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Repair of cartilage defects in BMSCs via CDMP1 gene transfection

G. Wu[1], Y. Cui[2], Y.T. Wang[2], M. Yao[2], J. Hu[2], J.X. Li[2], Y. Wang[2] and B. Zhang[2]

[1]Department of General Surgery,
The First Affiliated Hospital of Liaoning Medical University,
Jinzhou, Liaoning Province, China
[2]Department of Otolaryngology,
The First Affiliated Hospital of Liaoning Medical University,
Jinzhou, Liaoning Province, China

Corresponding author: Y. Cui
E-mail: yingcuicn@126.com

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ABSTRACT. This study aimed at investigating the ability of cartilage-derived morphogenetic protein 1 (CDMP1) gene-transfected bone marrow mesenchymal stem cells (BMSCs) loaded on the poly(lactic-co-glycolic acid) (PLGA) scaffold for the repair of laryngeal cartilage defects and make a preliminary assessment of its repair effect. The mRNA and protein expressions of hCDMP1 were detected by reverse transcriptase-polymerase chain reaction and Western blotting. The expression of type II collagen (Col II) and glycosaminoglycan (GAG) were detected by immunohistochemistry. The cytoskeletal culture systems before and after transfection were transplanted into the rabbit full-thickness defects of thyroid cartilage for observation of the repair of cartilage defects from general and histological aspects. The exogenous hCDMP1 gene could be successfully transplanted into BMSCs through adenovirus infection to obtain a stable expression. Compared with the control group, hCDMP1 gene-transfected BMSCs

had enhanced secretory abilities of Col II, GAG, and other cartilage-specific matrices, with a trend of promoting cartilage differentiation. The transfected cytoskeletal complexes could more effectively repair laryngeal cartilage defects. hCDMP1 gene-transfected BMSCs/PLGA 3-D biological scaffold compounds transplanted into animal bodies could effectively repair laryngeal cartilage defects.

Key words: Cartilage tissue engineering; Adenovirus transfection; Cartilage-derived morphogenetic protein 1; Bone marrow mesenchymal stem cells

INTRODUCTION

It has been difficult to repair cartilage defects for surgeons in departments of orthopedics, otorhinolaryngology-head, and neck surgery. Over the years, the autologous cartilage and allogeneic cartilage have been used to repair cartilage defects, but the repair could not reach the ideal level. The emergence of cartilage tissue engineering technology provided a new way and method to solve this problem. At present, the main problem in the cartilage tissue engineering is how to improve the cartilage cell differentiation. Bone marrow mesenchymal stem cells (BMSCs) are the currently accepted and ideal seed cells for cartilage tissue engineering. Recent studies have demonstrated that transforming growth factor, bone morphogenetic protein, and growth differentiation factor play important regulatory roles in inducing the differentiation of BMSCs into chondrocytes. However, the growth factors selection is still uncertain for regulating and ensuring continuous and efficient function to stimulate cartilage differentiation (Arnalich-Montiel et al., 2008; Ghosh et al., 2009; Zhang et al., 2009; Sun et al., 2010). Cartilage-derived morphogenetic protein 1 (CDMP1), a recently identified polypeptide growth factor, is a member of the transforming growth factor β superfamily and is a new subtype of the bone morphogenetic protein family (Miyamoto et al., 2007; Park and Na, 2008; Yin et al., 2010; Dines et al., 2011). This family of proteins mainly promotes the adhesion, aggregation, and differentiation of mesenchymal chondrocytes at the early stage and significantly promotes the maturation and hypertrophy of chondrocytes in the late stage, with specific capacity to form chondrocytes induced by ectopic. CDMP1 has been applied in cartilage tissue engineering at home and abroad, but most scholars added the CDMP1 protein to *in vitro* study systems or injected the CDMP1 protein *in vivo*. Although considerable results have been achieved, the short action time, fast degradation of the CDMP1 protein, the additional and expensive cost, and the repeated and multivarious operation have limited its application. Gene therapy using an exogenous CDMP1 gene transferred into cells to obtain stabile expression could solve these challenges. Therefore, this study tried to transfer the exogenous CDMP1 gene into BMSCs through genetic engineering technology and achieve stabile and efficient expression. The recombinant hCDMP1 adenovirus-infected BMSCs/PLGA [poly(lactic-co-glycolic acid)] compound was transplanted into animals *in vivo* to repair laryngeal cartilage defects, providing a new method for repair of cartilage defects in genetic engineering. hCDMP1 gene-transfected BMSCs/PLGA 3-D biological scaffold compounds transplanted into animal bodies could effectively repair laryngeal cartilage defects.

MATERIAL AND METHODS

Animals

One-month-old healthy New Zealand white rabbits were supplied by the Animal Center of Liaoning Medical University [SYXK (Liao) 2009-0004]. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the First Affiliated Hospital of Liaoning Medical University.

Separation and culture of BMSCs

The femoral bone marrows of 1-month-old healthy New Zealand white rabbits were removed and filtered through a 100-mesh screen under sterile conditions. The filtrate, lymphocyte separation medium, was transferred to a centrifuge tube for gradient centrifugation (Park and Na, 2008). The mononuclear cell layer was removed and washed twice with serum-free L-DMEM (Sigma, Germany), followed by 2% trypan blue staining and cell counting, and then inoculated in the culture bottle at a concentration of 4 x 105/mL. Half medium was changed for the first time after 48 h, and subsequently, the medium was changed every 2-3 days.

Gene transfection

The BMSCs of the third-generation rabbits were taken and inoculated on a 6-well plate at a concentration of 5 x 105/well for 24 h, and the supernatant was removed after the cells had adhered. BMSCs were transfected according to 4 values, the multiplicity of infection (MOI) = 50, 100, 200, and 300, leaving 2 wells for the negative control. After 3 h of incubation at 37°C, the medium was replaced with complete medium for 48-72 h of conventional culture. eGFP was detected under an inverted fluorescence microscope, and the best MOI value of the adenovirus was considered the greatest MOI without causing obvious cytopathic effect. The third generation of BMSCs had good growth conditions and was grown to approximately 80% confluence and then randomly divided into 3 groups. Transfection group: infected with the Ad-CMV-hCDMP1-IRES-eGFP (group A) (Cyagen, Guangzhou, China); non-transfection group: infected with the Ad-CMV-eGFP (group B) (Cyagen); control group: not infected with the virus (group C). BMSCs were transfected with each group of virus solution, with the best MOI value. The infection effects were observed under an inverted fluorescence microscope after 48-72 h, and the infection efficiencies were calculated.

RT-PCR

The total RNA was extracted for 1% agarose gel electrophoresis analysis, and the hCDMP1 expression was detected by RT-PCR. The primer sequences are shown in Table 1.

Western blot

The expression of the CDMP-1 protein was detected by Western blotting; specifically,

the preparation of protein samples, determination of protein content, calculation of protein concentration according to a standard curve, loading of samples, electrophoresis, transferring proteins to a membrane, analysis of the target band, and inner reference on the membrane using gel imaging system.

Table 1. Sequence of primer.

Enlarged segment	Primer sequence	Length (nt)
β-actin (541 bp)	5'-GGGACCTGACTGACTACCTC-3'	20
	5'-TCATACTCCTGCTTGCTGAT-3'	20
hCDMP1 (408 bp)	5'-CAGAGCGGGCCTTAATCT-3'	18
	5'-CGTGGTCAGGAAGCAGAG-3'	18

Immunohistochemistry

The above described 3 groups of cell climbing sheets (10 pieces in each group) were removed after 72 h of infection and fixed with acetone. The expression of type II collagen was detected using an immunohistochemical method (Univ-Bio, Shanghai, China). The absorbance of the staining was measured using a medical imaging analysis system. Three positive visions in the sections were randomly taken under a 200X magnification fluorescence microscope for measuring the absorbance, the mean of which was taken to represent the strength of protein expression in this specimen.

Animal experiments

Twelve New Zealand white rabbits (3-month-old, male or female, weighing 2.0-2.5 kg) were randomly divided into 3 groups (4 rabbits in each group). Group I: transfected cells and PLGA compound were transplanted (Dai Gang, Jinan, China); group II: BMSCs and PLGA compound were transplanted; group III: simple PLGA was transplanted. The animal model of local thyroid cartilage defects was established (Miyamoto et al., 2007), with a 0.8 x 0.8-cm full thickness cartilage defect in the left side, without penetrating the laryngeal mucosa. The 3 groups of cytoskeletal compounds were transplanted into the defect positions and slightly fixed with suturing. Penicillin (1.6 million units) was injected daily into the ear marginal vein postoperatively for a continuous 3 days. The animals underwent postoperative observation of breathing, activities, and feeding, and were each painlessly sacrificed on the postoperative 4th or 8th week. The engineered cartilage underwent general observation and histological examination.

Statistical analysis

Each group was statistically analyzed using the SPSS version 17.0 software (SPSS, Chicago, IL, USA). Data are reported as means ± SD.

RESULTS

Cell culture

There were blood cells confounded in the BMSCs in the early inoculation, with not

easily distinguishable compositions. After the first exchange of medium 3 days later, there were triangular, polygonal, or short spindles of adherent cells visible, part of which appeared as a small colony of cells, ranging from several to dozens. The adherent cells underwent a volume increase after 6-7 days, with the same cell morphology, a typical fibroblast morphology. After 10-12 days, the adherent cells were gradually increased and connected into pieces, which were in a fish-like arrangement. The components of the subcultured cells were more pure than the primitive cells, in which there were most cells adherence with stretch cell morphology visible after 2 days, significantly increased cell proliferation with typical long spindle after 3-4 days, and a large number of cells covered the bottom of the bottle after 7-8 days (Figure 1A and B).

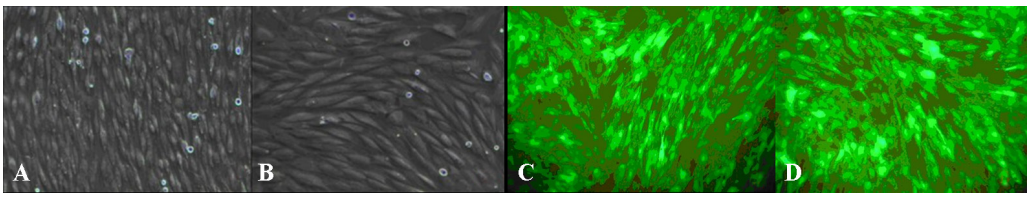

Figure 1. Separation and culture of rabbit BMSCs and the expression of eGFP transfected by adenovirus after 7 days. **A. B.** BMSC morphology: **A.** Primary BMSCs after culturing for 12 days; **B.** fifth passaged BMSCs after culturing for 8 days; **C. D.** expression of eGFP: **C.** transfected group: transfected by Ad-CMV-hCDMP1-IRES-eGFP; **D.** untransfected group: transfected by Ad-CMV-eGFP.

hCDMP1 gene expression after infection

There was a small amount of green fluorescence visible in the cells of groups A and B under an inverted fluorescence microscope after 24 h of infection, which were significantly increased after 48 h, and reached a large number after 72 h, consistent with the cell profiles, with more than 90% of the transfection rates for more than 14 days (Figure 1C and D). There was no green fluorescence visible in the cells of group C.

RT-PCR

A band at 443 bp appeared during electrophoresis of group A, consistent with the size of hCDMP1 cDNA, but no electrophoresis bands appeared for groups B and C (Figure 2A).

Western blot

A band appeared at 55.6 kDa in the transfection group (consistent with the molecular mass of the hCDMP1 protein), but no electrophoresis band appeared in non-transfection and control groups (Figure 2B). The internal reference, β-actin, was 43 kDa.

Immunohistochemistry

Clear expression of type II collagen appeared in the cytoplasm of the transfection group, but weaker coloration appeared in the non-transfection group and in the control group (Figure 3).

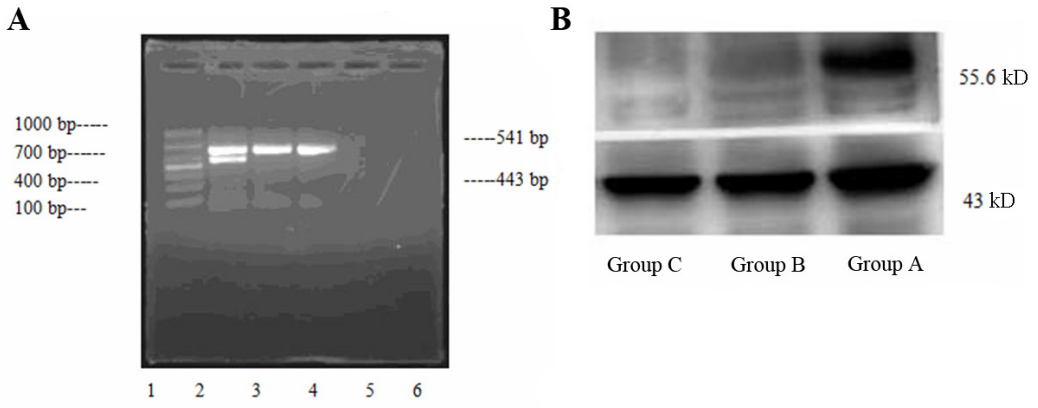

Figure 2. Expression of hCDMP1 mRNA and proteins of BMSCs after transfection, monitored by RT-PCR and Western blotting. **A.** Expression of hCDMP1 mRNA monitored by RT-PCR: *lane 1* = molecular marker; *lane 2* = group A (transfected by Ad-CMV-hCDMP1-IRES-eGFP); *lane 3* = group B (transfected by Ad-CMV-eGFP); *lane 4* = group C (untransfected group); *lanes 5* and *6* = controls. **B.** Expression of the hCDMP1 protein monitored by Western blotting: group A = transfected by Ad-CMV-hCDMP1-IRES-eGFP; group B = transfected by Ad-CMV-eGFP; group C = untransfected group.

The expression of each group of type II collagen, presented as the integral absorbance density, is shown in Table 2, based on computer image analysis.

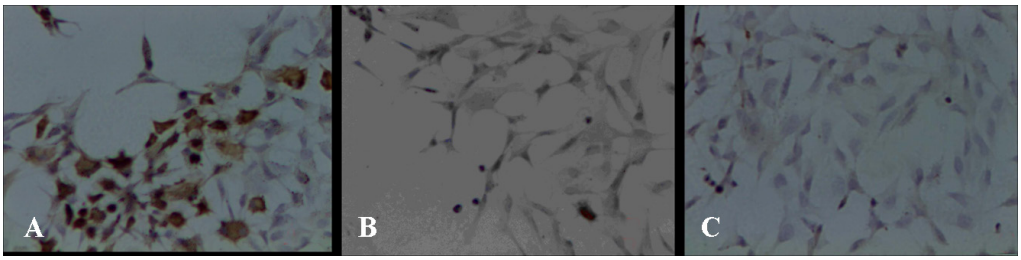

Figure 3. Expression of collagen type II in BMSCs after transfection, monitored by immunohistochemistry (200X). **A.** Group A (transfected by Ad-CMV-hCDMP1-IRES-eGFP); **B.** group B (transfected by Ad-CMV-eGFP); **C.** group C (untransfected group).

Table 2. Analysis of integral absorbance density of BMSCs after transfection.

Groups	Collagen II	GAG
Transfected group	35.17 ± 1.07*	38.34 ± 1.28*
Untransfected group	10.32 ± 1.01	11.44 ± 1.09
Control group	10.24 ± 0.90	10.45 ± 1.11

Data are reported as means ± SD for 10 pieces in each group. *P < 0.05, compared with other groups.

Animal experiments

All animals were allowed to feed 4-8 h after surgery. The animals proceeded with

normal activities after 24 h, with the first stage of healing of the neck incisions. Each group of animals was sacrificed on the postoperative 4th and 8th weeks for observation of the repair area of the cartilage defects. Four weeks after surgery, there was a light red color and smooth surfaces, substantially abreast with the surrounding cartilages, in the defect positions visible in group I. There were no obvious difference in the general form of the defect positions in groups II and III, with obvious wound surfaces and dark red color and clear boundaries in the surrounding normal cartilages. The defect positions were soft tissues, without obvious infection and necrosis in the wound surfaces. Eight weeks after surgery, there were slightly gray wound surfaces with cartilage-like appearance and smooth surfaces, with fuzzy boundaries with the surrounding normal cartilages, in the defect positions in group I; there were light red, hollow, tough wound surfaces, with clear boundaries with the surrounding normal cartilages in the defect positions, in groups II and III (Figure 4).

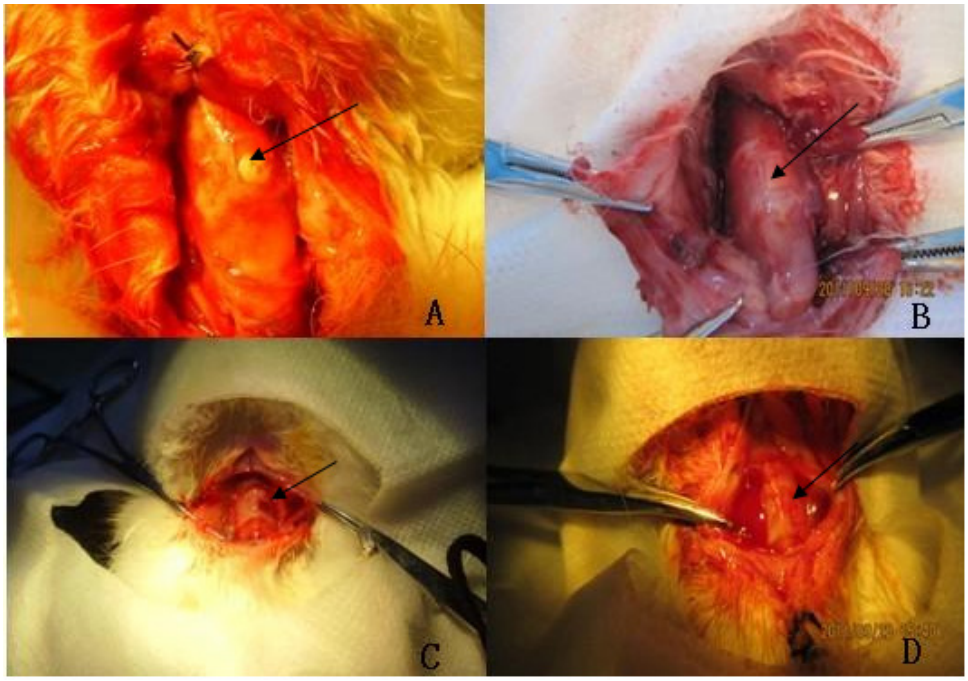

Figure 4. Transfected and untransfected cell scaffold culture systems implanted into the rabbit thyroid cartilage defects, followed by analysis at the gross level. **A.** 4-week transfected group; **B.** 4-week untransfected group; **C.** 8-week transfected group; **D.** 8-week untransfected group.

The obtained tissue-engineered cartilages were subjected to histologic observation. On the 4th week, there were no obvious inflammatory reactions in the defect positions; there were immature chondrocytes formation but no obvious cartilage lacuna in the neck defect positions in group I, with positive type II collagen and toluidine blue staining. There was no obvious chondrocyte formation in the neck defect positions of groups II and III, with negative type II collagen and toluidine blue staining. On the 8th week, there were significantly increased cartilage-like cells and a large number of cartilage lacuna formations in the neck de-

fect positions in group I, with positive type II collagen and toluidine blue staining; there were basically fibrous scar tissues instead in the defect positions in groups II and III, with negative type II collagen and toluidine blue staining (Figure 5; Table 3).

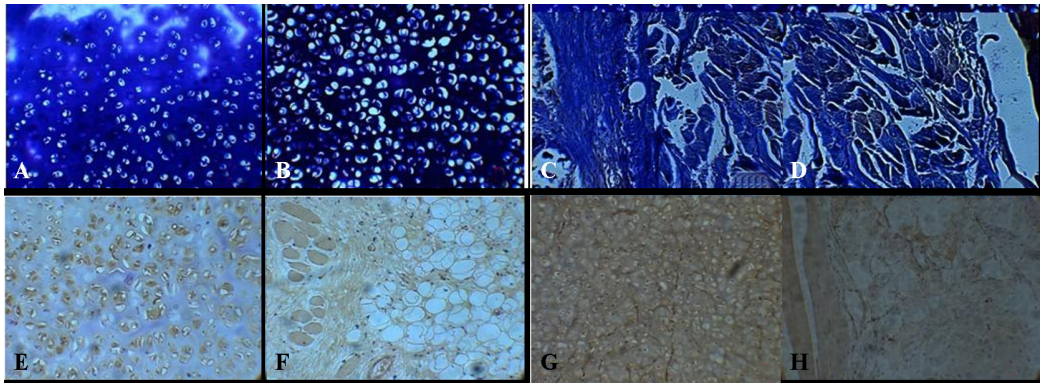

Figure 5. Expression of glycosaminoglycans (GAGs) and collagen II in cell scaffold culture system (200X). **A.-D.** Expression of GAGs determined by alcian blue staining: **A.** 4-week transfected group; **B.** 8-week transfected group; **C.** 4-week untransfected group; **D.** 8-week untransfected group. **E.-H.** Expression of collagen II by immunohistochemistry: **E.** 4-week transfected group; **F.** 8-week transfected group; **G.** 4-week untransfected group; **H.** 8-week untransfected group.

Table 3. Analysis of glycosaminoglycans (GAG) and collagen II-positive area and intensity of different groups.

Groups	4 weeks	8 weeks
GAG		
Group I	41.36 ± 1.62*	85.75 ± 1.93*
Group II	7.57 ± 0.42	8.67 ± 0.52
Group III	6.71 ± 0.48	7.31 ± 0.61
Collagen II		
Group I	37.99 ± 2.39*	84.62 ± 2.63*
Group II	6.01 ± 0.44	6.38 ± 0.25
Group II	5.09 ± 0.49	5.53 ± 0.32

Data are reported as means ± SD for 10 pieces in each group. *P < 0.05, compared with other groups.

DISCUSSION

In this study, the exogenous CDMP1 gene was transfected into rabbit BMSCs using the adenovirus transfection method and got a stable expression. The transfected cells and scaffold complex transplanted into animal *in vivo* could effectively repair the local thyroid cartilage defects. On the 8th week after transplantation, there were significantly increased cartilage-like cells and a large number of cartilage lacuna formations in the thyroid cartilage defects, with positive type II collagen and toluidine blue staining, basically consistent with the research results by other scholars (Nochi et al., 2004; Tian et al., 2007; Yeh and Lee, 2010). Tian et al. (2007) transfected the CDMP1 gene into rabbit BMSCs using liposome transfection to repair rabbit articular cartilage defects. After CDMP1 had been transfected into homologous BMSCs and transplanted into rabbit articular cartilage defects for 8 weeks, the surface morphology of the repaired cartilages could be compared with the maturely differentiated

hyaline cartilages. The subchondral tissues were fully repaired by the new thick layer of bone tissues close to the host subchondral bone. The liposome-mediated method successfully transfected the hCDMP1 gene into the rabbit BMSCs and induced the BMSCs into chondrocytes, but the obtained engineered cartilages were smaller than the normal cartilages. In this study, the adenovirus vector missed the E1 region, i.e., lack of viral replication, so that as a vector for gene therapy, it would not be continuously replicated *in vivo* or integrated into the host chromosomes. Therefore, it was relatively safe for the body. However, the controllability of gene expression needed to be further improved. In this study, the target gene was confirmed to be stably and continually expressed for more than 2 weeks by the successful implementation of adenovirus carried hCDMP1 gene into rabbit BMSCs, RT-PCR, and Western blotting. The results of the MTT test proved that the BMSCs infected with adenovirus carried the hCDMP1 gene and did not cause an excessive proliferation or inhibition of the BMSCs (data not shown), indicating that adenovirus transfection was relatively safe, laying a foundation for the application of gene transfection in cartilage tissue engineering.

PLGA has good mechanical strength, biocompatibility, controllability, safety, low foreign body reaction and immunogenicity, stable physical and chemical properties, providing suitable 3-D space for the proliferation and differentiation of BMSCs, which have been widely used in tissue engineering and as drug slow-release materials (Yao et al., 2009; Li et al., 2011; Subhash et al., 2012).

The interstitial chemical composition of the chondrocytes mainly includes collagen and proteoglycan. The collagen in the cartilage tissues is mainly type II collagen, accounting for more than 90% of the total amount of collagen. Therefore, the type II collagen and glycosaminoglycans (GAGs) are hallmarks of chondrocytes. In this study, the gene-transfected BMSCs had strengthened abilities of synthesis of extracellular matrix, and compared with the control group and non-transfection group, the expression levels of type II collagen and GAGs were significantly improved in the transfection group, thereafter prompting that hCDMP1 could induce BMSCs to differentiate towards the chondrogenic phenotype. It was observed from animal experiments that on the 4th week, the new tissues in the neck defect positions were with positive type II collagen and toluidine blue staining in group I; and the new tissues in the neck defect positions were with negative above staining in groups II and III. On the 8th week, there were increased chondrocyte-like cells and areas of positive type II collagen and toluidine blue staining in the defect area in group I; the defect areas in groups II and III were for fibrous tissue repair, without significant positive above staining. The secretions of type II collagen and GAGs in group I were stronger than the other 2 groups, indicating that BMSCs infected with adenovirus that carried the hCDMP1 gene could sustain expression of hCDMP1 with biological effects.

In this study, the transfected BMSCs infected with adenovirus carried with hCDMP1 gene and PLGA 3-D biological scaffold complex was used to repair the laryngeal cartilage defects, which confirmed the cartilage-like formation and successful repair of laryngeal cartilage defects in the defect area from the morphology and histology. However, this study did not discuss the related indexes of the scaffold. In subsequent studies, we will assess new scaffold materials, electrostatic spinning (Agarwal et al., 2008; Janjanin et al., 2008; Wright et al., 2010), and cell-chip technology (Kaneshiro et al., 2006; Mitani et al., 2009; Tani et al., 2010; Weder et al., 2010), and in more detail compare the properties of the engineered cartilages with normal cartilages.

In conclusion, the tissue-engineered cartilages obtained from the transfected BMSCs infected with a higher transfection efficiency of adenovirus carried with hCDMP1 gene and PLGA 3-D biological scaffold complex could effectively repair the local thyroid cartilage defects. These experimental results provided a new method to reconstruct cartilage, providing a new idea for the repair of cartilage tissue defects in clinical as well as a theoretical basis for the combination and clinical applications of cartilage tissue engineering and gene engineering technology, with a wide range of social and economic significance.

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