Biotechnology and Applied Biochemistry

TGF-β1 gene-engineered mesenchymal stem cells induce rat cartilage regeneration using nonviral gene vector

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Abstract.

This study evaluated the potential of utilizing transfected *pTGFβ-1* gene-engineered rat mesenchymal stem cells (MSCs) using nonviral vector to promote cartilage regeneration. Pullulan–spermine was used as the nonviral gene vector and gelatin sponge was used as the scaffold. MSCs were engineered with *TGF-β1* gene with either the three-dimensional (3D) reverse transfection system or the two-dimensional (2D) conventional transfection system. For the 3D reverse transfection system, pullulan–spermine/*pTGF-β1* gene complexes were immobilized to the gelatin sponge, followed by the seeding of MSCs. Pullulan–spermine/*pTGF-β1* gene complexes were delivered to MSCs cultured in the plate to perform the 2D conventional transfection system, and then MSCs were seeded to the gelatin sponge. Then, *TGF-β1* gene-transfected MSC seeded gelatin sponge was implanted to the full-thickness cartilage defect. Compared with the control group, both groups of *TGF-β1* gene-engineered MSCs improved cartilage regeneration through optical observation and histology staining. So, with pullulan–spermine as the nonviral vector, *TGF-β1*-gene engineered MSCs can induce cartilage regeneration *in vivo*.

Volume 59, Number 3, May/June 2012, Pages 163–169 •
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Keywords: cartilage defect, nonviral gene vector, reverse transfection, three dimensional, mesenchymal stem cells

1. Introduction

Articular cartilage is vulnerable to injuries but it has limited self-repair ability because of the sparse distribution of highly differentiated, nondividing chondrocytes, low supply of progenitor cells, and lack of vascular supply [1]. A variety of methods have been developed to treat injured cartilage and can be mainly classified into repair, reconstruction, and regeneration techniques [2]. The repair technique, such as subchondral drilling and microfractures, helps to form fibrocartilaginous tissue by stimulating the bone marrow and facilitating the access of blood vessels and osteoprogenitor cells [3]. Reconstruction methods are to fill the defect with autologous articular cartilage (osteochondral autograft transfer, mosaicplasty) transplantation or allografts. Regenerative methods develop hyaline cartilage tissue by bioengineering techniques (autologous chondrocyte implantation [ACI] and mesenchymal stem cells [MSCs]) [4]. Although good results have been reported with ACI, it suffers from the limited supply of the chondrocytes, and chondrocytes can dedifferentiate into fibroblasts when cultured *in vivo* [5]. Compared with the autologous chondrocytes, bone marrow-derived MSCs are easier to obtain and can be manipulated for multiple passages [6],[7]. However, as MSCs are scarcely distributed in the bone marrow and are often not powerful therapeutically, they are always expanded and genetically modified *in vitro* before they are introduced to the body [8]. For chondrogenic induction of adult MSCs, cells are collected into high-density pellets or in appropriate 3D biomaterials and treated with specific growth factors. Growth factors most commonly used for chondrogenic differentiation of MSCs belong to the TGF-β superfamily [9]. *In vitro* and *in vivo* studies have shown that TGF-β1 stimulates chondrogenesis, chondrocyte proliferation, and prevents cartilage hypertrophy [10].

In this study, we used the nonviral vector, which has several advantages, such as the ease of synthesis, cell/tissue targeting, low immune response, and unrestricted plasmid size, and can be a good alternative for gene transfection compared with the viral vector [11]. In the previous study, we

Abbreviations: MSCs, mesenchymal stem cells; H&E, hematoxylin and eosin; β-TCP, β-tricalcium phosphate; DMEM, Dulbecco's modified Eagle's medium.

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Received 7 September 2011; accepted 10 January 2012
DOI: 10.1002/bab.1001
Published online 4 March 2012 in Wiley Online Library (wileyonlinelibrary.com)

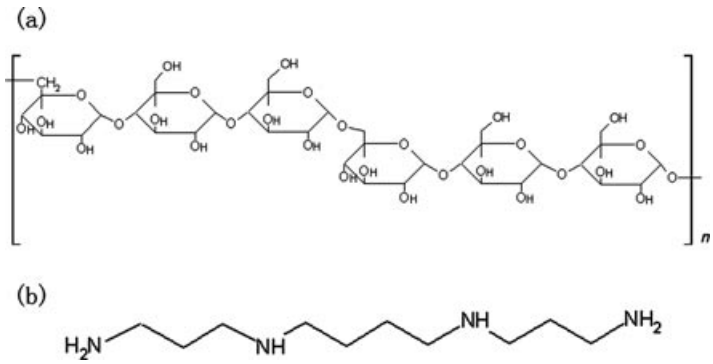

Fig. 1. Chemical structure of pullulan (a) and spermine (b).

successfully developed a transfection system based on the reverse transfection and three-dimensional (3D) transfection system using pullulan–spermine (Fig. 1) as the nonviral gene vector [12]. In reverse transfection, pullulan–spermine/gene complexes are first fixed to the 3D scaffolds before the cells are seeded, whereas in the conventional transfection, cells are seeded on the culture plates for complete attachment prior to the addition of gene complexes. As compared with the conventional transfection method, which is serum resistant for nonviral gene delivery to various cell types, reverse transfection induces no decrease of the transgene levels with the addition of serum. Also, 3D scaffold provides a good environment for cell proliferation and shows enhanced gene expression as compared with the two-dimensional (2D) system in the long run.

2. Materials and methods

2.1. Preparation of gelatin sponge

Gelatin sponges were prepared according to the methods reported by Takahashi et al. [13]. Briefly, 4.29 wt% aqueous solution of gelatin was mixed with β-tricalcium phosphate (β-TCP) at 5,000 rpm at 37 °C for 3 Min by using a homogenizer (ED-12; Nihonseiki Company, Tokyo, Japan). After addition of 2.17 wt% of glutaraldehyde aqueous solution, the mixed solution was further mixed for 15 Sec by the homogenizer. The resulting solution was cast into a polypropylene dish followed by leaving it at 4 °C for 12 H for gelatin cross-linking. Then, the cross-linked gelatin hydrogels with β-TCP were placed into 100 mM of aqueous glycine solution at 37 °C for 1 H to block the residual aldehyde groups of glutaraldehyde. Following complete washing with double-distilled water, the hydrogels were freeze-dried and cut into cubes of $5 \times 5 \times 3$ mm^3.

2.2. Characterization of gelatin sponge

The gelatin sponges were sputter coated with gold for scanning electron microscope (SEM) observation (JSM 6500F; JEOL, Japan). The average pore size was measured by calculating the average pore size of five SEM images. The porosity of sponges was calculated from the weight before and after swelling in phosphate-buffered saline (PBS) for 24 H. The weight of the sponge before swelling was set as *a*, and the weight of that after swelling was set as *b*. The porosity can be expressed as $(b - a)/b$ [14].

In vitro compression resistance of freeze-dried gelatin sponges was evaluated by measuring their compression moduli at a rate of 1 mm/Min (AG-5000B; Shimadzu, Kyoto, Japan). The load-deformation curve was obtained and the compression modulus of sponges was calculated from the initial slope of load-deformation curve. Measurement was done five times for each sample to calculate the average value and the SD of the mean [13].

2.3. Gene transfection

Mesenchymal stem cells were isolated from the bone shaft of femurs of 3-week-old male Sprague–Dawley rats as described in the previous study [12]. Briefly, both ends of rats' femurs were cut off from the epiphysis, and the bone marrow was flushed out using a syringe (21 gauge needle) with 1 mL of Dulbecco's modified Eagle's medium (DMEM). The cell suspension was placed into two 25 cm^2 flasks and cultured in 10 mL of DMEM supplemented with 10% fetal calf serum (FCS), L-glutamine, penicillin (50 U/mL), and streptomycin (50 U/mL) at 37 °C in 5% CO_2. The medium was changed on day 4 of the culture and every 3 days thereafter. When the cells of the first passage became subconfluent (usually 7–10 days after seeding), the cells were detached from the flask after treatment with PBS solution containing 0.25 wt% trypsin and 0.02 wt% ethylenediaminetetraacetic acid (EDTA). MSCs were subcultured with DMEM supplemented with 10% FCS, L-glutamine, penicillin (50 U/mL), and streptomycin (50 U/mL) and MSCs at the third passage were used in this study. For 2D conventional transfection, MSCs were seeded on plates (100 mm diameter) and incubated at 37 °C in 5% CO_2 for 24 H. After this, the medium was replaced with DMEM containing pullulan–spermine/*pTGF-β1* gene complexes. Pullulan–spermine were prepared using an *N*,*N*′-carbonyldiimidazole (CDI) activation method as previously reported [12]. The molar extent of spermine introduced to the hydroxyl groups of pullulan was 12.3 mol%. After 6 H incubation, the complex was removed and the cells were further incubated in DMEM with 10% serum and further cultured for 18 H.

For reverse 3D transfection, the gelatin sponges were coated with the anionic gelatin, pronectin and pullulan–spermine/pDNA complex (pDNA coding for luciferase [pGL3]) or pTGF-β1). After the coating of the complex, 100 μL of MSCs (1×10^6 cells per scaffold) were seeded to the scaffold and incubated for 2 H for cell attachment. Then, 2 mL of medium was added slowly to the plate wells and cultured for 24 H.

2.4. Cartilage defect model and treatment

Sprague–Dawley male rats were supplied by Zhejiang University Experimental Animal Center, People's Republic of China. All animals were maintained under constant conditions (temperature 25 ± 1 °C) and had free access to a standard diet and drinking water. All of the experimental procedures were in accordance with the Zhejiang University guidelines for the welfare of experimental animals. The male rats with the body weight of 180 g were anesthetized by the intraperitoneal injection of 10%

chloral hydrate (4 mL/kg). A full-thickness cartilage defect (2 mm in diameter and 3 mm in depth) was created through the articular cartilage and into the subchondral bone of the patellar groove in rats using an electric drill equipped with a 2-mm-diameter drill bit.

Twenty-four rats were divided into six groups. In group A, healthy rats were used as the control. The full-thickness cartilage defects were created as described above in both of the two knees of the remaining 20 rats. Defects were not filled in group B as an empty control. In group C, the defects were implanted with gelatin sponges incorporating pullulan–spermine/*pTGF-β1* gene complexes. In group D, MSCs were transfected with the reverse 3D transfection method using *pGL3* as the plasmid, and implanted into the defect after 24 H *in vitro* culture. For group E, MSCs were similarly treated as group D, but using *pTGF-β1* as the plasmid. In group F, MSCs were transfected with the conventional 2D transfection method as described above, and then detached from the flask after treatment with PBS solution containing 0.25 wt% trypsin and 0.02 wt% EDTA and seeded into the gelatin sponge. After *in vitro* culture in the gelatin sponge for 24 H, the MSC-seeded scaffold was implanted into the defect.

2.5. Surface observation

The rats were sacrificed 2 months after the treatment. The entire knee was dissected and examined macroscopically and photographed.

2.6. Histology staining

The rats were anesthetized to death 2 months after the treatment. All knees were retrieved and washed in PBS, fixed in 4% phosphate-buffered paraformaldehyde at 4 °C for 24 H, decalcified in formic acid–hydrochloric acid–ammonium chloride solution for 1 week, dehydrated through a graded series of ethanol, embedded in paraffin, and sectioned at 10-mm thickness on the slides. For histological analysis, slides were stained with hematoxylin and eosin (H&E), Safranin O and Alcian blue. Images were acquired with a light microscope [15–17].

3. Results

3.1. Characterization of gelatin sponge

As seen from the SEM images (Fig. 2), the average pore size of the gelatin sponge was 315 nm, with the porosity around 96.7%, which was reported to be suitable for cell infiltration, proliferation, and the flow of metabolites [18],[19]. The mechanical property of the gelatin sponge was weak, with the compression modulus of 0.149 MPa, which was a common weakness of the natural derived materials.

3.2. Macroscopic appearance of the defect area

Animals recovered quickly from the bilateral surgical procedure and regained full movement within 2 weeks. Two months after implantation, the cartilage defect area of all the groups were filled with the repair tissue with a color similar to that of the surrounding normal cartilage tissue (Fig. 3). The surface of the cartilage treated with *pTGF-1* gene-transfected MSCs using the conventional method (group f) appeared smooth and well incorporated with the adjacent host cartilage, and there was not much difference in the macroscopic appearance of the defects compared with the healthy rats (control group a). The surface of the defects treated with cell-free *pTGF-β1*-incorporated scaffold (group c) and reverse 3D-transfected MSCs with *pTGF-β1* gene(group e) appeared smoother than control group b, but the repaired tissue still showed an irregular surface. The surface of the repair tissue in group d that was treated with *pGL3*-transfected MSCs appeared rough and uneven, and severe inflammation could be observed.

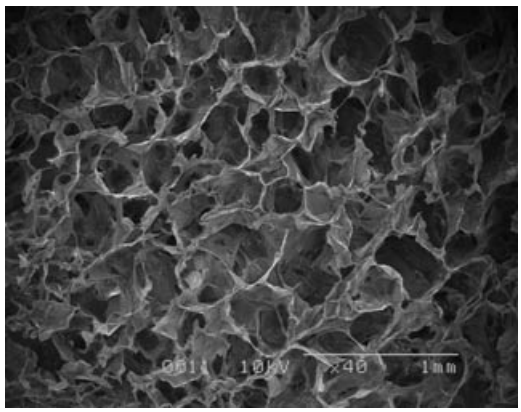

Fig. 2. SEM image of the gelatin sponge.

3.3. Histology staining

H&E staining revealed that the surface of the defects treated with *pTGF-1* gene transfected MSCs using the conventional method (group f) and MSC seeding in *pTGF-β1*-incorporated gelatin sponge (group e) appeared smoother than control groups b, c, and d, but the repaired tissue still showed an irregular surface. Meanwhile, some chondrocytes-like cells could be found in groups f and e. The articular surface appeared to be more irregular and the chondrocytes cells were scarcely distributed in the defect area of groups b, c, and d (Fig. 4). The surface of the repair tissue in group d, which was treated with *pGL3*-transfected MSCs, appeared rough and uneven, and severe inflammation could be observed.

Groups f and e contained MSCs transfected with *TGF-β1* gene, whereas groups b and d were *TGF-β1* gene free, indicating the important role of therapeutic gene playing in the treatment. Although no exogenous MSCs were implanted in group c, it still showed better cartilage regeneration than groups b and d.

Safranin-O staining and Alcian blue staining can show that accumulation of sulfated glycosaminoglycans and the distribution of the cartilaginous matrix [6],[20]. As seen from Figs. 5 and 6, the thicker repaired cartilaginous layer could be observed in groups e and f rather than groups b, c, and d.

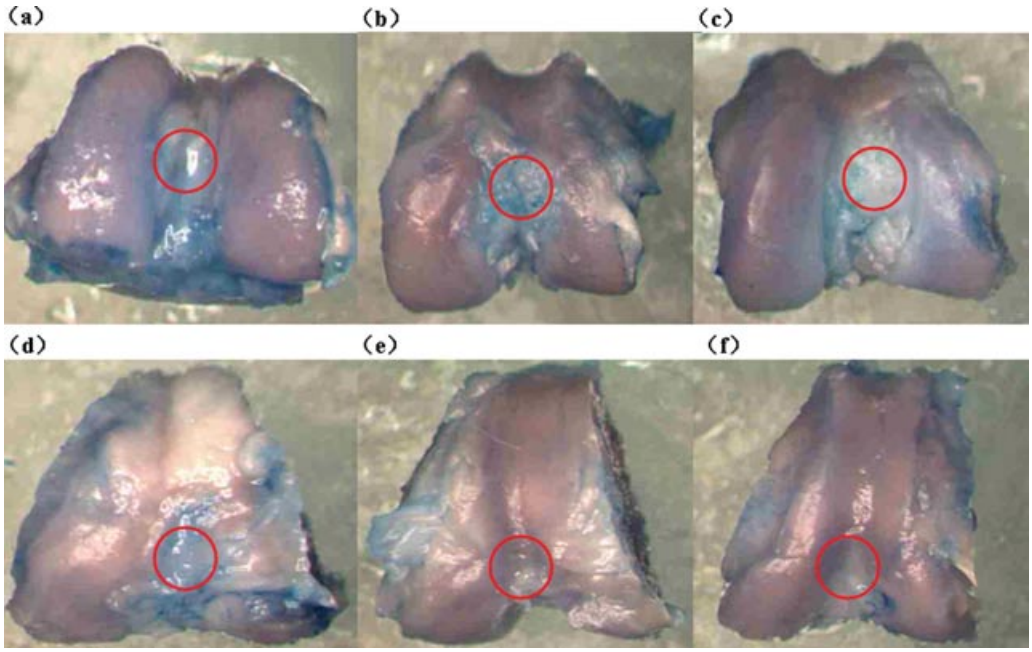

Fig. 3. Macrophotographs of the articular cartilage repair *in vivo* taken after 2 months. (a) No-treatment control group. (b) Nothing in articular cartilage defect. (c) *pTGF-β1*-incorporated scaffold group. (d) MSC-seeded in *pGL3*-incorporated gelatin sponge group. (e) MSC seeded in *pTGF-β1*-incorporated gelatin sponge group. (f) *TGF-β1*-transfected MSC-seeded gelatin sponge group.

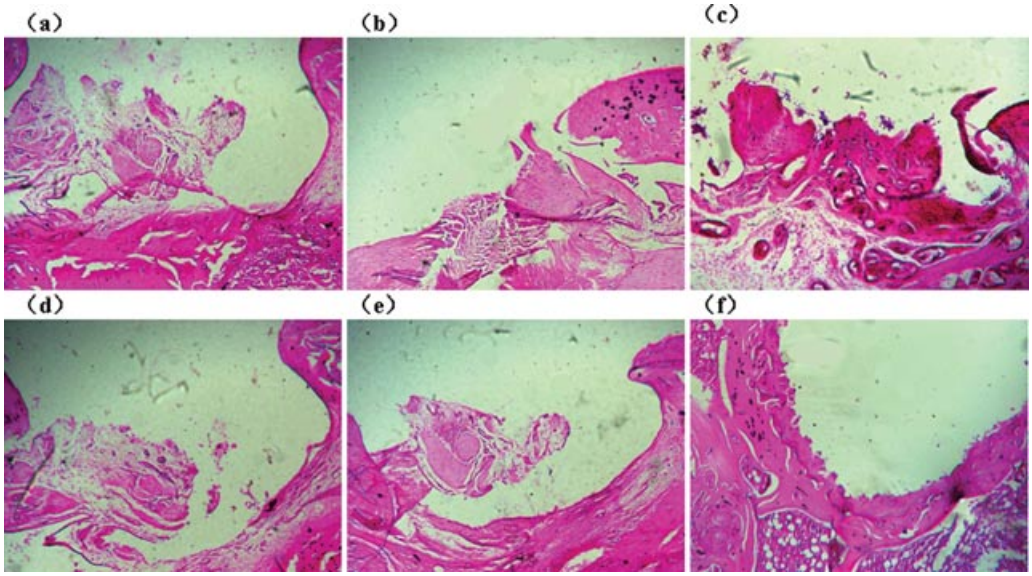

Fig. 4. H&E staining of the cartilage repair *in vivo* taken after 2 months. (a) No-treatment control group. (b) Nothing in articular cartilage defect. (c) *pTGF-β1*-incorporated scaffold group. (d) MSC seeded in pGL3-incorporated gelatin sponge group. (e) MSC seeded in *pTGF-β1*-incorporated gelatin sponge group. (f) *TGF-β1*-transfected MSC-seeded gelatin sponge group.

4. Discussion

Cell source, signaling molecules, and scaffolds are important parameters for cartilage tissue engineering. MSCs, a kind of multipotent stem cells, are used in the present study because MSCs can overcome the limited supply of primary chondrocytes and be manipulated for multiple passages as well. Members of the TGF-β superfamily play a major role in cartilage development and repair. A variety of studies have reported that TGF-β1 can promote chondrogenesis of MSCs [21–23]. The sustained release of growth factors is desirable, but the half-life of the growth factor is short [24]. Recent strategies focusing on ways

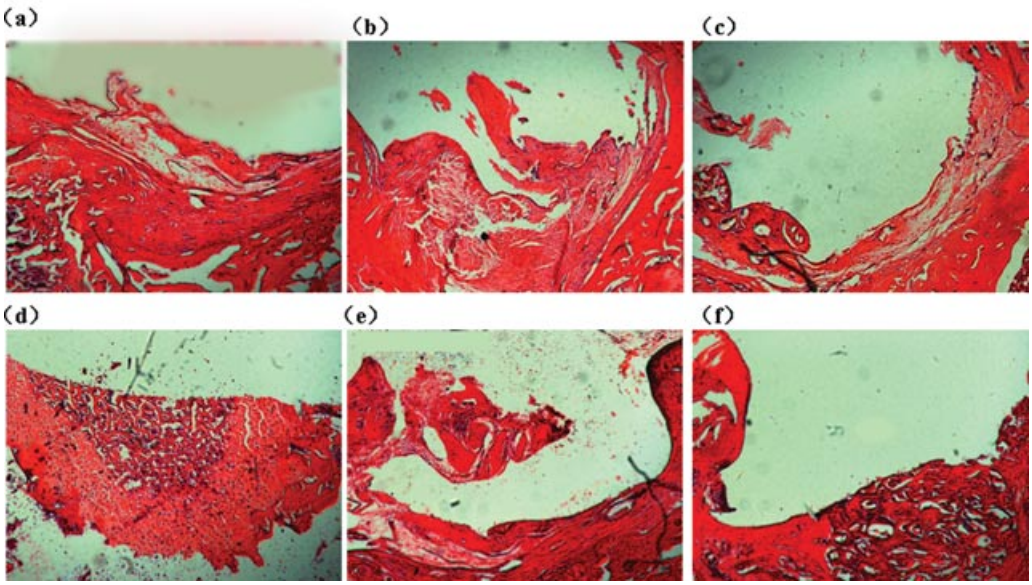

Fig. 5. Safranin-O staining of the cartilage repair *in vivo* taken after 2 months. (a) No-treatment control group. (b) Nothing in articular cartilage defect. (c) *pTGF-β1*-incorporated scaffold group. (d) MSC seeded in *pGL3*-incorporated gelatin sponge group. (e) MSC seeded in *pTGF-β1*-incorporated gelatin sponge group. (f) *TGF-β1*-transfected MSC-seeded gelatin sponge group.

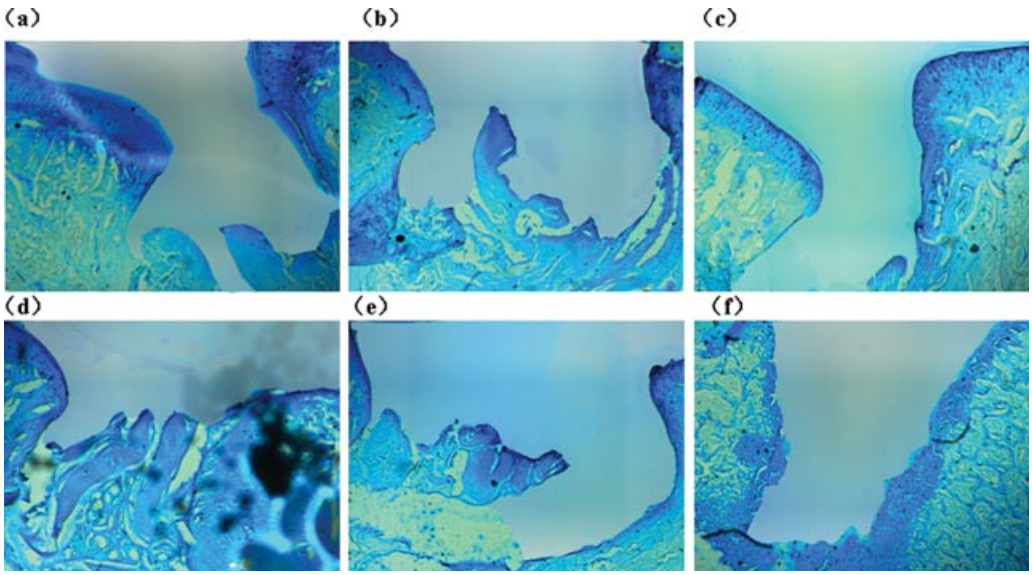

Fig. 6. Alcian blue staining of the cartilage repair *in vivo* taken after 2 months. (a) No-treatment control group. (b) Nothing in articular cartilage defect. (c) *pTGF-β1*-incorporated scaffold group. (d) MSC seeded in *pGL3*-incorporated gelatin sponge group. (e) MSC seeded in *pTGF-β1*-incorporated gelatin sponge group. (f) TGF-β1-transfected MSC-seeded gelatin sponge group.

to temporally control the release of bioactive growth factors for enhancing MSC chondrogenic differentiation include incorporation of factor-loaded microparticles into the scaffolds, covalent binding, or bioconjugating the factors to the matrix [25],[26]. Transfection of MSCs with growth factor genes, which can result in endogenous *in situ* constitutive production of growth factor, is an alternative for devising the controlled-release system of growth factor. Recently, Chen et al. formulated a bilayered gene-activated osteochondral scaffold consisting of *pTGF-β1*-activated chitosan–gelatin scaffold for chondrogenic layer and pBMP-2-activated hydroxyapatite/chitosan–gelatin scaffold for osteogenic layer. MSCs seeded in each layer of the bilayered gene-activated scaffold showed high expression of TGF-β1 and BMP-2, respectively, and could simultaneously support the articular cartilage and subchondral bone regeneration in the rabbit knee ostochondral defect model [27]. In another study, MSCs were transfected with *pTGF-β1* in monolayer culture using the liposome as the vector (Lipofectamine 2000, Invitrogen, USA),

Table 1
Summary of the cartilage defect treatment experiment

Group	Number of rats	Description	Results
A	4	Healthy (control)	Healthy
B	4	Defects without treatment (empty control)	Rough surface; chondrocytes cells scarcely distributed in the defect area; thin repaired cartilaginous layer
C	4	Defects implanted with pullulan–spermine/p*TGF-β1* gene complexes incorporating gelatin sponges	Smoother surface compared with group B, but still showed irregular surface; chondrocytes cells were scarcely distributed in the defect area; thin repaired cartilaginous layer
D	4	Defects implanted with gelatin sponge and MSC transfected with *pGL3* using reverse 3D transfection method	Surface appeared rough and uneven with severe inflammation; chondrocytes cells were scarcely distributed in the defect area; thin repaired cartilaginous layer
E	4	Defects implanted with gelatin sponge and MSC transfected with *pTGF-β1* using reverse 3D transfection method	Smoother surface compared with group B, but still showed irregular surface; some chondrocytes-like cells could be found; a thicker repaired cartilaginous layer
F	4	Defects implanted with gelatin sponge and MSC transfected with *pTGF-β1* using the conventional transfection method	Surface appeared smooth and well incorporated with the adjacent host cartilage; some chondrocytes-like cells could be found; a thicker repaired cartilaginous layer

then seeded into chitosan scaffolds, and finally implanted into the full-thickness articular cartilage defects of rabbits' knees. Twelve weeks after implantation, the defects were filled with regenerated hyaline-like cartilage tissue [28]. Herein, we use pullulan–spermine, a kind of cationic polymer prepared by conjugation of pullulan and spermine, as the gene vector to transfect MSCs because of its high transfection capability and low toxicity at the proper ratio [12].

The main factors need to be considered in scaffold design are pore size, porosity, and mechanical properties. Gelatin is denatured collagen product, and has been widely used in the field of medicine with its biodegradability [29–32]. In addition, some experiments show that coating the surface of the cell culture dish can promote cell attachment and proliferation [33],[34]. So, gelatin sponge was prepared for the *in vivo* implantation as a scaffold because of its favorable biocompatibility and biodegradability. Pore size of the scaffold affects the waste and nutrient exchange, and porosity is important in providing a large area for cell adhesion. So, the pore size and porosity of the gelatin sponge were investigated to prepare a porous gelatin sponge with the pore size large enough. The biomechanical properties of the scaffold should be similar to that of the native tissue because the support of the scaffold plays an important role in the initial stage. However, scaffolds made of natural materials typically suffer from inferior mechanical strength. Although β-TCP was added during the fabrication of the gelatin sponge, the mechanical strength still did not improve. The synthetic materials, which have the advantage of easy control and modification, could be combined with the natural materials to tailor the hybrid scaffold with favorable mechanical properties, and we will try this in future study.

By observing the surface of the defects with the microscope, the situation of cartilage repair can be grossly assessed. But, as the interior composites of defects cannot be revealed, the quality of repaired cartilage was evaluated by cartilage-specific extracellular matrix in defect with histological staining.

Our previous studies have investigated the transgene expression using either conventional transfection or the reverse 3D transfection method. We found that the expression level of the conventional transfection group was higher than that of the reverse 3D transfection group on the 5th day after transfection [12]. It was also found that 3D scaffold, which can provide a good environment for cell proliferation, showed enhanced *in vitro* gene expression in the long run as compared with the 2D system. In the present study, the MSCs in group f transfected with the conventional method were seeded into the 3D scaffold 24 H after being transfected, and then implanted to the defect after another 24 H *in vitro* culture, indicating that MSCs here need not suffer from the limited space of a 2D system. Thus, it is shown that group f displayed a better repair effect than group e because the transfection efficiency of the conventional 2D transfection was higher than that of the reverse 3D transfection; similarly, MSCs in group f share a similar culture environment with the 3D system in group e.

pGL3 was used in group d as a control plasmid to demonstrate whether the repair effect of group e was a function of *pTGF-β1*. As summarized in Table 1, MSCs transfected with a report gene (group d) did not show the potential to repair the defects. It is hypothesized by us that cells in the implantation site transfected by *TGF-β1* gene could release TGF-β1, which can stimulate the autologous mesenchymal cells to differentiate into chondrocytes cells. Further studies need to be carried out to demonstrate this hypothesis. Some researchers have done studies investigating *in vivo* plasmid delivery at other sites and have typically identified cell types such as macrophage fibroblasts, and endothelial cells as the primary cell responsible for expressing the plasmid [35–37]. In the Salvay et al. [38] study, *in vivo* implantation of the DNA-incorporated scaffolds at the subcutaneous site can induce long-term transgene expression localized to the site of implantation that persisted. Histological analysis indicated that both macrophages and muscle cells adjacent to the implant were transfected.

5. Conclusions

Our results demonstrate that *TGF-β1* gene-engineered MSCs using nonviral vector and 3D reverse transfection system are beneficial for *in vivo* cartilage tissue engineering and have the potential for clinical application in cartilage regeneration.

Acknowledgements

This work was financially supported by National Natural Science Foundation of People's Republic of China (30873173 to Jian-Qing Gao, 81001410 to Yu-Lan Hu), Fundamental Research Funds for the Central University (81001410), Zhejiang Provincial Natural Science Foundation of People's Republic of China (R2090176), People's Republic of China–Japan Scientific Cooperation Program (81011140077) supported by both NSFC (People's Republic of China) and JSPS (Japan), and Zijin Program of Zhejiang University (188020-544802[78]).

We thank Ms. Hong-Yu Lu for technical assistance. We thank Yang Zhou from Iowa State University for polishing this article.

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