Extracellular matrix characteristics of co-cultured zonal chondrocytes and bone marrow mesenchymal stem cells

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Abstract: Zonal structures of engineered cartilage are thought to be essential for cartilage repair. Engineering zonal cartilage with zonal chondrocytes may improve the functions of engineered cartilage. However, zonal chondrocytes must be expanded to obtain sufficient cells, leading to a loss of zonal characteristics. Co-culture could replace most zonal chondrocytes with bone marrow mesenchymal stem cells (BMSCs), allowing unexpanded or low passaged zonal chondrocytes to be used without losing zonal characteristics. We intend to determine whether co-cultured zonal chondrocytes and BMSCs exhibit zonal extracellular matrix composition characteristics. Superficial zone chondrocytes (SZCs), middle/deep zone chondrocytes (MDZCs) and BMSCs were encapsulated in alginate as single cell groups, and SZCs or MDZCs were mixed with BMSCs at 1:2 ratios as co-culture groups. Alginate beads were either cultured in chondrogenic media or implanted subcutaneously into nude mice and collected for analyses. According to the results of gene expression assays, biochemical analyses and histological staining, the co-culture groups showed zonal extracellular matrix composition characteristics at days 1 and 7 in vitro and week 2 in vivo. Besides, co-culture groups produced higher level of extracellular matrix than single chondrocyte groups when normalized to initial seeded zonal chondrocytes which means reducing chondrocytes needed. Overall, zonal chondrocytes co-cultured with BMSCs hold great promise for engineering zonal cartilage.

Keywords: Zonal cartilage, zonal chondrocytes, co-culture, bone marrow mesenchymal stem cells, extracellular matrix

Introduction

Articular cartilage is a heterogeneous tissue, the structure and composition of which vary with depth. It is typically divided into three distinct zones: the superficial, middle and deep zones. The superficial zone has the highest amount of collagen, the lowest amount of sulfated glycosaminoglycans (GAGs) and secretes the highest amount of proteoglycan 4 (PRG4), also known as superficial zone protein (SZP). With increasing depth, the collagen content and PRG4 secretion decreases, whereas GAG content increases [1]. Furthermore, collagen fiber alignment differs among zones [1]. Zonal variations in the collagen and proteoglycans result in significant differences in biomechanical characteristics [2]. Additionally, SZP synthesized and secreted by superficial zone cartilage is known to function as a boundary lubricant in articular cartilage and reduces the coefficient of friction [3]. Therefore, there are multiple differences among the superficial, middle and deep zones, especially regarding the extracellular matrix composition, and the characteristics of each zone are thought to be essential for the optimal function of articular cartilage.

Due to the avascular and minimal biosynthetic activities of mature cartilage, articular cartilage lesions caused by trauma or age-related degeneration may not always heal on their own [4]. Recently, several cell-based therapies have been used in clinical treatments [5-7]. Articular cartilage implantation (ACI) is the most common and effective therapy for treating cartilage defects [8]. However, ACI does not consider the natural variations in articular cartilage. Increasing attention has been paid to engineering zonal articular cartilage rather than homogenous cartilage [9, 10].
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Engineering cartilage with zonal chondrocytes is one such approach. Different zonal chondrocytes can maintain their zonal characteristics during in vitro culture [11], and zonal constructs that use zonal chondrocytes can mimic the characteristics of native cartilage in vitro [12]. Unfortunately, over the first few monolayer passages, zonal chondrocytes lose their zonal characteristics and chondrogenic phenotype, which are not substantially reacquired [13, 14]. Studies that employed zonal chondrocytes as seeds almost always used low passages or unexpanded zonal chondrocytes. However, primary chondrocytes must be passaged several times to obtain a sufficient amount of chondrocytes in clinical scenarios [5]. Therefore, a method to engineer zonal articular cartilage with low passages or unexpanded zonal chondrocytes is needed.

Co-culture strategies have raised the possibility of substituting single chondrocyte therapies [15]. Low passages or unexpanded chondrocytes isolated from non-weight-bearing areas co-cultured with bone marrow mesenchymal stem cells (BMSCs) were shown to induce a similar level of chondrogenesis as single chondrocyte therapies [16]. Therefore, this approach could reduce the number of chondrocytes needed, which could reduce or even circumvent the dedifferentiation processes required to obtain sufficient amounts of chondrocytes. Without dedifferentiation, zonal characteristics may be able to be maintained. Moreover, with co-culture techniques, cartilage repair may become a one-step procedure if chondrocytes expansion is not necessary. However, whether zonal chondrocytes co-cultured with BMSCs show zonal characteristics remains unclear.

In this study, we employed a commonly used alginate 3-dimensional (3D) culture system to investigate whether zonal chondrocytes co-cultured with BMSCs show zonal extracellular matrix characteristics in vitro and in vivo.

Materials and methods

Zonal chondrocyte isolation

For zonal chondrocyte isolation, we first confirmed the thickness of each zone. Lateral femur condylar and tibial plateau osteochondral plugs from one-month-old New Zealand rabbits were stained with Alcian blue (the methods are presented below).

According to Alcian blue staining, the femur condylar cartilage is thicker and easier to obtain than tibial plateau cartilage, and therefore, we decided to isolate zonal chondrocytes from the femur condylar cartilage using the method described previously [3]. In brief, 100 μm of superficial zone cartilage was isolated using a dermatome, and the middle and deep zone cartilage was obtained using a scalpel blade. Cartilage tissues were minced into small pieces after washing with phosphate buffer saline (PBS) three times and digested with 0.2% type II collagenase in Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12, Hyclone, USA) supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin (PS, Gibco, USA) at 37°C for 6-8 hours. Harvested cells were filtered through a 70 μm cell strainer (BD Biosciences, USA) and rinsed with DMEM/F-12.

The isolated cells were used for the following experiments.

BMSCs isolation and multi-lineage differentiation

BMSCs were harvested according to the protocol described previously [17]. Briefly, bone marrow was aspirated into a 10 ml syringe containing 4000 U/ml heparin from the tibia and femur of the knees of anaesthetized one-month-old New Zealand rabbits under aseptic conditions. Bone marrow was washed once with PBS and
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re-suspended onto a 10 cm flat plate in growth media consisting of DMEM/F12 supplemented with 10% fetal bovine serum (Hyclone, USA) and PS. Unattached cells were removed after 72 hours. For further expansion, BMSCs were passaged at 10^4 cells/cm^2 until 90% confluence. Passage 3 (P3) BMSCs were used for the following experiments.

BMSCs were incubated in six-well plates at a density of 5×10^4 cells/well with StemPro® Adipogenesis differentiation medium (Gibco, USA) for one week for adipogenesis or with StemPro® Osteogenesis differentiation medium for two weeks for osteogenesis differentiation. For chondrogenic differentiation, 4×10^5 BMSCs were centrifuged for 5 min at 500 g to form a pellet in a 15 ml centrifuge tube containing 500 µl serum free chondrogenic media (Cyagen, China) consisting of Dulbecco’s modified Eagle medium-high glucose (DMEM-HG), 1% ITS+Premix, 10 ng/ml transforming growth factor β3 (TGF-β3), 10^-7 M dexamethasone, and 50 μg/mL ascorbic acid for three weeks. Adipogenic differentiation was assessed via oil red O staining, osteogenic differentiation was assessed via Alizarin Red S staining, and chondrogenic differentiation was assessed via collagen II immunohistochemistry staining.

Zonal chondrocyte 2-dimensional proliferation assay

For the 2-dimensional (2D) proliferation assay, primary SZCs and MDZCs were plated in a 96-well plate (n = 6) at 2000 cells/well in growth media. At days 3, 5, 7, and 10, 10 µl Cell Counting Kit-8 (CCK-8, Dojindo, Japan) reagent in 90 µl growth media was added to each well and incubated at 37°C for 1 h. The absorbance was measured using a microplate reader (Multiskan Spectrum, Thermo Fisher, USA) at 450 μm.

Alginate 3D culture in vitro and in vivo

Primary zonal chondrocytes and P3 BMSCs were re-suspended in sterile, low viscosity alginate gel (2% w/v, Sigma, USA) alone or mixed at ratio of 1:2 at a final concentration of 6×10^6 cells/ml. All experiment groups are listed in (Table 1). The alginate/cell suspension was slowly dropped into a 102 mM CaCl2 (Sigma-Aldrich, USA) gelation solution through a 23-gauge needle. After gelation for 20 min, the newly formed alginate beads were washed three times with DMEM-HG. In the in vitro experiments, alginate beads were transferred to 12-well culture plates (3 beads/well) containing 800 µl chondrogenic media, and alginate beads were incubated in a humidified atmosphere of 5% CO_2 at 37°C with their media changed two or three times per week. Samples were harvested at days 1, 7, and 21 for the following analyses. For in vivo experiments, alginate beads fabricated according to the protocols described above. The beads were then immediately implanted into 14 male 6-week-old nu/nu mice using the method described previously [18]. Briefly, two separate incisions were made along the central line of the spine after anaesthetizing with 10% sodium pentobarbital (80 mg/kg, Solarbio, China). Four separate subcutaneous dorsal pockets were prepared by blunt dissection, after which the beads were implanted into these pockets. After 2 and 8 weeks, the animals were killed, and the samples were used for histologic and biochemical analyses. Animal experiments were carried out with approval of the animal ethical committee.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

To determine the gene expression characteristics of zonal chondrocytes, total RNA was extracted from 2×10^5 primary SZCs and MDZCs followed by lysing with 1 ml RNAiso Plus (Takara, Japan). Three alginate beads (n = 3) from each group at days 1, 7 and 21 were dissolved in 55 mM citric acid sodium for 10 min to release cells from the alginate beads. The released cells were collected with centrifuged at 400 g for 10 min. The collected cells were lysed using 1 ml RNAiso Plus, and the total RNA was extracted. The extracted RNA was reverse transcribed using a PrimeScript™ RT reagent Kit (Takara, Japan). After cDNA synthesis, real-time PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), collagen type II (COL2A1), aggrecan (ACAN), proteoglycan 4 (PRG4) was performed using an SYBR Premix EX Taq II Kit (Takara, Japan). After cDNA synthesis, real-time PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), collagen type II (COL2A1), aggrecan (ACAN), proteoglycan 4 (PRG4) was performed using an SYBR Premix EX Taq II Kit (Takara, Japan) on a Stepone plus real-time PCR machine (Applied Biosystems, USA). The primer sequences are listed in (Table 2). All procedures were performed according to the manufacturer’s instructions. Relative gene expression values were compared using the 2^-ΔΔCt method.

Biochemical evaluation

For the in vitro study, alginate beads were harvested (n = 3) at days 1, 7 and 21. For the in
In vivo study, alginate beads were harvested (n = 3) at weeks 2 and 8. After washing with PBS three times, alginate beads were digested overnight at 56°C in a solution containing 250 μg/mL papain (Merck, USA) in sterile 0.1 M PBS, pH 6.0, with 5 mM cysteine-HCl (Solarbio, China) and 5 mM Na2EDTA (Sigma-Aldrich, USA) [19]. Alginate beads from day 1 were used for DNA quantification; beads from the other time points were used for DNA and GAG quantification. For in vivo alginate beads, samples were frozen in -80°C following digestion by papain. Quantification of total DNA was performed with Hoechst 33258 (Sigma-Aldrich, USA) [20] using a spectrofluorometer (Infinite M200 Pro, Tecan, Switzerland) at an excitation of 348 nm and an emission of 457 nm. Calf thymus DNA was used as the standard. The amount of GAG was determined by dimethylmethylene blue dye (PH1.75, Sigma Aldrich, USA) using a microplate reader (Multiskan Spectrum, Thermo Fisher, USA) [19]. The
amount of GAG was calculated using a standard of chondroitin sulfate C (Solarbio, China) by measuring the absorbance at 525 nm.

**Histologic evaluation of the extracellular matrix**

Osteochondral plugs were obtained from the central region of the lateral femur condylar and tibial plateau of the knee joints of one-month-old New Zealand rabbits using a 3 mm-diameter dermal punch. The 3 mm-diameter cartilage disks were dehydrated and embedded in paraffin and then cut into 4-μm sections and mounted on glass slides. To determine the thickness of the femur condylar and tibial plateau cartilage, cartilage sections were deparaffinized and stained with Alcian blue for 30 min and then stained with fast red for 10 min using an Alcian blue staining kit (Keygene, China). Alcian blue-stained samples were viewed under a Leica light optical microscope (DMIL LED, Leica, USA). Lubricin and type II collagen (col2) were detected by immunofluorescence staining [21]. In brief, sections were deparaffinized, and antigen retrieval was performed using sodium citrate. Then, blocked with 1% goat serum and incubated with mouse type II collagen antibody (diluted 1:50, Santa Cruz, USA) and rabbit lubricin antibody (diluted 1:50, Bioss, China) at 4°C. The primary type II collagen antibody and lubricin antibody were detected by goat anti-mouse-PE (Abbkine, USA) and goat anti-rabbit-FITC (Proteintech, USA) secondary antibodies, respectively, in the dark for 1 hour, and nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Molecular Probes, USA). The fluorescent signals were visualized using a fluorescent microscope (BX51, Olympus, Japan). At 2 and 8 weeks of in vivo culture, alginate beads were fixed in 4% paraformaldehyde for 24 h. The samples were dehydrated and embedded in paraffin and cut into 4 μm sections. After deparaffinization and antigen retrieval, lubricin and col2 were also detected via immunofluorescence staining using the method described above.

**Statistical analysis**

All quantitative data were presented as the means ± standard deviation. All data were analyzed by analysis of variance (ANOVA) and post-hoc Tukey’s tests. P-values <0.05 were considered statistically significant. Means and standard deviations are shown on each figure. Statistical analysis was performed using SPSS-19 (IBM, USA).

**Results**

**Isolation of zonal chondrocytes and identification of zonal characteristics**

To isolate zonal chondrocytes, we first confirmed the thickness of the femur condylar and
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Figure 3. SZC co-culture group and MDZC co-culture group showed gene expression differences at days 1 and 7 in vitro. Expressions of ACAN (A), COL2A (B) and PRG4 (C) expression of each group were measured by qRT-PCR at days 1, 7 and 21 in vitro. *indicates a statistically significant difference between the MDZC group or MDZC co-culture group and the corresponding SZC group or SZC co-culture group; †indicates a statistically significant difference between the co-culture groups and the corresponding single zonal chondrocyte groups; and & indicates a statistically significant difference between the BMSC group and all other groups (P<0.05).

Figure 4. MDZC co-culture group deposited more GAG content than SZC co-culture group at day 7 in vitro and week 2 in vivo and co-culture group deposited more GAG content than corresponding single chondrocyte group when normalized to the initial number of seeded primary zonal chondrocytes both in vitro and in vivo. GAG content of in vitro 3D cultures at days 7 and 21 (normalized to DNA content) (A), GAG content of in vitro 3D culture at days 7 and 21 (normalized to the initial number of seeded primary zonal chondrocytes) (B). GAG content of in vivo 3D culture at weeks 2 and 8 (normalized to DNA content) (C), GAG content of in vivo 3D culture at weeks 2 and 8 (normalized to the initial number of seeded primary zonal chondrocytes) (D). *indicates a statistically significant difference between the MDZC group or MDZC co-culture group and the corresponding SZC group or SZC co-culture group; †indicates a statistically significant difference between the co-culture groups and the corresponding single zonal chondrocyte groups; and & indicates a statistically significant difference between the BMSC group and all other groups (P<0.05).

The full thickness of the tibial plateau cartilage is approximately 1.2 mm, the superficial layer is approximately 100 μm, and the full thickness of the superficial layer is approximately 0.6 mm, and the superficial layer is approximately 60 μm (Figure 1A). The femur condylar cartilage is thicker and easier to obtain than tibial plateau cartilage, and thus, we decided to isolate zonal chondrocytes from the femur condylar cartilage using method described above. Immunofluorescence staining suggested that lubricin and col2 fluo-
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Immunofluorescence signals decreased with depth (Figure 1B). In accordance with the immunofluorescence results, qRT-PCR performed using cDNA synthesized from RNA extracted from isolated primary zonal chondrocytes revealed that the expression levels of PRG4 and COL2A were higher in the SZCs and that ACAN expression was higher in the MDZCs (Figure 1C). The CCK-8 assay suggested that MDZCs proliferated faster than SZCs in 2D culture (Figure 2A). However, there were no significant differences in DNA content between zonal chondrocytes during in vitro (Figure 2B) and in vivo (Figure 2C) 3D culture.

Isolation and identification of BMSCs

After removing unattached cells 72 hours later, the cells adhered to the plate showed a fibroblastic morphology (Figure 1D). To identify the multi-lineage differentiation abilities, P3 BMSCs were induced to adipogenic, osteogenic and chondrogenic differentiation. The histology results suggested that the P3 BMSCs could differentiate into adipocyte, osteocyte and chondrocyte lineages (Figure 1D). These results demonstrated that the isolated cells were BMSCs.

Zonal extracellular matrix characteristics maintenance at early time points

Upon supplementation with chondrogenic growth factor TGF-β3, all groups exhibited significant chondrogenesis in vitro. Not only the single chondrocyte groups but also the co-culture groups exhibited zonal characteristics at early time points. As shown in (Figure 3A), ACAN

Figure 5. SZC co-culture group expressed higher level of Lubricin than MDZC co-culture group at week 2 in vivo. Immunofluorescence staining showed the expression of Lubricin in SZC group (A), MDZC group (B), SZC co-culture group (C), MDZC co-culture group (D) and BMSC group (E) at weeks 2 and 8 in vivo, scale bar = 500 μm.
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Expression in the MDZC co-culture group was higher than in the SZC co-culture group at days 1 and 7. The same trend was found in the MDZC and SZC groups. In contrast, the SZC and SZC co-culture groups expressed higher levels of PRG4 than the MDZC and MDZC co-culture groups, respectively, at days 1 and 7 (Figure 3C). Although the primary SZCs exhibited higher expression levels of COL2A than the primary MDZCs (Figure 1C), significant differences were not found between the SZC co-culture group and the MDZC co-culture group throughout the in vitro 3D culture period (Figure 3B). The same result was found for the single chondrocyte groups. In addition, the MDZC co-culture and MDZC groups showed higher GAG content (normalized to DNA content) than the SZC co-culture and SZC groups, respectively, at day 7 in vitro (Figure 4A). In accordance with the in vitro study, the MDZC and MDZC co-culture groups deposited more GAG than the SZC and SZC co-culture groups, respectively, after 2 weeks in vivo (Figure 4D). Although the single zonal chondrocyte groups had higher GAG contents (normalized to DNA content) than the respective co-culture groups, when normalized to the initial seeded zonal chondrocytes, the co-culture groups had GAG contents similar to the respective single zonal chondrocyte groups in vitro (Figure 4B) at day 7 and higher GAG contents than the respective single zonal chondrocyte groups in vivo (Figure 4D) at week 2. Furthermore, the SZC and SZC co-culture groups had higher levels of lubricin than the MDZC and

Figure 6. SZC co-culture group and MDZC co-culture group showed no significant difference in vivo according to col2 Immunofluorescence staining. Immunofluorescence staining showed the expression of col2 in SZC group (A), MDZC group (B), SZC co-culture group (C), MDZC co-culture group (D) and BMSC group (E) at weeks 2 and 8 in vivo, scale bar = 500 μm.
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MDZC co-culture groups, respectively, at week 2 in vivo, which was confirmed by immunofluorescence staining (Figure 5).

Zonal extracellular matrix characteristics disappeared at late time points

At day 21 in vitro, no differences in gene-expression were observed between the co-culture groups (Figure 3A-C) or among the single chondrocyte groups. The in vitro MDZC co-culture and SZC co-culture groups contained similar GAG contents at day 21. The same trend was found between the MDZC and SZC groups (Figure 4A). In vivo, GAG synthesis (normalized to DNA content) was similar at the end of 8 weeks (Figure 4C). The single zonal chondrocyte groups exhibited greater GAG content (normalized to DNA content) than the respective co-culture groups in vitro (Figure 4A). Unlike the in vitro culture, the co-culture group and the single zonal chondrocyte groups had similar GAG contents (normalized to DNA content) at week 8 (Figure 4C). However, when normalized to the initial seeded zonal chondrocytes, the co-culture groups had much more GAG content than the respective single zonal chondrocyte groups (Figure 4B, 4D) both in vitro and in vivo. Moreover, there were no significant differences in lubricin and col2 immunofluorescence signals between the co-culture groups or among the single zonal chondrocyte groups at week 8 (Figures 5, 6). Interestingly, the cells in all groups in vivo showed more direct cell-to-cell contact than the in vitro culture (Figures 5, 6).

Discussion

Recently, increased efforts have been dedicated to repairing cartilage by mimicking native cartilage. Engineering multilayer cartilage with zonal chondrocytes has been shown to be successful in vitro. Unfortunately, only low passage or unexpanded zonal chondrocytes maintain zonal characteristics. However, low numbers of passages or leaving zonal chondrocytes unexpanded do not provide enough cells for clinical applications. Co-culture makes it possible to repair cartilage with fewer zonal chondrocytes. We sought to determine whether the mixture of zonal chondrocytes and BMSCs show zonal characteristics in vitro and in vivo.

Articular cartilage is a heterogeneous tissue with many differences between zones. However, because zonal chondrocytes do not have specific markers, zonal cartilage can be separated only based on histology. Zonal chondrocytes isolated using a dermatome demonstrate various differences. We found that MDZCs had a higher proliferation rate than SZCs in 2D culture, which is in agreement with another study [22]. However, during in vitro and in vivo 3D alginate culture, there were no significant differences among zonal chondrocytes. These results may be related to the alginate culture system because chondrocytes encapsulated in alginate proliferate slowly. In addition, we found that the SZCs expressed higher levels of COL2A and PRG4 and lower levels of ACAN, which was in accordance with our histology results and with a previous report by other researchers [23]. In summary, we found that the zonal chondrocytes that we isolated exhibited multiple differences.

Although the exact interactions among co-cultured cells remain unclear, some mechanisms have been proposed to explain these beneficial effects. Most researchers have reported that these beneficial effects are partly derived from the paracrine soluble factors secreted by each type of cell [24]. These trophic factors promote robust chondrogenesis. Although numerous studies have focused on the interaction of mixed cells, the interaction of zonal chondrocytes and BMSCs has not been investigated. Only one study has investigated the beneficial effects of zonal cartilage to BMSCs in an indirect culture system [25]. They found that superficial zone cartilage had more chondrogenic differentiation effects than middle and deep zone cartilage and can also induce higher expression levels of PRG4 via soluble factors. These results indicated that zonal cartilage may interact in different manners with BMSCs.

As expected in our study, the gene expression results, biochemical analysis and histological appearances revealed that the co-culture groups demonstrated zonal extracellular matrix characteristics at early time points. However, these characteristics disappeared by the end of the culture period. Another study [26] reported that SZCs deposited a lower amount of GAG than MDZCs at day 21 in a 3D alginate culture system. However, the GAG content was equal at day 28, which may be because we used a different concentration of TGF-β than the previously reported studies. The TGF-β family is the most commonly used chondrogenic growth factor. The concentration of TGF-β influences its effects on zonal chondrocytes [27]. Overall, the zonal chondrocytes co-cultured with BMSCs
showed zonal extracellular matrix characteristics, but these zonal characteristics disappeared with prolonged culture time. These results indicated that zonal characteristics play a dominant role in chondrogenesis at early time points. However, at later stages, the microenvironment and trophic factors may play more important roles in chondrogenesis.

In addition to soluble factors, the mixed cells also interacted in other manners. Some have reported that direct cell-cell contact plays a role in these beneficial effects. Windt et al. [28] observed less chondrogenesis in a limited cell-cell contact 3D culture system, such as in an alginate culture system, than in a direct cell-cell contact 3D culture system. In addition, they proved that BMSCs stimulate chondrogenesis partly through gap junctions. In line with that research, we found limited cell-to-cell contact in our in vitro study (data not shown). Although direct cell-to-cell contact increased at the end of the in vitro culture, the co-culture groups still produced lower amounts of GAG than the single chondrocyte groups during the in vitro culture. In contrast to the cells cultured in vitro, we found that the cells grown in the alginate beads in vivo exhibited more direct cell-to-cell contact, which may explain why the GAG content of the single chondrocyte groups and co-culture were similar at the end of 8 weeks. Apparently, seeding mixed cells in a direct cell-to-cell manner accelerates extracellular matrix deposition. Whether zonal chondrocytes co-cultured with BMSCs maintain zonal characteristics in direct cell-to-cell systems requires further investigation.

Several studies have demonstrated that the matrix deposition in co-culture is primarily from chondrocytes [19, 29]. Furthermore, the number of BMSCs decreases with culture time [29]. In other words, BMSCs in co-culture mainly act as trophic factors to stimulate chondrocyte proliferation and matrix deposition instead of undergoing chondrogenic differentiation. To determine the trophic effects exerted by the BMSCs, we normalized GAG content to initially seeded primary zonal chondrocytes. The results suggested that the co-culture groups deposited more GAG than the single chondrocyte groups, which reflects the stimulating effects of the BMSCs.

In addition to engineering zonal articular cartilage with zonal chondrocytes, some other approaches have been proven valid. Because there are apparent mechanical differences between zones, some studies have employed zonal scaffolds to engineer zonal cartilage [9, 10, 30]. Moreover, oxygen tension [2], cell density [31] and other cues have also been investigated. Although, according to our study, zonal chondrocytes co-cultured with BMSCs retained zonal characteristics only during the early time points after seeding, we still believe that co-culture is a promising approach that could be used to create more stable zonal cartilage when combined with other strategies. Furthermore, other zonal-specific compositions are also important for cartilage function [32]. Whether other zonal-specific compositions would retain their characteristics among zones requires further studies.

In conclusion, we demonstrated that BMSCs stimulated chondrogenesis in co-culture and that zonal extracellular matrix characteristics were found at the beginning of co-culture in vitro (day 1, day 7) and in vivo (week 2). We demonstrated that the co-culture of zonal chondrocytes with BMSCs is a promising approach for engineering zonal cartilage combined with other zonal constructs engineering strategies.

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Disclosure of conflict of interest

None.

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