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## Effects of Chondrogenic Microenvironment on Construction of Cartilage Tissues Using Marrow Stromal Cells *In Vitro*

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**Objective:** To investigate whether it is feasible to use the chondrogenic microenvironment provided by cartilage cells to construct cartilage tissues *in vitro* with bone marrow stromal cells (BMSC). **Materials and Methods:** We isolated and cultured BMSC and cartilage cells from Sprague Dawley rats (SD rats). The supernatant of cartilage culture was used as inducing solution to cause differentiation of BMSC from the second generation of cells cultured *in vitro*. Cells were examined seven days later, using immunohistochemistry to determine the expression of collagen specific to type II cartilage. RT-PCR was used to detect the expression of type II collagen and aggrecan mRNA. BMSC and cartilage cells were isolated from SD rats and cultured *in vitro*. The BMSC and cartilage cells in culture were mixed evenly in an 8:2 ratio and inoculated into a polyglycolic acid/poly(lactic acid) (PGA/PLA) scaffold to a final concentration of  $5.0 \times 10^7$  cells/ml. PGA/PLA preparations with pure cartilage cells or pure BMSC served as the positive and negative controls, respectively. The control group of low-concentration cartilage cells consisted of PGA/PLA preparations containing cartilage cells at 20% of the abovementioned concentration ( $1.0 \times 10^7$  cells/ml). Samples were collected eight weeks later, at which time general observations, wet weight, and glycosaminoglycan (GAG) levels were determined, and histological and immunohistochemical examinations were performed. **Results:** Immunohistochemistry showed the induction of BMSC type II collagen, and RT-PCR indicated the expression of type II collagen and aggrecan mRNA. In the mixed-cell group and the positive control group, pure mature cartilage cells were produced after eight weeks of culture *in vitro*, and the size and shape of the scaffold were maintained throughout the culture period. The two groups gave rise to newly generated cartilage cells essentially identical in appearance and histological properties. The immunohistochemical results showed that the cartilage cells of both groups expressed abundant cartilage-specific type II collagen. The average wet weight and GAG content were more than 70% of the values in the positive control group. Only an extremely small amount of immature cartilage tissue formed in local regions in the BMSC-only sample, and the scaffold was obviously shrunken and deformed. Although the wet weight of newly generated cartilage tissue in the low-concentration cartilage cell sample reached 30% of the value of the positive control group, the scaffold was obviously shrunken and deformed. Only regional and discontinuous cartilage tissues were formed, and the amount of newly generated cartilage was obviously less than in the co-culture and positive control groups. **Conclusions:** Cartilage cells can provide a microenvironment for cartilage formation to some extent, and also effectively induce BMSC to differentiate into cartilage cells and form tissue-engineered cartilage *in vitro*.

**Keywords:** Cartilage tissue engineering, cartilage cell, bone marrow stromal cell, chondrogenic microenvironment

### INTRODUCTION

Cartilage defects or damage are a constant problem in surgical clinics. Cartilage tissue engineering may provide a new method for replacing defective or damaged cartilage [1]. However, the scarcity of cartilage sources severely inhibits the application of cartilage tissue engineering in clinics. Auto-cartilage cells are limited in non-functional areas, and cartilage collection causes great damage to the human body [2]. In addition, cartilage allografts are

vulnerable to immunological rejection, and they cannot survive in the human body for long.

In recent years, research has focused on the differentiation of bone marrow stromal cells (BMSC) into cartilage cells, and many growth factors have been applied to induce BMSC to differentiate into cartilage cells [3,4]. The main problem with this method is that a large amount of growth factor is needed, making the procedure too expensive for large-scale production of cartilage. An early study indicated that it is very important

for BMSC to differentiate into cartilage cells and chondrocytes [5]. In this study, we investigated whether it is feasible to use the chondrogenic microenvironment provided by cartilage cells to construct cartilage tissues *in vitro* using bone marrow stromal cells. The cartilage tissues obtained in this way were compared with cartilage tissues constructed on their own *in vitro*.

## MATERIALS AND METHODS

### Preparation and Culture of BMSC

The femur and tibia from SD rats were collected under sterile conditions. The marrow cavity was flushed with Dulbecco's modified eagle's medium (DMEM). Bone marrow was centrifuged and inoculated using published methods for culturing bone marrow [6]. DMEM supplemented with 10% calf serum was added (Gibco, Grand Island, NY, USA), and the mixture was directly inoculated into a culture dish at a concentration of  $6 \times 10^5$  nucleated cells/cm<sup>2</sup>. Cells (P0) were cultured at 37°C with 50 ml/L CO<sub>2</sub> and 100% saturated humidity. When 90% of the cells in the P0 culture had fused, digestion passage was performed with 0.25% trypsin (containing 0.02% EDTA) and the resulting cultures were marked as P1. The next passage was performed after cell fusion, and cultures were marked P2. Subsequent passages were performed as described above.

### Preparation and Culture of Cartilage Cells

Rib cartilage was collected from both sides of the SD rats under sterile conditions, cut into tissue blocks of 1 mm<sup>3</sup>, then digested with 0.2% type II collagenase for 8–12 h. The solution was filtered, centrifuged, and washed, and cells were counted. Cells were then resuspended in DMEM containing 10% calf serum, and inoculated into a culture dish at  $1 \times 10^6$  cells/60 mm. Cells were cultured at 37°C in a 5% CO<sub>2</sub> atmosphere with 100% saturated humidity. After the cells grew to the point of fusion, they were digested and collected.

### Induction of BMSC Differentiation into Cartilage Cells

Cellular supernatant was collected from P1, filtered and sterilized, and stored at 4°C. When 80% of the P1 BMSC had fused, the cells were digested and collected, and inoculated into a culture dish at  $1.5 \times 10^4$  cells/cm<sup>2</sup>. A glass slide was placed in the culture dish prior to inoculation, and the dish was stored overnight with

DMEM containing 10% calf serum. After the cells had attached, the culture solution was aspirated and the supernatant from cartilage cells culture was added to induction differentiation. This was the induction group. The non-induction group consisted of BMSC cultured without the addition of cartilage cells. Half of the medium was changed daily, and the slide was taken out after seven days of culture. The glass slide was fixed with 95% cold acetone, and subjected to immunohistochemistry. The rest of the cells were collected in a culture dish for use in RT-PCR analysis.

### Preparation of PLA/PGA Compound Materials

PGA (10 mg) was uniformly loaded into the prepared silica gel column (internal diameter, 9 mm; height, 3 mm), 0.3 ml of PLA (1% in dichloromethane) was added dropwise onto the PGA fibers, and fibers were removed after they had dried naturally, which were prepared into a compound with the PLA/PGA scaffold (diameter 9 mm, height 3 mm). The compound material was immersed in 75% ethanol for 30 min, washed twice with PBS, and dried and stored for later use.

### Experimental Grouping and Culture In Vitro

The experiment consisted of four groups. In Group A, BMSC and cartilage cells were mixed evenly in the ratio 8:2, and inoculated into PLA/PGA scaffold at a final concentration of  $5.0 \times 10^7$  cells/ml. In Group B, pure cartilage cells of the same concentration were inoculated. Group C contained pure BMSC of the same concentration. In Group D, cartilage cells were inoculated to a final concentration of  $1.0 \times 10^7$  cells/ml; this group served as the control. All the groups were analyzed during the eighth week of culture *in vitro*.

### Analysis

#### Determination of type II collagen

After seven days of induction, slides were taken out and immunohistochemical staining of type II collagen was performed on the induction group and the non-induction group. Cartilage cells were used as the positive control. The expression of type II collagen in all groups was examined using rabbit anti-mouse type II collagen monoclonal antibody (diluted 1:100) and the PV two-step immunohistochemistry kit (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd).

### RT-PCR analysis

Trizol was used to extract total mRNA from all groups, and reverse transcription was performed to obtain cDNA according to the manufacturer's instructions (Shanghai Bioengineering Co., Ltd.). Col II and aggrecan primer sequences were designed and synthesized: upstream Col II primer, 5'-GTCCAGATGACTTTCCTCCGTC-3'; Col II downstream primer, 5'-TGTCCACACCAAATT CCTGATC-3' (PCR product: 325 bp); aggrecan upstream primer, 5'-GGAATCCCT AGCTGCTTAGCAG-3'; aggrecan downstream primer, 5'-GAGTCATTGGAGCG-AAGGTTC-3' (PCR product: 458 bp). PCR conditions were as follows: for Col II, 95°C for 3 min, then 30 cycles of 95°C for 30 sec, 64°C for 30 sec, 72°C for 1.5 min, then 72°C for 10 min; for aggrecan, 95°C for 3 min, then 30 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 1.5 min, then 72°C for 10 min. PCR products were analyzed by electrophoresis in 1% agarose gels.

### General observations

The *in vitro* cultures were observed for changes in size, shape, quality, and color of the cell-material compound, and results were compared between groups.

### Determination of wet weight

After eight weeks of cell culture, the wet weight of new tissue in each group was determined, and results were compared between groups.

### Histological determination

The newly formed tissues were fixed with 10% formaldehyde for 24 h, embedded in paraffin, sliced and subjected to staining with HE, Safranin O, Massion, or Alcian blue.

### Determination by immunohistochemistry

The expression of type II collagen in all groups was examined using rabbit anti-mouse type II collagen monoclonal antibody (diluted 1:100) and the PV two-step immunohistochemistry kit (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd).

### Determination of GAG content

Alcian blue staining was used to determine GAG content in all samples, and the results for different groups were compared.

## RESULTS

### Determination of Type II Collagen

In the induction group, type II collagen immunohistochemistry showed that cells with cytoplasm stained brown were positive for type II collagen (Figure 1A), whereas no cells in the non-induction group tested positive for type II collagen (Figure 1B).

### RT-PCR

After the inducer cells were amplified by RT-PCR, two distinctive bands could be observed, one between 300 and 500 bp and another migrating near this region. These results suggest that the induced cells express both type II collagen and aggrecan mRNA, whereas no such expression was observed in the control group (Figure 2).

### Combination of Cell Material and Culture

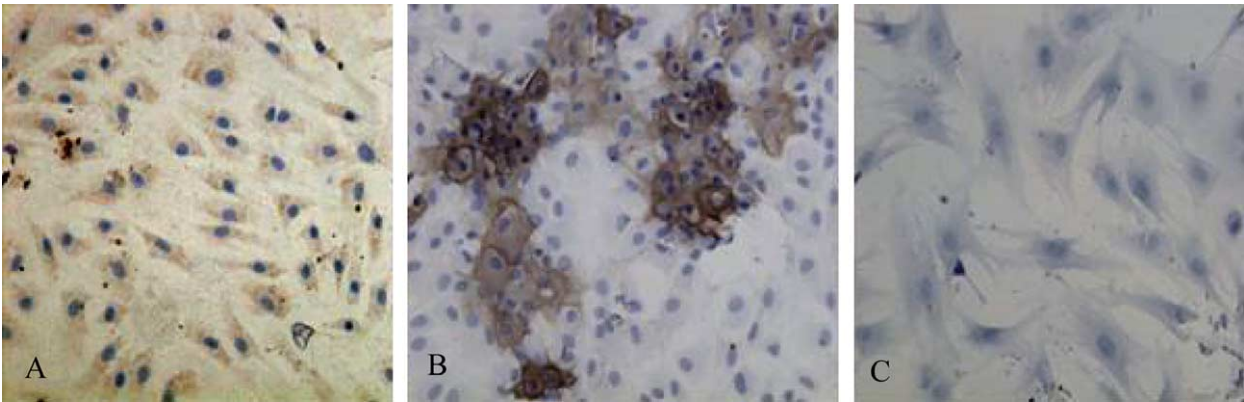
After the PGA/PLA scaffold was dried and removed from the silica gel model, the column shape was maintained (Figure 3), and the material showed good hydrophilicity following sterilization with ethanol and washing with BPS. The cells adhered tightly to the bracket after inoculation (Figure 4).

### General Observations

The morphology of the initial cell-material compound is shown in Figure 5A. After eight weeks of culture *in vitro*, the compounds in group A (co-culture group, Figure 5B) and group B (positive control, pure cartilage cells at the same concentration, Figure 5C) showed the same diameter and thickness as the original material, a normal shape, a white ivory appearance, and elasticity. In group C (less concentrated cartilage cell group, Figure 5E), the structure was relatively loose, the whole compound structure was visibly shrunken and deformed, and it showed no elasticity.

### Histological Examination

HE staining showed that by the eighth week, the histological characteristics of the co-culture group (Figure 6A) and positive control group (Figure 6B) had been established. All of the cultures contained mature and continuously linked cartilage tissues, and many mature cartilage lacunae could also be observed. The scaffold materials had been degraded, and no fiber-like tissues had formed. The



**Figure 1.** The results of Col II specific staining ( $\times 200$ ). A: Col II specific staining of induced cells; B: Col II specific staining of rat chondrocytes; C: Col II specific staining of rat BMSC.

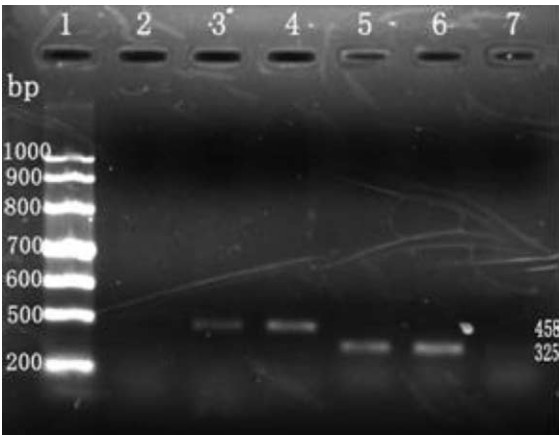
BMSC group (Figure 6C) mainly showed fibrous tissue, the structure was very loose, and a small amount of cartilage-like tissue had formed along the edges of the culture. Although the cartilage lacunae were not distinctive, the color of the extracellular matrix was similar to that of cartilage. The less concentrated cartilage cell group (Figure 6D) showed mature cartilage tissues along the edges, but the succession of cartilage was poor, and the tissue density and amount of newly generated cartilage were clearly lower than in the culture group.

The other stains provided additional information. Safranin O staining revealed no obvious difference between the co-culture group (Figure 7A) and the positive control group (Figure 7B). The extracellular matrix was stained deep red and many vacuole-like cartilage lacunae were visible. Massion staining revealed a large number of green collagen sediments in the

co-culture group (Figure 8A) and the positive control group (Figure 8B). The nuclei were stained black, and many mature cartilage lacunae were visible. Alcian blue staining showed no obvious difference between the co-culture group (Figure 9A) and the cartilage cell group at the same concentration (Figure 9B). Cartilage lacunae were obvious, and the extracellular matrix was stained blue and reflected active secretion by the cultured cells.

**Immunohistochemistry**

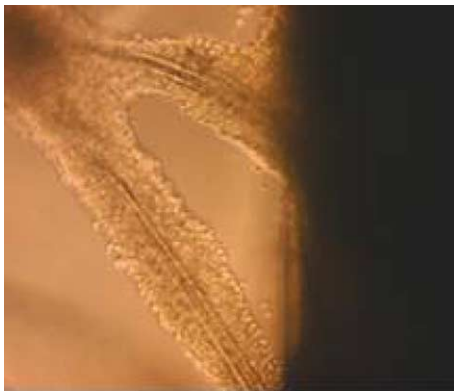
Type II collagen immunohistochemistry staining in the eighth week indicated that in the co-culture and cartilage cell groups, strong positive reactions (brown yellow) occurred in the areas surrounding cartilage lacunae, indicating robust secretions of cartilage protein-specific type II collagen. In the group with the lower cartilage cell concentration, strong reactions were observed only in local areas, while there was only a weak expression of type II collagen along the compound borders in the BMSC group (Figure 10).



**Figure 2.** Detection of collagen type II and aggrecan mRNA expression by RT-PCR. 1: RNA marker; 2: Col II expression of BMSCs without induction; 3: Col II expression of induced cells; 4: Col II expression of chondrocytes; 5: aggrecan expression of induced cells; 6: aggrecan expression of chondrocytes; 7: aggrecan expression of BMSCs without induction.



**Figure 3.** PGA/PLA scaffolds.



**Figure 4.** Cell attachment on scaffold.

### Determination of Wet Weight

Except for groups C and D, significant differences (multi-sample *t* test) existed between all combinations of other groups ( $P < 0.05$ ). Group D, which served as the control to the group with low cartilage cell concentration, had a wet weight that was approximately 30%–50% of that of group B.

### Determination of GAG Content in Newly Generated Cartilage

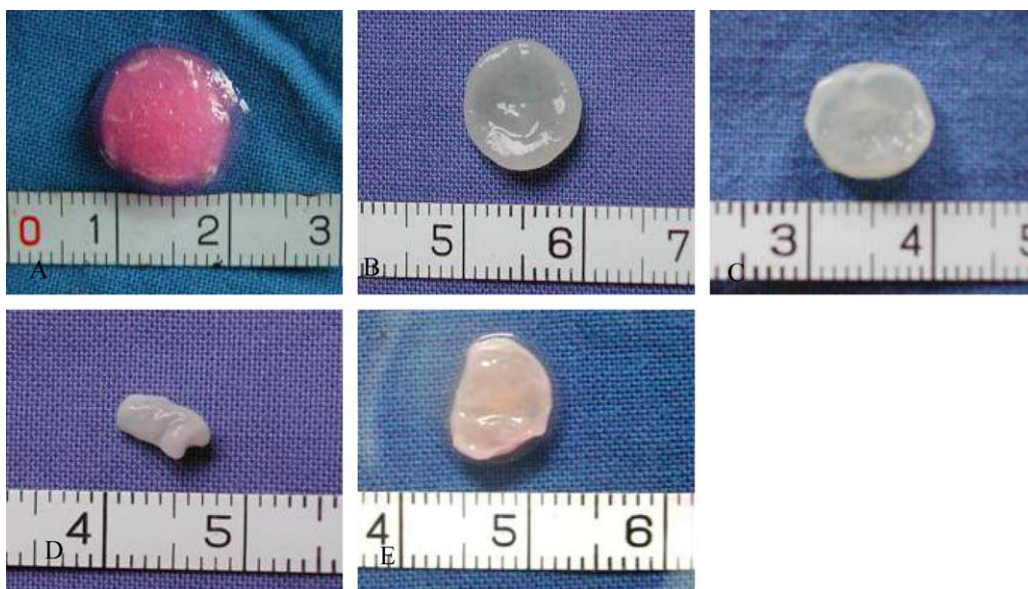
Except for groups C and D (multi-sample *t* test), significant differences existed between all pairings of the other groups ( $P < 0.05$ ).

## DISCUSSION

Many studies have demonstrated that BMSC can express large amounts of cartilage extracellular matrix after being jointly induced with dexamethasone and transforming growth factor  $\beta 1$  (TGF $\beta 1$ ). After the induced BMSC and PGA/PLA scaffolds are implanted into the affected joint, the articulation injury can be completely restored. When the same cells are injected subcutaneously into animals, fibrous tissues form with obvious vascularization [5,7,8]. These results suggest that the chondrogenic microenvironment is potent for inducing the differentiation of BMSC into cartilage cells and the formation of cartilage tissues.

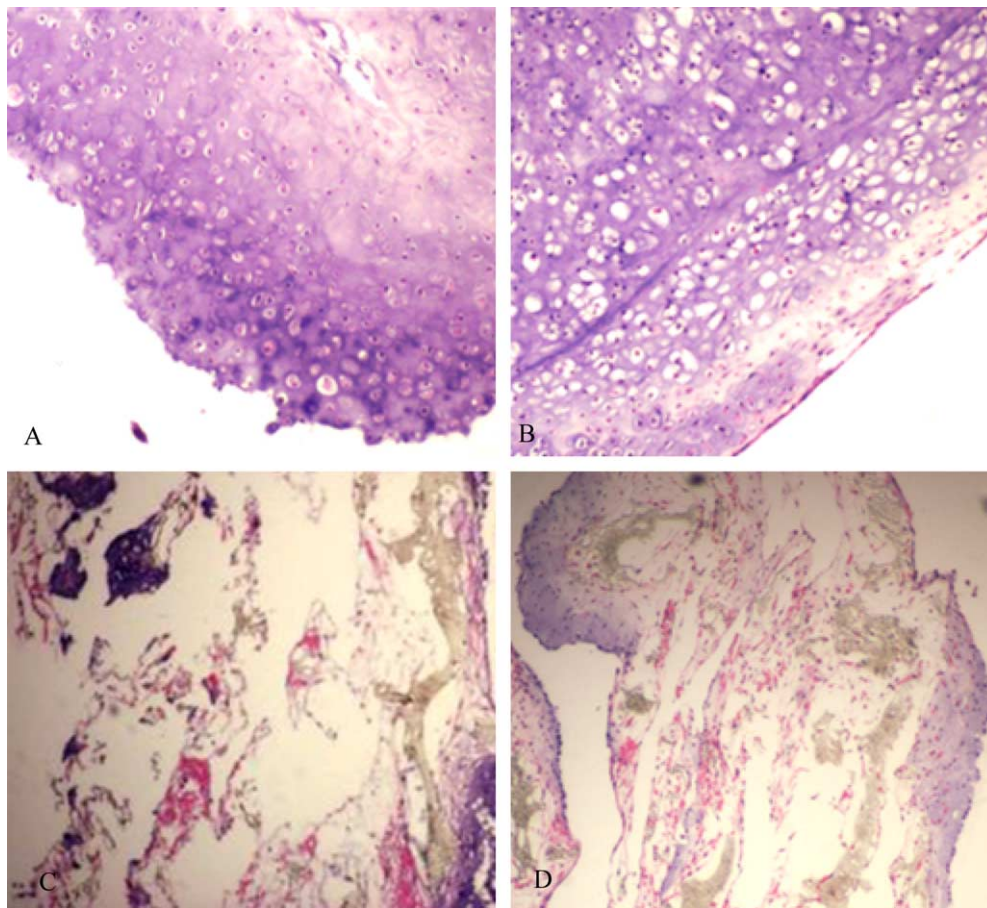
The results of this study indicate that the supernatant from cartilage cell cultures can induce BMSC to transform into cartilage cells. Immunohistochemical examination of type II collagen after seven days of induction showed that the cartilage cells had secreted an abundance of cartilage-specific type II collagen into the extracellular matrix. RT-PCR was used to detect the expression of type II collagen and aggrecan mRNA in cells before and after the experiment. The results indicate that type II collagen and aggrecan mRNA are expressed in the induction group, but not in the control group. These results strongly suggest that BMSC differentiated into cartilage cells after induction.

We co-cultured 20% cartilage and various amounts of BMSC on 3D scaffolding, leading to the formation of mature cartilage tissue by the eighth week of culture. Compared to the newly-generated cartilage produced by pure cultures of cartilage cells at the same concentration as in the co-cultures, the newly produced cartilage cells in the co-cultures all maintained the primary shape and size



**Figure 5.** Gross view of specimens in all groups cultured *in vitro* for 8 weeks. A: Cells and scaffold (24 h); B: co-culture group; C: Chondrocyte group; D: BMSC group; E: Low chondrocytes group.



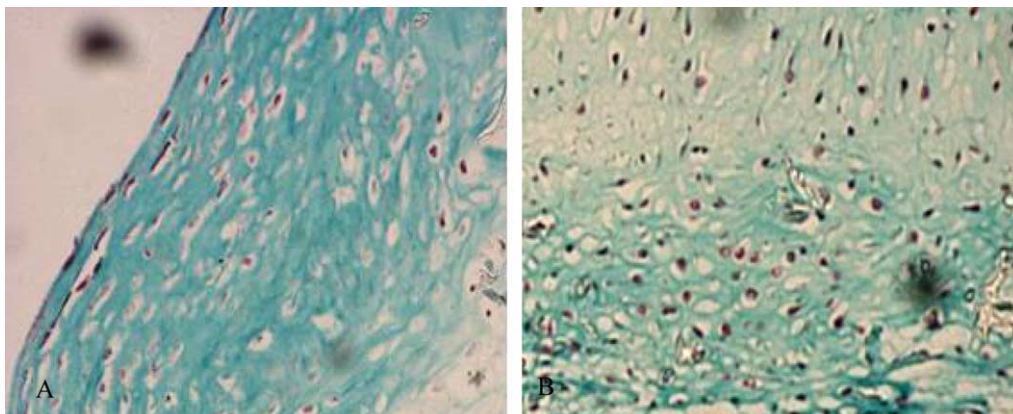


**Figure 6.** Histology of specimens in all groups cultured *in vitro* for 8 weeks. A: co-culture group; B: Chondrocyte group; C: BMSC group; D: Low chondrocytes group.

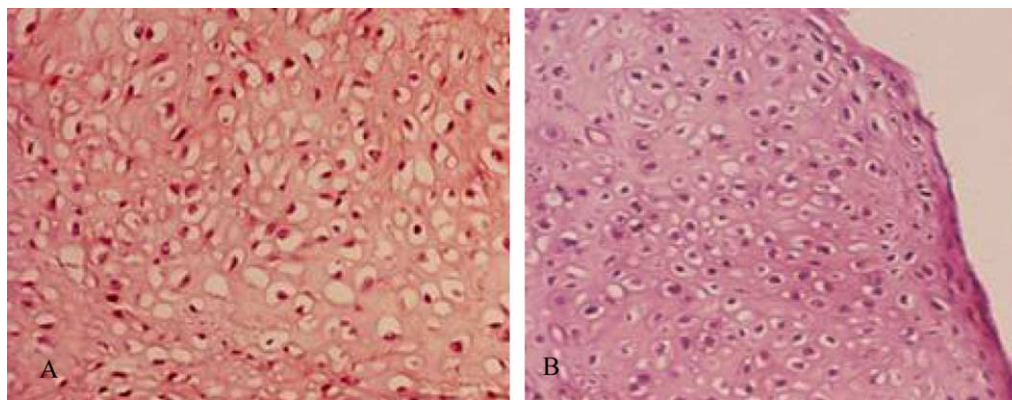
of the scaffold and a certain level of elasticity. Many mature cartilage lacunae were observed in histological and immunohistochemical examinations, and cells secreted abundant extracellular matrix showing the characteristics of cartilage-specific matrix. Our measurements found that both the wet weight and GAG levels in the co-

culture group reached 70–80% of the levels of the positive control group.

In contrast, in the control group little or no cartilage formed when the biological materials were inoculated with only BMSC or only cartilage cells at a lower concentration than in the co-culture group. These results indicate that,



**Figure 7.** Massion strain of some specimens *in vitro* for 8 weeks. A: co-culture group; B: Chondrocyte group.

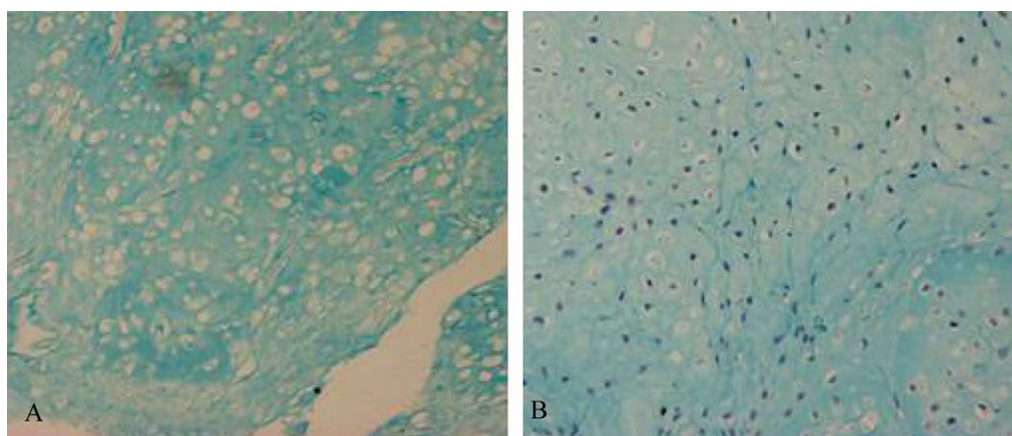


**Figure 8.** Safranin O stain of some specimens *in vitro* for 8 weeks. A: co-culture group; B: Chondrocyte group.

without induction by cartilage, BMSC have little tendency to form mature cartilage *in vitro*. Furthermore, when a small number of cartilage cells is used to induce the BMSC, complete cartilage tissues cannot be constructed *in vitro*. In the co-culture group, the quality and quantity of newly produced cartilage were all significantly higher than in the low-concentration cartilage cell group; the two groups had the same numbers of cartilage cells, but the co-culture group also had BMSC. This demonstrates that a small relative number of cartilage cells *in vitro* (20%) can induce a large proportion of BMSC (80%) to mature into cartilage cells and form cartilage tissue. This result also provides robust confirmation that cartilage cells can induce BMSC to differentiate into cartilage cells and form mature cartilage tissues. This will reduce the amount of cartilage cells needed in the construction of cartilage and obviates the need for expensive growth factors.

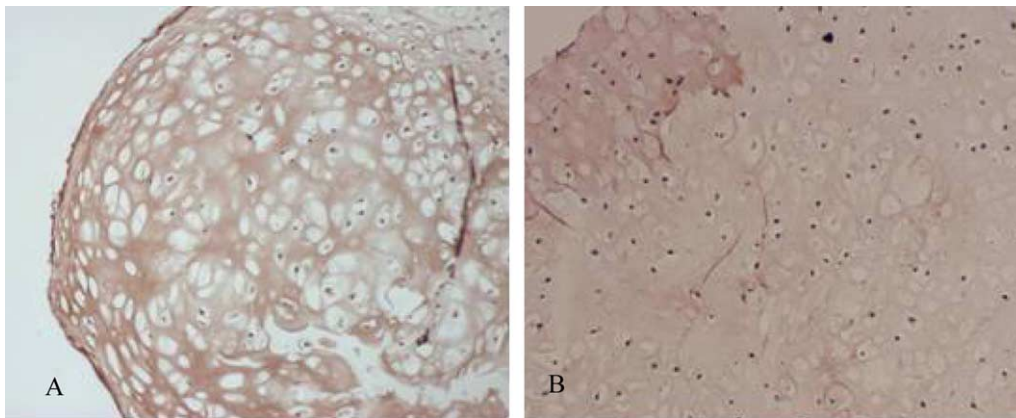
Previous studies have shown that the chondrogenic microenvironment can strongly affect the differentiation of BMSC into cartilage, and that even without induction by growth factors, BMSC can form mature cartilage tissues in the affected joints [8]. Therefore, detailed analysis of induction by the articular microenvironment will be

helpful in understanding how cartilage cells induce BMSC to differentiate into cartilage. Recently, studies have demonstrated that co-culture of sheep MSCs with synovial cells [30] or human MSCs with bovine chondrocytes [11] can induce chondrogenesis. Similarly, human embryonic stem cells (hESCs) can be induced to differentiate to osteoblasts when co-cultured with bone-derived cells [12] or to chondrocytes in the presence of limb bud cells, precursors of chondrocytes [13]. This response is not confined to chondrocytes, because cardiomyocytes were generated when hESCs were co-cultured with visceral endodermal-like cells in serum-free cultures, and CD34+ hESCs efficiently produced hematopoietic cells when co-cultured with OP9 mouse stromal cells [14]. These co-culture experiments all used stem cells: human embryonic or mesenchymal. In the articular environment, factors that affect cartilage differentiation include activation factors secreted by cartilage cells or the synovium into the synovial fluid, mechanical stimulation in the movements of the articulation, and the non-vascularized state around the articulation. Several studies have indicated that cartilage cells can secrete growth factors such as TGF- $\beta_1$  and IGF-1, which are commonly used to induce the



**Figure 9.** Strain of some specimens *in vitro* for 8 weeks. A: co-culture group; B: Chondrocyte group.





**Figure 10.** Collagen type II immunohistochemistry of some specimens cultured *in vitro* for 8 weeks. A: co-culture group; B: Chondrocyte group.

differentiation of BMSC into cartilage cells. These findings together provide a basis for using cartilage cells to induce the differentiation of BMSC.

In general, the microenvironment provided by cartilage cells strongly induces the differentiation of BMSC, and the relevant inducing factors including diverse secreted soluble factors, extracellular matrix proteins, and cell surface molecules. If through further study we can completely reproduce the chondrogenic microenvironment in the joint, perhaps by providing mechanical stimulation with a biological reactor, we may be able to improve the degree of maturation and the mechanical characteristics of the cartilage constructed through co-culture *in vitro*.

**Declaration of interest:** The authors declare no conflicts of interest.

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