Bcl-xL mutant promotes cartilage differentiation of BMSCs by upregulating TGF-β/BMP expression levels

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Abstract. Bcl-xL is a transmembrane molecule in the mitochondria, with apoptosis-related and pro-metabolic functions, that also plays a role in chondrogenesis and differentiation. A Bcl-xL mutant, in which the GRI sequence is replaced by ELN, has no anti-apoptotic effect, while other biological functions of this mutant remain unchanged. The present study investigated the impact of this Bcl-xL mutant on cartilage differentiation and the expression levels of TGF-β and bone morphogenetic protein (BMP). Human bone marrow mesenchymal stem cells (BMSCs) were transfected with Bcl-xL and Bcl-xL mutant (ΔBcl-xL) overexpression vectors. The cells were divided into four groups: Control (not subjected to any transfection), EV (empty pcDNA3.1-Bcl-xL vector), OV (Bcl-xL overexpression) and ΔOV (ΔBcl-xL overexpression). Saffron and toluidine blue staining was performed to observe cartilage tissue formation. Flow cytometry was conducted to measure BMSC apoptosis. The expression levels of TGF-β and BMP were evaluated using reverse transcription-quantitative PCR (RT-qPCR) and western blotting. Compared with that in the control group, the expression levels of Bcl-xL in the OV group increased significantly ($P<0.05$). Western blotting and RT-qPCR results revealed that OV and ΔOV treatment increased the expression levels of TGF-β and BMP in transfected cells, compared to their expression in the control and EV groups ($P<0.05$). Saffron and toluidine blue staining results showed that cartilage formation was increased in the ΔOV and ΔOV + Bax-/Bak-groups to similar degrees. Cell apoptosis in the ΔOV group did not change compared with that in the control group. The Bcl-xL mutant promoted cartilage differentiation of BMSCs and upregulated TGF-β/BMP expression. This enhancement of chondrogenic differentiation was not related to the expression of Bax and Bak. Taken together, these findings provided for improved application of bone tissue engineering technology in the treatment of articular cartilage defects.

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Introduction

Articular cartilage has complex biomechanical characteristics and high durability (1). However, due to its limited repair activity, irreversible damage to its structure and function can result from external injuries or natural degeneration. Currently, clinical treatment methods for articular cartilage injury include micro-fracture, autologous chondrocyte transplantation and cartilage transplantation (2). However, long-term therapeutic effects are not ideal due to challenges with the application, such as difficulty in obtaining materials and etc. (3). Bone marrow mesenchymal stem cells (BMSCs) have multi-directional differentiation potential and have been widely used as ideal seed cells in bone tissue engineering (4,5). Currently, studies on factors promoting differentiation mainly involve cytokines, intermediate molecules of cartilage differentiation signaling pathways and non-coding RNAs, with satisfactory results being obtained regarding the differentiation of BMSCs into chondrocytes (6,7).

Bcl-xL is a transmembrane molecule in the mitochondria, which belongs to the Bcl-2 family. The classical anti-apoptotic pathway of Bcl-xL plays a role in chondrogenesis and differentiation (8). Nakagami *et al* (9) applied angiotensin II to promote cartilage healing in a fracture model and found that the expression of Bcl-xL mRNA increased significantly. Moreover, Bcl-xL is upregulated in chondrosarcoma with abnormal chondrocyte proliferation (10). A previous study revealed that in addition to apoptosis-related effects, Bcl-xL also functions in an independent pro-metabolic process known as the non-classical Bcl-xL pathway. In this pathway Bcl-xL does not bind to Bax; rather, it directly dissociates from the mitochondrial membrane, enters the nucleus and exerts its role in mediating cellular metabolism by regulating corresponding cytokines (11). Compared with the wild-type Bcl-xL, the Bcl-xL mutant (where the GRI sequence is replaced by ELN) has no anti-apoptotic effect; however, its other biological functions remain unchanged (12). Our previous study found that the expression of TGF-β3 in

BMSCs transfected with Bcl-xL mutant expression vectors was significantly increased (13,14). TGF-β3 is a member of the TGF-β superfamily and plays a significant role in promoting chondrogenic differentiation (13,14). Members of the bone morphogenetic protein (BMP) family also promote cartilage repair (15). BMP-2 enables for the migration and aggregation of mesenchymal stem cells into clusters, maintains them in a tight state, stimulates Smad phosphorylation, enhances Sox9 expression and promotes mesenchymal stem cell differentiation into chondrocytes (16). BMP-7 phosphorylates SMAD1 and SMAD5, and induces the transcription of a variety of osteoblastic and chondrogenic genes (17).

The present study investigated whether Bcl-xL promotes chondrogenic differentiation of BMSCs in the microenvironment of articular cartilage damage through its dual roles in anti-apoptosis and TGF-β/BMP upregulation. The aim was to provide a potential improvement to the application of bone tissue engineering for the treatment of articular cartilage defects.

Materials and methods

Isolation and identification of BMSCs. From January 1st 2018 to September 30th 2018, bone marrow samples from 20 patients (healthy volunteers with lower limb fractures; age, 18-60 years; 10 males and 10 females) were collected from Pu Ai Hospital affiliated to Tongji Medical College, Huazhong University of Science and Technology. All operations were approved by the Ethics Review Committee of Pu'ai Hospital affiliated with Tongji Medical College of Huazhong University of Science and Technology, and all patients signed written informed consent to participate in this study and for their samples to be used for subsequent experiments. After skin preparation, disinfection and toweling in the patient's crotch area, 15 ml of bone marrow solution was obtained and treated with 15 ml of lymphocyte separation solution (cat. no. XY08001T; Shanghai Xinyu Biological Technology Co., Ltd.). The solution was centrifuged at 400 x g for 15 min at 4˚C, resuspended in PBS and centrifuged again using the same conditions. After the supernatant was discarded, the cells were suspended in a complete medium (DMEM/F12 (cat. no. SH30023.01; HyClone; GE Healthcare Life Sciences) containing 10% FBS (cat. no. 10270-106; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin and streptomycin (cat. no. PAB180056; Bioswamp Wuhan Beinle Biotechnology Co., Ltd.) and the cell concentration was adjusted to $1x10^6$ cells/ml. The cells were then cultured in a polylysine-coated culture plate in an atmosphere containing 5% CO_2 and 95% air, at 37˚C for 48 h. The medium was replaced every 72 h and the cells were subcultured or cryopreserved when the confluence reached 80-90%. BMSCs were identified using light microscopy (200x magnification) and flow cytometry (CD34, CD45, CD73, CD90 and CD105) as previously described (18,19).

Vector construction and transfection. The Bcl-xL sequence (NM 138578.1) was obtained from the National Center for Biotechnology Information database. Since cDNA cannot enter the cell directly without a corresponding vector (20), the gene fragment was introduced into BMSCs to make it stable and abundantly expressed. The cDNA of the human Bcl-xL gene was inserted into the pcDNA3.1-Bcl-xL vector (Addgene, Inc.) with *Xho*I and *Nhe*I restriction sites. The digested PCR gene fragments and linearized vector were ligated at 16˚C overnight and the resulting Bcl-xL overexpression vectors were transformed into competent DH5α cells. Target plasmids were extracted from the bacterial liquid according to the manufacturer's instructions. The transfection efficiency was determined using reverse transcription-quantitative PCR (RT-qPCR) as subsequently described.

The cDNA sequence corresponding to the GRI amino acid sequence of Bcl-xL at positions 138-140 was replaced with a sequence encoding ELN using the QuickChange Lighting Site-Directed Mutagenesis kit (Agilent Technologies, Inc.). The pcDNA3.1-ΔBcl-xL plasmid was constructed using pcDNA3.1-Bcl-xL as a template and following the experimental procedures used in the construction of the parent vector.

The following Bax and Bak primer sequences were used: Bax forwards, 5'-GGATGCTCTGAGCAGATCATGAAGATT CAAGAGATCTTCATGATCTGCTCAGAGCTTTTTTCC-3' and reverse, 5'-GAATAAAAAAGCTCTGAGCAGATCATG AAGATCTCTTGAATCTTCATGATCTGCTCAGAGCTC-3'; Bak forwards, 5'-GGATGGCAGAGAATGCCTATGAGTATT CAAGAGATACTCATAGGCATTCTCTGCCTTTTTTCC-3' and reverse, 5'-GAATAAAAAAGGCAGAGAATGCCTATG AGTATCTCTTGAATACTCATAGGCATTCTCTGCCTC-3'. The cDNA for the human Bax and Bak genes were inserted into the pSuper-Bax and pSuper-Bak vectors (Addgene, Inc.), respectively, using *Xho*I and *Bam*HI restriction sites. The target plasmids were extracted from the bacterial liquid and transfected using the Lipofectamine 2000 reagent according to the manufacturer's instructions.

The BMSCs were divided into four groups: Control (not subjected to transfection), EV (empty pcDNA3.1-Bcl-xL vector), OV (Bcl-xL overexpression) and ΔOV (ΔBcl-xL overexpression).

Saffron and toluidine blue staining. The cells ($7.5x10^5$ per group) were resuspended in a 15-ml centrifuge tube and centrifuged at 150 x g for 5 min at 4˚C. The supernatant was aspirated and the cells were resuspended in the complete cartilage differentiation induction medium (RASMX-9004; Cyagen Biosciences, Inc.). The cells were centrifuged at 150 x g for 5 min at 4˚C and incubated at 37˚C in 5% CO_2. After two weeks of continuous induction, the resultant cartilage was fixed with 10% formalin at 4˚C for 48 h, subjected to saffron O staining (cat. no. PAB180084; Wuhan Beinlai Biotechnology Co., Ltd.) for 2 min at 60˚C and then observed under a light microscope (200x magnification).

Cartilage slices were incubated at 65˚C for 1 h and placed in xylene (15 min, 4˚C) and a concentration gradient of alcohol (5 min, 4˚C). After two washes with double-distilled water for 2 min each, the slices were placed in toluidine blue staining solution (cat. no. G3668; Beijing Solarbio Science & Technology Co., Ltd.) for 30 min. The cells were then washed with double-distilled water, sealed with neutral gum and observed under a light microscope (MD1000; Leica Microsystems, Inc.) to detect the integrated optical density values in each group.

Flow cytometry. The cells ($1x10^6$ per group) were resuspended in 100 μl of flow buffer (cat. no. PAB180076; Bioswamp Wuhan

Beinle Biotechnology Co., Ltd.) in an Eppendorf tube and 2 μl of CD45-FITC (cat. no. 11-9459-42, eBioscience; Thermo Fisher Scientific, Inc.), CD34-FITC (cat. no. CD34-581-01; Invitrogen; Thermo Fisher Scientific, Inc.), CD73-FITC (cat. no. 11-0739-42r; eBioscience; Thermo Fisher Scientific, Inc.), CD90-FITC (cat. no. 11-0903-82; eBioscience; Thermo Fisher Scientific, Inc.) or CD105-FITC (cat. no. MA1-19594; Invitrogen; Thermo Fisher Scientific, Inc.) was added. The cells were incubated in the dark for 45 min at 4˚C, following which, 400 μl of flow cytometry dyeing buffer (cat. no. PAB180076; Wuhan Beinlai Biotechnology Co., Ltd.) was added to each tube. The cells were subjected to flow cytometry (CytoFLEX S; Beckman Coulter, Inc.) and the results were analyzed using the CYEXPERT software (CXP Analysis 2.0; Beckman Coulter, Inc.).

Cells were cultured for 24 h at 37˚C, harvested, treated with 1 ml of pre-cooled PBS and centrifuged at 1,000 x g for 5 min at 4˚C. Subsequently, 10 μl of Annexin V-FITC and 10 μl of PI were added. The cell samples were then analyzed using flow cytometry as aforementioned. A one-step fluorescence compensation strategy was used to eliminate interference with the FITC channel (21).

RT-qPCR. Total RNA was extracted from $1x10^6$ cells using Trizol® reagent (according to the manufacturer's procedures), and cDNA was synthesized using a Reverse Transcriptase kit (Takara Bio, Inc.). qPCR was performed using a real-time PCR system (Bio-Rad Laboratories, Inc.) using the SYBR Green PCR kit (cat. no. KM4101; Kapa Biosystems; Roche Diagnostics). Each qPCR reaction was performed in duplicate: 95˚C for 3 min; followed by 39 cycles of 95˚C for 5 sec, 56˚C for 10 sec, 72˚C for 25 sec; 65˚C for 5 sec and 95˚C for 50 sec for final extension, using GAPDH as a housekeeping gene. The results were analyzed using the $2^{-\Delta\Delta Cq}$ method (22). The primers were designed and configured by Nanjing Kingsy Biotechnology Co., Ltd. (Table I).

Western blotting. The protein (20 μg) extracts prepared by cell lysate (cat. no. PAB180006; Bioswamp Wuhan Beinle Biotechnology Co., Ltd.) from BMSCs which had been cultured for 24 h, and the concentration was measured by BCA protein assay kit (cat. no. PAB180007; Bioswamp Wuhan Beinle Biotechnology Co., Ltd.). Total protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes (EMD Millipore). The membranes were blocked with 5% milk in Tris-buffered saline (pH 7.6) containing 0.1% Tween-20 for 2 h at 24˚C. Subsequently, they were incubated overnight at 4˚C with specific primary antibodies against TGF-β1 (1:1,000; cat. no. ab92486; Abcam), BMP2 (1:1,000; cat. no. ab14933; Abcam) and GAPDH (1:1,000; cat. no. 2118; Cell Signaling Technologies, Inc.). After three washes with PBS/Tween 20, the membranes were incubated with a horseradish peroxidase-conjugated secondary goat anti-rabbit IgG (1:10,000; cat. no. PAB150011; Wuhan Beinlai Biotechnology Co., Ltd.) for 2 h at 4˚C. Protein bands were visualized using ECL color detection (Tanon-5200; Tanon Science and Technology Co., Ltd.) and analyzed using the AlphaEase FC gel image analysis software (version 4.2; Tanon Science and Technology Co., Ltd.).

Table I. Primer sequences.

Primer	Sequence (5'→3')
Bcl-xL-F	GCCACTTACCTGAATGACC
Bcl-xL-R	TGAGCCCAGCAGAACC
TGF-β-F	ATTCCTGGCGATACCTCA
TGF-β-R	GGCGAAAGCCCTCAAT
BMP2-F	TGACGAGGTCCTGAGCG
BMP2-R	CCTGAGTGCCTGCGATA
GAPDH-F	CCACTCCTCCACCTTTG
GAPDH-R	CACCACCCTGTTGCTGT

BMP, bone morphogenic protein; F, forward; R, reverse.

Statistical analysis. Data are expressed as the mean ± SD (n=3). To analyze the differences between groups, data comparisons were performed using one-way ANOVAs and subsequent Tukey's post-hoc tests. $P<0.05$ was considered a statistically significant difference.

Results

Isolation and identification of BMSCs. The morphology of BMSCs was observed under a microscope. As shown in Fig. 1A, BMSCs were morphologically consistent and arranged as ordered fibroblast-like cells. The expression of BMSC surface markers was further evaluated (Fig. 1B) and it was found that CD34 and CD45 were negatively expressed (1.03 and 0.78%), while CD73, CD90 and CD105 showed positive expression (88.26, 90.78 and 93.44%, respectively), suggesting that the BMSCs were successfully isolated.

Transfection efficiency of the overexpression and interference vector. The expression of Bcl-xL mRNA in the control, EV and OV groups was assessed to confirm the transfection efficiency of the Bcl-xL overexpression vector. Compared with the control and EV groups, expression of Bcl-xL in the OV group was significantly increased ($P<0.05$) (Fig. 2A). As shown in Fig. 2B, the expression of Bax and Bak in the interference groups was significantly decreased compared with the control group and EV group ($P<0.05$), implying that the respective interference vectors were transfected successfully (Fig. 2).

Effect of the Bcl-xL mutant on BMSC apoptosis and cartilage differentiation. To investigate the effect of Bcl-xL mutant on apoptosis and cartilage differentiation in BMSCs, flow cytometry, toluidine blue staining and saffron staining were used to detect the apoptotic rate. As shown in Fig. 3A, the rate of apoptosis in the OV group was significantly decreased ($P<0.05$) compared with that in the control group, while that of the ΔOV group did not change significantly ($P>0.05$). Evaluation of cartilage differentiation showed that the staining area of the OV and ΔOV groups increased ($P<0.05$) compared with that of the control group (Fig. 3B). To further investigate whether promotion of cartilage differentiation in the Bcl-xL mutant was dependent on the expression of Bax/Bak, plasmids capable of silencing Bax/Bak were constructed to create Bax-/Bak-cells.

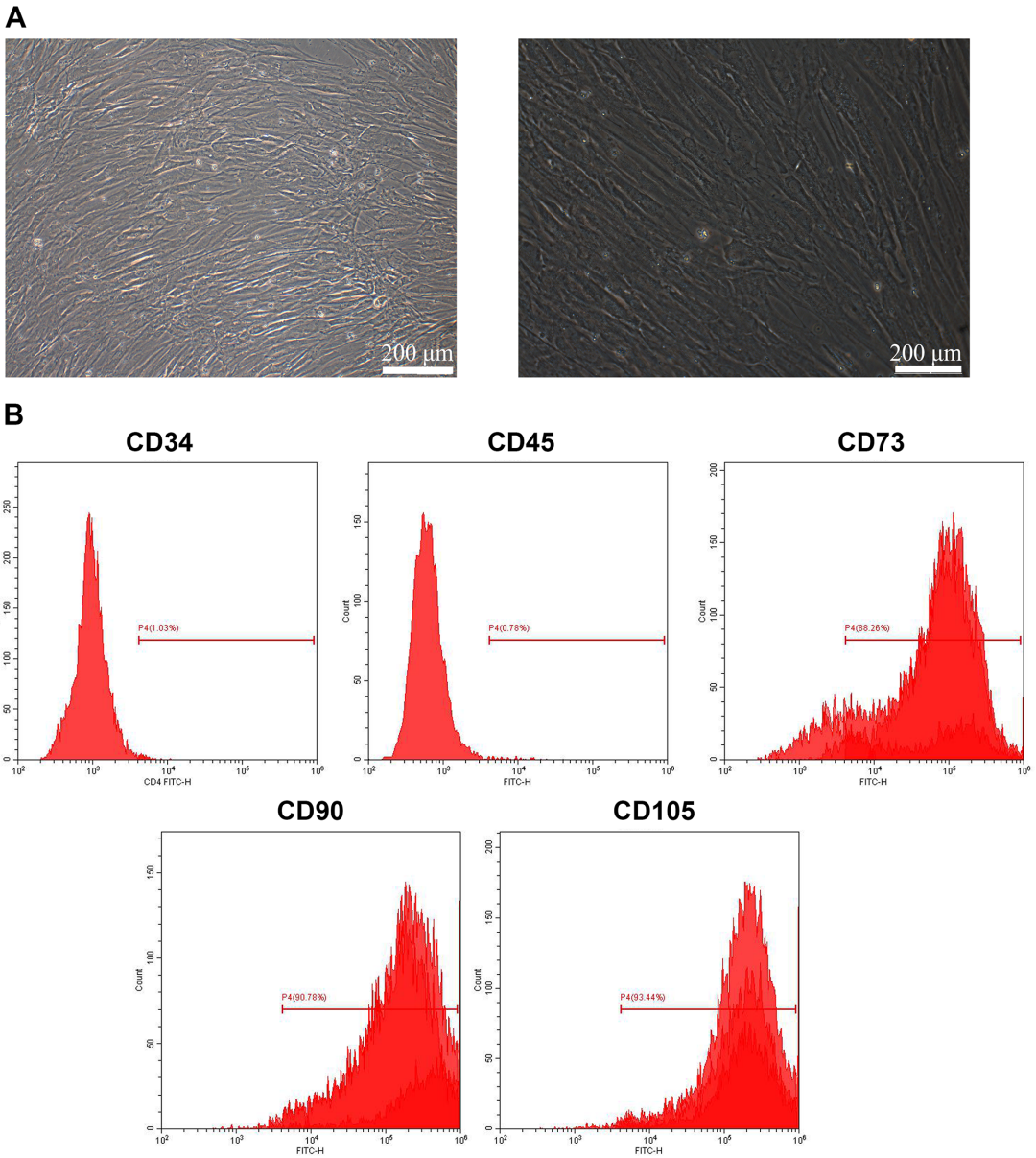

Figure 1. Culture and identification of bone marrow mesenchymal stem cells. (A) Light microscopy of BMSCs (scale bar=100 μm for the left image and 200 μm for the right image). (B) Percentage of CD34-, CD45-, CD73-, CD90- and CD105-positive BMSCs were detected by flow cytometry. BMSC, bone marrow mesenchymal stem cell.

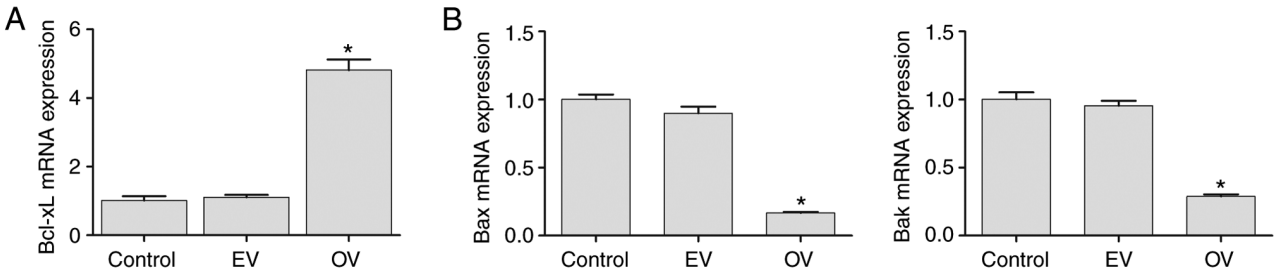

Figure 2. Identification of transfection efficiency. (A) Expression of Bcl-xL after vector construction. (B) Expression of Bax and Bak after pSuper-Bax and pSuper-Bak vector construction. *P<0.05 vs. control (n=3). EV, empty vector group; OV, overexpression group.

Compared with that in the control group, the area of ΔOV and ΔOV+Bax-/Bak-staining increased (P<0.05), while there was no significant difference in cartilage formation (indicated by saffron and toluidine blue staining) between ΔOV and ΔOV+Bax-/Bak- (P>0.05) (Fig. 3B). These observations suggested that the Bcl-xL mutant promoted the differentiation of BMSCs into cartilage without affecting BMSC apoptosis

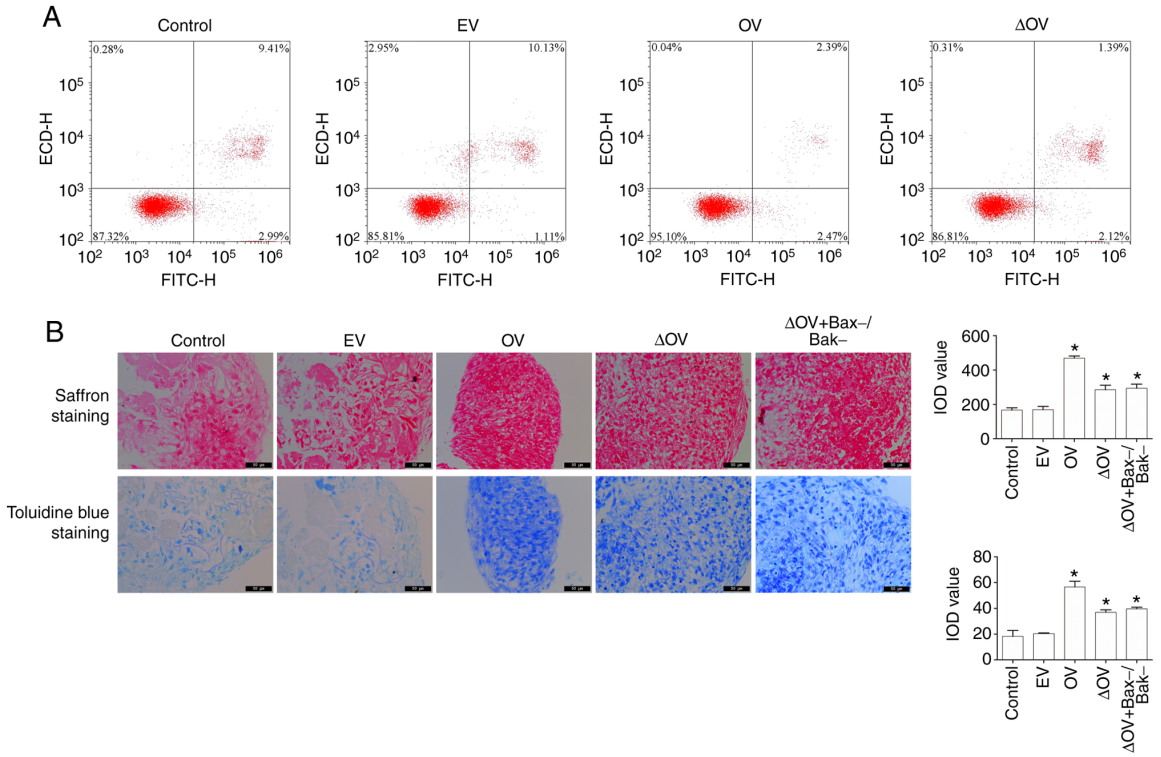

Figure 3. Effect of Bcl-xL mutant on BMSC apoptosis and cartilage differentiation. (A) Detection of apoptosis by flow cytometry. (B) Cartilage tissue morphology was observed using saffron and toluidine blue staining (scale bar=50 μm) and the IOD values of each group were statistically analyzed. *P<0.05 vs. control (n=3). EV, empty vector group; OV, overexpression group; ΔOV, ΔBcl-xL overexpression group. IOD, Integrated optical density.

and that the effect of promoting cartilage differentiation of BMSCs is not dependent on Bax-/Bak-.

Effect of the Bcl-xL mutant on the expression of TGF-β and BMP. Western blotting and RT-qPCR were conducted to assess the expression of TGF-β1 and BMP2. Fig. 4A and B show that OV and ΔOV induced higher protein and mRNA expression levels of TGF-β1 and BMP2 than those observed in the control and EV groups (P<0.05). Compared with the OV group, the expression of TGF-β1 and BMP2 in ΔOV group was significantly decreased (P<0.05), suggesting that Bcl-xL mutant improved the expression of TGF-β1 and BMP2.

Discussion

Previous studies have shown that BMSCs are suitable for clinical applications owing to their straightforward isolation and differentiation from a variety of tissues (23,24). In addition, BMSCs are considered to be good vectors for cell-mediated gene therapy, as they are relatively easy to handle *in vitro* (25). At present, most clinical methods for repairing articular cartilage injury are invasive; therefore, the development and application of new methods employing more conservative approaches are urgently needed. Direct intra-articular injection of BMSCs is a potentially conservative cell therapy for the repair of cartilage defects (26). There are two primary methods for applying BMSCs in treatment: One utilizes a suitable cell matrix as a carrier for the implantation of an *in vitro* stem cell scaffold, while the other involves the *in vivo* differentiation of BMSCs by co-culturing with target cells. Jin (27) demonstrated the

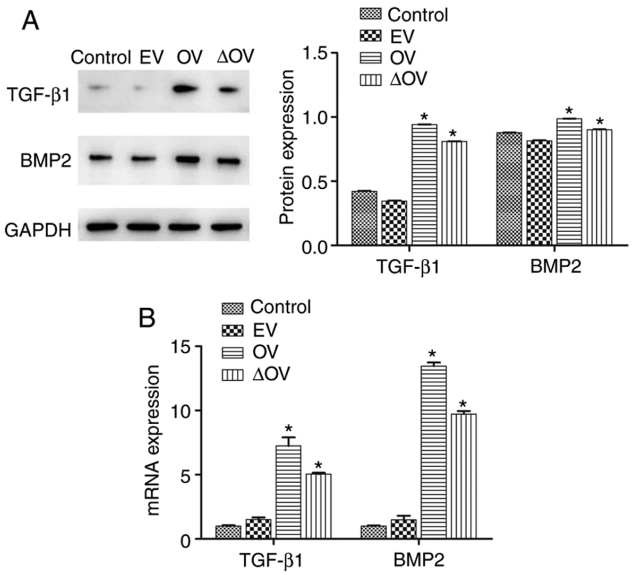

Figure 4. Effects of Bcl-xL mutants on the expression of TGF-β1 and BMP2. (A) Western blotting evaluation of TGF-β and BMP protein expression levels. (B) mRNA expression of TGF-β1 and BMP2 was measured using reverse transcription-quantitative PCR. *P<0.05 vs. control (n=3). EV, empty vector group; OV, overexpression group; ΔOV, ΔBcl-xL overexpression group. BMP, bone morphogenic protein; IOD, Integrated optical density.

feasibility and high efficiency of autologous exostromal BMSC scaffolds in cartilage tissue engineering, with both *in vivo* and *in vitro* experiments. Hu (28) also confirmed that BMSC and chondrocyte aggregation co-cultures improved cartilage repair in a study on rabbit knee articular cartilage defect.

However, the targeted cartilage differentiation of BMSCs is dependent on the local microenvironment and the synergistic effects of various inducing factors (29). The effective induction of BMSC differentiation into chondrocytes and initiation of cartilage tissue formation has become a challenging process that needs to be addressed.

Bcl-xL is an important anti-apoptotic molecule in the Bcl-2 family that inhibits the pro-apoptotic molecule, Bax, preventing the apoptosis caused by the release of cytochrome C (30). Bcl-xL also blocks apoptosis by inhibiting the binding of Apaf-1 and caspase-9 downstream of Bax activation (31), as well as by inhibiting apoptosis induced by the Fas-FasL pathway (32). Therefore, Bcl-xL exerts important anti-apoptotic effects by blocking various apoptotic pathways stimulated by pro-apoptotic factors, such as hypoxia and inflammation, thereby demonstrating its role in promoting chondrogenic differentiation (33). Wang *et al* (34) reported an increase in cartilage formation during fracture repair in mice with Bax gene deletion, suggesting that Bcl-xL continues to promote cartilage production and repair during chondrogenesis, even in the absence of the anti-apoptotic effect of Bcl-xL (presumably through alternative pathways). Mutants are defined as individuals with mutations showing phenotypes that differ from the wild type. A previous study have shown that the Bcl-xL mutant, where the GRI amino acid sequence at positions 138-140 is replaced by ELN, has no anti-apoptotic effect, while its other biological functions remain similar to those of wild-type Bcl-xL (12).

The present study examined the anti-apoptotic effect of the Bcl-xL mutant on cartilage differentiation of BMSCs. it was demonstrated that the Bcl-xL mutant promoted cartilage differentiation of BMSCs without affecting BMSC apoptosis, whereby this effect was not related to the expression of Bax and Bak. The potential mechanism of action of the Bcl-xL mutant on promoting cartilage differentiation of BMSCs was further examined. The results showed that the expression of TGF-β and BMP increased significantly after Bcl-xL and Bcl-xL mutant intervention, revealing that the Bcl-xL mutant may promote cartilage differentiation of BMSCs by upregulating TGF-β/BMP expression levels.

A limitation of the present study was that the differentiation experiments were performed after a long term culture. BMSCs can differentiate to chondrocytes within two weeks. As such, analyzing the effects of overexpression of Bcl-xL longitudinally, examining the expression profiles of this protein in the various groups to show the kinetics of expression, is necessary. However, the main aim of the present study was to observe the effect of the Bcl-xL mutant on cartilage differentiation and the expression of TGF-β and BMP. As such, further studies should examine the effects of Bcl-xL in the various BMCS treatment groups in subsequent experiments.

In conclusion, the present study demonstrated that Bcl-xL mutants promoted cartilage differentiation of BMSCs and upregulated TGF-β/BMP expression levels, whereby this enhancement of chondrogenic differentiation was not related to the expression of Bax and Bak.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KX, LY and WX contributed to the conception of the study. KX and XG designed and performed the experiments, analyzed the data and wrote the manuscript. RH and MX analyzed the data and provided technical support. KX and LY confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All operations were approved by the Ethics Review Committee of Pu'ai Hospital affiliated with Tongji Medical College of Huazhong University of Science and Technology [approval no. (2017)IEC(S118)]. Informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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