

# Lentiviral-mediated multiple gene transfer to chondrocytes promotes chondrocyte differentiation and bone formation in rabbit bone marrow-derived mesenchymal stem cells

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**Abstract.** The aim of the present study was to provide a theoretical and experimental foundation on the differentiation of stem cells through the induction of multiple genes. The lentiviral vector carrying *TGF-β1* and *IL-10* genes was transfected to bone marrow-derived mesenchymal stem cells (BMSCs) which differentiated into chondrogenesis. Healthy New Zealand white rabbits, 2-3 months of age were used in the present study. A 6-8 ml of bone marrow was isolated from the iliac and tibial shaft of each rabbit. The BMSCs suspension was aspirated following centrifugation of the bone marrow by percoll separating medium. The BMSCs were primarily cultured and subcultured *in vitro*, then divided into four groups according to the difference of lentivirus vectors: group A, receiving transforming growth factor β1 (TGF-β1); group B, receiving TGF-β1 and Interleukin-10 (IL-10); group C, empty vector transfection; and group D, receiving no cell growth factor. Fluorescence expression was detected 12 h after transfecting the lentiviral vector carrying the *TGF-β1* and *IL-10* gene to BMSCs. The transfection efficiency was approximately 70% with a MOI=100 after 96 h. Expression of SOX-9 aggrecan and Type II collagen in groups A-E on day 7 and 14 was detected by RT-PCR and western blot analysis. The expression level of three genes expressed in groups A and C were higher compared to the expression in groups B, D and E. The expression level of

the three genes expressed in group B was higher compared to the expression in group D. The expression level of three genes expressed in group A and C showed no statistical difference. Cytokines therefore play an important role in cell proliferation and chondrogenic differentiation. TGF-β1 has a synergistic effect in the differentiation. In addition, IL-10 may have a protective role in the restoration of cartilaginous tissue.

## Introduction

Damage to articular cartilage is the source of considerable disability in the form of arthritis and trauma (1). Adult articular chondrocytes lack an effective intrinsic capacity for self-repair. Loss of articular cartilage substance results in damage that is generally permanent and often progressive (2). A number of studies have shown that TGF-β1 can induce mesenchymal stem cells (MSCs) to differentiate into cartilage cells, promote the proliferation and maturation of the cartilage cells, and enhance the synthesis of the cartilage cells and the secretion of proteoglycan and type II collagen. It is optimal to induce the differentiation of stem cells into cartilage cells (3). Cytokine IL-10 was identified in 1989 by Fiorentino *et al* (4) as an immune mediator that is secreted by mouse type 2 T-helper cell clones (Th2) and inhibits the synthesis of IL-2 and interferon (IFN) in Th1 cell clones. Since it was initially identified, IL-10 has been extensively investigated. The key function appears to be the inhibition of innate and acquired immune responses by means of suppression of monocyte/macrophage activity as well as inhibition of the development of effector T-cells. In addition to these effects, IL-10 regulates the function and/or differentiation of B cells, NK cells, mast cells, granulocytes, dendritic cells and some connective tissue cell types (5). Accumulating evidence has identified the role of IL-10 in regulating connective tissues homeostasis and extracellular matrix (6-8). Cartilage, a typical connective tissue that mainly consists of an abundant collagen type II-rich and proteoglycan-rich extracellular matrix, is synthesized by chondrocytes. Only 3-5% of the cartilage volume is represented by chondrocytes, the single cell type in cartilage that is responsible for maintaining extracellular

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matrix integrity. Chondrocytes are protected from the immune system, because they are separated from exogene stimuli by their encapsulation with a dense impenetrable extracellular cartilage matrix. However, chondrocytes are highly activated by cytokines. Although previous findings have identified a protective role of IL-10 in the inflamed joint, the particular impact of IL-10 on cartilage and chondrocyte biology is poorly understood. Under inflammatory conditions, IL-10 may exhibit immunoregulatory effects on the activated chondrocytes in articular cartilage.

Regional gene therapy has the potential to be developed as one aspect of a comprehensive tissue engineering strategy to manage a variety of bone repair scenarios (15-17). The advantages of regional gene therapy are the duration of protein production that can be determined by the vector selected and theoretically multiple transgenes that may be delivered to a specific anatomic site (15-19). Lentivirus is a common transgenic carrier used in laboratories due to its high infection efficiency and stable expression of transfected genes (20-22). It was previously reported that the treatment effect of the Lv-BMP-mediated hBMP2 gene that was overexpressed in osteoporotic rBMSCs formed by *in vitro* genetic infection on local osteoporosis was more beneficial than that performed by *in vivo* genetic infection (9). Focus has been directed towards the development of alternative vectors, such as lentivirus, which is based on the human immunodeficiency virus (11). Lentivirus is known to transduce dividing and non-dividing cells and incorporate them into the host genome (ref?). Furthermore, lentiviral vectors have demonstrated high levels of stable gene transfer and target gene expression (10). Additionally, minimal immunogenicity is associated with the lentiviral vectors (11), a significant advantage over gene therapy systems involving adenovirus. However, similar to adenoviral vectors, the safety profile of lentivirus needs to be addressed before these vectors are to be used to treat patients with non-lethal conditions (12).

In the present study, we investigated the role of TGF- $\beta$ 1, IL-10 and IL-1 $\beta$  in chondrocyte differentiation using New Zealand white rabbits and lentiviral vectors carrying multiple genes transfected to BMSCs.

## Materials and methods

**Isolation, culturing and induction of rBMSCs.** Bone marrow was harvested from New Zealand white rabbits aged 2-3 months and weighing 1.8-2.4 kg under sterile conditions as described in a previous study (?). The two ends of the femora were separated at the epiphysis and the marrow was washed using low glucose Dulbecco's modified Eagle's medium ( $\alpha$ -MEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT, USA). To obtain a single-cell suspension, the marrow was gently kneaded in L-DMEM containing 10% FBS. Cell activity prior to cell culturing was analyzed using trypan blue (Guangzhou Genebase Bioscience Co., Ltd., Guangzhou, China) staining. The percentage of cell activity was calculated as per the formula: cell activity (%) = [(total cells - stained cells)/total cells] x 100%. The percentage of cell activity was >96% in all the groups. Experimental procedures were carried out at a facility accredited by the Institutional Animal Care and Use Committee of Shandong University. The culture medium was

initially changed on the 4-5th day after which non-adherent cells were discarded. After 90% confluence was reached, the BMSCs were excreted from the culture substratum using trypsin/EDTA (0.25% w/v trypsin, 0.02% EDTA) (Hyclone) and were transferred to the culture dishes (10 cm in diameter) at  $1.0 \times 10^5$  cells/ml in 10 ml. The characterization of rabbit BMSCs was confirmed using a flow cytometry assay, as described in a previous study (?).

**Transduction of rBMSCs with lentivirus-delivered IL-10.** The full-length coding sequence of IL-10 [1306 base pairs (bp), accession no: NM\_010548.2] was cloned into a lentivirus-based plasmid construct (pHR'CS-IL-10). A vesicular stomatitis virus glycoprotein pseudotyped lentiviral vector was generated by transient transfection of three plasmids into human embryonic kidney 293T cells with Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The viral vector was examined by Southern blot analysis on genomic DNA isolated from infected U2OS cells.

After the multiplicity of infection (MOI) and the infection efficiency were determined, the lentivirus hosting the PHR'CS-IL-10 was introduced into MSCs at a MOI of 10 in  $\alpha$ -MEM for 24 h with 10  $\mu$ g/ml polybrene (Sigma-Aldrich, St. Louis, MO, USA). Infected MSCs were washed twice after 12 h, and bright green fluorescence was observed under an inverted fluorescence microscope (Leica, Mannheim, Germany). The culture supernatant containing secreted IL-10 was examined for 7 days. IL-10 expression was validated further by an enzyme-linked immunosorbent assay (ELISA).

**ELISA for TGF- $\beta$ 1 and IL-10.** TGF- $\beta$ 1 and IL-10 were quantified using an ELISA kit (Quantikine, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The cells were lysed to determine the total protein content for the standardization of TGF- $\beta$ 1/IL-10 and the total protein content was determined using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard. The adherent RBMCs were rinsed twice with 1% PBS and then lysed using a cell culture lysis reagent while still adherent to the culture dish. The lysed cell debris was transferred to a microcentrifuge tube and the content was microcentrifuged for 30 sec. The supernatant was incubated with 100 ml D-luciferin and luciferase activity was quantified on a luminometer. Each sample was repeated in triplicate and the results were reported as luciferase activity/total protein/24 h.

**Toluidine blue staining for cartilage cell identification.** The cartilage cells were induced 14 days after transfection. A solution consisting of 70% ethanol and 0.2% toluidine blue was used to stain the cartilage cells overnight. Subsequently, gradient alcohol dehydration was performed, dimethylbenzene was transparent and neutral gum was used for sealing. The cells were fixed in 4% paraformaldehyde for 15 min and observed for the secretion of glycosaminoglycan using an inverted microscope.

**RT-PCR detection of the expression of genes associated with the differentiation into cartilage cells.** The expression of genes associated with the differentiation in cartilage

Table I. PCR primer sequences and product lengths.

Gene	Forward primer	Reverse primer	Length (bp)
<i>Aggrecan</i>	CCTACCAGGACAAGGTCTCG	CCGATAGTGGAAACACAACACC	179
<i>SOX-9</i>	CTGGAGACTGCTGAACGAGAG	CCATTCTTCACCGACTTCCTC	129
<i>Type II collagen</i>	GCCTGTGTCTGCTTTTTCTGT	AAGATTCCAGTTGTTGGGTCA	120
<i>GAPDH</i>	ATGGGGAAGGTGAAGGTCG	TAAAGCAGCCCTGGTGACC	119

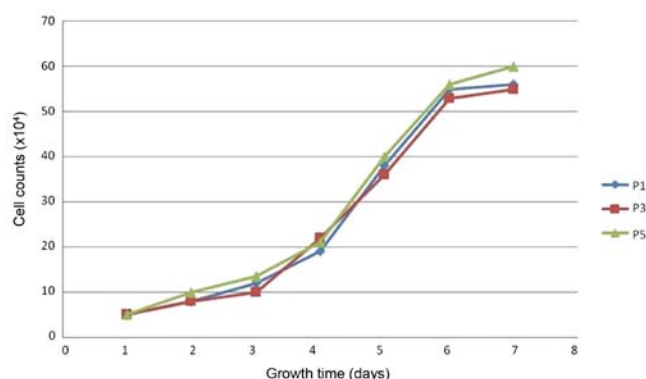


Figure 1. Growth curve of BMSCs of P1, P3 and P5 generation.

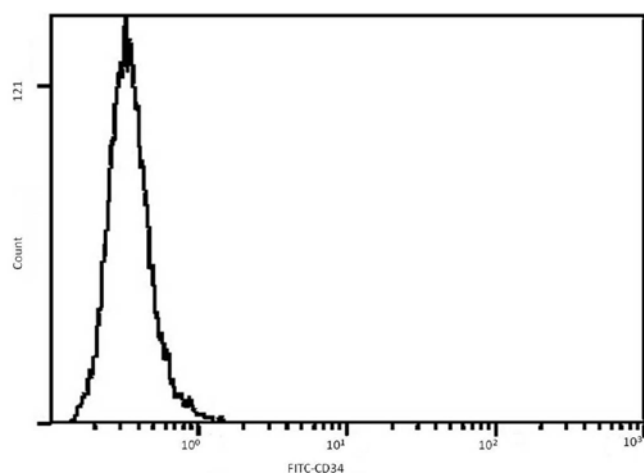


Figure 2. Flow cytometry of FITC-CD34 in the P3 generation.

cells was determined by RT-PCR analysis using standard methods. Total RNA was extracted from cell pellets using the TRIzol reagent and amplified by reverse transcription-PCR (RT-PCR) according to the manufacturer's instructions, respectively. A housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), was used as an endogenous control. *GAPDH* and related genes primers were constructed by using Primer 5 Software and verified by DNA sequencing analysis. *GAPDH* (1382 bp) primers are provided in Table I.

**Western blot detection of type II collagen secreted by cartilage cells.** The cells were washed with PBS three times

and digested by 0.25% trypsin for 2 min on days 7, 14 and 21. The digestion was terminated by the addition of culture medium containing 10% FBS. After blowing for 3 min, the cell suspension was transferred to a centrifugal tube for centrifugation at 1,500 rpm for 3 min. The supernatant was discarded and Trizol was added to extract the total protein of the digestive cells. *GAPDH* was used as positive reference. The 10% SDS-PAGE (Bio-Rad vertical electrophoresis system) was conducted under the conditions of 10  $\mu$ l sample, 110 V and 60 min. Then 15 V was applied for trans-blotting for 70 min (Bio-Rad Trans-blot SD semi-dry transfer). Anti-rabbit type II collagen polyclonal antibody (Calbiochem, San Diego, CA, USA) of 5  $\mu$ l was diluted with 5 ml 10% TBST 1:1,000. The cells were incubated at 4°C overnight. After being washed with 10% TBST three times (5 min/time), 1:1,000 mouse anti-rabbit IgG was added. After 1 h of incubation, the cells were washed by 10% TBST three times (5 min/time). ECL staining and scanning were conducted for quantitative analysis.

**Statistical analysis.** Quantitative results were presented as mean  $\pm$  standard deviation (SD). Experiments were performed in triplicate. Independent sample t-test and one way analysis of variance (ANOVA) followed by Student Newman Keuls (SNK) post hoc analysis were performed with SPSS, v.13.0. Values were considered statistically significant at  $P < 0.05$ .

## Results

**Growth curve of rabbit BMSCs.** BMSCs of P1, P3 and P5 generation were counted and a growth curve was drawn. The results showed that the first 2 days were the slow growth period, while 3-6 days were the rapid growth period and on the 7th day it entered the platform period (Fig. 1).

**Detection of cell surface markers on rabbit BMSCs.** Flow cytometry was used to detect cell surface markers on BMSCs of P3. The positive rate of CD34 was 2.0% (Fig. 2) and of CD44 was 97.6% (Fig. 3).

**Determination of the viral titer of 293T cells.** 293T cells were plated and ~60-70% of the cells were fused on the following day. Subsequently, the virus buffer was added and the cells were observed under the microscope after 24 h. Only viable cells were observed in each well using visible light. Fluorescence expression was detected after 96 h of growth (Fig. 4). Fluorescence microscopy showed that the number of fluorescent cells decreased with the corresponding increase of the dilution ratio of the virus buffer. The viral titers of the viral stock solution (TGF- $\beta$ 1 and IL-10) in the last

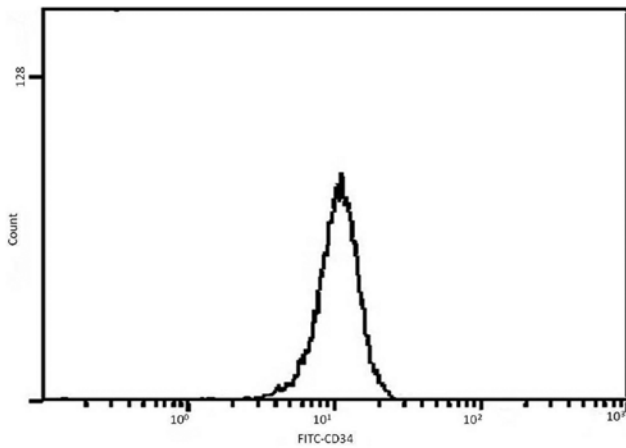


Figure 3. Flow cytometry of FITC-CD44 in the P3 generation.

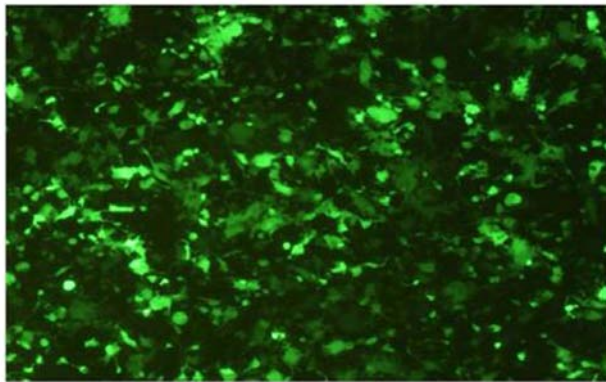


Figure 4. Determination of the viral titer of 293T cells.

two wells of the 96-well plate were obtained by counting the number of fluorescent cells in the last two wells divided by the corresponding dilution ratios.

**MOI of rabbit BMSCs.** The virus group with a MOI=1, 10, 100 and the polybrene group with a MOI=1, 10, 100 were observed under the microscope after 24 h. A small amount of dead cells was resuspended (Figs. 5 and 6) and there was no obvious change of BMSCs cell morphology. In the above two groups with a MOI=200, more resuspended dead cells were observed. BMSCs were shrunk and transformed, with unclear nuclei and disappeared nucleoli. A few cells expressed fluorescence under the fluorescent microscope after 24 h of liquid change, especially when MOI=100. The expression rate of GFP or RFP was increased after 96 h. Under fluorescence microscopy, the maximum efficiency was measured in polybrene with MOI=100 and the transfected efficiency, as determined by FCM, was 70.2% which was the optimal MOI (Figs. 7 and 8).

**Single- or double-gene-transfected BMSCs mediated by lentiviral vector.** The cells were divided into four groups based on the lentiviral vectors: group A: TGF- $\beta$ 1; group B: TGF- $\beta$ 1+ IL-10; group C: blank vector; and group D: blank control group. After 24 h, the virus was added and the groups were observed. A small amount of resuspended cells was evident in group A (TGF- $\beta$ 1), group B (TGF- $\beta$ 1+

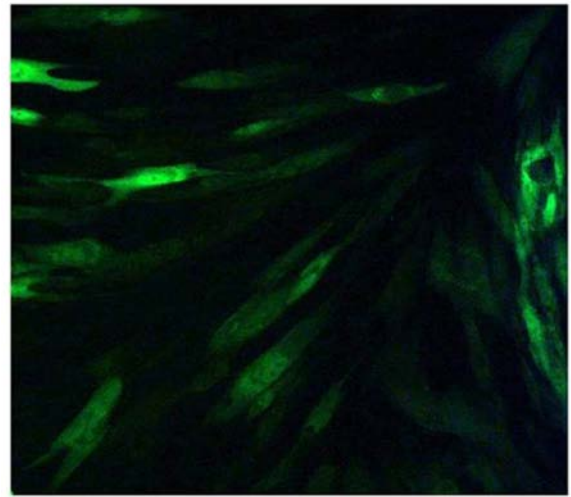


Figure 5. Fluorescence microscopy of BMSCs affected with lenti-TGF $\beta$ 1-GFP (MOI=10 after 96 h.

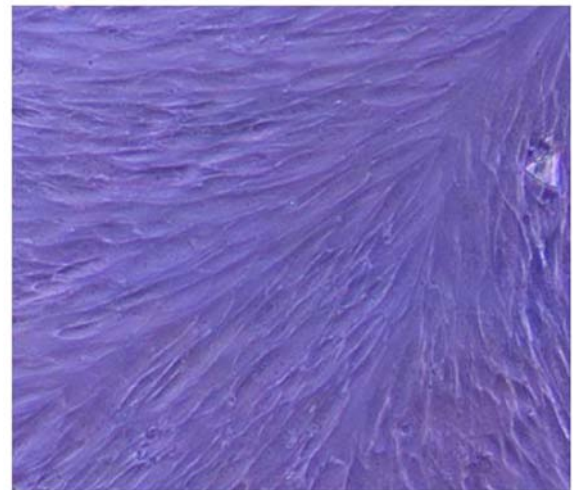


Figure 6. Cell morphology of BMSCs affected with lenti-TGF $\beta$ 1-GFP (MOI=10) after 96 h. No obvious change of cell morphology was observed and there were almost no dead cells.

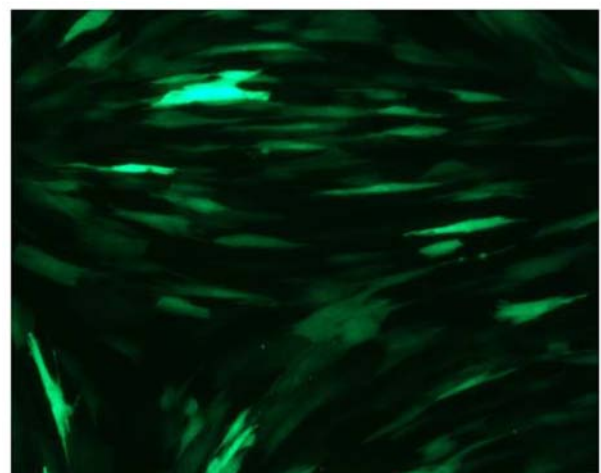


Figure 7. Fluorescence microscopy of BMSCs affected with lenti-TGF $\beta$ 1-GFP (MOI=100) after 96 h.



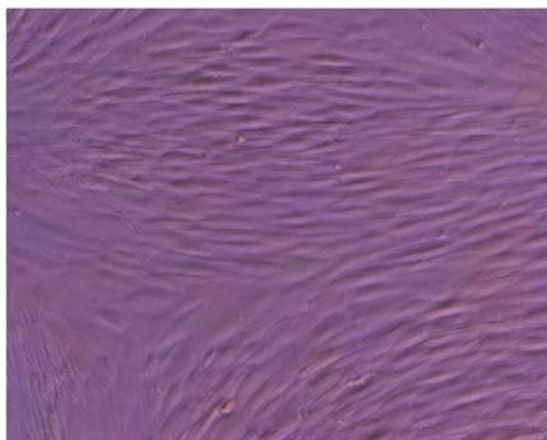


Figure 8. Cell morphology of BMSCs affected with lenti-TGF- $\beta$ 1-GFP (MOI=100) after 96 h. No obvious changes were evident in cell morphology and only a few cells died.

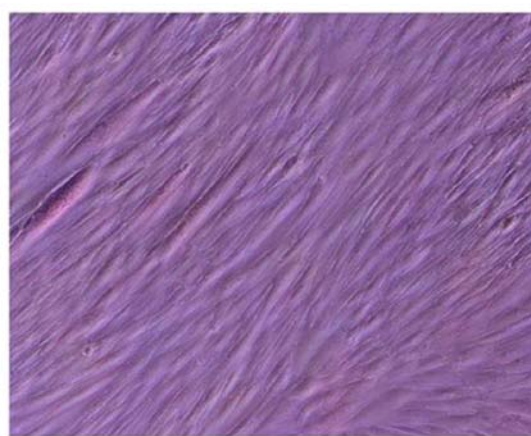


Figure 10. BMSCs treated with lenti-TGF- $\beta$ 1-GFP and lenti-IL10-RFP (MOI=100) after 96 h. No morphological changes were observed.

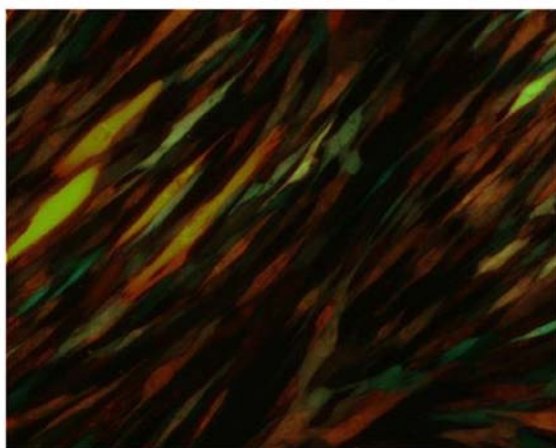


Figure 9. BMSCs treated with lenti-TGF- $\beta$ 1-GFP and lenti-IL10-RFP (MOI=100) after 96 h.

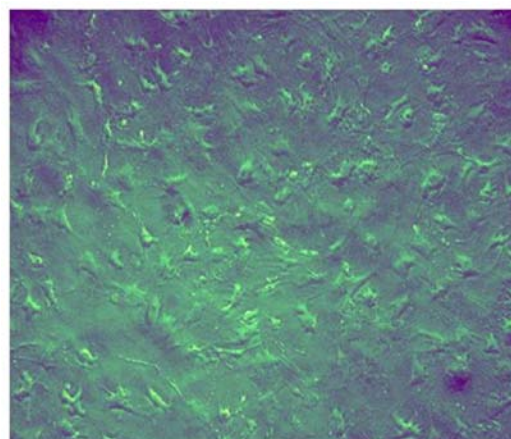


Figure 11. Phase contrast microscopy of BMSCs in group A (single-transfected group). After 3-4 days after induction of chondrogenesis a small amount of cells were shorter and the protuberance was reduced.

IL-10) (Figs. 9 and 10) and group C (blank vector group), although no obvious changes were evident in cell morphology. No cells died in group D (control group). Fluorescence microscopy showed that only a few cells expressed GFP or RFP following the addition of the lentiviral vector. The expression levels of GFP or RFP were observed daily and the signals gradually increased. The infected efficiency was ~70-75% after 96 h as determined by FCM.

**Expression level of targeted TGF- $\beta$ 1 and IL-10 genes in the supernatants.** An ELISA method was used to detect the level of TGF- $\beta$ 1 in the supernatant. The level of TGF- $\beta$ 1 was ( $24.78 \pm 2.75$ ) pg/ml in group A (TGF- $\beta$ 1) and ( $25.02 \pm 3.43$ ) pg/ml in group B (TGF- $\beta$ 1+ IL-10). In group C (blank vector group) and group D (control group), there was no gene expression detected. Statistical analysis revealed that there were significant differences between gene-transfected groups and non-gene-transfected groups ( $P < 0.05$ ).

An ELISA method was used to detect the level IL-10 in the supernatant, which was ( $32.08 \pm 2.95$ ) ng/l in group B (TGF- $\beta$ 1+IL-10). In group A (TGF- $\beta$ 1), group C (blank vector

group) and group D (control group), there was no gene expression detected. Statistical analysis revealed that there were differences between group B and the all other groups ( $P < 0.05$ ).

**Cell morphology.** Phase contrast microscopy showed that 3-4 days after induction a small amount of cells was shorter and the protuberance was reduced. After 7 days of culturing, cell proliferation was accelerated and most cells were deformed. The protuberance was significantly decreased and some cells were round shaped (Fig. 11). After 14 days of culturing, cell proliferation was highly accelerated and the majority of the cells were round shaped. Specifically, the protuberance disappeared, which was similar to the cartilage cells (Fig. 12).

**Alcian blue staining after the induction of chondrogenesis of BMSCs.** The cells gradually aggregated 2-3 days after induction and differentiation of chondrogenesis. Patchy or nodular cell clusters with high density were evident in sites where cells accumulated until the 7th day. After 14 days of induction the number of aggregated cell clusters increased in number and volume. Highly aggregated cell clusters had a patchy or nodular shape. The smooth surface was colloid and the surrounding

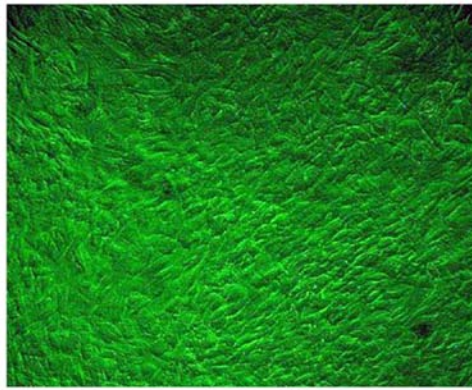


Figure 12. Phase contrast microscopy of BMSCs in group A (single-transfected group) 14 days after induction of chondrogenesis. Cell proliferation was highly accelerated and the majority of the cells were round shaped. The protuberance was disappeared, which was similar to the cartilage cells.

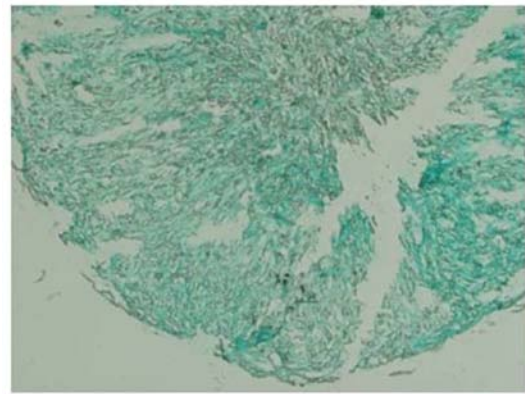


Figure 13. Alcian blue staining after induction of chondrogenesis of BMSCs in group A (single-transfected group). The nodular and surrounded aggregated cells are in pale blue.

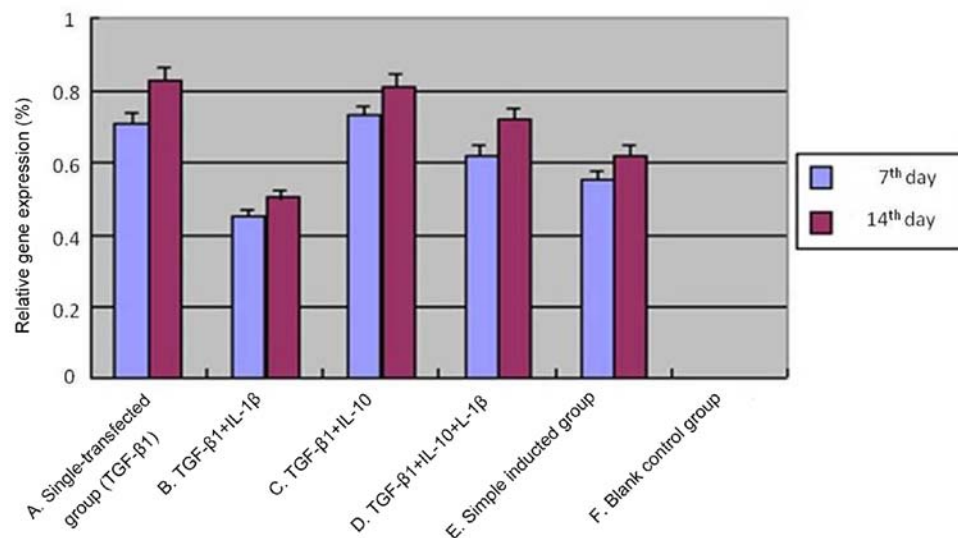


Figure 14. Relative quantification of the gene expression of SOX-9.

cells were radial. The nodular and surrounded aggregated cells were pale blue subsequent to Alcian blue staining (Fig. 13).

**mRNA expression of BMSCs-induced chondroblasts.** Previous results showed that the three targeted genes were all expressed in group A-E, but there was no expression in group F (blank control group) after 14 days of culturing. The expression levels of SOX-9, aggrecan and Type II collagen were significantly higher in group A and C compared to group B and D (containing IL-1β) and the blank vector group, and the differences were statistically significant. In group D (TGF-β1+IL-10+IL-1β), the expression levels of the target genes were higher than those of group B (TGF-β1+IL-1β), with statistical significance ( $P < 0.05$ ). No significantly statistical differences of targeted genes between group A (TGF-β1) and group C (TGF-β1+IL-10) ( $P > 0.05$ ) were observed (Figs. 14-16).

**Protein expression of BMSCs-induced chondroblasts.** Western blot analysis of Type II collagen after 21-days induction of BMSCs showed that there was no significantly

statistical difference of grey levels between groups A, C and E ( $P > 0.05$ ). Grey levels of group D (TGF-β1+IL-10+IL-1β) were higher than that of group B (TGF-β1+IL-1β) and there was a statistical significance ( $P < 0.05$ ) (Fig. 17).

## Discussion

Several cytokines, hormones and growth factors are known to influence the anabolic and catabolic processes of chondrocytes. Therefore, a number of growth factors, including transforming growth factor (TGF-β), insulin-like growth factor (IGF-1), bone morphogenetic proteins (BMPs) and to a lesser extent fibroblast growth factors (FGFs) and epidermal growth factor (EGF), have been used in cartilage tissue engineering studies *in vitro* to promote the chondrogenic phenotype and to stimulate ECM production and to promote chondrogenesis of MSCs. Several members of the TGF-β superfamily play a major role in cartilage development and repair. Mainly, the isoforms TGF-β1, 2 and 3 enhance chondrocyte proliferation and increase ECM synthesis by

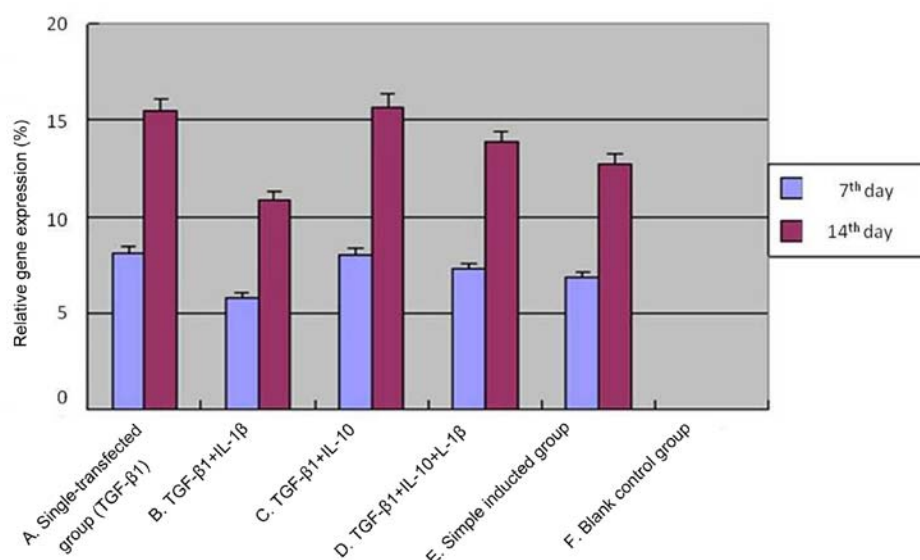


Figure 15. Relative quantification of the gene expression of aggrecan.

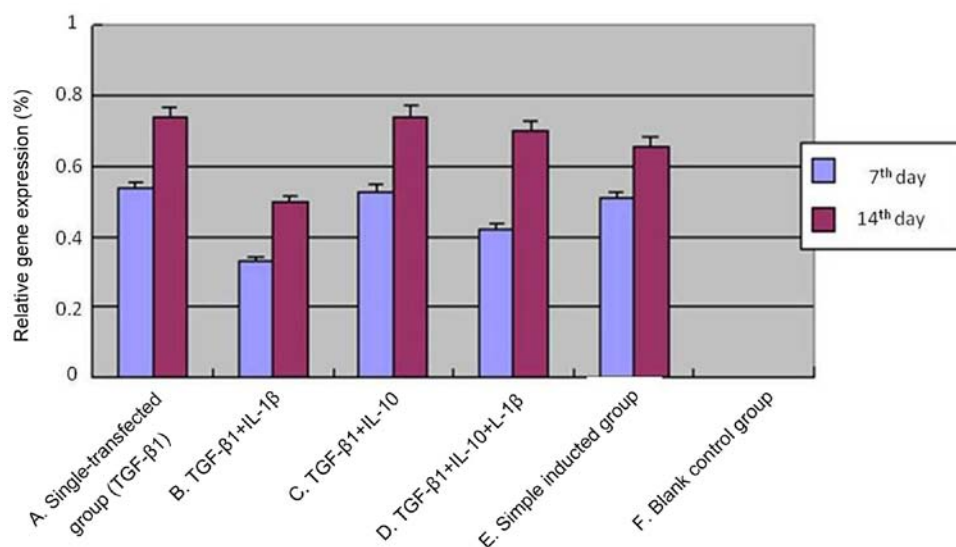


Figure 16. Relative quantification of the gene expression of Type II collagen.

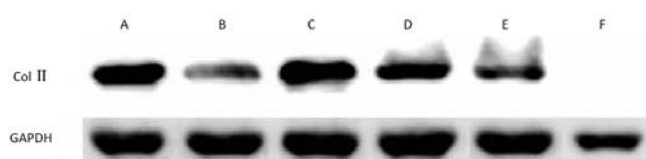


Figure 17. Western blot analysis of Type II collagen (130 kDa) in the different groups. GAPDH was used as a loading control.

chondrocytes. Furthermore, TGF-β1 and 3 promote chondrogenesis of MSCs (ref?). IGF-1 can stimulate the anabolic activity of chondrocytes and can induce chondrogenesis of MSC cell types. BMPs, especially BMP-2 and BMP-7, promote chondrogenesis of MSCs and increase matrix production by chondrocytes and MSCs.

Pro-inflammatory cytokines such as IL-1β and tumor necrosis factor (TNF)-α play a crucial role in the pathogenesis of osteoarthritis mediating a cascade of catabolic and pro-inflammatory events (23-25). Chondrocyte apoptosis, a typical feature in osteoarthritic cartilage (26), is accelerated by TNF-α where mitochondria-dependent apoptotic pathways may be activated (27). Elevated concentrations of the immunoregulatory cytokine IL-10 have been demonstrated in osteoarthritic cartilage (28). IL-10 inhibits some catabolic and inflammatory processes in the inflamed joint (29-32). However, its specific role involves the interplay with pro-inflammatory cytokines such as TNF-α, whereas its effects on chondrocyte apoptosis in cartilage remains unclear. Regulation of apoptosis by IL-10 has been shown in other cell systems (33,34). IL-10 has recently been found to interfere with TNF-α-induced apoptotic pathways in epithelial cells and macrophages (35,36). Wang and



Lou (37) indicated a direct protective effect of IL-10 from IL-1 $\beta$ -induced chondrocyte death. TNF- $\alpha$  elicits a wide range of biological responses including inflammation, cell proliferation, differentiation and apoptosis. Chondrocyte apoptosis is induced by TNF- $\alpha$  (38-41) through its binding to the TNF receptor (TNF-R)I (p60, 55 kDa) (42). Chondrocytes are normally resistant to the induction of apoptosis by IL-1 $\beta$  stimulation (43). It has also been reported that IL-1 $\beta$  protects chondrocytes from CD-95-induced apoptosis by a mechanism that was independent of IL-1 $\beta$ -induced NO (43). However, conflicting results have been reported showing IL-1 $\beta$ -induced apoptosis in human articular chondrocytes (38). The induction of chondrocyte apoptosis by TNF- $\alpha$  is also arguable because different reports yielded different results. The resistance to cell death of normal chondrocytes exposed to TNF- $\alpha$  or IL-1 $\beta$  is thought to rely on the ability of TNF- $\alpha$  and IL-1 $\beta$  signaling to upregulate protective cellular genes.

In the present study, we have demonstrated that: i) Cultured BMSC grew stably *in vitro*, proliferated rapidly, had high suitability and could be used as the seed-cells of bone tissue engineering. Bone marrow stromal cells can be induced to differentiate into chondrocytes and osteoblasts under appropriate conditions. ii) Expression of target genes in BMSCs by lentiviral vectors was highly efficient and stable. iii) Cytokines play an important role in cell proliferation and chondrogenic differentiation.

Notably, we observed that cytokines are important in cell proliferation and chondrogenic differentiation. TGF- $\beta$ 1 has synergistic effects in the differentiation. In addition, IL-10 may have a protective role in the restoration of cartilaginous tissue. This observation is consistent with several studies on bone marrow cell therapy, which demonstrated important features for tissue healing, although the precise mechanism by which genetically engineered BMSCs confer a therapeutic benefit in the treatment of tissue engineered cartilage remains to be examined.

In conclusion, in the present study, we have identified an optimal Lentiviral-mediated multiple gene transfer system that is safe and capable of transfecting TGF- $\beta$ 1 and IL-10, which, when transplanted to meniscal tissue *in vitro*, continues to express the transgene. These data may be of value for combined cell transplantation and gene therapy approaches using growth factors that promote cartilaginous tissue repair.

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