Recapitulating Developmental Condensation and Constructing Self-organised Cartilaginous Tissue for Cartilage Regeneration

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Objective: *To develop a novel chondrocyte condensation culture strategy recapitulating developmental condensation and construct self-organised cartilaginous tissue for cartilage regeneration.*

Methods: *Cell-condensation aggregate (CCA) was generated using the condensation culture method by sequential cell seeding. The chondrification capacities and biocompatibilities of CCA were assessed by comparison with the cell–scaffold complex (CSC), which was constructed by cell–scaffold coculture. Preclinical studies including implantation into nude mice subcutaneously and cartilage defect repair in rabbits were performed.*

Results: *CCA constructed by condensation culture exhibited a morphology of self-organised cartilaginous tissue. Meanwhile, the condensation culture inhibited or abolished expression of HOX genes including HOXC4 and HOXD8, which was partially consistent with developmental HOX gene expression patterns and associated with enhanced regeneration capacities. Compared with CSC, CCA showed a higher capacity for chondrification and regeneration of rabbit cartilage defects.*

Conclusion: *The therapeutic assessments indicate that CCA is an efficient therapeutic tool for cartilage regeneration, providing a new strategy for tissue engineering by mimicking developmental events.*

Key words: *cartilage defects, cartilage regeneration, cell condensation, HOX genes*

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Millions of patients worldwide suffer from pain and misery due to articular cartilage defects[1], especially in the temporomandibular and knee joints. The repairing techniques, such as autologous chondrocyte implantation (ACI), which involves delivering chondrocytes to the cartilage defect site, cannot meet the needs of clinical application, mainly due to the limited regeneration potential of the suspended cells in vivo[1-4]. Meanwhile, various new strategies based on the cell–scaffold complex (CSC) are performed, such as matrix-induced autologous chondrocyte implantation (MACI) using a collagen bilayer seeded with chondrocytes for cartilage regeneration[5]. MACI can provide a scaffold for the survival and localisation of the implanted cells. These strategies have been demonstrated to improve clinical and radiological outcomes in patients[6,7]; however, they are merely performed by mimicking native structures or components typically lacking physiological formation and cell junctions and are far from the native tissue

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morphology and functions[2,8]. This evidence highlights cell self-organisation strategies, which can mimic spontaneous biological processes and construct morphological structure and organisation of native tissue.

Tissue morphogenesis depends on a series of events during development, indicating that recapitulating developmental events may be an efficient strategy to construct self-organised tissue in vitro[9-11]. A previous study demonstrated that recapitulating transient vascularisation during cartilage development led to the construction of avascular elastic cartilage tissue from in vitro cultured cells[12]. Cartilage development is initiated by the aggregation of undifferentiated mesenchymes into a condensed mass of cells which then undergo differentiation to form condensed chondrocytes and deposit cartilage-specific extracellular matrix (ECM)[12-14]. This indicates that functional cartilaginous tissue could be constructed by recapitulating the physiological developmental process of cell condensation in vitro. However, it is still unknown whether recapitulating condensation using chondrocytes could restore their potential for tissue formation and construct cartilaginous tissue in vitro.

Cells that gained self-renewal and self-assemble ability to form neotissue during the entire development process are regulated by various genes including Wnt and TGF-β[15-17]. HOX genes also encode transcription factors with important roles in tissue development, some of which are globally expressed in condensing cells while others are turned off at a certain developmental stage[18]. HOXA2 is switched off or downregulated to initiate condensation in cartilage development[19], and HOXD11 and HOXD13 affect both condensation and growth in a predominantly negative manner[20]. Specifically, negative expression of HOX genes reflects a higher level of self-renewal capacity and tissue regeneration capacity in totipotent human stem cells[21,22]. Moreover, chondrocytes with negative HOX gene expression show an enhanced capacity for cartilaginous tissue regeneration[23].

Herein, we found that the condensation culture endowed chondrocyte aggregate with a similar HOX gene expression pattern to developmental cartilage tissue, and finally constructed cell-condensation aggregate (CCA) for cartilage regeneration in preclinical animal models.

Materials and methods

Primary chondrocyte isolation and culture

Cartilage tissue was harvested from 2-month-old New Zealand white rabbit articular cartilage. Briefly, the cartilage specimens were mechanically minced into 0.5-mm to 1-mm pieces with scalpels, then were enzymatically digested in serum-free Dulbecco's Modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) that contained 0.2% collagenase type II at 37°C for 6 to 8 hours. The filtered digested chondrocytes were washed with DMEM and centrifuged at 600 × g for 5 minutes, and plated at a density of 1×106 cells/culture dish following resuspension in H-DMEM supplemented with 10% foetal bovine serum (FBS; HyClone, Logan, UT, USA) and 2 mm L-glutamine and antimicrobials (100 U/ml penicillin and 100 µg/ml streptomycin). The plates were then kept at 37°C and maintained as a high-density monolayer prior to the construction of CCA.

CCA construction

For CCA construction, high density (2.5×10^6 cells/cm^2) primary chondrocytes were reseeded on monolayer chondrocytes into 24-well plates three times to generate a multilayer engineering construction. The chondrocytes were briefly plated in a well to form a monolayer and then the same density of chondrocytes was reseeded on the lower layer every 20 hours, three times per well. All constructions were maintained in a humidified incubator at 37°C with 5% CO_2 in H-DMEM supplemented with 20% FBS, 60 µg/ml ascorbate, 2 mm L-glutamine and antimicrobials (100 U/ml penicillin and 100 µg/ml streptomycin). The CSC construction was performed as previously described[24].

Gene expression analysis

Total RNA was isolated from chondrocytes or the tissue engineering construction using RNA isolation reagent (Takara Bio, Kusatsu, Japan). Then, 1 µg total RNA was converted to cDNA using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara Bio). The RT-qPCR was performed using an SYBR Premix Ex Taq II kit (Takara Bio) with a quantitative PCR System (Bio-Rad, Hercules, CA, USA). β-Actin was used to normalise the samples. The results were evaluated using CFX Manager software (Bio-Rad). Primers used in the present study can be provided on request.

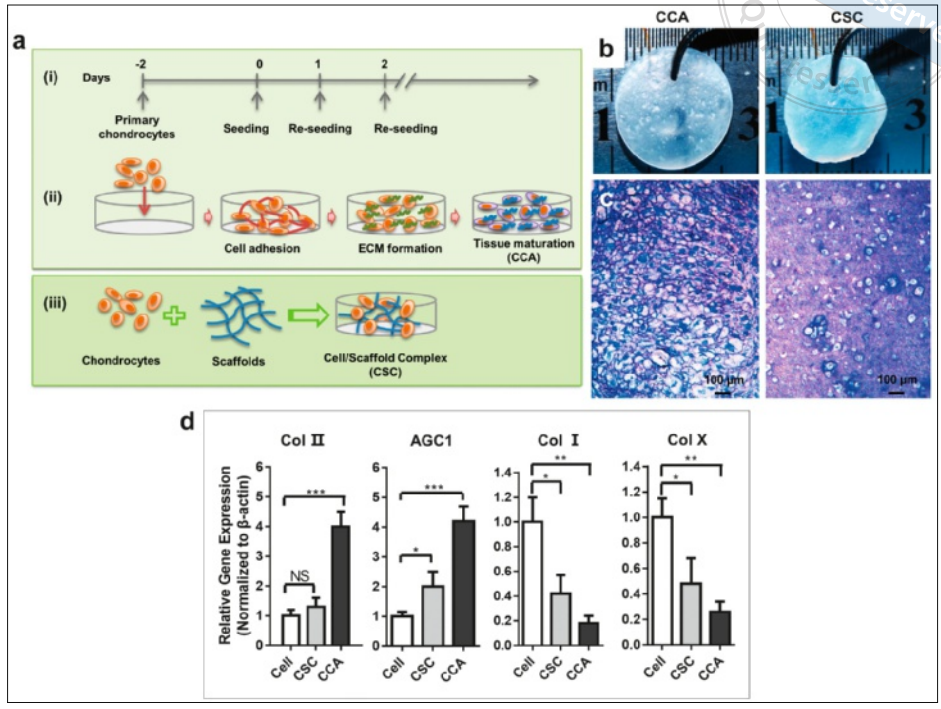

Fig 1 Characteristics of CCA. **(a)** Schematic diagram of the construction process of CCA (i, ii) and CSC (iii). **(b)** Gross morphology of CCA and CSC. **(c)** Toluidine blue staining (scale bar 100 μm). **(d)** Relative gene expression analysis of chondrocyte differentiation by RT-qPCR. The data were presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA. NS, not significant.

Ectopic implantation of CCA in immunodeficient mice

To test the chondrification capacities, CCA and CSC were subcutaneously implanted into the dorsal surface of 8-week-old immunocompromised mice as described previously[25]. Animal experiments were approved by the Ethics Committee of the Fourth Military Medical University and performed in accordance with the guidelines of the Intramural Animal Use and Care Committee of the Fourth Military Medical University. Specimens were harvested after 2 and 6 weeks. The morphology of the transplantation was evaluated and these specimens were assessed by Safranin-O and toluidine blue staining.

Articular cartilage defect repair in rabbits

The 6-month-old New Zealand white rabbits were randomly divided into three groups (seven rabbits per group): the CCA group, CSC group and a negative control group. A cartilage defect, 4 mm in diameter and 2 mm deep, was created in the area of the medial trochlea of each rabbit using a biopsy punch. Transplantation of the CSC graft was performed as previously described[26,27]. In the CCA group, the cartilage defect was filled with a multiple-folded CCA which was stabilised with fibrin glue. Specimens were harvested after 2, 6 and 12 weeks, respectively. The samples were fixed in 4% paraformaldehyde for 48 hours and incubated in decalcifying solution for 1 month. They were then rinsed, embedded, sectioned and stained with Safranin-O. The histological scoring of these specimens was performed using the O'Driscoll 24-point scoring system.

Statistical analysis

The data were expressed as mean ± standard deviation (SD). Statistically significant differences were determined with a two-tailed Student *t* test between two groups and a one-way analysis of variance (ANOVA) between more than two groups. $P < 0.05$, $P < 0.01$ or $P < 0.001$ was considered statistically significant.

Results

Characterisation of CCA

The construction process for CCA is illustrated in Fig 1a. Multiple construction strategies, including different cell numbers and seeding time intervals, were determined. We found that a certain cell number (2.5×10^6 cells/cm^2) and time interval (20 hours) and addition of vitamin C could ensure both cell condensation density and survival, and successfully constructed a ECM-rich CCA. To determine the properties of self-organised CCA, a CSC constructed by cell seeding in scaffold (collagen I) was used as a control. The CSC construction used the same contents and methods (Fig 1a) as the cartil-

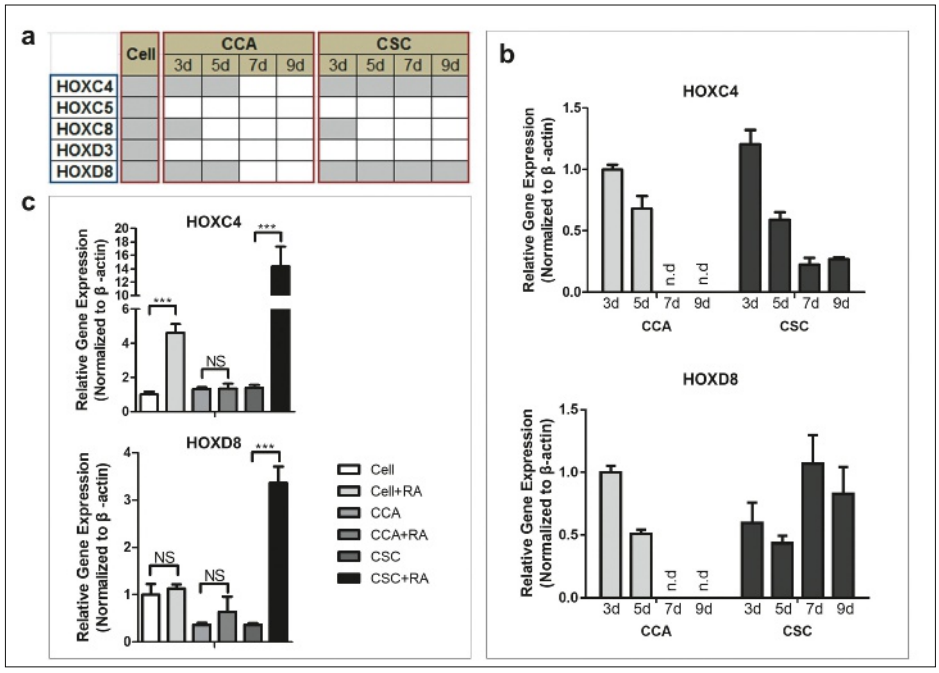

Fig 2 Self-organisation capacities and HOX gene expression in CCA. **(a)** HOX gene expression in chondrocytes, CCA or CSC. Actively expressed genes are depicted in grey, whereas genes whose expressions were under the limits of detection are shown in white. **(b)** RT-qPCR analysis of HOXC4 and HOXD8 in CCA or CSC at different time points of culture. n.d., below limits of detection. **(c)** RT-qPCR analysis of HOXC4 and HOXD8 in chondrocytes, CCA or CSC after 11 days of culture with retinoic acid (RA) treatment. The data were presented as mean ± SD. $^{***}P < 0.001$ by one-way ANOVA.

age regeneration system (CaReS), which has been used in clinical applications[24]. Obviously, CCA had a gross morphology with tenacity, which was highly similar to the native tissue, while the appearance of CSC was irregular (Fig 1b). Histological toluidine blue staining showed the chondrocytes of CCA were condensed in a self-driven manner which was similar to the native tissue, while the cell distribution of CSC was irregular (Fig 1c). To investigate the maintenance of chondrocyte phenotypes and the chondrocyte-specific ECM components, gene expressions of various cartilage-related genes were examined. The expression levels of Collagen II and AGC1 in CCA and CSC were significantly higher than in cultured chondrocytes, while CCA was higher than CSC (Fig 1d). The expression of type I and X collagen, which represent fibraocartilage and hypertrophic cartilage respectively, was significantly lower in CCA than in CSC or cultured chondrocytes. These results suggest that recapitulating developmental condensation facilitated good self-organised cartilaginous tissue formation, and had great potential for chondrification. These results also provided further evidence that self-organisation facilitates tissue construction in vitro[9].

Self-organisation capacities of CCA

To determine whether condensation culture facilitates tissue formation and HOX gene expression changes, we investigated HOXC4, HOXC5, HOXC8, HOXD3 and HOXD8 expression. Compared with cultured chondrocytes, after 3 days of culture, CCA and CSC showed negative HOXC5 and HOXD3 expression (Fig 2a). Moreover, HOX gene expression in CCA and CSC showed a different pattern (Fig 2a). HOXC4 and HOXD8 could not be detected in CCA after 7 days of culture but were still expressed in CSC (Figs 2a and b), indicating the greater tissue regeneration and self-renewal capacity of CCA. We then confirmed whether the HOX gene expression in CCA was inhibited by treating chondrocytes, CCA and CSC with retinoic acid (RA), a HOX gene activator. The results showed that the expression of HOXC4 and HOXD8 could be activated in culture chondrocytes and CSC, but could not be activated in CCA (Fig 2c). These data indicated that recapitulating condensation probably facilitates cartilaginous tissue construction via downregulation of HOX genes.

Chondrification potential of CCA

To identify the chondrification potential of the constructions, CCA and CSC were implanted into nude mice subcutaneously. CCA exhibited a white oval shape with a glossy surface, which was similar to native cartilage at 2 weeks posttransplantation (Fig 3a). Evident lacunae and dense cartilage-specific ECM proteins were then detected by toluidine blue and Safranin-O staining (Fig 3a), which demonstrated that abundant mature hyaline cartilage had formed. In contrast, the surface of the CSC graft

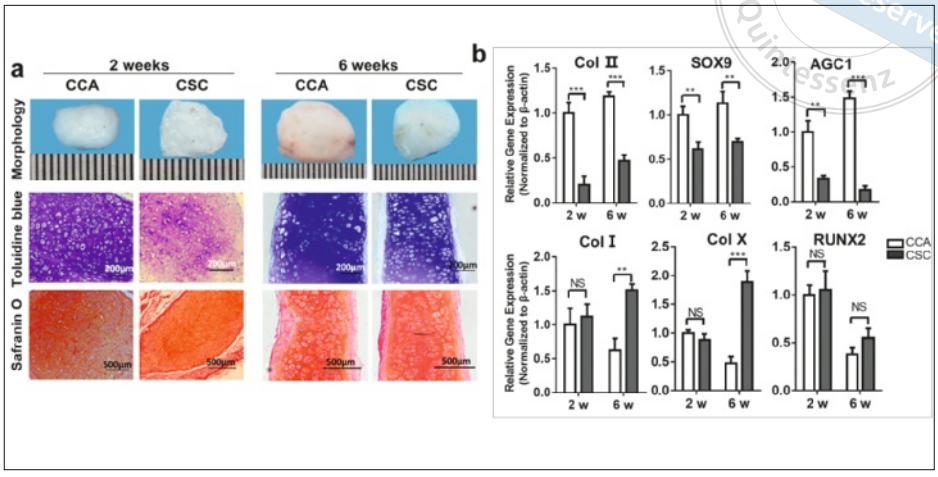

Fig 3 Chondrification potential of CCA in subcutaneous tissue of nude mice. CCA and CSC were implanted into the nude mice subcutaneously and harvested after 2 (n = 6) or 6 weeks (n = 7) of implantation. **(a)** Morphology, toluidine blue and safranin-O staining of representative implanted grafts. **(b)** RT-qPCR assessed the expression of specific genes related to chondrogenic ability. The data were presented as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ by *t* test.

was not as glossy as that of CCA (Fig 3a). Toluidine blue and Safranin-O staining also demonstrated that mature cartilage had not formed well in the CSC graft at 2 weeks posttransplantation as compared with CCA. By 6 weeks, both grafts had completely formed mature hyaline cartilage, and did not differ based on visual examination or histological analysis (Fig 3a).

Consistent with the histological assessment, the expression of Col-II, SOX9 and AGC-1, which are markers of chondrogenesis, were higher in CCA compared with CSC at 2 weeks or 6 weeks posttransplantation (Fig 3b). The expression of Col-I, Col-X and RUNX2 was lower or not significantly different in CCA compared with CSC posttransplantation (Fig 3b). Although no differences in the formed cartilage morphology with a longer implant time were observed histologically, the high expression of chondrogenic genes demonstrated that CCA was more suitable for retaining chondrocyte regenerative potential at ectopic sites.

Regenerative potential of CCA in rabbit articular cartilage defects

Chondral defects in rabbit trochleae were used to evaluate the therapeutic potential of CCA by comparison with CSC. At 2 weeks, the defects treated with CCA graft were nearly completely filled with tissue, but the defects in the CSC graft treatment group were not, as indicated by palpation (Fig 4a). By 6 weeks, the defect boundaries had decreased, and the colour and texture were more similar to those of the surrounding cartilage. Although cartilage had formed and filled defects in the CSC group, obvious defect boundaries remained. At 12 weeks, the defect boundaries and colours for all the rabbits in the CCA groups could barely be distinguished, indicating integration of the newly formed hyaline cartilage into the surrounding tissue (Fig 4a).

Histological assessments revealed that the defects treated with CCA were filled with regular, mature cartilage, and the boundaries between newly formed cartilage and surrounding tissue disappeared gradually during the whole process (Fig 4b). In contrast, the defect boundaries were obvious in the CSC-treated group (Fig 4b). Quantitative scoring of histological sections was performed using a modified O'Driscoll 24-point scoring system[28]. In accordance with the histological assessments, the average score for the CCA group was significantly higher than the CSC group at 2 weeks and 6 weeks, while there was no significant difference between them at 12 weeks (Fig 4c). These data indicated that, compared with CSC, CCA accelerated the repair of the cartilage defects.

Discussion

The events of cartilage development have been studied extensively. In this process, the progenitor cells initiate condensation to form an aggregate, and finally develop into fully mature cartilage with ECM[12]. Unlike previous studies using undifferentiated mesenchymal cell condensation[14,29], we used differentiated chondrocytes for condensation culture in the present study. We found that the cells regained regenerative potential and could form self-organised tissue without any exogenous support in vitro, suggesting that condensation culture may provide a developmental microenvironment to facilitate the regaining of cell functions in the early development stages.

Various signalling pathways including Wnt and TGF-β play important roles in cartilage development[15-17]. Meanwhile, switching off or downregulating HOX genes is associated with the condensation pro-

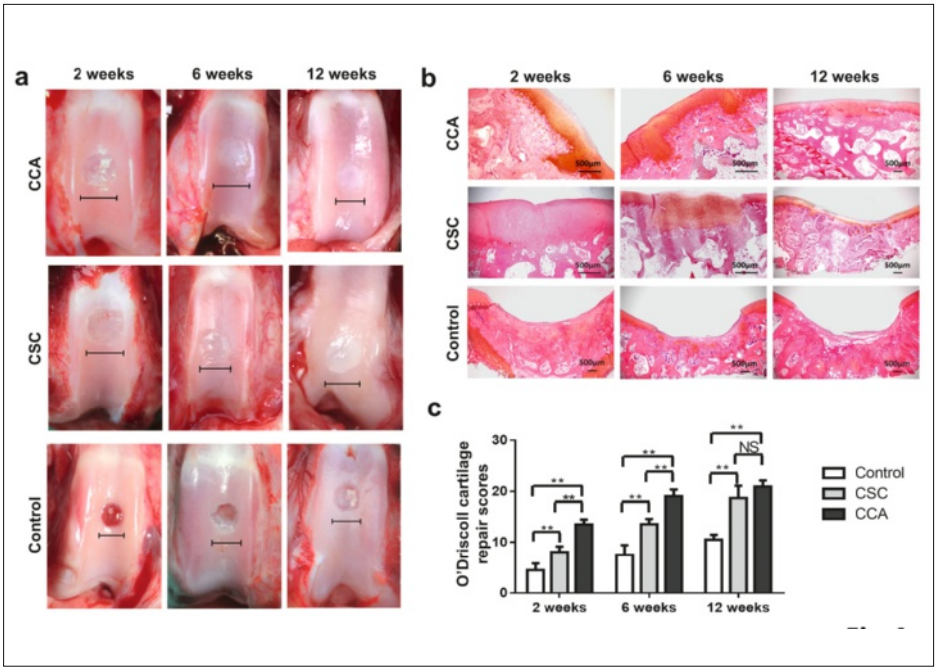

Fig 4 Regenerative potential of CCA in rabbit articular cartilage defects. **(a)** Gross observation of articular defects treated with implants after 2, 6 and 12 weeks respectively (n = 7). **(b)** Histological analysis using safranin-O staining at 2, 6 and 12 weeks after implantation. **(c)** Quantitative scoring of histological sections using a modified O'Driscoll 24-point scoring system. The data were presented as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA.

cess and indicates the early developmental stages of chondrocytes[18-20]. HOX genes negatively expressed in adult human neuroectoderm-derived nasal chondrocytes (NC) directly contribute to cartilage repair[23]. Similarly, we found that the condensation culture decreased or abolished HOX gene expression in CCA in the same manner as with chondrocytes in development, indicating that downregulation of HOX genes may be a new way to enhance chondrocytes' potential for regeneration and tissue construction in vitro.

Although cell/scaffold assembling strategies have been used[4], it remains difficult to construct tissue with native morphology and functions[9]. Here, we constructed well-organised cartilaginous tissue CCA by recapitulating developmental condensation and demonstrated the therapeutic effects generated by CCA in preclinical models. The process of mimicking developmental spontaneous condensation is crucial to CCA construction, resulting in mature neotissue with higher accumulation of ECM and superior bioactivity. Multiple methods have been used to enhance the regenerative potential of cells[12,23]. This study provided a new self-organisation construction strategy that could be adopted extensively with different sources of cells for tissue engineering. Further understanding of the developmental spontaneous condensation mechanism will help to generate more innovative engineering constructions.

Conclusion

In this study, the chondrification regeneration potential of CCA and CSC was evaluated in ectopic implantation in immunodeficient mice and an articular cartilage defect model in rabbits. CCA showed better regenerative potential than CSC at an early stage, but they showed similar regenerative potential in the later stage. This may be because the rodent model has different regenerative potential compared with larger animals or humans, and the host implantation microenvironment and regeneration may affect the observation of the differences in regenerative potential between CCA and CSC. In future studies, large animal models with cartilage defects need to be used to determine the regenerative potential of CCA in the long term.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Drs Li Li BAO and Si Ying LIU contributed to the study design, data acquisition and interpretation and drafting of the manuscript; Drs Xin Yu QIU, Zhi Quan TIAN and Yong Jie ZHANG contributed to the animal experi-

ments, data analysis and interpretation; Dr Shi Yu LIU contributed to the study conception and design and drafting of the manuscript.

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