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Original Article

Bcl-xL expression improves the therapeutic effect of human umbilical cord stem cell transplantation on articular cartilage injury in rabbit

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Abstract: Background: To investigate the therapeutic effect of transplantation of B-cell lymphoma-extra large (Bcl-xL) gene modified human umbilical cord blood stem cells (HUCSCs) on rabbit articular cartilage injury. Materials and methods: HUCSCs were isolated and identified. Lentiviral encoding Bcl-xL was applied to modify HUCSCs. The effects of Bcl-xL overexpression on apoptosis and related gene expression after differentiation induction of HUCSCs were detected. Additionally, the efficiency of transplantation of Bcl-xL gene modified HUCSCs on articular cartilage injury were evaluated. Results: HUCSCs could differentiate into chondrocytes after induction. Compared with control group, the apoptosis after induction was significantly elevated, but reduced by Bcl-xL gene overexpression. The differentiation of HUCSCs into chondrocytes was displayed by expression of type II collagen (CII), but accompanying with expression of caspase-3 and matrix metalloproteinase-3 (MMP-3). By contrast, Bcl-xL gene overexpression reduced caspase-3 and MMP-3 expression, but further increased CII expression. Pathological staining showed that Bcl-xL gene modified HUCSCs could obviously repair cartilage injury. Compared with sham control group, the expression of caspase-3 and MMP-3 in model group was significantly up-regulated, while the expression of CII was significantly down-regulated. Transplantation of HUCSCs could ameliorate the injury, while Bcl-xL modification could improve the therapeutic effect of transplantation of HUCSCs. Moreover, Bcl-xL modification could further decrease cartilage injury-induced expression of caspase-3 and MMP-3, and improve the expression of CII compared with transplantation of normal HUCSCs. Conclusions: Bcl-xL gene modification decreases cell differentiation-induced apoptosis and improves the efficiency of HUCSCs transplantation in the repairing of cartilage injury.

Keywords: Bcl-xL, human umbilical cord stem cells, transplantation, articular cartilage injury

Introduction

Articular cartilage injury is a common joint disorder, with a poor self-repairing capacity, due to the deficiency of undifferentiated articular cartilages and blood supply. Articular cartilage injury often causes severe degeneration of articular cartilages [1-3]. The most effective treatment of articular cartilage injury is the transplantation of tissue or stem cell [4, 5]. However, bone allograft rejection often restricts the application [6]. Tissue engineer provides a new choice for the repair of articular cartilage. Seed cells are cultured *in vitro* and grown in a three-dimensional scaffold, which can be easily degraded [7]. The amplified cells are then transplanted back into the injury site, leading to formation of functional articular tissues.

Stem cells from human umbilical cord blood are a kind of stem cell with strong proliferative and multi-directional differentiation ability. However, the construction of tissue engineering cartilages needs a large number of seed cells. *In vitro* amplification could to some extent improve the cell number of seed cells. However, apoptosis during *in vitro* culture could restrict the application of seed cells [8-10]. Therefore, anti-apoptosis method is useful to improve the stem cell therapy.

B-cell lymphoma-extra large (Bcl-xL) is one member of Bcl-2 protein family, which regulate apoptosis [11]. It has been documented that Bcl-xL could inhibit apoptosis induced by multiple factors, including nutrition deficiency, radiation, etc. Moreover, Bcl-xL has a certain anti-

inflammatory activity in various diseases [12-14]. In this study, we constructed lentivirus encoding Bcl-xL to transfect human umbilical cord stem cells (HUCSCs). The effects of Bcl-xL on apoptosis in the process of differentiation of HUCSCs and the efficiency of *in vivo* transplantation of HUCSCs in articular cartilage injury were evaluated. This study provides experimental evidence for clinical treatment of articular cartilage injuries.

Materials and methods

Preparation of HUCSCs

Umbilical cord blood was collected from 5 normal pregnant women (gestation: 36-42 weeks) from the First Affiliated Hospital of Anhui Medical University. All protocols were approved and supervised by the Ethics Committee of Anhui Medical University. Umbilical cord blood was treated by anticoagulant citrate, phosphate and glucose (CPD). The lymphocyte separation liquid was used to suspend umbilical cord blood mononuclear cells and adherent culture method was applied to obtain mesenchymal stem cells. The mononuclear cells and mesenchymal stem cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum. The surface markers were detected by flow cytometry with the antibodies of CD45-PE, CD90-FITC, CD29-PE, CD34-FITC (BD, USA). The differentiation of HUCSCs into chondrocytes was induced by induction solution (100 mL/L FBS, H-DMEM, 0.05 mM AA, 6.25 μg/mL insulin, 10 ng/mL TGF-β1) for 14 days and confirmed by toluidine blue staining. The *in vitro* experiment was divided into control, induction, induction + Bcl-xL overexpression and induction + vector groups.

Cell transfection

When cell confluence attained to 50-70%, HUCSCs were transfected with Bcl-xL lentivirus or vector (Jiman Biotech, Shanghai, China). 6 h later, the medium was changed back to DMEM containing 10% fetal bovine serum in 5% CO_2 incubator at 37°C. 48 h later, Bcl-xL expression was confirmed by real-time PCR and Western blotting.

Flow cytometry

The cells were seeded on the 6-well plate. When confluence reached 50-70%, the cells were transfected with the virus or vector. 48 h later, the cells in each group were treated following the induction procedure. After that, the cells were collected by digestion, and incubated with Annexin V-FITC/PI for 30 min at room temperature. Flow cytometry was performed within 1 h according to the instruction of Annexin V-FITC/PI cell apoptosis kit by BD FACSCalibur (BD, USA).

Animal model and treatments

Sixty Japanese white rabbits (three-month old) were obtained from Nanchang Long Ping Rabbit Industry Co. Ltd. (SCXK(Gan)2014-0005). Articular cartilage injury model was produced as following. Briefly, after anesthesia by 3% pentobarbital sodium (1.5 ml/kg, ear vein injection), skin of the knee joint in hind legs was prepared using an electric shaver knife. Bilateral knee joint in articular surface was drilled with incisions of 4-mm diameter and 3-mm depth. The subchondral bone was penetrated until the fresh blood of articular cavity was observed. The patella was sutured layer by layer and sterilized by iodophor. Penicillin was injected intramuscularly three days after operation, and the infection was monitored. The experiments were divided into 5 groups (n=12 in each group): a sham control group, an articular cartilage injury model model group, a HUCSCs transplantation group, a Bcl-xL modified HUCSCs transplantation group, and a vector modified HUCSCs group. HUCSCs were grown on alginate carrier material, as previously described [15]. The cells were locally injected into joint cavity (0.2 ml) at the eighth day after surgery. Eight weeks after the injection, all the animals were killed. Articular tissue was collected for the staining and biochemical experiments.

Immunohistochemistry, Masson staining and Safranin O-fast green

Immunostaining of histological sections was performed using monoclonal antibodies against caspase-3 (1:50, Abcam, USA), MMP13 (1:200, Abcam, USA), type II Collagen (1:200, Abcam, USA). Endogenous peroxidase activity was blocked with 3% (vol/vol) H_2O_2 for 5 minutes. After that, tissues were incubated with primary antibody overnight at 4°C followed by a 30-minute incubation with secondary antibody (Dako, Carpinteria, CA) and visualization with DAB chromagen for 3 minutes. Safranin O-fast green staining was carried out to indicate gly-

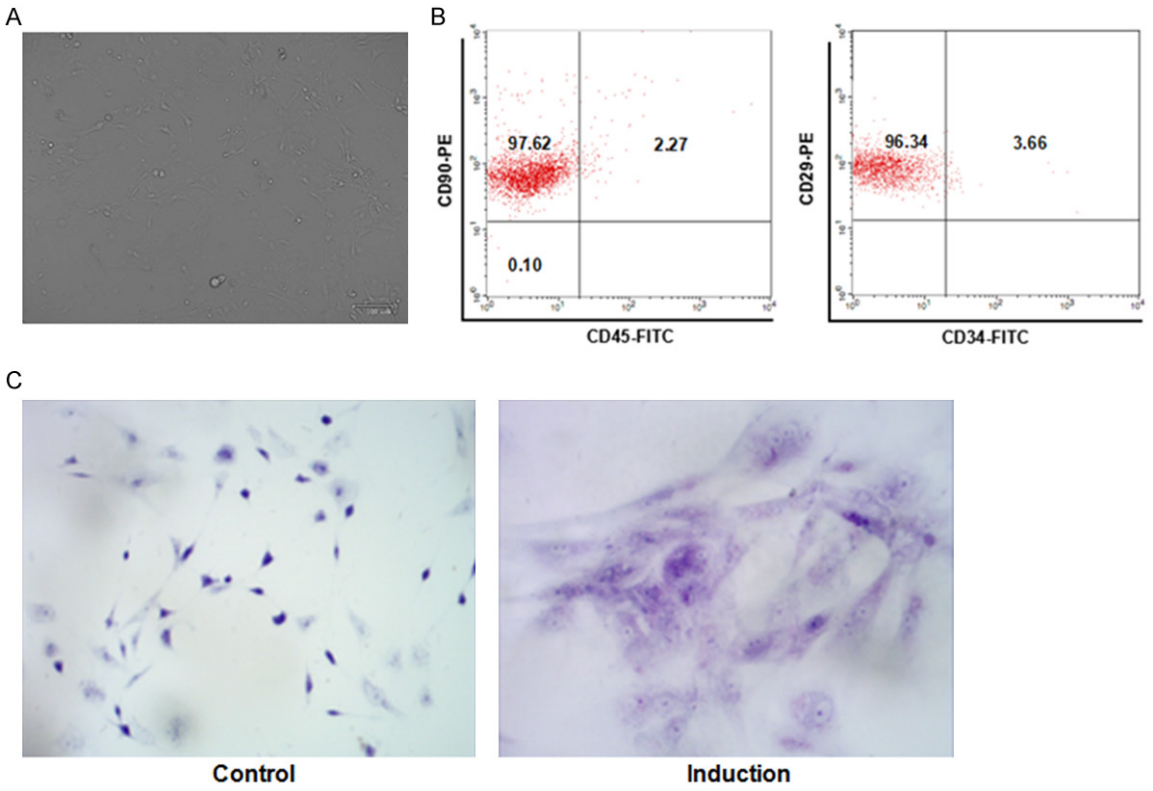

Figure 1. Identification of HUCSCs. A: HUCSCs were observed under light microscope. B: The cell markers were detected by flow cytometry. C: HUCSCs could be induced into chondrocytes.

cosaminoglycans in the articular cartilage. Collagens were stained by Masson staining.

Real-time PCR

Total RNA was extracted from articular tissues or cultured cells using Trizol reagent. RNA concentrations were determined spectrophotometrically, and 1 μg total RNA was reversely transcribed using an avian myeloblastosis virus reverse-transcriptase kit (Promega, Madison, WI, USA). PCR primers were listed as follows:

Type II Collagen-F: AACACTGCCAACGTCCAGAT, Type II Collagen-R: CTGCAGCACGGTATAGGTGA; MMP-3-F: CGGTGGCTTCAGTACCTTTC, MMP-3-R: ACCTCCTCCCAGACCTTCA; Caspase-3-F: GCACTGGAATGTCAGCTCGCA, Caspase-3-R: GCCACCTTCCGGTTAACACGA; BCL-xL-F: AAGAGTGAGCCCAGCAGAACC, BCL-xL-R: CTGTGCGTGGAAAGCGTAGAC; β-actin-F: TCGTCCTCCTCTGGTGCTCT, β-actin-R: CCACTTTGTGAAGCTCATTTCCT.

The amplification reactions were carried out with a 7500 real-time PCR system (Applied Biosystems), with initial hold step (95°C for 10 min) and 40 cycles of a two-step PCR (95°C for 15 s and 58°C for 1 min). The comparative computed tomography method was used to determine the amount of target, normalized to an endogenous reference (β-actin) and relative to a calibrator ($2^{-\Delta\Delta Ct}$) as previously described [16].

Western blotting

Protein was extracted from articular tissues or cultured cells for western blotting as previously described [17]. The antibodies, including anti-caspase-3 (1:50, Abcam), anti-MMP-3 (1:200, Abcam), anti-type II collagen (1:200, Abcam), anti-β-actin (1:1000) (Cell Signaling Technology, Beverly, USA) were incubated overnight at 4°C. The ECL reagent kit was applied to assist the staining. The blots were scanned by ChemiDocTM XRS (Bio-Rad, USA). The grey density was analyzed by Image J 7.0 software.

Statistical analysis

Data were presented as means ± standard deviations. One-way analysis of variance with

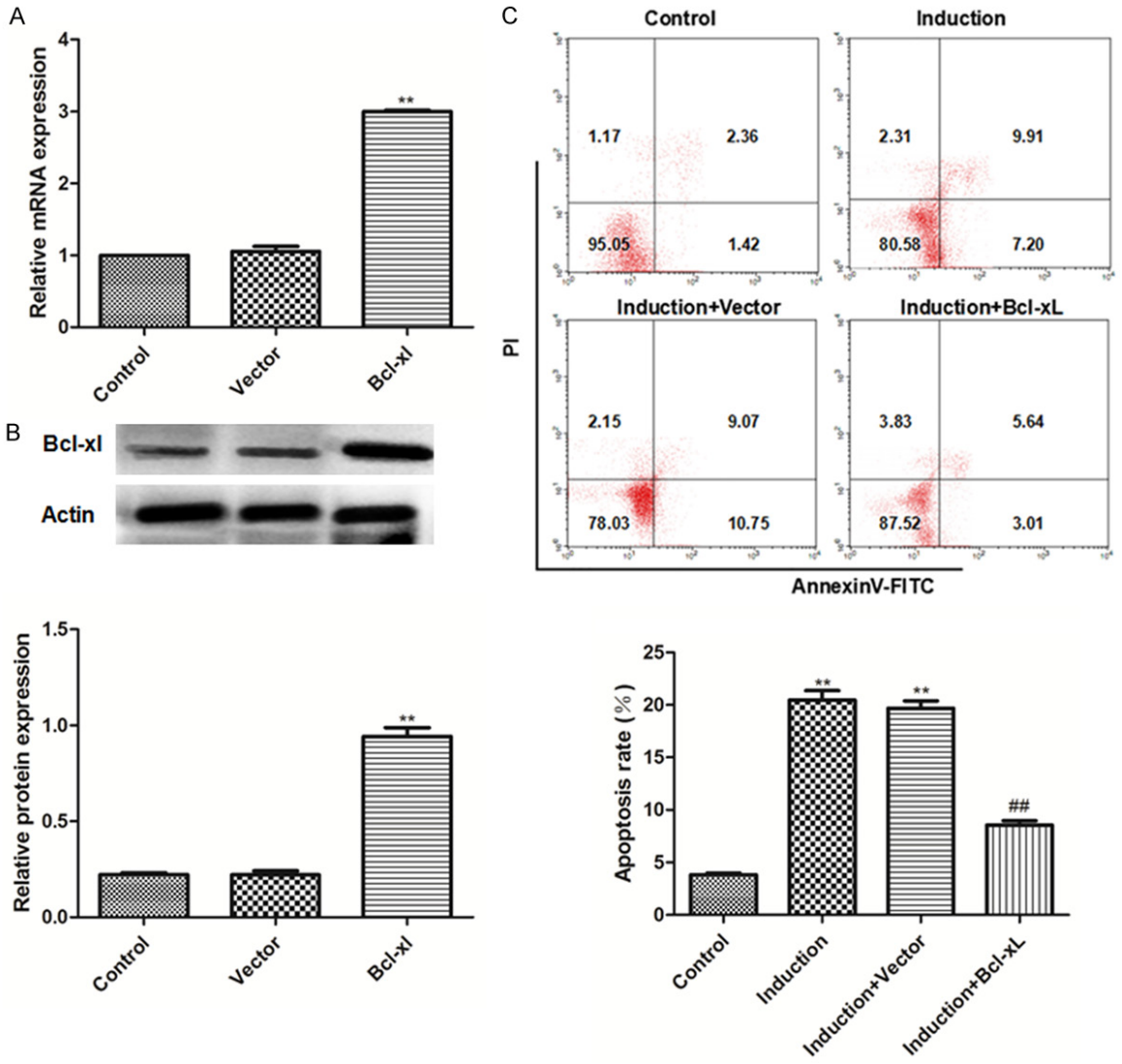

Figure 2. Bcl-xL overexpression reduced chondrocyte differentiation-induced apoptosis. A: Real-time PCR confirmed that lentiviral encoding Bcl-xL increased Bcl-xL expression in HUCSCs. B: Western blotting confirmed that lentiviral encoding Bcl-xL increased Bcl-xL expression in HUCSCs. C: Bcl-xL overexpression reduced apoptosis induced by chondrocyte differentiation. Upper panel were representative images of flow cytometry. Down panel was the quantification data of apoptosis. **P<0.01 compared with control. ##P<0.01 compared with induction (One-way ANOVA with *post-hoc* Bonferroni).

post-hoc Bonferroni test for multiple comparisons was performed. P<0.05 was considered significant difference.

Results

Preparation and identification of HUCSCs

The image of HUCSCs was taken under a phase-contrast microscope (**Figure 1A**). Flow cytometry analysis showed that 95% of the cells were $CD45^-$ and $CD34^-$, but $CD90^+$ and $CD29^+$ (**Figure 1B**). After differentiation induction, most of cells displayed the characteristics of cartilage chondrocytes (**Figure 1C**).

Viral encoding Bcl-xL reduced differentiation-induced apoptosis

Our results showed that viral encoding Bcl-xL significantly up-regulated Bcl-xL expression in both of mRNA and protein levels (**Figure 2A, 2B**). Viral encoding vector did not increase Bcl-xL expression. As shown in **Figure 1C**, induction solution could lead to the differentiation of HUCSCs into chondrocytes. However, typical

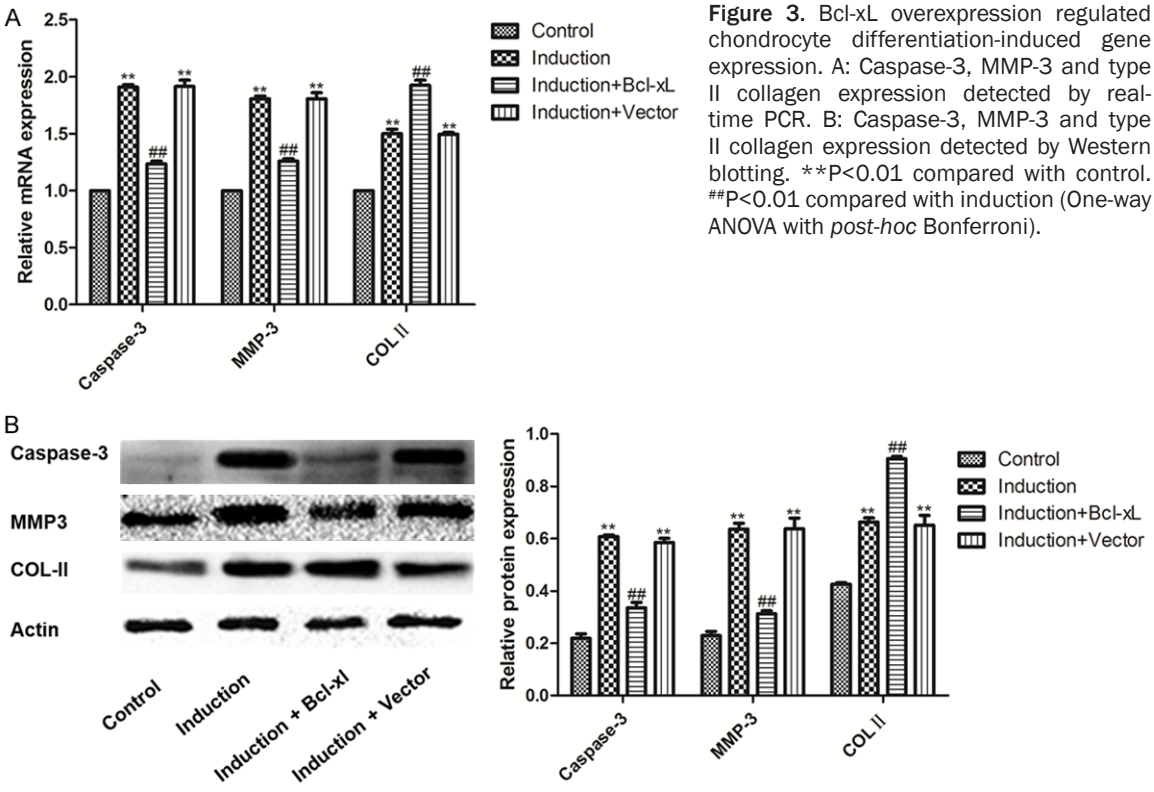

Figure 3. Bcl-xL overexpression regulated chondrocyte differentiation-induced gene expression. A: Caspase-3, MMP-3 and type II collagen expression detected by real-time PCR. B: Caspase-3, MMP-3 and type II collagen expression detected by Western blotting. **P<0.01 compared with control. ##P<0.01 compared with induction (One-way ANOVA with *post-hoc* Bonferroni).

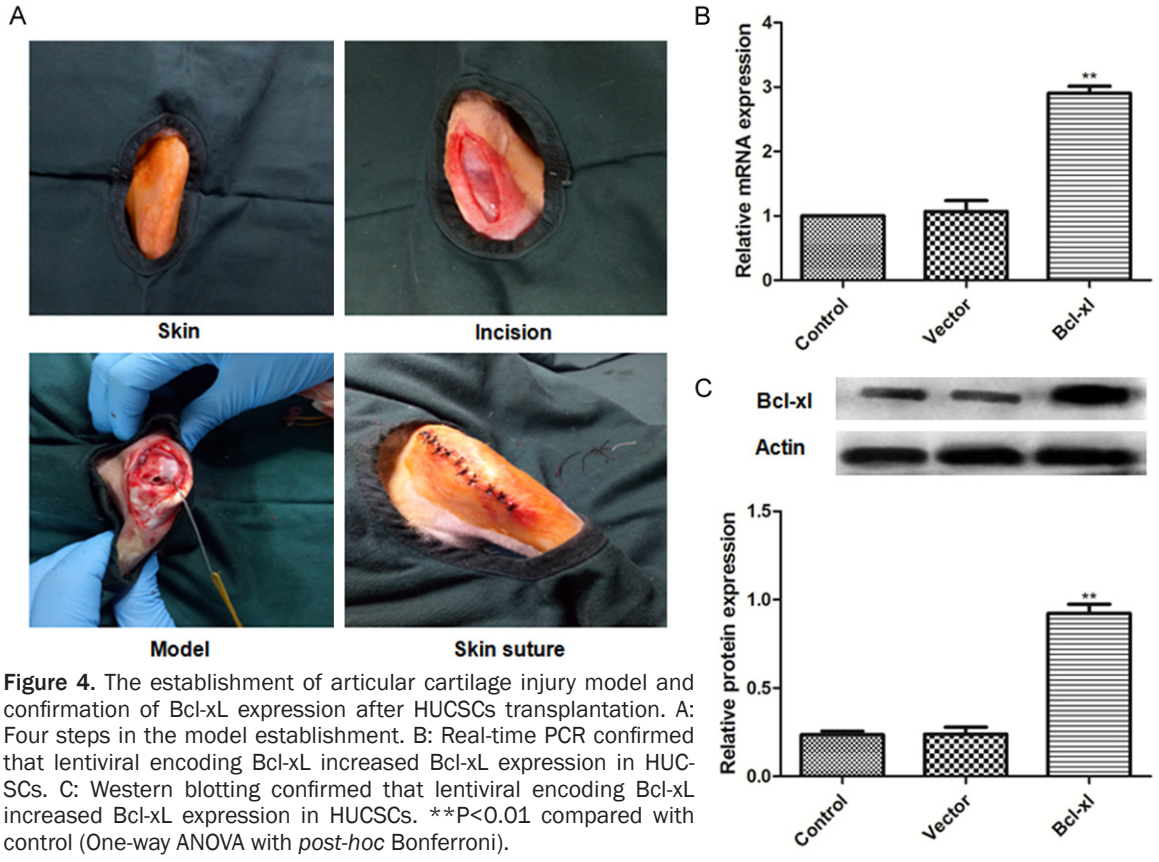

Figure 4. The establishment of articular cartilage injury model and confirmation of Bcl-xL expression after HUCSCs transplantation. A: Four steps in the model establishment. B: Real-time PCR confirmed that lentiviral encoding Bcl-xL increased Bcl-xL expression in HUCSCs. C: Western blotting confirmed that lentiviral encoding Bcl-xL increased Bcl-xL expression in HUCSCs. **P<0.01 compared with control (One-way ANOVA with *post-hoc* Bonferroni).

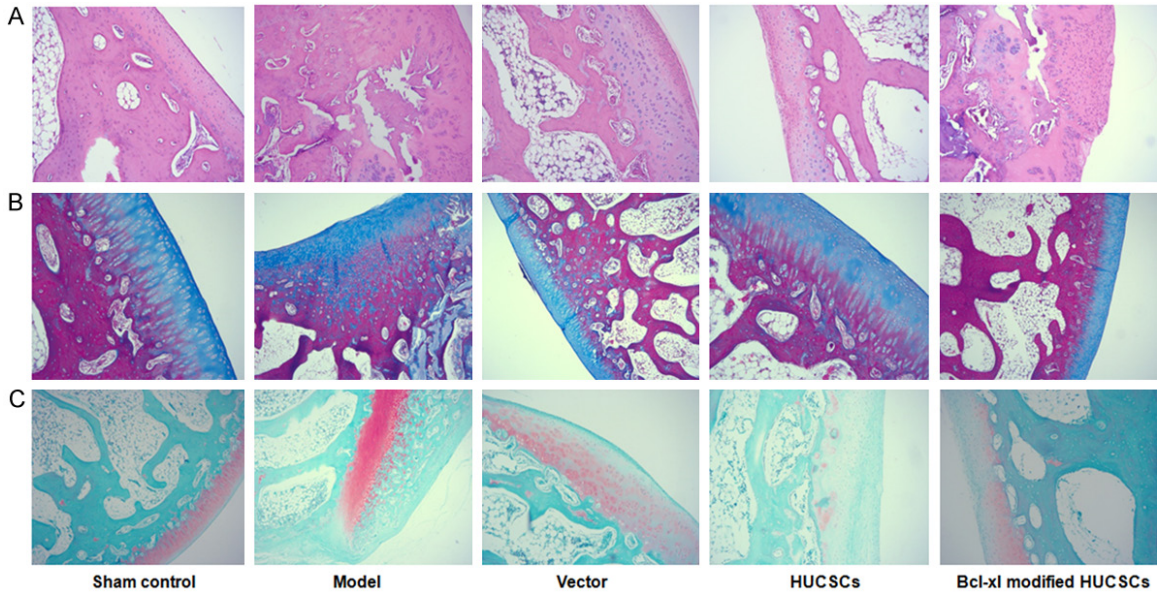

Figure 5. Pathological staining of the articular tissue. A: H&E staining. B: Masson staining. C: Safranin O-fast green staining.

apoptosis was observable after induction (**Figure 2C**). Importantly, viral encoding Bcl-xL, but not vector significantly reduced the differentiation-induced apoptosis (*vs* induction group, P<0.05).

Viral encoding Bcl-xL altered differentiation-induced expression of caspase-3, MMP-3 and type II collagen

As shown in **Figure 3A**, chondrocyte differentiation promoted caspase-3 expression in both of mRNA and protein levels. By contrast, viral encoding Bcl-xL significantly reduced caspase-3 expression compared with induction group. As shown in **Figure 3B**, chondrocyte differentiation also promoted MMP-3 expression, but reduced by viral Bcl-xL expression. A typical feature of chondrocyte differentiation is the expression of type II collagen. As shown in **Figure 3C**, chondrocyte differentiation induced type II collagen expression in both of mRNA and protein levels. Bcl-xL modification further increased the type II collagen expression (*vs* induction, P<0.05).

Transplantation of Bcl-xL modified HUCSCs improved the therapy of HUCSCs

In addition to the *in vitro* effects, the activity of Bcl-xL overexpression was also evaluated in articular cartilage injury model. As shown in **Figure 4A**, articular cartilage injury model was established in rabbits. After modeling, the rabbits are in good condition and there was no death during the modeling process. After transplantation of HUCSCs, Bcl-xL expression was detected in the articular cartilages. As shown in **Figure 4B**, **4C**, Bcl-xL expression was significantly up-regulated in the articular cartilages after transplantation of Bcl-xL modified HUCSCs, compared with control.

As shown in **Figure 5A**, articular structures from Bcl-xL-modified HUCSCs and control group are complete and revealed mature trabecular bone. Cartilages occupied a large area, with clear bone plate boundaries. By contrast, the bone structure of the model group is incomplete. There were a lot of granulation tissues in the defect and a small amount of new bone was formed, while the trabecular bone is arranged irregularly, and the new tissue was not closely linked with the surrounding bone. As shown in **Figure 5B**, a small blue bone was formed in the rabbits from HUCSCs (Bcl-xL overexpression) and control group. Newborn, trabecular bone and lamellar bone tissue were regularly arranged. By contrast, a large blue area occupied articular area and collagen fibers were irregularly arranged in model group. As shown in **Figure 5C**, red area occupied a large area and mature cartilages were observable in the rabbits from HUCSCs (Bcl-xL overexpres-

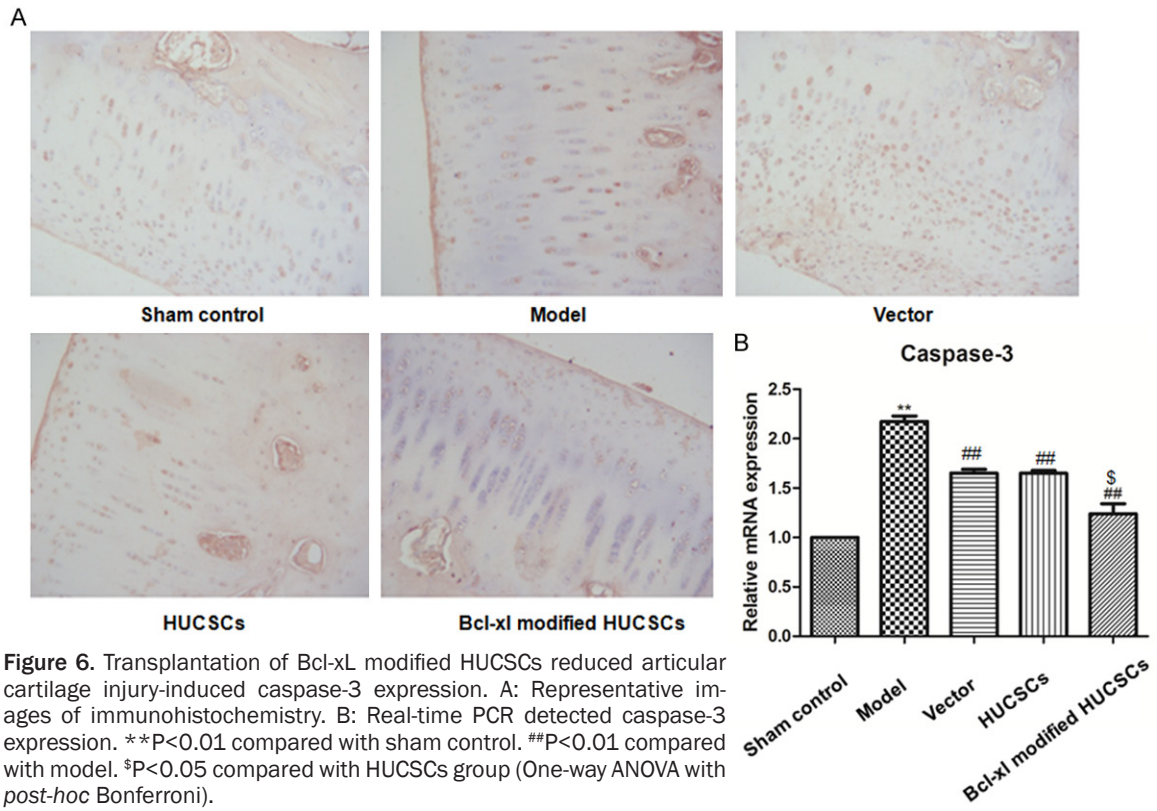

Figure 6. Transplantation of Bcl-xL modified HUCSCs reduced articular cartilage injury-induced caspase-3 expression. A: Representative images of immunohistochemistry. B: Real-time PCR detected caspase-3 expression. **P<0.01 compared with sham control. ##P<0.01 compared with model. $P<0.05 compared with HUCSCs group (One-way ANOVA with *post-hoc* Bonferroni).

sion) and control group. By contrast, model group was revealed by gray green, with small red area, which indicated that complete cartilage tissue has not been formed and the growth of cartilage layer was slow.

Transplantation of Bcl-xL modified HUCSCs reduced articular cartilage injury-induced expression of caspase-3, MMP-3 and type II collagen

As shown in **Figure 6**, articular cartilage injury promoted caspase-3 expression in both mRNA and protein levels. By contrast, transplantation of HUCSCs significantly decreased caspase-3 expression, compared with model group. Interestingly, transplantation of HUCSCs (Bcl-xL overexpression) further decreased caspase-3 expression.

MMP-3 expression was also detected. As shown in **Figure 7**, articular cartilage injury promoted MMP-3 expression, which was reduced by transplantation of HUCSCs. Interestingly, transplantation of HUCSCs (Bcl-xL overexpression) further decreased MMP-3 expression.

As shown in **Figure 8**, articular cartilage injury significantly decreased type II collagen expression in both mRNA and protein levels, which were improved by transplantation of Bcl-xL modified HUCSCs. Interestingly, transplantation of Bcl-xL modified HUCSCs further increased type II collagen expression.

Discussion

In this study, we provided data demonstrating that Bcl-xL overexpression in HUCSCs prevented *in vitro* chondrocyte differentiation-induced apoptosis, and transplantation of Bcl-xL modified HUCSCs could further promote the therapy of HUCSCs in articular cartilage injury model. The mechanisms were related to caspase-3, MMP-3 and type II collagen.

Articular cartilage belongs to hyaline cartilages and covers the surface of synovial joint. The function of articular cartilage was related to reduce joint frictional resistance [18]. Due to the poor cartilage repairing ability, cartilage damage is thought to be an irreversible pathological process [19]. HUCSCs are a type of seed cells, which can be easily obtained. Previous report has demonstrated the cartilage repairing activity of HUCSCs [20]. Transplanted HUCSCs could differentiate into osteogenic and

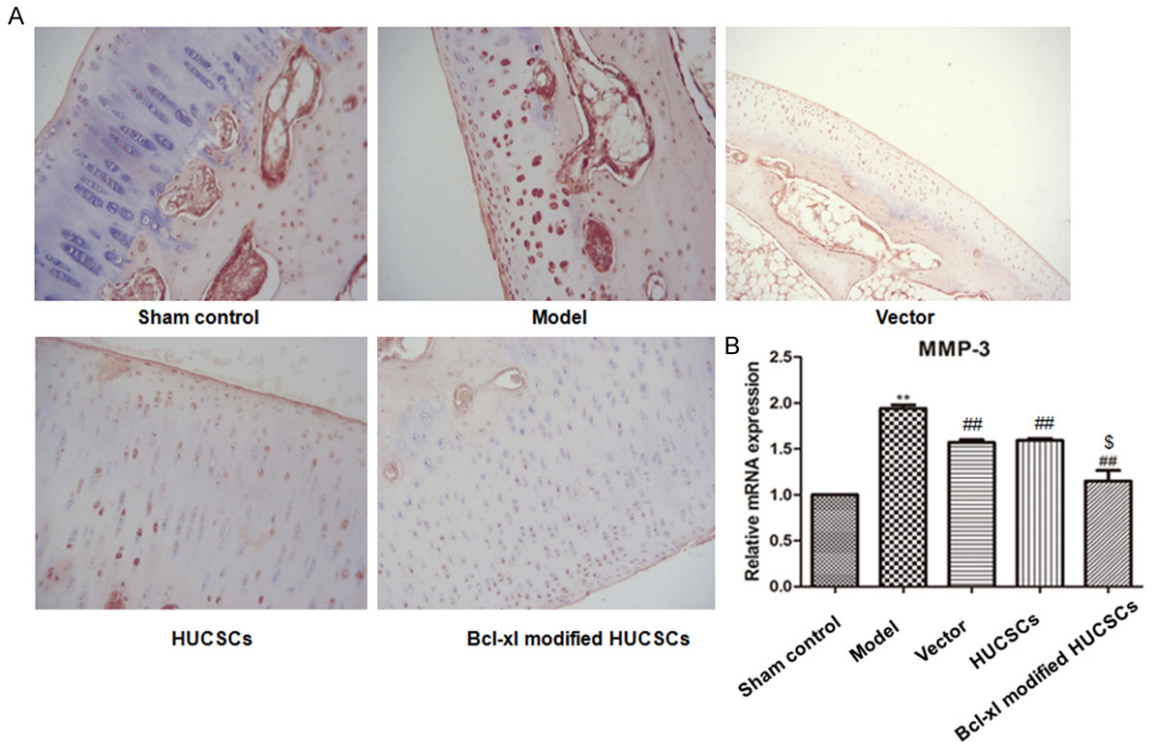

Figure 7. Transplantation of Bcl-xL modified HUCSCs reduced articular cartilage injury-induced MMP-3 expression. A: Representative images of immunohistochemistry. B: Real-time PCR detected MMP-3 expression. **P<0.01 compared with sham control. ##P<0.01 compared with model. $P<0.05 compared with HUCSCs group (One-way ANOVA with *post-hoc* Bonferroni).

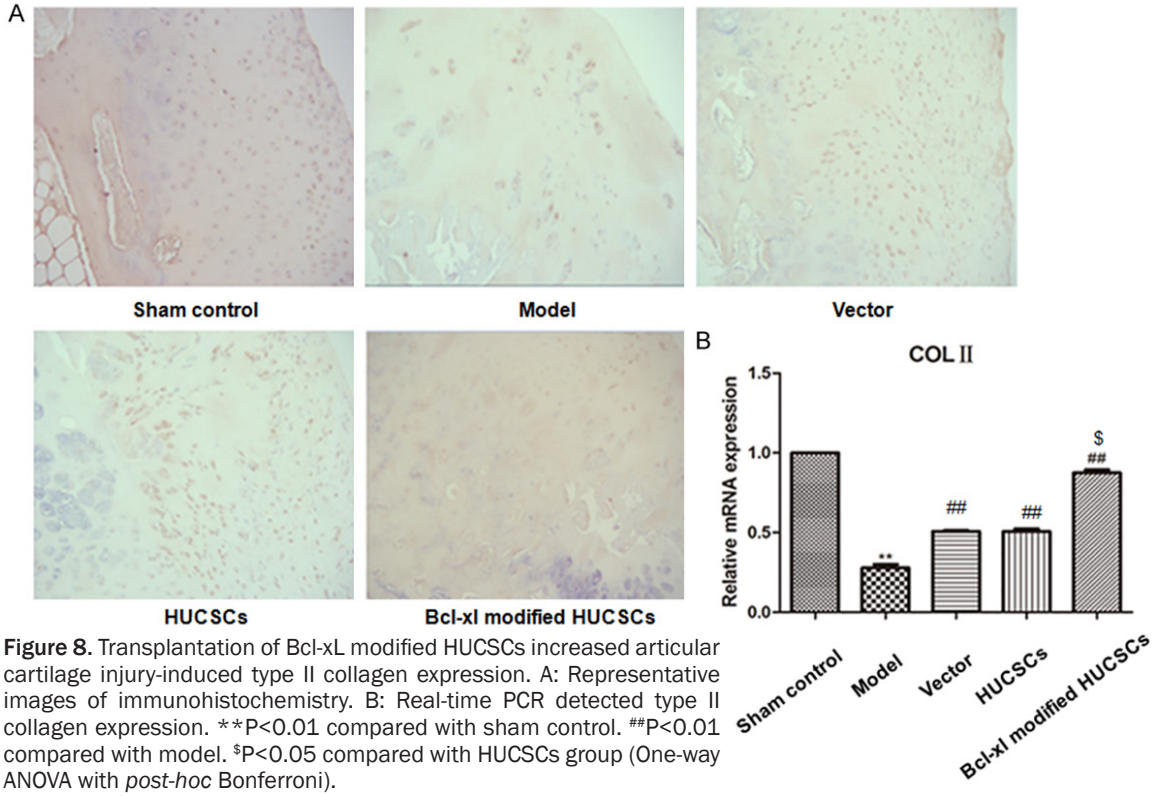

Figure 8. Transplantation of Bcl-xL modified HUCSCs increased articular cartilage injury-induced type II collagen expression. A: Representative images of immunohistochemistry. B: Real-time PCR detected type II collagen expression. **P<0.01 compared with sham control. ##P<0.01 compared with model. $P<0.05 compared with HUCSCs group (One-way ANOVA with *post-hoc* Bonferroni).

chondrogenic cells, and repair the damage of cartilage and subchondral bone.

The surface markers were utilized to identify HUCSCs. The cells in our study were $CD45^-$ and $CD34^-$, but $CD90^+$ and $CD29^+$, which showed that the isolated cells were indeed HUCSCs. We also confirmed the differentiation ability of HUCSCs by toluidine blue staining. The cell size and shape of the cells were uniform, and the cells were in spindle shape. Although HUCSCs can be easily obtained, the number of cells required for tissue engineering is still pretty large. In the process of transplanting seed cells, apoptosis is more likely to occur, and reduce the efficiency of stem cell transplantation. In our study, we reported a new method to improve the repairing ability of HUCSCs, by overexpressing Bcl-xL in HUCSCs.

Bcl-xL gene belongs to anti-apoptotic members, and has anti-apoptotic activity through Bcl-2 dependent and independent pathways [11]. Bcl-xL could inhibit multiple factors-induced apoptosis. In addition to the anti-apoptotic effect, Bcl-xL also has anti-inflammatory effects and is involved in cell regeneration [11]. In our study, Bcl-xL lentivirus could increase the expression of Bcl-xL protein and significantly reduce the apoptosis of chondrocytes. Caspases play a key role in apoptosis [21]. When the cells were stimulated by apoptosis inducers, cytochrome c from the mitochondria will release and activate apoptotic protease activating factor-1 (Apaf-1) in the presence of dATP, subsequently activating caspase-9 and caspase-3 [22, 23].

Matrix metalloproteinase-3 (MMP-3) is helpful for matrix degradation [24]. Moreover, a correlation between MMP-3 and TIMP-1 determines the development and morphology formation of chondrocytes. In the disease conditions, the balance of MMP-3 and TIMP-1 is destroyed [25-27]. The increase of MMP-3 leads to proteoglycan formation in articular cartilage, forming a unique network structure that supports the function of chondrocytes [28]. The results of this current study demonstrated that the induction of HUCSCs into cartilage cells promoted MMP-3 and caspase-3 expression, which might modulate the apoptosis. The increased expression of type II collagen indicated the differentiation of HUCSCs into cartilage cells. The viral Bcl-xL expression decreased the expression of caspase-3 and MMP-3, which indicated that Bcl-xL had anti-apoptotic effects and promoted the survival of seed cells.

In order to further explain the role of stem cells in cartilage injury repairing and the anti-apoptotic effect of Bcl-xL, a rabbit cartilage injury model was constructed and successfully transfected into tissue engineered materials adsorbed by Bcl-xL stem cells. The results demonstrated that the expression of Bcl-xL was significantly increased after transfection with Bcl-xL lentivirus. Special staining also confirmed that Bcl-xL modified HUCSCs could significantly improve the repair the injury of cartilage. Consistently, the expression of caspase-3 and MMP-3 was increased in articular cartilage injury. The expression of type II collagen was also decreased, which subsequently caused destruction of the cartilage matrix. However, the use of Bcl-xL modified HUCSCs significantly reduced the expression of caspase-3 and MMP-3, and significantly increased type II collagen, thereby enabling the repair of damaged cartilage, and ultimately accelerating cartilage repairing.

Conclusions

Bcl-xL overexpression in HUCSCs prevented *in vitro* chondrocyte differentiation-induced apoptosis, and transplantation of HUCSCs with Bcl-xL overexpression could further promote the therapy of HUCSCs in articular cartilage injury model. The mechanisms are related to expression of caspase-3, MMP-3 and type II collagen. Our data provide experimental evidence supporting the application of gene modification to promote the stem cell therapy.

Disclosure of conflict of interest

None.

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