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Application of tissue-engineered cartilage with BMP-7 gene to repair knee joint cartilage injury in rabbits

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Abstract Injured articular cartilage has a poor capacity for spontaneous healing. So far, satisfactory solution to this subsistent problem has not been found, but transgenic therapy may be a promising way. This study aims to evaluate the effectiveness of a tissue-engineered cartilage that was transfected with morphogenetic protein 7 (BMP 7) in repairing the cartilaginous defects of rabbit knee joints. Chondrocytes were transfected with BMP-7 gene (5×10^6 cells/ml), inoculated into the collagen-fibrin gel scaffolds, and cultured for 14 days. Then, the scaffolds were implanted onto the created defects (5.0 mm in diameter) in rabbits' knee joints. After 12 weeks, the rabbits were sacrificed and histological sections were evaluated using modified O'Driscoll cartilage scores; In situ hybridization and immunohistochemistry were performed to detect the expression of BMP-7 mRNA and BMP-7 at the implanted site while the content of DNA and GAG was determined as well. A better quality of repairs was observed at the 12th week after implantation when compared to the control group using histological analyses. The content of DNA and specific secretion of GAG in the treatment group is statistically significant different compared with the control group. Gene therapy may be a promising treatment method, but the novel therapy approach needs further studies with respect to a longer follow-up period.

Keywords Bone morphogenetic protein-7 (BMP-7) · Tissue-engineering cartilage · Cartilage defect · Gene

Introduction

Articular cartilage degradation, including focal osteochondral, chondral defect and osteoarthritis, as well as models of degeneration in intervertebral disc cartilage is common. Focal cartilage and osteochondral injuries typically affect a young, athletic population. Full-thickness defects of articular cartilage in the knee have a poor capacity for repair. They may progress to osteoarthritis, which is a major cause of disability among the adult population in the United States [5]. Chronic back pain can be caused by degenerative disc disease (DDD). Because cartilage defects have extremely limited intrinsic healing capacity [18], with the ultimate aim of increasing the healing potential, experimental and clinical approach in various models were introduced by scientists and orthopaedic surgeons. Bone marrow stimulation, mosaicplasty and autologous chondrocyte implantation (ACI) have been used to stimulate biological repair of cartilage in clinical practice [14]. Nevertheless, they all have inherent problems, leaving many patients with inadequately treated cartilage lesions. The use of mesenchymal stem cells (MSC) transplantation could be another treatment method as reported by Wakitani, but this method is yet to be studied and validated [26]. Recently, a novel approach in cell delivery system using magnetically labeled MSC for

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cartilage repair was reported by Kobayashi [12], but chondrogenic differentiation capacity of MSC cannot be fixed without specific growth factors or bioactive molecules. Cell-based approaches using tissue engineering and regenerative medicine techniques have offered a new therapy [16]. Bone morphogenetic proteins (BMPs), as members of the human transforming growth factor-β superfamily, induce new cartilage and bone formation in vitro and in vivo [7], particularly osteogenic protein-1 (OP-1) (also called BMP-7), has demonstrated a great potential as cartilage anabolic factors because of their ability to induce matrix synthesis and promote repair in cartilage [5]. BMP-7 increased expression of collagen type II, the major collagen component of normal hyaline cartilage [25]. Importantly, BMP-7 maintains in its chondrocytic phenotype since there is no type I or type X collagen synthesis or markers of chondrocytes differentiation detected after cultured in the presence of BMP-7 [4, 7]. Furthermore, BMP-7 is able to counteract catabolic changes caused by interleukin-1, fibronectin and collagen fragments [11, 13] in human and bovine articular cartilage. Currently, there are only a few studies done on using both tissue engineering and gene therapy in treating defected cartilage. In addition, the effectiveness of using a chondrocyte inoculated collagen-fibrin gel scaffold to repair rabbit knee joint cartilage defects had been tested clinically with reasonable follow-up [2, 20]. Based on these assumptions, we hypothesize that combining collagen-fibrin gel scaffold with BMP-7 gene transfected chondrocytes will generate a better cartilage repair response than curing with ACI in rabbits. The purpose of this study was to evaluate collagen-fibrin scaffold and chondrocytes with BMP-7 gene will improve cartilage healing compared to the same scaffold and chondrocytes without BMP-7 gene in an established model of an osteochondral injury in the rabbit knee.

Materials and methods

Goat-anti-human BMP-7 antibodies were obtained from Santa Cruz Biotechnology, USA. A BMP-7 in situ hybridization kit was obtained from Wuhan Boster Biological Technology Ltd., China. HPR-conjugated rabbit, anti-goat IgG was from Sigma Company, USA.

The 27 adult New Zealand White rabbits (body weights 2.0–2.5 kg) in this study were treated in accordance with the Medical Science Association of Heilongjiang Province, China, of animal care guideline. The rabbits were randomized to the following three groups. (1) Spontaneous healing group (empty group): rabbits were subjected to subchondral drilling only. (2) Scaffold with chondrocytes group (control group): rabbits were subjected to subchondral drill and treated by collagen-fibrin gel scaffold with autologous chondrocytes. (3) Scaffold with chondrocytes and BMP-7 gene group (treatment group): rabbits treated by autologous chondrocytes transfected with BMP-7 gene in a collagen-fibrin gel scaffold. The animals were allowed to move freely in their cages immediately after surgery, and most animals were able to bear weight on both extremities immediately. The animals were fed in their cages after the procedure without any dietary restrictions.

Creation of the defect method

Fourteen days after cells seeding into the scaffold, 27 rabbits were subjected to surgery in both knees under sterile. For each rabbit, barbital sodium (1%, 30 mg/kg of animal body weight) was injected into the ear vein, and the anesthetized animal was placed in a fixed position on an operating table. An incision was made in the medial patella to dislocate the lateral patella and to expose the patellar joint cartilage at the femoral distal end. A 5.0 mm diameter and 3.0 mm deep defect was made by drilling into the surface of joint cartilage using an orthopedic manual drill to reach the lower osseous lamella as shown in Fig. 1. The removed cartilage tissue is used as biopsies for autologous chondrocyte cultures. In vitro tissue-engineered cartilage, which was cultured for 14 days, was cut to match the shape of the created cartilage defect before implantation. The collagen-fibrin gel scaffold containing cartilage with or without BMP-7 gene was implanted in one rabbit (two knees, left and right side were alternated). A total of 54 defects were treated. After implantation, 60 passive flexion/extension repetitions of the knees were made. Subsequently, we checked that the implants were still in place. Patellae were repositioned after the procedure, joint capsule and skins were sutured. The animals were kept alive and there were no infections at the surgical sites during this part of the experiment. Then, the effectiveness of the repair

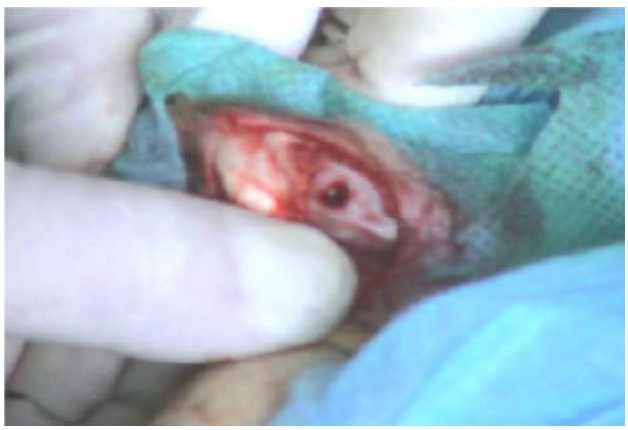

Fig. 1 Photograph of created defect in rabbit knee, 5.0 mm diameter and 3 mm deep

at the defected sites was evaluated 12 weeks after the implantation procedure.

The collagen-fibrin gel scaffold were cut into small cubes (8 × 8 × 3 mm) and were transferred to a 24-well tissue culture plate and then pre-sterilized with 0.22-μm filtered 70% (v/v) ethanol solution at 4°C for 3 h. Residual ethanol was removed by N_2 flow and washed several times using the culture media. Sterilized collagen-fibrin gel was pre-wetted with fresh culture media by incubating at 37°C for 2 h in a humidified atmosphere composed of 5% CO_2. After removing this media, 20 μl of cell suspension (chondrocytes and chondrocytes transfected with BMP-7 gene) containing 5×10^6 cells was loaded onto each scaffold and allowed to penetrate into the scaffold. The scaffold was left undisturbed in an incubator at 37°C under 5% CO_2 condition for 4 h to allow the attachment of cells to the scaffold. In addition, 1 ml of culture media was added to each well and kept in the incubator afterwards. Culture medium was changed every 2–3 days.

Samples were obtained from the distal end femur and fixed with 4% paraformaldehyde/0.1 M PBS (pH 7.0–7.4, 1/1000 DEPC) for 2–4 h. The specimen was soaked in 20% EDTA, swaying for 7 days, during which 20% EDTA was changed four times. Then, hybridization was carried out. Probes for BMP-7 were 5′-TGCGG CGCCG CACAG CTTCG TGGCG CTGTG-3′; 5′-CCTAC AAGGC CGTCT TCAGT ACCCA GGGCC-3′; 5′-CTGGA TGGGC AGAGC ATCAA CCCCA AGTTG-3′. Tissues were stained with DAB and viewed by microscope to observe any color changes in chondrocytes.

Samples obtained as described earlier were fixed with 4% paraformaldehyde, decalcified, embedded and cut perpendicularly. Slides were incubated with 50 μl of goat anti-human BMP-7 IgG diluted with PBS (1:200) at 4°C overnight followed by incubation with 50 μl of biotin-labeled rabbit anti-goat IgG diluted with PBS (1:500) for 30 min.

DNA and GAG production were determined for the phenotypic analysis. The tissue-engineering cartilage (100 mg) was taken at 0, 4, 8, and 12 weeks after transplantation.

A total volume of 250 μl of each type of cartilage culture grown for 4, 8, and 12 weeks was added to 96-well plate. After determining the weight of cartilage in the cultures, 100 mg of cartilage samples per 2 ml of 125 μl/ml type II collagenase (Sigma) were added to a centrifuge tube for digestion at 60°C for 12 h followed by centrifugation at 1000 rpm for 5 min. The, the supernatant was then stained using the Hoechst 33258 fluorescence stain, and the fluorescence intensity was measured using Spectrofluorophotometer (ex wavelength at 360 nm, em at 450 nm) (Fig. 6). Its content was determined via standard curve based on bovine thymus DNA [21].

Samples of tissue-engineered cartilage cultures grown for 4, 8, and 12 weeks were cut into pieces and placed in a centrifuge tube. Between 0.5 and 1.0 ml of Pronase E (Beijing Solarbio Ltd., Beijing, China) was added and the cultures were added to 100 μl of the supernatant fluid, mixed thoroughly and warmed for 10 min in water bath at 100°C. Next, 0.2 ml of 1.25% carbazole dehydrated alcohol was added and well mixed at room temperature; the tube was then warmed for 15 min in 100°C water bath. The glycosaminoglycan (GAG) content was determined by comparing the optical density at 540 nm against a galacturonic acid standard curve [21].

The specimens were fixed in 10% paraformaldehyde solution for 48 h, decalcified, and embedded in paraffin. Sections were stained with haematoxylin/eosin (H&E) and Toluidine Blue. A minimum of four sections from the central portion of defects were subjected to semiquantitative histological analyses. To characterize the quality of the repair, we selected parameters (modified from the O'Driscoll score by Løken [17]) and were given a score of 0 being the lowest, and 1 or 2 being the highest): the scores were reported for each parameter separately, without combining them to a total score. The evaluation was performed repeatedly at two occasions separated by 3 weeks by two observers (G. Z. Li and X. D. Bai), who were blinded to the type of surgery performed. If a discrepancy occurs in the scoring process for one parameter in a specimen, this parameter would be re-evaluated by the same observer and a final consensus was made.

Statistical analysis

Data are presented as mean and standard deviation. Statistical analysis was performed by using SPSS for Windows (Version 13.0) employing Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

Results

Animals from all three groups had relatively low activities, but normal flexion and jumping posture for both knee joints after 3–5 days of implantations. Their activities increased during the following weeks, and all animals were restored to normal activities within 2 weeks. No rabbit's knees were left out or replaced. No animals died and there were no infections at surgical sites during the experimental period. On the 12th week after the transplantation, macroscopically, the wounded surfaces for the spontaneous healing group rabbits appeared to be white, indented with red edges and had a clear demarcation from normal tissues; the surfaces for the scaffold with chondrocytes group were filled with white implanted material that had a height equal to the

normal joint, a smooth surface fused with normal tissues without any fractures. In the scaffold with chondrocytes and BMP-7 gene group, the surface were filled with implanted material that had a color similar to that of normal tissue, had a smooth surface and equal height with the normal tissue, showed no clear boundaries with the normal tissue and did not have any fractures. These observations demonstrated that there was not a cartilage repair sign in the spontaneous healing group but there was a cartilage repair sign in both the scaffold with chondrocytes and the scaffold with chondrocyte and BMP-7 group. Obviously, better pronounced repair effect was showed in the scaffold with chondrocyte and BMP-7 group (Fig. 2).

Chondrocytes nucleus appeared with a dark red color in the treatment group, as visualized by in situ hybridization and immunochemistry after 12 weeks of the procedures. The expression of BMP-7 mRNA and BMP-7 was readily detected. In contrast, no dark red color was observed and no BMP-7 mRNA and BMP-7 was detected for chondrocyte nucleus from the control group (Figs. 3 and 4).

DNA and GAG production determination was used for the phenotypic analysis. The results showed that the DNA expression of chondrocytes increased with the culture period (Fig. 5), and GAG was produced in the three-dimensional (3D) scaffold, indicating that the BMP-7 gene enhanced the ability of chondrocytes synthesizes GAG with increasing cultivation time (Fig. 6). These results demonstrated chondrogenic differentiation in both treatment and control groups.

At 12 weeks after the operations, the wound surfaces in the control group rabbits were filled with fibrous tissues with apparent dents as shown in Fig. 6. A few round cells were observed that were localized in the dent, and there were some thick regenerated bone tissues at the bottom of

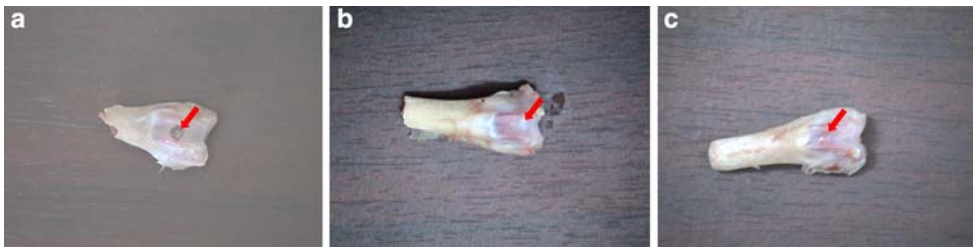

Fig. 2 Photographs of cartilage deficiency section of rabbit knee at 12 weeks after transplantation. **a** empty group: soft tissue repair only no cartilage repair; **b** control group: some degree cartilage repair; **c** treatment group: complete cartilage repair

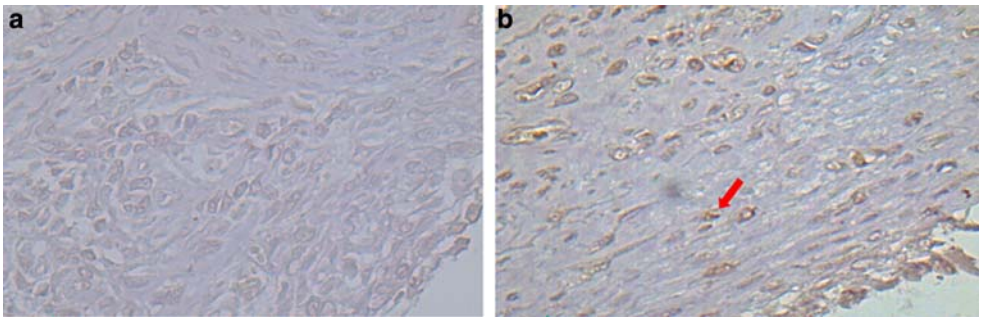

Fig. 3 Light micrographs showing hybridization in situ staining of restoration sections at 12 weeks after transplantation (high power magnification ×100). **a** control group; **b** treatment group. *Red arrow* points to positive cell

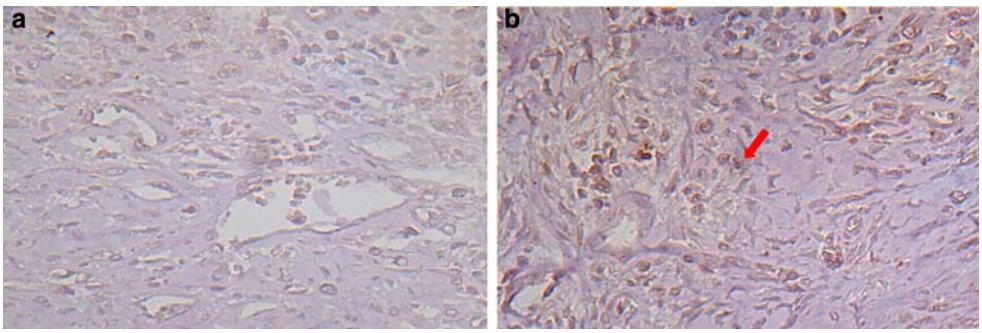

Fig. 4 Immunohistochemical staining demonstrates restoration sections at 12 weeks after transplantation (×100). **a** control group; **b** treatment group. *Red arrow* points to positive cell

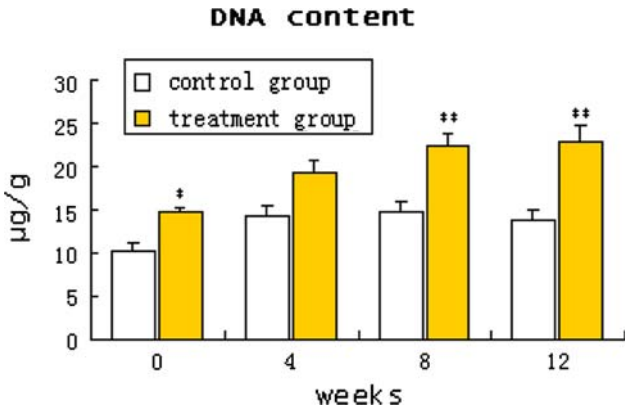

Fig. 5 Quantitative determination of DNA synthesis from non-BMP-7 gene-transfected cartilage restoration group (control) and BMP-7 gene-transfected group (treatment) analyzed by the spectrofluorophotometer. *Error bars* mean standard deviations ($n = 7$, $^{*}P < 0.05$, $^{**}P < 0.01$)

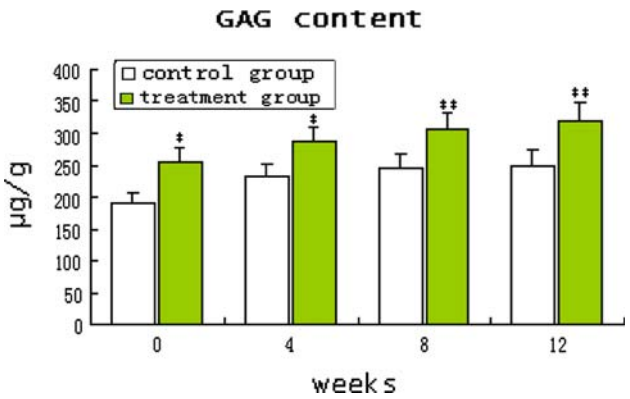

Fig. 6 Quantitative determination of GAG synthesis between non-BMP-7 gene-transfected cartilage restoration group and BMP-7 gene-transfected group analyzed by spectrofluorophotometer. *Error bars* mean standard deviations ($n = 7$, $^{*}P < 0.05$, $^{**}P < 0.01$)

the wound surface. In the control group rabbit, the wound surfaces appeared to be smooth, a little lower than the normal cartilage surfaces and were filled with hyaline cartilage, as shown by Toluidine Blue and H&E staining. Massive amounts of chondrocytes were seen localized in specific niches along with endogenous chondrocytes. The cell density of the wound surface was higher than that of the normal tissue and with clear boundaries. There was no obvious infiltration of inflammatory cells. Trabeculas were apparent in newly formed bones. For the treatment group rabbits, the wound surfaces were filled with hyaline cartilage that had a similar coloration to the normal surrounding tissues. They had smooth surfaces and were fused with surrounding tissues. The cell density was nearly the same as that of the normal tissue with no clear boundaries. There was no obvious infiltration of inflammatory cells. The formation and repair of trabeculas were readily observed at the bottom of the wound surface, and were more obvious than those of the control group rabbit.

Semiquantitative data on the quality of the repair are shown in Table 1. There was a significantly higher score for the degree of surface regularity and integration at borders (i.e. more regularity and integration, $P = 0.001$ and 0.006, respectively) in the defects treated with scaffold of cartilage transfected with BMP-7 gene compared to scaffold with only cartilage. There were NS differences in score for the parameters hyaline like cartilage, amount of necrosis and cluster formation. No traces of the biomaterial were seen. Selected histology samples are shown in Fig. 7.

Discussion

The most important finding of the present study was BMP-7 should be considered playing an important role in this restoration procedure. There were an increased surface regularity and integration at borders in the cartilage with BMP-7 gene treated defects in the semi-quantitative analysis, but no statistical difference in the other parameters. Although cluster formation in BMP-7 gene treated group is not statistically different from the control group, the score value which was in line with the study of Løken [17] was high. Cluster formation is a sign of repair in early osteoarthritis [8], and in cartilage repair, cluster formation may be interpreted as a positive phenomenon as cell proliferation is central to new tissue formation. Chondrocytes were cultured in the 3D collagen-fibrin gel scaffold giving a cell density of 5×10^6/ml. When the cell density reached a certain level, secretion of chondrocytes including GAG and collage II reduced, the capabilities of proliferation and differentiation in cultured gene-modified chondrocytes decreased, cell senescence is undergoing because it is hard to get enough nutrients in the middle of the 3D structure scaffold. Chondrocytes density of 5×10^6/ml was cultured

Table 1 Qualitative analysis of the cartilage repair (modified from O'Driscoll) [mean (SD)]

	Hyaline cartilage	Surface regularity	Necrosis	Integration at borders	Cluster formation
Treatment group	1.2 (0.09)	1.3 (0.09)	1.4 (0.10)	1.3 (0.09)	1.2 (0.13)
Control group	1.2 (0.09)	1.0 (0.09)	1.4 (0.07)	0.9 (0.20)	1.0 (0.86)
P value	0.300	0.001	1.000	0.006	0.053

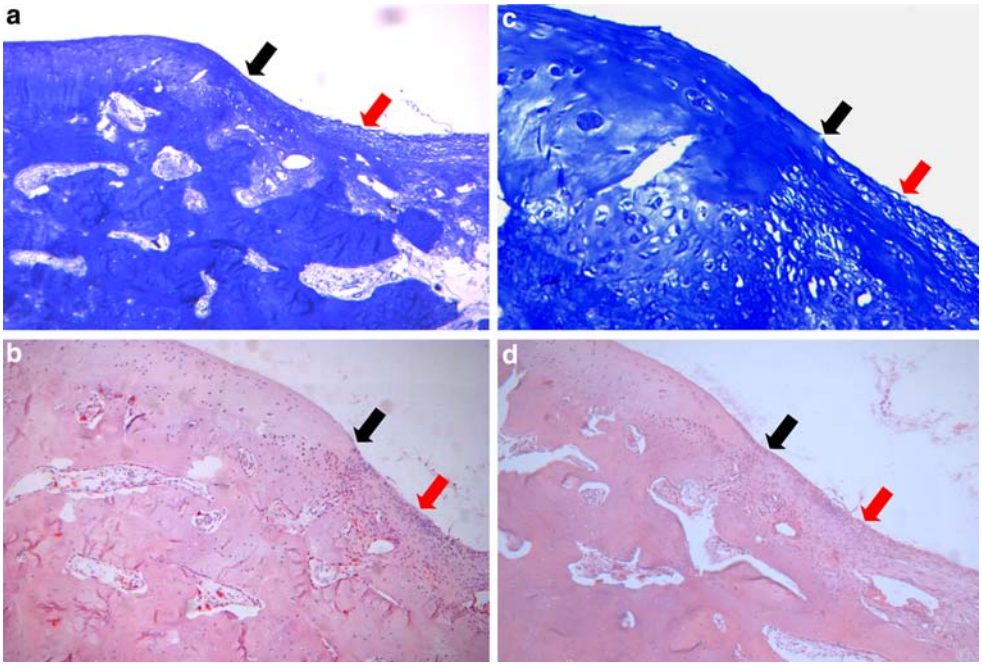

Fig. 7 Light micrographs of selected histology samples 12 weeks after operation (×100). **a**, **b** Distal end of femur defects treated with scaffold of cartilage transfected with BMP-7 gene by Toluidine Blue staining. **c**, **d** Distal end of femur defects treated with scaffold of cartilage by H&E staining. *Black arrows* indicate the interface between repair tissue and native cartilage, and *red arrow* illustrates transplant section

in the 3D collagen-fibrin gel scaffold for 14 days and are chosen as the optimal cultured gene modified chondrocytes in vitro in this study. Active BMP-7 expressed in chondrocytes could promote the differentiation, proliferation of chondrocytes and synthesis of cellular matrix such as GAG and collagen II. Gene-modified chondrocytes cultured in vitro have almost same characteristics as that of normal cartilage tissues. The similarity of the two kinds of chondrocytes facilitated implantation to complete integration of the regenerated cartilage with the surrounding cartilage and underlying bone, and prevented immunological rejection of exogenous materials caused by the invasion of cells from blood, nerve and lymphatic vessels.

Synthesis of GAG is one of the important functions of chondrocytes and plays a significant role in regulating the chondrocyte phenotype. Culturing chondrocytes in the 3D scaffold led to a marked production of GAG, as shown in Figs. 4 and 5. These outcomes of this experiment indicated that cartilage with BMP-7 gene apparently enhance the ability of GAG synthesis.

The current animal model was chosen because the rabbit knee is widely used in experimental cartilage repair studies. Cartilage at the distal end of femur bears more pressure and has greater attrition than any other sites of the body; therefore, they are easily led to joint damage and osteoarthritis over time. Since cartilage defects in a rabbit model within 3.0 mm diameters can be self-repaired [23], it is difficult to create a cartilage defect with dimensions greater than 5.0 × 5.0 × 3.0 mm at the distal end of femur due to the small size of the rabbit knee joint. To limit the impact of this confounding factor, 5 mm defects were chosen in this experiment. It was difficult to fix a tissue-engineered cartilage implant on the distal end of femur that was found in previous studies. A degree of damage to the implant was caused by a state of constant attrition, leading to uneven surfaces of repaired sites, poor fusion with the host and irregular surface by histological examination after a long period of time following the operation. Thus, the distal end of femur was not so suitable for tissue-engineered cartilage implantation therapy. This is the reason why the patellar distal end, which could obtain nutrients from joint synovial fluids and withstand some mechanical pressure, was chosen as experimental model system. In addition, the place of postgraft was protected by the patella, which helps the implant to locate in a sound surface, and prevented some damage caused by surrounding tissues. At the implanted site, chondrocytes could proliferate and differentiate into cartilage tissues. This process is divided into three stages: proliferation, maturation and reconstruction. Active BMP-7 secreted by the gene-modified chondrocytes not only promoted the proliferation of chondrocytes within the tissue-engineered cartilage, but also diffused to surrounding wound surfaces, which resulted in differentiation of mesenchymal cells induced into chondrocytes and proliferation of chondrocytes. It was reported that [6] BMP-7 can also inhibit the immune

response of foreign materials by promoting the synthesis of an extracellular matrix, thus facilitating the repair process. As mentioned previously, current surgical techniques have been developed to stimulate biological repair, include bone marrow stimulation, mosaicplasty and ACI. The latter is the most commonly used cell-based therapy for the treatment of cartilage defects in the young people [22]. However, some potential limitations exist in these procedures such as leakage of transplanted cells, invasive surgical method, hypertrophy of periosteum [9, 15] and loss of chondrogenic phenotype of expanded chondrocytes in monolayer culture [3]. As for the first generation ACI, the newborn regenerated cartilage often consists of fibrous tissues [10, 24] possibly due to limited number of chondrocytes and their low proliferation potential. Bony overgrowth results in thinning of the regenerated cartilage. Furthermore, this method still do damage to healthy cartilage. These aspects limit ACI in the treatment of large defects. The second generation of ACI was born to resolve these problems. Biomaterials such as collagen type I gel, hyaluronan-based scaffold and collagen type I/III membrane were applied to secure cells in the defect site, to restore chondrogenic phenotype by way of 3D culture and to replace the periosteum as defect coverage. There are still NS differences between original and second generation ACI in the short-term clinical outcomes as mentioned by two studies [1, 19].

Conclusion

In summary, there was a tendency for a better quality of repair with the BMP-7 gene treated knees than that of without BMP-7 gene from the tissue score. Especially, the content of GAG, which is essential for the repair of skeletal trauma, was significantly different between BMP-7 gene group and without BMP-7 gene group. Four weeks after implantation, histological evaluation using Toluidine Blue and H&E staining was more intense in gene-modified cartilage than that of non-gene modified cartilage, but was weaker than that of the normal cartilage. It could be due to insufficient repair time or that the implanted material had not reached the differentiation state of normal cartilage tissue. Nevertheless, 12 weeks after implantation, gene tissue-engineered cartilage with transfected BMP-7 gene appeared to have a smooth surface with obvious trabeculas and height equal to normal cartilage, demonstrating a better repair effect than implanted cartilage without BMP-7 gene. These findings support that collagen-fibrin gel scaffold in combination with BMP-7 gene therapy regenerate superior cartilage in rabbit's knee. This new approach is preponderant comparing to other methods and may open novel treatment avenues toward repairing cartilage defects. However, the technique is still in the state of preclinical study, and comparative clinical study with other surgical study is needed to evaluate the effectiveness of BMP-7-based therapy for cartilage repair.

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Conflicts of interest The authors have no conflicts of interest to declare in relation to this article.

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