ossification tendency and their limited capacity for chondrogenic differentiation depending on the donor age limit their clinical application. Cartilage stem/progenitor cells are ideal seeding cells, as cartilage stem/progenitor cells from auricular cartilage and the perichondrium have the inherent advantages of chondrogenesis capacity and an easy and nontraumatic harvesting process, displaying promise for applications. The identification and comparison of cartilage stem/progenitor cells from auricular cartilage and the perichondrium in vitro were explored in our previous study, but the in vivo chondrogenesis of these cells has not been fully examined. In the current study, we explored the ectopic chondrogenesis of cartilage stem progenitor/cells from auricular cartilage and the perichondrium after chondrogenic induction in vitro. Our results suggest that stem/progenitor cells from auricular cartilage exhibit significantly better chondrogenesis than those from the perichondrium in vivo, with upregulated chondrogenic genes and a stable cartilage phenotype, as well as good mechanical properties, indicating that stem/progenitor cells from auricular cartilage could be one type of ideal seeding cells for cartilage tissue engineering.

Keywords: Chondrogenesis, cartilage stem/progenitor cells, auricular cartilage, perichondrium, in vivo

Introduction

The auricle is a surface organ of the human body and is of great significance to human facial aesthetics. When an ear becomes deformed and damaged, patients have a strong desire to reconstruct their auricle [1]. However, this reconstruction is a major challenge in the field of plastic and reconstructive surgery due to the limited self-repair and regenerative ability of auricular cartilage, which lacks blood vessels and nerves [2]. At present, approaches for repairing auricular cartilage defects include transplantation of autologous cartilage, Medpor (artificial prosthesis) or other implants [3]. The most common treatment for auricular reconstruction is to harvest rib cartilage from the patient and sculpt this cartilage into the shape of an ear [4]. However, this strategy not only causes considerable trauma and many complications but also needs highly skilled surgeons to meet aesthetic demands [5]. In addition, the reconstructed ear with autologous costal cartilage can become calcified, contracted and deformed or be absorbed over time [6]. All of these problems have limited the application of autologous cartilage transplantation. Fortunately, the emergence of cell-based tissue engineering offers an alternative strategy that could overcome the obstacles mentioned above.

Seeding cells are the key factors in cartilage tissue engineering because their quantity and quality are directly related to the success of reconstructive surgery [7, 8]. Stem cells and chondrocytes have potential as seeding cells for tissue-engineered cartilage [9, 10]. Chondrocytes are the preferred seeding cells for cartilage tissue engineering, but their dedifferentiation in long-term culture in vitro may lead to an insufficient amount of functional chondrocytes [11]. Dedifferentiated chondrocytes lose their ability to form stable phenotypic cartilage [12]. The reasons above limit the applications of chondrocytes are for cartilage tissue engineering. Establishing the best seeding cell applica-

tion strategy can provide a theoretical basis and technical support for auricular cartilage tissue engineering to overcome current barriers.

Stem cells, with their wide variety of sources, self-renewal capacity, immune-regulatory capacity, and multidifferentiation ability, have been the focus of cartilage tissue engineering research [13]. Stem cells can be divided into three groups: mesenchymal stem cells (MSCs), induced pluripotent stem cells (iPSCs), and embryonic stem cells (ESCs) [14-16]. Although iPSCs and ESCs are pluripotent, these cells have an associated risk of tumor formation and ethical problems [17]. Adult MSCs can be obtained from various adult tissues, such as bone marrow, synovium, and adipose tissue. Bone mesenchymal stem cells (BMSCs) have stimulated extensive and deep research, owing to their multipotency, low immunogenicity, high expansion rates, easy harvesting process, and immunoregulatory capacity [18]. However, BMSCs tend to undergo vascularization and ossification rather than forming stable phenotypic cartilage [19]. Cartilage stem/progenitor cells (CSPCs) have been isolated and identified in recent years [20], and these tissue-specified stem cells displayed high colony-forming capacity, multipotential ability, and high replicative potential expression of surface markers of MSCs.

Auricular cartilage often regenerates in patients who undergo auricular cartilage resection for rhinoplasty. It is unclear whether CSPCs or perichondrium stem/progenitor cells (PSPCs) guide the repair process in vivo. In our previous experiments, we successfully isolated stem cells from porcine auricular cartilage and ear cartilage perichondrium [8]. Accordingly, exploring the chondrogenic capacity of CSPCs and PSPCs in vivo will help us to understand the effect of CSPCs in the regeneration and repair of auricular cartilage defects.

Materials and methods

All procedures involving animal tissues and cells were approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine.

Isolation and culture of CSPCs and PSPCs

After neonatal pigs were anesthetized and disinfected, both external ears of the pigs were harvested, and the ears were soaked in 75% ethanol solution for 15-30 min. Ophthalmic scissors were employed to remove extra soft tissue, and the auricular cartilage perichondrium was separated from ear cartilage tissues. The separated ear cartilage perichondrium and cartilage were cut into pieces of approximately 1×1 cm^2 and transferred to 50 ml centrifuge tubes. The samples were soaked in sterile chloramphenicol solution for 30 min and then rinsed with sterile PBS solution and sterile 0.20% collagenase solution. The samples were digested in a constant temperature shaker for 4-6 hours. A 100 μl sterile cell strainer was used to filter the digested cell suspension, the undigested tissue fragments were discarded, and the filtrate was centrifuged at 1500 rpm/min for 5 min. The pellet was resuspended and plated in a fibrin-coated culture dish for a density of 1-2×10^5 cells/cm^2. After incubation for 20 min in the incubator, the nonadherent cells were removed, and 10 ml of warm fresh low-glucose complete culture medium containing 10% fetal bovine serum and 1% penicillin-streptomycin was added. The cells were cultured in conditions with carbon dioxide and at a concentration of 2×10^5 cells/cm^2. The cells were trypsinized and subcultured until reaching at least 80% confluence.

FACS analysis

The auricular CSPCs and PSPCs at passages 1, 3 and 5 were used for FACS analysis to assess the characteristics of cells. A total of 1×10^6 cells were washed in PBS and incubated for 1 hour at 4°C with fluorescence-conjugated mouse anti-human monoclonal antibodies (CD29, CD34, CD45, and CD90). The cells were centrifuged at 2000×g, the supernatants were removed, and the cells were washed thrice in PBS. Finally, the labeled cells were resuspended in 1 ml of PBS and subjected to analysis with a Beckman Coulter FC 500 flow cytometer.

Cell differentiation

CSPCs and PSPCs were seeded at a density of 5000 cells/well in 12-well culture plates and cultured in different induction media to evaluate their osteogenic, adipogenic, and chondrogenic differentiation. Cells were induced in osteogenic induction medium (DMEM containing 10% FBS, 0.01 μM 1,25-dihydroxyvitamin D_3, 50 μM ascorbate-2-phosphate, 10 mM β-glyc-

erophosphate, 1% antibiotic/antimycotic; all reagents were purchased from Sigma company, USA) for 16 days, and the culture medium was replaced every 2 days. To evaluate osteogenesis, cells in a 12-well plate were washed with PBS, fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature, and incubated with 1% Alizarin Red S solution for 10 min at room temperature using the Alizarin Red Stain Kit (Sigma-Aldrich Co., St Louis, MO, USA).

Cells were induced in adipogenic induction medium (DMEM containing 10% FBS, 0.5 mM isobutyl-methylxanthine, 1 μM dexamethasone, 10 μM insulin, 200 μM indomethacin, 1% (v/v) antibiotic/antimycotic; all reagents were purchased from Sigma company, USA) for 14 days, and the culture medium was replaced every 2 days. To evaluate adipogenesis, cells in a 12-well plate were fixed with 4% PFA for 30 min at room temperature and stained with oil red O solution for 10 min at room temperature using the Oil Red O Stain Kit (Sigma-Aldrich Co., St Louis, MO, USA).

For chondrogenesis, cells were seeded at a density of 5000 cells/well in a 12-well culture plate and cultured using high glucose DMEM containing 10% FBS, 10 ng/ml transforming growth factor-1, 10^{-7} M dexamethasone, and 40 ng/ml insulin-like growth factor-1. After two weeks of induction, the cells were stained with toluidine blue.

Cell/collagen construction

After collecting CSPCs and PSPCs at passage 3, the cell concentration was adjusted to 50×10^6 cells/ml and placed on ice for use. Twelve microliters of 100 mM NaOH solution was added to 200 μl of type I rat tail collagen and mixed quickly. Then, 23 μl of prechilled sterile 10×PBS was added to the above mixture, and 760 μl of the cell suspension was mixed with the above mixture. The solution was mixed gently and rapidly to avoid bubble generation, and this mixture was then transferred to a sterile 15 ml centrifuge tube (200 μl of mixture per tube). Afterward, the mixture was put them into a constant temperature incubator for 15 min for gelation, followed by the addition of 3 ml of preheated low-glucose DMEM and incubation at 37°C in an atmosphere of 5% CO_2 in air.

The culture medium was replaced with chondrogenic induction medium (high glucose DMEM containing 10% FBS, 10 ng/ml transforming growth factor-1, 10^{-7} M dexamethasone, and 40 ng/ml insulin-like growth factor-1) after 24 hours, and the chondrogenic induction medium was changed performed every 2-3 days. After 3 weeks of in vitro culture with chondrogenic induction medium, the samples were transplanted subcutaneously into nude mice (5 weeks old, male, weight of 24 to 28 g) for 6 weeks.

Histological and immunohistochemical analysis

The samples were fixed in 4% PFA and embedded in paraffin for slicing into 5 μm sections, and hematoxylin and eosin (H&E) and Safranin O staining were used to evaluate the histological structure and cartilage matrix deposition in the cell constructs.

To determine the expression of type I, type II and type X collagen in the constructs, some sections were subjected to two-step indirect immunohistochemical analysis as described previously [21]. Briefly, the secretion of collagen types I, II and X was studied by primary anti-collagen type I antibody (1:400; ab90395), anti-collagen type II antibody (1:50; ab34712), and anti-collagen type X antibody (1:500; ab49945), respectively, and horseradish peroxidase-conjugated anti-rat and anti-rabbit antibody (1:200 in 0.5% BSA; Goodbio Technology Co., Ltd.). Diaminobenzidine tetrahydrochloride (DAB; Maibio, Shanghai, China) was employed for color development, and a Nikon Y-FL 07-8077 microscope (Nikon, Tokyo, Japan) was used for visualization.

Glycosaminoglycan, total collagen and biomechanical analysis of engineered tissue in vivo

Six weeks after implantation, the total collagen content was analyzed by a hydroxyproline assay as previously described [22]. Briefly, the samples were prepared by alkaline hydrolysis, and free hydroxyproline hydrolysates were assayed. The hydroxyproline content was converted to total collagen on the basis of the mass ratio of collagen to hydroxyproline.

The glycosaminoglycan (GAG) content of the specimens was assayed by dimethyl methylene blue chloride [23]: Total GAG was precipitated by guanidinium chloride solution (0.98 mol/L),

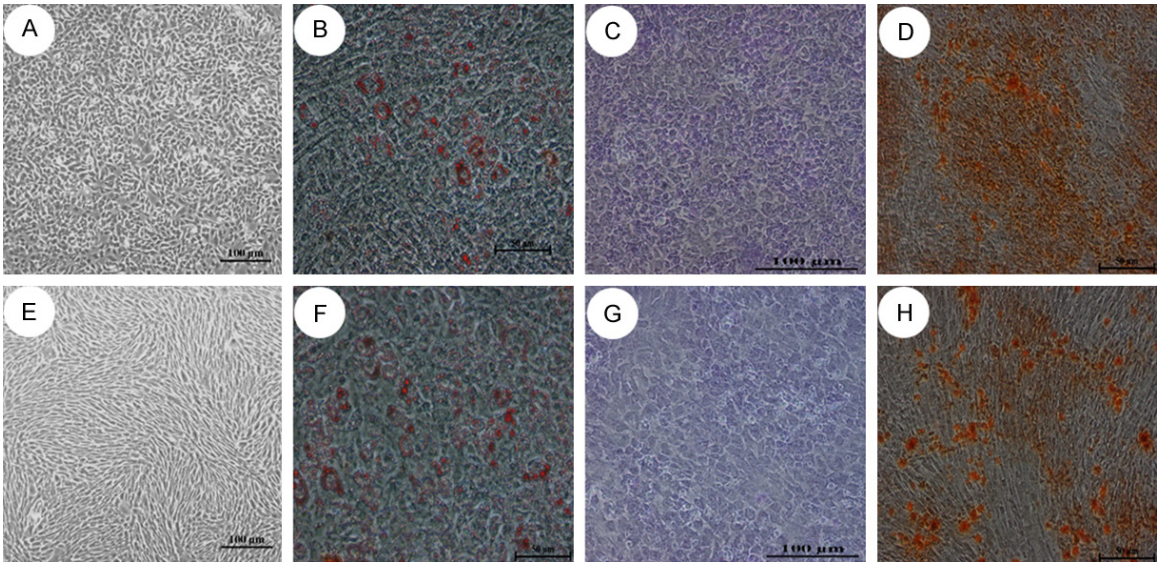

Figure 1. Cultivation and induced differentiation of CSPCs and PSPCs. A and E. Cytomorphology of the third-passage CSPCs and PSPCs. CSPCs in passage 3 culture were polygonal and spindle, while PSPCs showed short spindle at 3th passage. Scale bar: 100 μm. B and F. CSPCs and PSPCs were treated with adipogenic medium for 14 days. Red lipid drops stained by oil red O were observed in both CSPCs and PSPCs, which demonstrated their adipogenic capacity. Scale bar: 100 μm. C and G. Positive staining for toluidine blue displayed the chondrogenesis of the differentiated CSPCs and PSPCs. Scale bar: 100 μm. D and H. The osteogenic ability of CSPCs and PSPCs is shown by the formation of calcifying nodules with positive Alizarin Red S staining. Scale bar: 50 μm.

and the optical density (OD) was determined at 595 nm. A standard curve was established on the basis of the OD values of different concentrations of chondroitin-4-sulfate. The total GAG amounts were determined according to the standard curve and the OD value.

A biomechanical analyzer (Instron, Canton, MA) was used to evaluate biomechanical properties as previously described [24]. The samples in two groups were subjected to a constant compressive strain rate of 0.5 mm/min until 80% of the maximal deformation was achieved, and the compressive modulus of the tested tissue was calculated based on the force-displacement curve.

Western blot analysis

Western blot analysis was performed as described by Weng [25]. Primary antibodies specific to RUNX2 (1:1000, ab76956, Abcam, USA), SOX9 (1:1000, ab185966, Abcam, USA), collagen I (1:1000, ab6308, Abcam, USA), collagen II (1:1000, ab188570, Abcam, USA), collagen X (1:1000, ab182563, Abcam, USA), aggrecan (1:1000, ab36861, Abcam, USA), and GAPDH (1:1000, ab8245, Abcam, USA) were used.

Statistical analysis

The results are expressed as the mean ± standard deviation; The Student t test was employed to compare two groups of independent samples. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed with SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

Results

Cultivation and induced differentiation of CSPCs and PSPCs

The third-passage CSPCs and PSPCs (**Figure 1A** and **1E**) displayed short fusiform or polygonal shapes, but there was no apparent difference in their cytomorphology. After 14 days of adipogenic induction, both CSPCs and PSPCs changed shape from short fusiform or polygonal to oval, and oil red O-positive lipid droplets were found in the cells (**Figure 1B** and **1F**). After 2 weeks of chondrogenic induction, both groups were positive staining for toluidine blue, while the group of CSPCs displayed stronger staining than the group of PSPCs (**Figure 1C** and **1G**). After 16 days of osteogenic induction, CSPCs

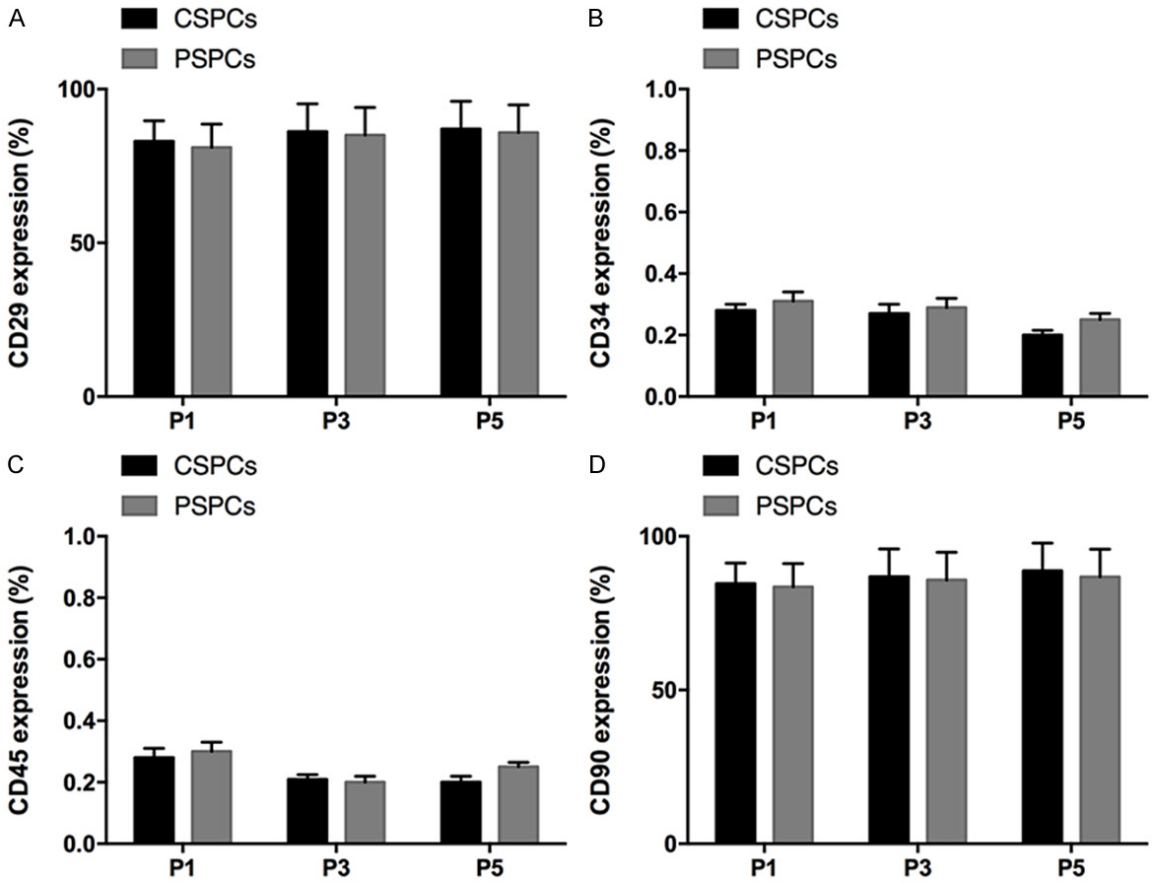

Figure 2. Flow cytometry analysis of the expression of cell-surface markers on CSPCs and PSPCs. CSPCs and PSPCs showed high expression of mesenchymal-derived stem cell positive surface markers (CD29 and CD90), while almost not express hematopoietic stem cell positive surface markers (CD34 and CD45).

and PSPCs formed red calcium deposits, and the CSPC group exhibited more calcium-containing mineralized nodules than the PSPC group (**Figure 1D** and **1H**).

Cell-surface marker expression of CSPCs and PSPCs

To characterize the cell-surface marker expression of CSPCs and PSPCs, we analyzed CSPCs and PSPCs with flow cytometry. The expression levels of cell-surface markers were similar between CSPCs and PSPCs. These cells highly expressed the MSC markers CD29 and CD90 (**Figure 2A** and **2D**) but did not express the hematopoietic stem cell markers CD34 and CD45 (**Figure 2B** and **2C**).

Ectopic cartilage formation of CSPCs and PSPCs in vivo

To explore the ectopic chondrogenesis capacity of CSPCs and PSPCs in vivo, we used a type I rat tail collagen scaffold system. After 6 weeks of implantation, the gross assay showed that both groups formed oyster white constructs, while there was no significant difference between CSPCs and PSPCs (**Figure 3A** and **3D**). However, H&E staining showed that the CSPCs formed more mature and uniform elastic cartilage-like tissue than the PSPCs (**Figure 3B** and **3E**). Safranin O staining was performed to assess the secretion of sulfated GAG and indicated that those matrixes were cartilaginous tissues (**Figure 3C** and **3F**). Both samples showed positive staining, which suggested that the CSPCs and PSPCs had the capacity to form ectopic cartilage. However, the CSPCs displayed stronger staining than the PSPCs, which was further indicated by the H&E test demonstrating that CSPCs possessed superior cartilage formation compared to that of PSPCs.

Immunohistochemical analysis

To further confirm the histological study, the samples were subjected to immunohistochemical tests. Similar to the results shown by histo-

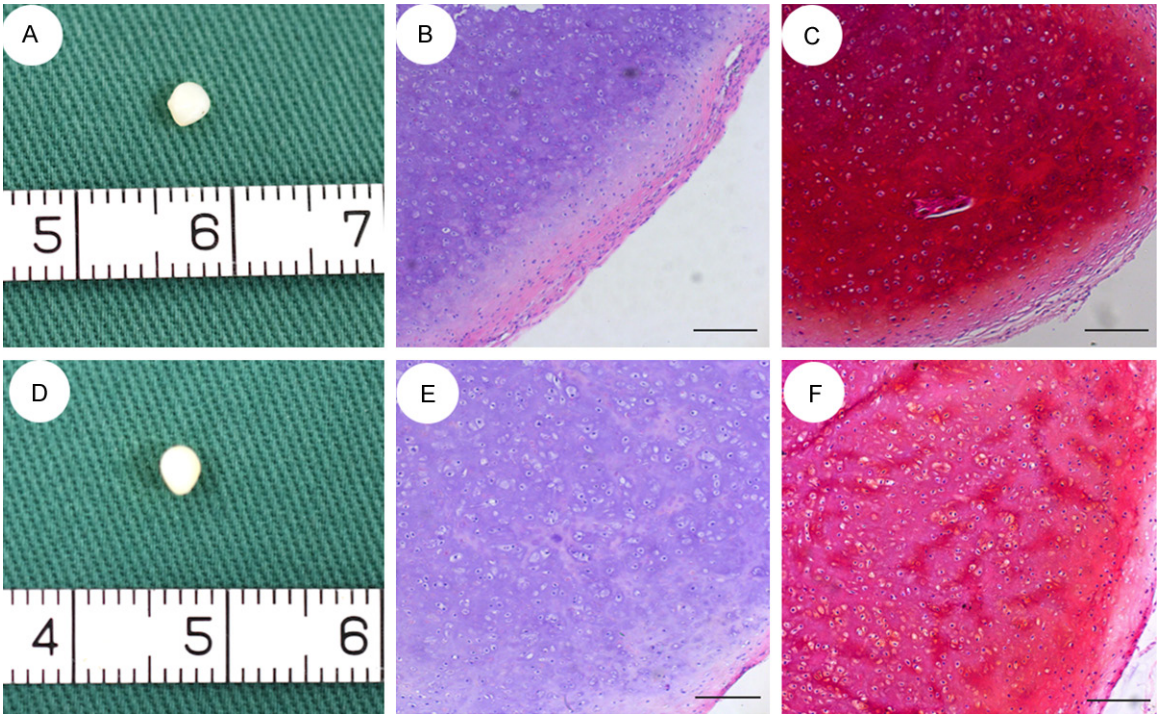

Figure 3. Capacity for cartilage formation by CSPCs and PSPCs in vivo. A and D. Gross assay of constructs formed by CSPCs and PSPCs. Both group displayed milky white appearance, which was similar to native cartilage tissue. B and E. Hematoxylin-eosin staining showed both group formed cartilage-like tissue with obvious cartilage lacuna, however, CSPCs formed cartilage that was more uniform and mature. Scale bar: 100 μm. C and F. Positive Safranin O staining showed that both of CSPCs and PSPCs formed cartilage like tissue. Copaired to group of PSPCs, strongly positive staining appeared in group of CSPCs. Scale bar: 100 μm.

logical staining, both groups showed the formation of type II collagen (**Figure 4A** and **4D**), and stronger staining was found in the CSPC group. In contrast, both groups were negative for type X collagen staining (**Figure 4B** and **4E**), indicating that CSPCs and PSPCs formed rather stable cartilage in our observation period. However, weak positive staining for type I collagen was observed in the PSPCs (**Figure 4F**) and negative staining was observed in the CSPCs (**Figure 4C**). These results together demonstrated that CSPCs had advantages over PSPCs.

Biochemical and biomechanical analysis

To further confirm the histological and immunological tests, we employed statistical analysis. The relative cartilage gene expression showed a significant difference between CSPCs and PSPCs (**Figure 5A-C**). Compared with PSPCs, CSPCs had upregulated expression of collagen II, aggrecan, and SOX9 and downregulated expression of collagen X, collagen I, and RUNX2, which are markers of calcification and ossification ($P<0.05$) (**Figure 5D-F**). In addition, the total collagen, GAG, and biomechanics tests showed significant differences between CSPCs and PSPCs ($P<0.05$) (**Figure 6**). Together, the quantitative measurements indicated that CSPCs could form better cartilage-like tissue than PSPCs, which was in line with the histological and immunological results.

Western blot analysis

The Western blot analysis results further validated the immunohistochemistry and Q-PCR results (**Figure 7**). Compared to PSPCs, CSPCs showed more obvious induction of collagen II and aggrecan, while collagen I was inhibited. In addition, the expression of other proteins was similar to that of collagen II in CSPCs.

Discussion

Ideal seeding cells play a vital role in constructing tissue-engineered cartilage with a stable phenotype. BMSCs have been extensively and

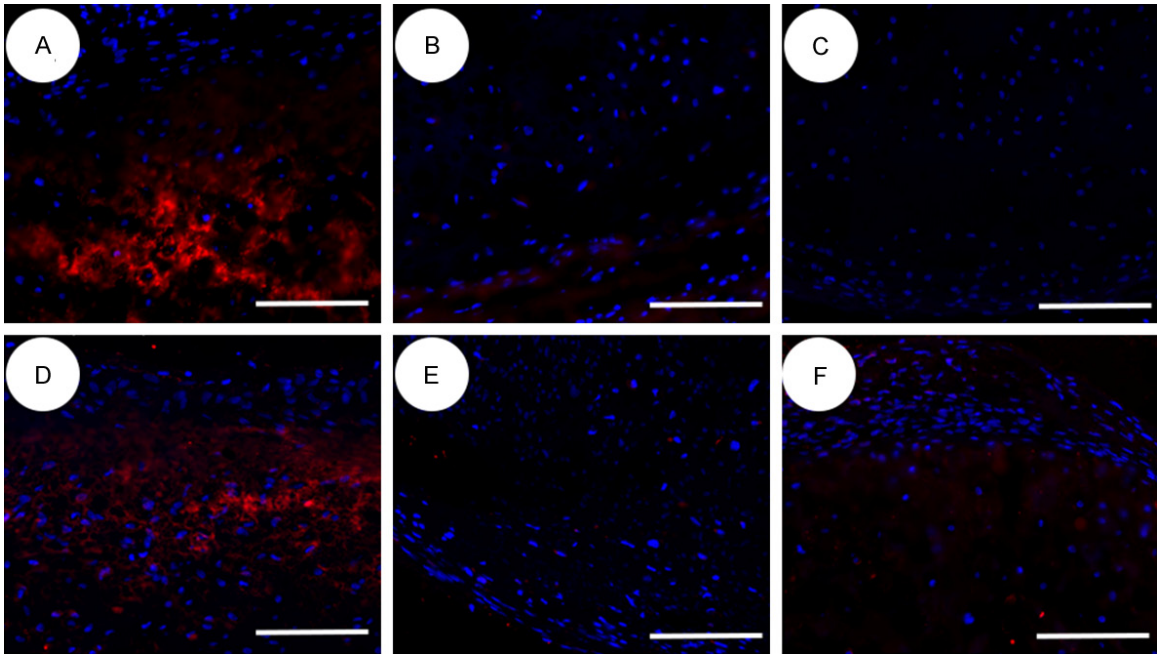

Figure 4. Immunohistochemical determination of cartilage formation of CSPCs and PSPCs in vivo. A and D. Collagen II staining showed that CSPCs secreted more type II collagen than PSPCs. Scale bar: 100 μm. B and E. Both group showed negative staining of Collagen X, one marker of hypertrophied cartilage. C and F. The formation of collagen I was also analyzed to compare chondrogenesis capacity between CSPCs and PSPCs. It could be founded that negative results for collagen I staining in PSPCs and weak positive staining in CSPCs. Scale bar: 100 μm.

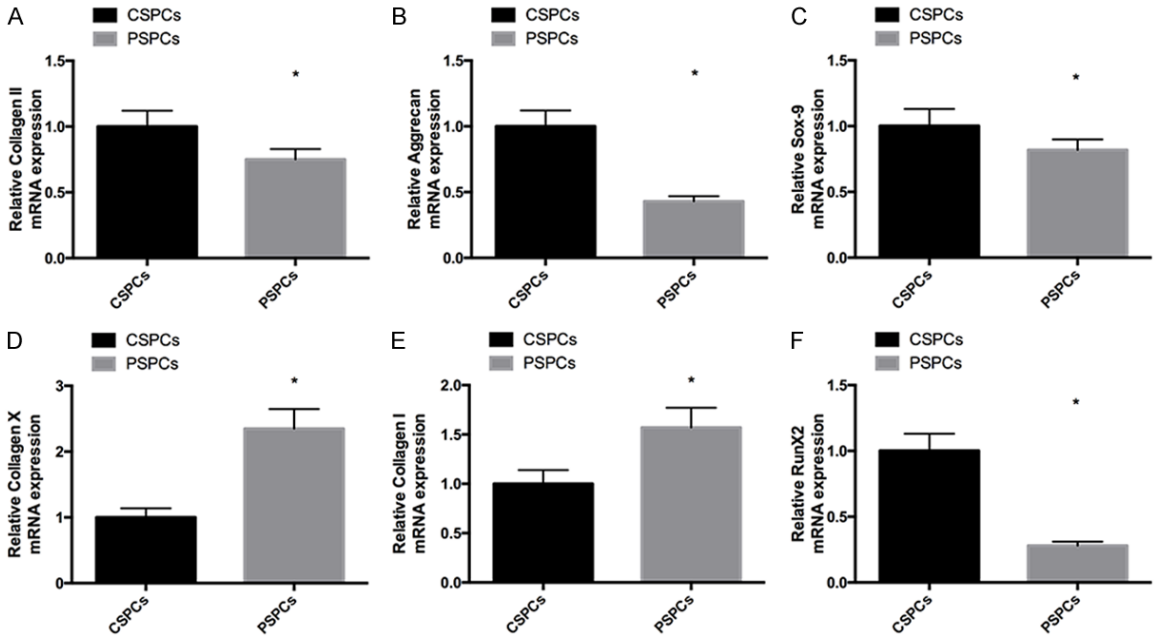

Figure 5. Gene expression of CSPCs and PSPCs in vivo after 6 weeks. The expression of the cartilage-related genes collagen II (A), aggrecan (B), and SOX9 (C) was upregulated in CSPCs compared with PSPCs, while the calcification gene collagen X (D) and the ossification genes collagen I (E) and RUNX2 (F) were downregulated. Data are presented as the mean ± SD (n=3), *P<0.05.

deeply studied in recent years, but tissue-engineered cartilage differentiated from BMSCs is very likely to ossify, which limits its clinical application [24]. In our previous study, we found

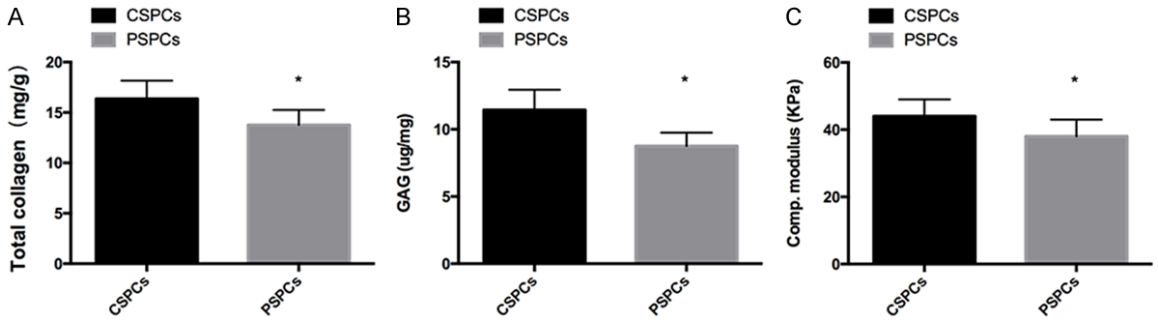

Figure 6. Total collagen, GAG and biomechanics tests of CSPCs and PSPCs in vivo. The secretion of total collagen (A) but not GAG (B) was greater in CSPCs than in PSPCs. Moreover, the constructs formed from CSPCs displayed better biomechanical properties than those formed from PSPCs (C). Data are shown as the mean ± SD (n=3), *P<0.05.

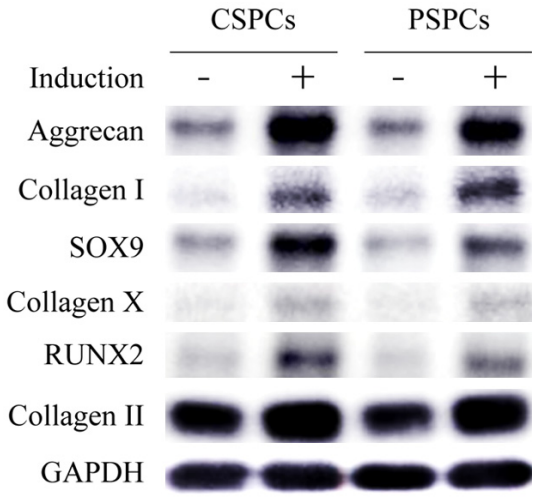

Figure 7. Visualization of tissue-specific protein expression by CSPCs and PSPCs. After CSPCs and PSPCs were exposed to chondrogenic solution, all the studied proteins except collagen I were upregulated; the expression of collagen II, aggrecan, SOX9, collagen X, and RUNX2 was upregulated in CSPCs compared to PSPCs. The GAPDH was taken as loading controls. +: cells cultured in chondrogenic induction medium. -: cells cultured in H-DMEM containing 10% fetal bovine serum.

that CSPCs possessed an inherent chondrogenic advantage, indicating that CSPCs are a promising cell source for cartilage regenerative medicine application [8, 26]. CSPCs from auricular cartilage and the perichondrium were isolated by a fibronectin differential adhesion assay, and the two cell populations expressed MSC surface markers, as indicated by flow cytometric analysis, and these cells could differentiate into osteogenic, chondrogenic and adipogenic lines under different induction conditions. In the current study, we explored the in vivo chondrogenesis of CSPCs from auricular cartilage and the perichondrium.

Our results showed that under chondrogenic induction conditions, the cell scaffold tended to form cartilage tissue after 4 weeks of in vitro induction plus 6 weeks in vivo culture, and the cell constructs formed relatively mature cartilage, as indicated by H&E and Safranin O staining. Furthermore, the phenotype of engineered cartilage that was differentiated from CSPCs from auricular cartilage and the perichondrium after subcutaneous ectopic implantation was identified.

Type I collagen contains specific combinations of amino acid sequences and has been reported to facilitate cell attachment and migration [27]. This type of collagen has been shown to help chondrocytes maintain their cobble-stone morphology and accumulate a cartilaginous extracellular matrix (ECM) [28]. It has been demonstrated that high-density treatment drove stem cells to differentiate into chondrocytes and accumulate more cartilaginous ECM [29, 30]. Therefore, type I rat tail collagen was used as the scaffold biomaterial in the current study, and high-density cultivation with exogenous growth factors was used to induce the adult progenitors to form chondrocyte-like cells.

Though there was no significant difference between the gross morphology of grafts differentiated from CSPCs and PSPCs, an obvious difference was observed in the histological analysis. In contrast with the PSPCs group, the CSPC group showed more mature cartilage-like tissue formation with obvious cartilage lacuna in

the histological examination, indicating that CSPCs accumulated more cartilage-like tissue than PSPCs. This difference may be because CSPCs derived from cartilage were more likely to undergo chondrogenic differentiation, which was consistent with our previous in vitro experimental results.

Apart from H&E and Safranin O staining, type II collagen is another specific marker of cartilage tissue and is an important characteristic of chondrogenic differentiation from stem cells [31, 32]. Both groups showed positive staining, while the CSPC group treated with growth factors displayed much stronger staining. It was speculated that on the one hand, stem cells derived from cartilage harbor the capacity to secrete cartilaginous matrix to some extent; on the other hand, there was some auricle chondrocyte contamination when isolating and harvesting CSPCs. The staining results of type II collagen suggested that CSPCs had a native chondrogenic advantage over PSPCs and further confirmed the H&E and Safranin O staining analyses.

To explore the stability of the cartilage phenotype differentiated from CSPCs and PSPCs, type I collagen and type X collagen were also evaluated by immunofluorescence. It is well known that the chondrocytes differentiated from MSCs undergo hypertrophy and ossification, as indicated by the expression of type I and X collagen [33, 34]. In our study, the staining of type I collagen was nearly negative in the CSPC group, while weak staining occurred in the PSPC group. Unlike the type I collagen results, the staining of type X collagen, an early marker of calcification, showed negative analysis, which was similar to the study of Takeshi. These results indicated the advantage of using CSPCs rather than PSPCs even prior to the formation of a stable cartilage tissue phenotype.

Q-PCR was performed to further confirm the histological results, and the gene expression results were in agreement with our histological and immunofluorescence analysis. There was higher chondrogenic gene expression in the CSPC group, which was also consistent with our previous study. In addition, the Western blot analysis results corresponded with the immunohistochemistry and Q-PCR results. Together, the current results demonstrated that the engineered cartilage in the CSPC group exhibited higher mechanical strength than that in the PSPC group. The extracellular matrix content, such as GAGs and collagen, mainly contributes to the mechanical properties of cartilage. We found that higher levels of GAGs and collagen in the CSPC group than in the PSPC group, which would contribute to the enhanced mechanical properties. Although we conducted this study with the aim of comparing the chondrogenic capacity between CSPCs and PSPCs, comparisons with other stem cell sources and auricle chondrocytes need to be performed.

In conclusion, CSPCs showed a significant advantage in chondrogenesis in vivo with upregulated chondrogenic genes and a stable cartilage phenotype, as well as good mechanical properties, indicating that CSPCs could be one type of deal seeding cells for cartilage tissue engineering.

Disclosure of conflict of interest

None.

Address correspondence to: Ke Xue and Kai Liu, Department of Plastic and Reconstructive Surgery, Shanghai 9th People's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200011, PR China. E-mail: drkexue@126.com (KX); drkailiu@163.com (KL)

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