Biochemical and Biophysical Research Communications 432 (2013) 444–450

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Differentiation of GFP-Bcl-2-engineered mesenchymal stem cells towards a nucleus pulposus-like phenotype under hypoxia *in vitro*

Zhong Fang [a,1], Qin Yang [b,1], Wei Luo [a], Guang-hui Li [a], Jun Xiao [a], Feng Li [a], Wei Xiong [a,*]

[a] Department of Orthopaedics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, PR China
[b] Department of Pathology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, PR China

ARTICLE INFO

Article history:
Received 16 January 2013
Available online 12 February 2013

Keywords:
Stem cell
Differentiation
Nucleus pulposus
Apoptosis

ABSTRACT

Differentiation of bone marrow-derived mesenchymal stem cells (MSCs) into a nucleus pulposus-like phenotype under hypoxia has been proposed as a potential therapeutic approach for intervertebral disc degeneration. However, limited cell viability under hypoxic conditions has restricted MSC differentiation capacity and thus restricted its clinical application. In this study, we genetically modified MSCs with an anti-apoptotic GFP-Bcl-2 gene and evaluated cell survival and functional improvement under hypoxia *in vitro*. Rat bone marrow MSCs were transfected by lentiviral vectors with the GFP-Bcl-2 gene (GFP-Bcl-2-MSCs). Cell proliferation and apoptosis were assessed, and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) was carried out to evaluate phenotypic and biosynthetic activities. In addition, Alcian blue staining was used to detect the formation of sulfated glycosaminoglycans (GAGs) in the differentiated cells. We found that the Bcl-2 gene protected MSCs against apoptosis. We also observed that Bcl-2 over-expression reduced apoptosis by 40.61% in non-transfected MSCs and 38.43% in vector-MSCs to 18.33% in Bcl-2-MSCs. At 3 days, the number of viable Bcl-2-MSCs was approximately two times higher than the number of MSCs or vector-MSCs under hypoxic conditions. RT-PCR showed higher expression of chondrocyte-related genes (Sox-9, aggrecan and type II collagen) in GFP-Bcl-2-MSCs cultured under hypoxia. The accumulation of proteoglycans in the pellet was 86% higher in GFP-Bcl-2-MSCs than in the control groups. Furthermore, the ratio of proteoglycans/collagen II in GFP-Bcl-2-MSCs was 6.2-fold higher compared to the MSC and vector-MSC groups, which denoted a nucleus pulposus-like differentiation phenotype. Our findings support the hypothesis that anti-apoptotic gene-modified MSCs can differentiate into cells with a nucleus pulposus-like phenotype *in vitro*, which may have value for the regeneration of intervertebral discs using cell transplantation therapy.

1. Introduction

Diseases related to intervertebral disc degeneration (IDD) are common in the clinic, and morbidity is increasing as a consequence of population aging [1]. Degeneration of the intervertebral disc is caused by a decrease in nucleus pulposus cells and a subsequent decrease in proteoglycans in the extracellular matrix (ECM) [2,3]. Current invasive and non-invasive treatments aim for relief of symptoms by temporarily decreasing the axial load of intervertebral discs or by removing the degenerated intervertebral discs and stabilizing the spine. Ideally, treatment of disc degeneration would accomplish full functional recovery [4]. For instance, treatment may repair (or regenerate) the nucleus pulposus (NP), producing a matrix with similar or improved biological and biomechanical properties compared with the original. Prior research has found that rat bone marrow mesenchymal stem cells (MSCs) can be differentiated into nucleus pulposus (NP)-like cells under hypoxic conditions [5].

Hypoxia is a key factor for *in vitro* differentiation and *in vivo* transplantation, but the high rate of cell apoptosis under hypoxia restricts MSC differentiation capacity and thus has hindered clinical application [6,7]. Therefore, it is necessary to reinforce MSCs against the arduous microenvironment associated with ischemia, the inflammatory response, and pro-apoptotic factors in order to improve the efficacy of stem cell therapy. The 26-kDa anti-apoptotic protein Bcl-2 belongs to the Bcl-2 family, which serves as a critical regulator of pathways involved in apoptosis [8]. Previous studies have demonstrated that Bcl-2 regulates the metabolic functions of mitochondria during ischemic conditions, which can contribute to both cardiac and neuronal protection under various stresses [6,9]. Over-expression of Bcl-2 can delay the onset of cell death and modestly augment viable cell growth in the first 48 h following hypoxia [10].

* Corresponding author.
E-mail address: hbwhfz@yahoo.com.cn (W. Xiong).
[1] These authors contributed equally to the work.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.01.127

In this study, adult rat bone marrow-derived MSCs were genetically modified to over-express GFP-Bcl-2 in order to increase stem cell viability and enhance their resistance to ischemic conditions. We hypothesized that genetic modification of stem cells with GFP-Bcl-2 would protect stem cells placed in a deteriorative ischemic microenvironment and improve viability in the early differentiation period, thereby enhancing their functional transformation in the later differentiation period.

2. Materials and methods

Fetal bovine serum (FBS), penicillin G, and L-glutamine TGF-β1 were purchased from Sigma Chemical, USA. Trypsin, Dulbecco's modified Eagle's medium (DMEM) and Lipofectamine 2000 were procured from Invitrogen, USA. PCR primers, Taq DNA polymerase, DNA ladder and Oligo dT were obtained from Sangon, China. The AgeI, EcoRI, and restriction enzyme HindIII were provided by NEB. The Plasmid DNA Extraction (Mini) Kit was provided by QIAGEN, UK. The *Escherichia coli* strain DH5a, the lentiviral vector pGCSIL-GFP, packaging system pHelper1.0 and pHelper2.0 were provided by GeneChem, China. HEK293T cells (ATCC#: CRL-11268) were used to generate lentiviral particles. Sprague–Dawley rats were obtained from the Experimental Animal Center of Tongji Medical College and were used following protocols approved by the Animal Care and Use Committee of Tongji Medical College of Huazhong University of Science and Technology (Permit number: 20051007).

2.1. Construction of plasmid vectors and lentiviral particles

To construct the eukaryotic expression vector, we used a clone containing the Bcl-2 gene (NM_005631) provided by Life Science College (Wuhan University, Wuhan, China). We designed the primers Bcl2-*Hind*III and Bcl2-*Age*I. A full-length sequence of the Bcl-2 gene, obtained by PCR, and pGCSIL-GFP were double digested with *Hind*III and *Age*I, respectively. After purification, the products of the enzyme digestions were connected directionally and transformed into DH5a-competent *E. coli* cells. A colony was selected for PCR, and the recombinant plasmids were extracted for sequence detection. Lastly, the endotoxin was removed from the recombinant eukaryotic expression vector (pGCSIL-GFP-Bcl2), and the plasmid was extracted.

We packaged the recombinant lentiviral vector for GFP-Bcl-2 with the highest interfering efficiency. After removing the endotoxins from the GFP-Bcl-2 lentiviral expression vectors and the packaging vectors (pHelper1.0 and pHelper2.0, respectively), we co-transfected the three types of vectors in 293T cell lines according to the instructions for Lipofectamine 2000. At 8 h after co-transfection, the cells were transferred to a complete medium. After 48 h, the lentiviral supernatant was harvested and concentrated. The virus titer was determined and calibrated in the 293T cell lines. The 293T cell culture medium was then centrifuged and concentrated to obtain the Bcl-2 lentivirus solution, which was named Lv-SIL-GFP-Bcl2 and kept in a collection cup at −80 °C. The concentrated virus stock was diluted 10× and then used to transfect 293T cells. To obtain the titer of the virus stock, we multiplied the number of fluorescent cells in 10% of the well and used the corresponding dilution.

2.2. Isolation, culture and genetic modification of MSCs

The isolation and cell culture of rats MSCs were described previously [11]. To achieve high rates of viral infection, we used a protocol involving two centrifugation steps. Cells from sub-confluent cultures were harvested by treatment with 0.05% (w/v) EDTA in phosphate-buffered saline (PBS) containing $MgCl_2$, $CaCl_2$ and 0.25% (w/v) trypsin. Cells were seeded at a density of 100,000 cells/cm^2 and centrifuged at 1000*g* at 37 °C for 10 min. The concentrated virus preparation was diluted 1:1.5 with DMEM medium and applied to the pre-centrifuged cells, which were then incubated at 37 °C for 40 min before a second centrifugation step for 60 min was performed. The infected cells were incubated under standard conditions overnight, followed by a media change. Bcl2-GFP reporter gene expression was analyzed after an incubation period of 24, 48 and 72 h. Viral titers were determined by flow cytometry [12]. Non-transfected MSCs and those transfected with Lv-SIL-GFP-Bcl2 or Lv-SIL-GFP are termed "MSCs," "Bcl-2-MSCs," and "vector-MSCs," respectively. All experiments and cell number determinations were performed in triplicate.

2.3. Differentiation of MSCs towards an NP-like phenotype under hypoxia

MSCs of the third passage were rinsed three times with PBS and harvested with 0.25% trypsin. Suspensions containing 5×10^5 cells were centrifuged at 20*g* for 5 min in 5 mL of growth medium in 15 mL polypropylene conical tubes to form an aggregated cell pellet [13]. To induce the differentiation of MSCs towards an NP-like phenotype, the pellets were cultured in differentiating medium (DMEM/HG supplemented with 10 ng/ml TGF-β1, 100 nmol/L dexamethasone, 50 μg/ml L-ascorbic acid 2-phosphate, 100 μg/ml sodium pyruvate, 40 μg/ml proline and 100× ITS-liquid media supplement). The pellets were cultured at 37 °C in a triphasic mini-incubator (Labnet International, USA) with 2% O_2 and 5% carbon dioxide. The pellets were harvested at days 7 and 14 for evaluation. The retrieved cells were then washed with PBS and used for RNA isolation. All cultures were conducted in triplicate.

2.4. Cell apoptosis assay

For the indicated times, cells were harvested using trypsin/EDTA, counted, and collected by centrifugation in PBS. Phosphatidylserine (PS) exposure on the outer leaflet of the plasma membrane was detected using the fluorescent dye Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, USA) according to the manufacturer's instructions. Similarly, TUNEL staining was performed with the Apo-BrdU Apoptosis Detection kit (BD Biosciences, USA) according to the manufacturer's instructions. All data were collected and analyzed by Lysis II software (BD Biosciences, USA). The experiments were repeated three times, and the results are presented as the mean ± SD.

2.5. DNA synthesis

For the indicated times, the cells were harvested, and the retrieved cells were exposed to 2 μCi/mL [^{3}H] thymidine for 2 h. After the cells were treated with trichloroacetic acid, they were dissolved with 0.1 mol/L NaOH, and the incorporated radioactivity was counted and normalized to the concentration of total cell protein, which was measured in parallel samples according to the method of Lowry [14].

2.6. Histology

Randomly selected pellets ($n = 3$ per data point) were fixed overnight at 4 °C in 4% paraformaldehyde, paraffin-embedded, and sectioned to 5 μm. Consecutive sections were stained with trichromate stain (hematoxylin, Alcian blue, picrosirius) for collagen (red) and proteoglycans (blue) as described by HE Grube [15].

Table 1
Design of primers and probes.

Gene	Gene primers: 5′–3′
Bax	F: tcc-ccc-cga-gag-gtc-ttt-t R: cgg-ccc-cag-ttg-aag-ttg
Bcl-2	F: aga-acc-ttg-tgt-gac-aaa-tga-gaa R: tac-cca-tta-gac-ata-tcc-agc-ttg-a
PBGD	F: ctg-gta-acg-gca-atg-cgg-ct R: gca-gat-ggc-tcc-gat-ggt-ga
Sox9	F: gac-ttc-cgc- gac-gtg-gac- R: cag-tac-ctg-ccg-ccc-aac
Collagen II	F: ggc-aat-agc-agg-ttc-acg-tac-a R: cga-taa-cag-tct-tgc-ccc-act
Aggrecan	F: cgc-gag-acc-tgg-gtg-gat-gc R: gaa-ggg-gca-ggc-tgg-ata-ttg-c

Abbreviations: F, forward; R, reverse.

2.7. Measurement of proteoglycan synthesis

To assess proteoglycan (PG) accumulation, the amount of sulfated glycosaminoglycan content in each of the pellets was quantified by dimethylmethylene blue dye assay (DMMB, Sigma, USA). Briefly, randomly selected pellets ($n = 3$ per data point) were digested using 10 times concentrated papain solution (200 mg/mL in 50 mmol/L EDTA, 5 mmol/L L-cysteine) for 24 h at 55 °C. Digested samples were mixed with DMMB buffer solution, and the absorption of these samples was measured with a spectrophotometer. DMMB dye reagent (200 μl) with 40 μl papain digested samples was added to the wells of a 96-well plate. The plate was read on an absorbance plate reader at 525 nm with the purified nasal septum D1 PG (Sigma, USA) to create a standard curve. An aliquot of papain digests was also used to measure DNA content by uptake of [^{3}H]-thymidine in order to obtain PG/per ng DNA data.

2.8. RT-PCR analysis

The total RNA was extracted from different samples using Trizol reagent to detect the expression of apoptosis-related genes (Bcl-2 and Bax) and chondrocyte marker genes (Sox-9, aggrecan and type II collagen). Porphobilinogen dehydrogenase gene (PBGD) served as an endogenous control. The PCR procedure was carried out with the primers indicated in Table 1. The samples were preheated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C (Bcl-2 and Bax), then 1 cycle at 94 °C for 1 min, 30 cycles at 94 °C for 30 s, 55 °C for 10 s, 72 °C for 40 s and 1 cycle at 72 °C for 5 min (Sox-9, aggrecan and type II collagen). Finally, the PCR product was visualized by a Bio-Imaging System (Bio-Rad Co, USA), and the ratio of target genes/PBGD was calculated and used as an indicator of relative expression levels.

2.9. Statistical analysis

The experiments were repeated three times. All data are represented as the mean ± SD, and statistical analyses were carried out using the SPSS software package (Version 12.0). The data were analyzed using the independent-samples t test or a paired t test. Bax/Bcl-2 data were not normally distributed and were therefore tested using the Wilcoxon signed-rank test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Over-expression of Bcl-2 in genetically modified MSCs

The transfection efficiency for genetically modified MSCs was evaluated by the expression of GFP using fluorescence microscopy

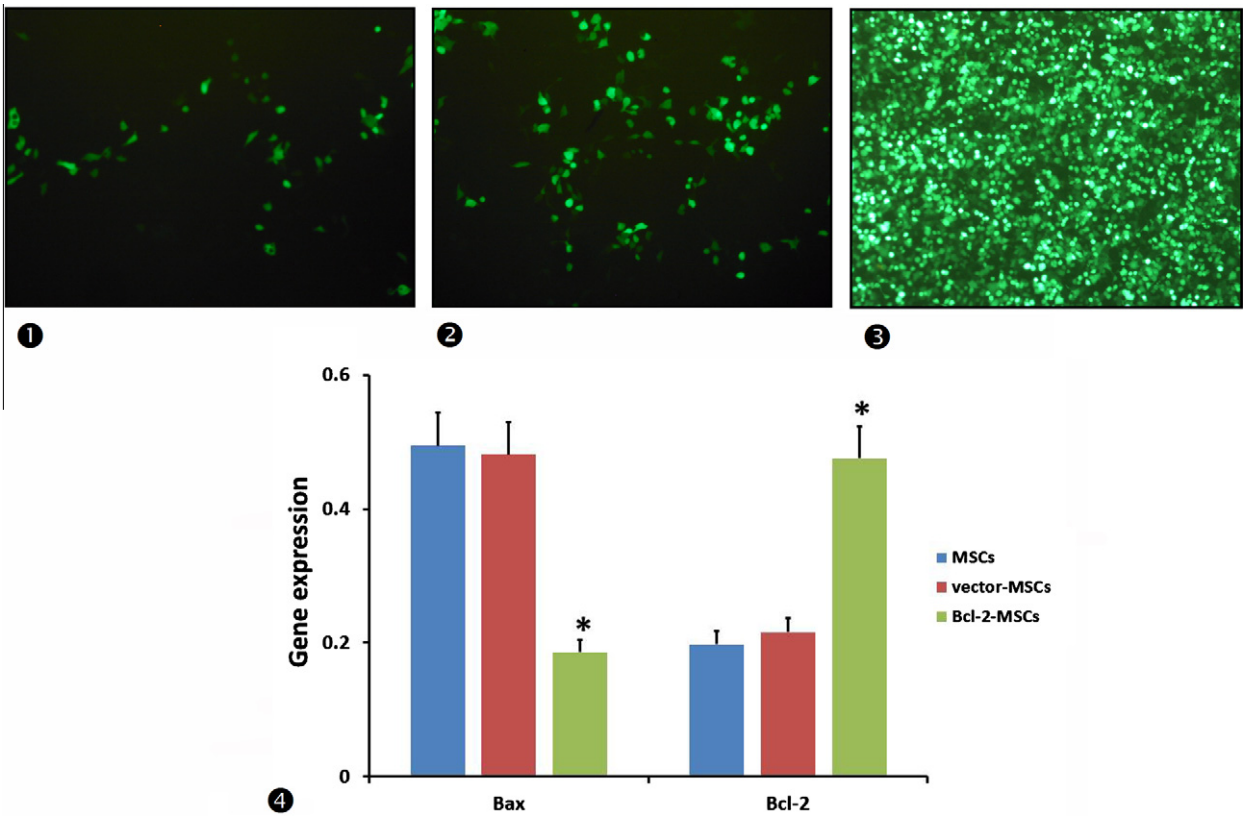

Fig. 1. Over-expression of Bcl-2 in genetically modified MSCs. Determination of transfection efficiency was performed by fluorescence microscopy after transfection (24 h (1), 48 h (2), 72 h (3), 200-fold magnification). (4) Bcl-2 and Bax gene transfection efficiency for genetically modified MSCs was detected on day 3. $^*p < 0.05$, Bcl-2-MSCs vs controls.

(Fig. 1-1–3). At 3 days, the transfection efficiency peaked at almost 83%. To evaluate the expression of the Bcl-2 gene in the genetically modified MSCs *in vitro*, RT-PCR was performed on cell samples at various time points post-transfection (24, 48, and 72 h). Compared with MSCs and Vector-MSCs, significant expression of Bcl-2 was observed in Bcl-2-MSCs as early as 24 h and remained detectable on day 3 ($p < 0.05$; Fig. 1-4). In addition, significantly reduced expression of Bax was detected in Bcl-2-MSCs compared with the other two groups on day 3 ($p < 0.05$; Fig. 1-4).

3.2. Bcl-2 modified MSCs protect against apoptosis in vitro

To test the ability of Bcl-2-MSCs to protect against apoptosis *in vitro*, the modified MSCs were treated under hypoxic conditions for 3 days. Under these conditions, the rate of cell apoptosis in MSCs and vector-MSCs exceeded that of Bcl-2-MSCs by 2.2-fold, as determined by the detection of Annexin V/PI staining. Thus, Bcl-2 genetic modification significantly reduced the apoptotic cell number (Fig. 2-1). Quantitative analysis showed that the percentage of TUNEL-positive cells was decreased in Bcl-2-MSCs (18.33 ± 1.1%) compared to the percentage found in MSCs (40.61 ± 2.2%) or vector MSCs (38.43 ± 1.9%; $p < 0.005$; Fig. 2-2). Additionally, the number of cells in Bcl-2-MSC cultures was 79.0×10^5, approximately two times higher than that of MSC cultures (37.94×10^5) or vector-MSC cultures (43.4×10^5; Fig. 2-3). These findings demonstrated that the Bcl-2 modified MSCs could protect against apoptosis under hypoxic conditions.

3.3. Bcl-2 modified MSCs differentiated towards an NP-like phenotype under hypoxia

To identify the potential of Bcl-2-MSCs for differentiation towards an NP-like phenotype under hypoxia, we examined the secretion of proteoglycan (PG) with DMMB dye and the accumulation of collagen and proteoglycans using histology. We also measured chondrocyte-related (Sox-9, aggrecan and type II collagen) mRNA expression. MSCs, vector-MSCs, and Bcl-2-MSCs were maintained under hypoxic conditions in pellet culture for up to 14 days. PG accumulation, evaluated by measurement of S-GAG content, was significantly increased in the pellets seeded under hypoxia after 7 days in culture (MSCs: 28.5 ± 2.7 mg/mL; vector-MSCs: 31.4 ± 1.6 mg/mL; and Bcl-2-MSCs: 58.3 ± 3.4 mg/mL, $p < 0.001$; Fig. 3-1). More than an 86% increase in PG accumulation was detected in Bcl-2-MSCs compared with MSCs and vector-MSCs when the cells were cultured under hypoxic conditions. S-GAG/DNA in Bcl-2-MSCs was also significantly higher than in MSCs and vector-MSCs (MSCs: 1.6 ± 0.11, vector-MSCs: 2.4 ± 0.15, Bcl-2-MSCs: 7.8 ± 0.30, $p < 0.001$, Fig. 3-2).

Even though all pellets initially contained the same number of cells (5×10^5), the 14-day Bcl-2-MSC pellets were significantly greater in size than MSC and vector-MSC pellets. Collagen was stained red, and sulfated and carboxylated acid mucosubstances (proteoglycans) were stained blue. The concentrations of proteoglycans (blue) were readily discerned, and individual proteoglycan bundles could be observed in areas of lower proteoglycan density. Accumulation of collagen (red) was also readily observed. As shown in Fig. 3-3, there was a significant difference in staining between the pellets in different groups. Dense regions of proteoglycans were distinguishable from those with a sparser matrix. The focal matrix concentrations that were stained with red/blue were markedly reduced.

Semi-quantitative RT-PCR was employed to study the changes in gene expression between control and transfected samples. Under hypoxia, the relative mRNA expression levels of Sox-9, aggrecan, and type II collagen were significantly increased in

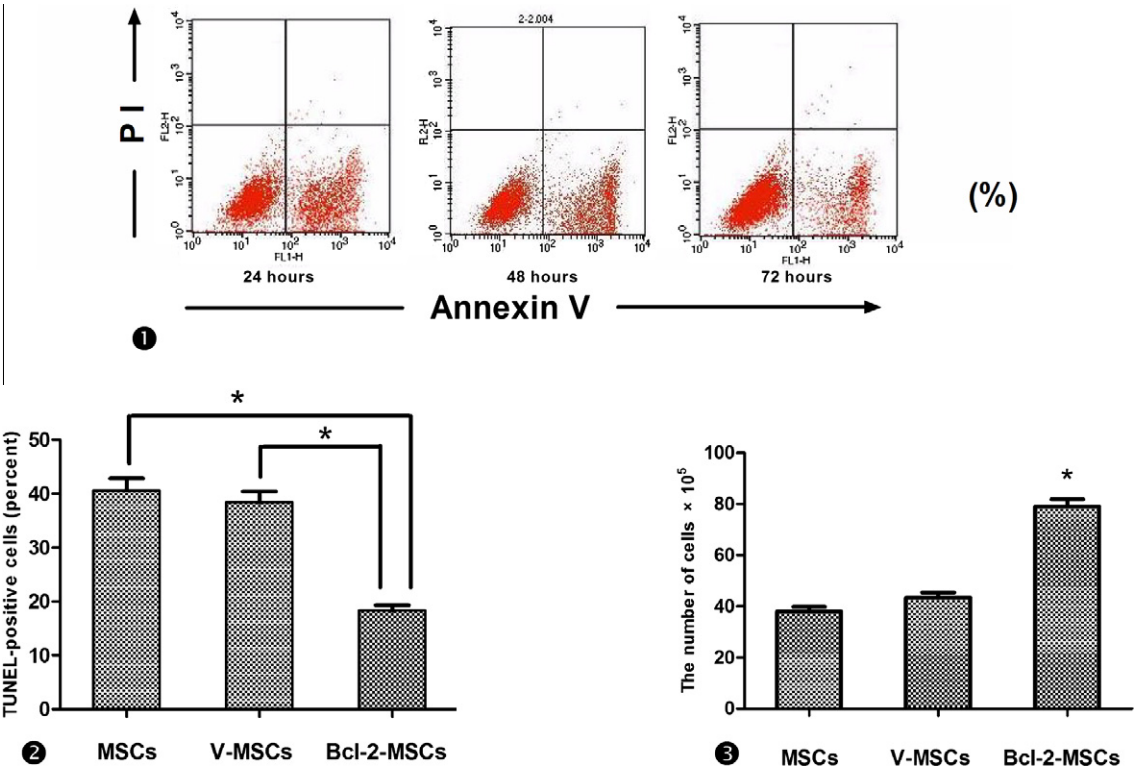

Fig. 2. Bcl-2 modified MSCs protect against apoptosis *in vitro*. (1) Apoptosis was quantified by FACS analysis after staining with Annexin V and PI. The Annexin V^+/PI^- cells are early in the apoptotic process. Viable cells are Annexin V^-/PI^-. (2) Quantitative analysis showed that the number of TUNEL-positive cells in Bcl-2-MSCs was significantly decreased compared with MSCs and vector-MSCs. (3) The number of cells in Bcl-2-MSCs was significantly more than the number of MSCs and vector-MSCs. *$p < 0.05$, Bcl-2-MSCs vs controls.

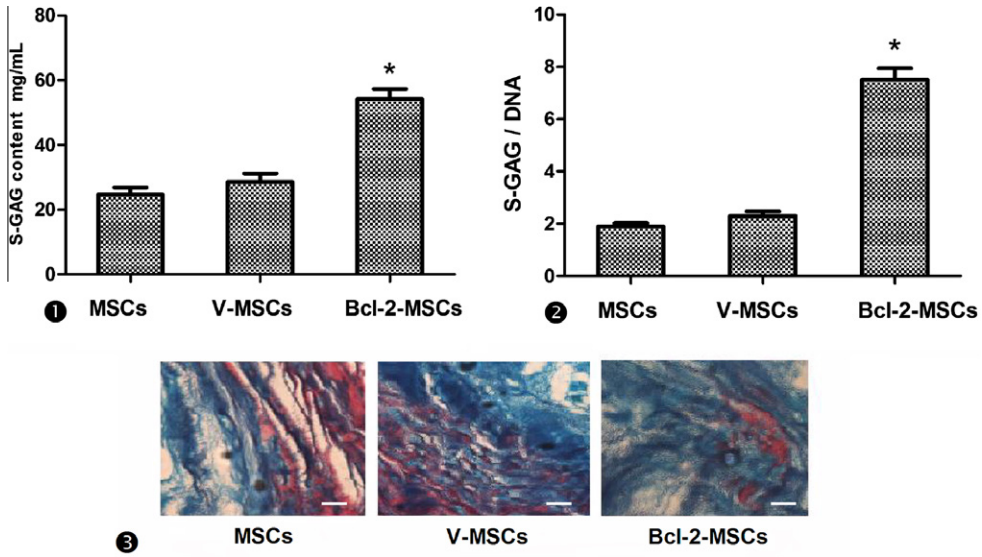

Fig. 3. Biochemical analysis of differentiated Bcl-2-MSCs. (1) Chondrogenic differentiation (S-GAG content) of the pellets from three groups. (2) Chondrogenic differentiation index was shown as the ratio of S-GAG to DNA. (3-3) Histology of pellet specimens from each group. Collagen was stained red, and proteoglycans were stained blue. Scale, bar = 100 μm. *$p < 0.001$ Bcl-2-MSCs vs controls. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Bcl-2-MSC cultures and reached peak expression on the 7th day ($p < 0.01$, Fig. 4-1 and 2). Furthermore, Bcl-2 gene transfection changed the expression of chondrocyte-regulating genes towards an NP-like phenotype, as indicated by a higher aggrecan/collagen II ratio (Fig. 4-3). The ratio of aggrecan/collagen II in Bcl-2-MSCs was 6.2-fold higher than in MSCs or vector-MSCs ($p < 0.01$).

4. Discussion

Tissue engineering of the nucleus pulposus has consistently been the goal of stem cell regeneration with regard to degenerated intervertebral discs. MSCs derived from adult bone marrow have been proposed as a promising cell source [4–7,13]. Research has

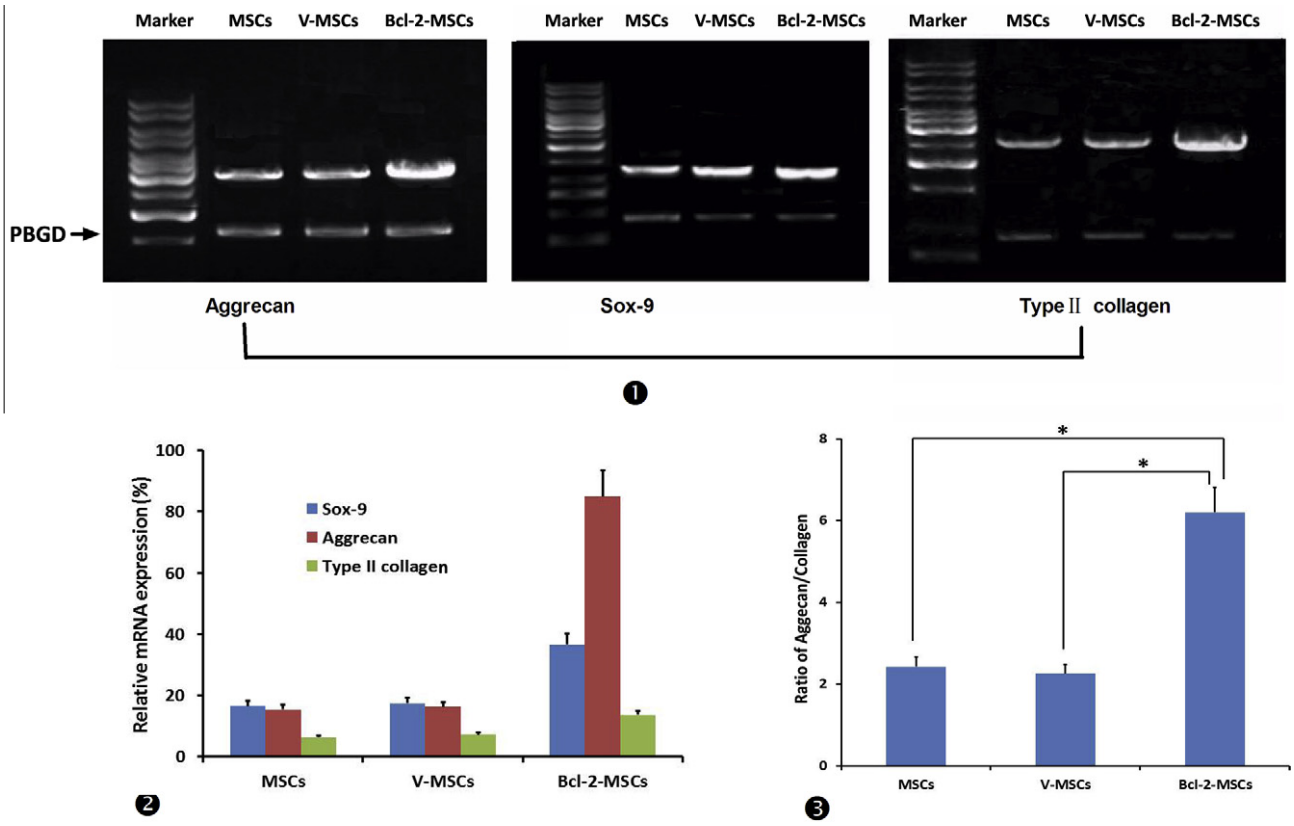

Fig. 4. Bcl-2 modified MSCs differentiated towards an NP-like phenotype under hypoxia. Fig. (4-1 and -2) Relative gene expression of chondrogenic differentiation marker in each group (7 days). (4-3) The ratio of aggrecan/collagen II gene expression in each group. *$p < 0.01$ Bcl-2-MSCs vs controls.

revealed two main factors that are important for the differentiation of MSCs towards a nucleus pulposus-like phenotype [16]. First, the three-dimensional culture environment of MSCs is a viable method for generating and transplanting differentiated cells. Various materials have been suggested for three-dimensional culturing including alginate, agarose, collagen gel, collagen sponges, fibrin gels, pellet culture, and microgravity environments [4,11,17,18]. Additionally, like chondrocytes, intervertebral disc cells maintained their native morphology and phenotype more readily when seeded in a three-dimensional culture system. The second factor is culturing under hypoxic conditions. Risbud et al. and others have shown that hypoxia (2% O_2) could drive MSC differentiation towards a phenotype consistent with that of the nucleus pulposus. Our earlier studies confirmed these data [5,6].

Hypoxia is a key factor for *in vitro* differentiation and *in vivo* transplantation; however, the high cell apoptosis rate under hypoxic conditions restricts differentiation capacity and thus hinders its clinical application [6,7,9,10]. Thus, it is necessary to reinforce MSCs against the arduous microenvironment incurred from ischemia, the inflammatory response, and pro-apoptotic factors in order to improve the efficacy of cell therapy. In this study, we identified key roles for Bcl-2 in protecting MSC survival under hypoxia–ischemia *in vitro*. Over-expression of Bcl-2 reduces MSC death and apoptosis under hypoxic conditions. In addition, Bcl-2-MSCs retained their proliferation capacity and could give rise to different lineages under hypoxic conditions, particularly the NP-like cell.

Previous studies [13,19–21] showed that genes for collagen II, Sox-9 and aggrecan were chondrocyte-specific. In this study, Bcl-2-MSCs in pellet culture, treated with differentiating medium under hypoxic conditions, displayed a significant increase in chondrocyte-specific gene expression compared with control groups (Fig. 4). Correspondingly, the Alcian blue staining was more intense in Bcl-2-MSCs. Thus, the Bcl-2-MSCs demonstrated the primary phenotype of chondrocyte-like cells in the proper differentiating medium under hypoxic conditions. Furthermore, the ratio of proteoglycan/collagen II can be used to differentiate the NP from cartilage [22]. A 27:1 ratio was found in the NP, but the ratio was only 2:1 in cartilage, demonstrating the importance of proteoglycans, particularly aggrecan, in the structure and function of the intervertebral disc [23]. The ratio of proteoglycan/collagen II after 7 days in Bcl-2-MSCs was 6.2 times higher than in the other groups (Fig. 4). Therefore, our study found that rat Bcl-2-MSCs could differentiate into functional NP-like cells under hypoxia.

In this study, three-dimensional pellets were used to imitate the micro-environment of the nucleus pulposus cells and to facilitate the detection of GAGs in the extracellular matrix. The pellet allows for appropriate three-dimensional interactions between neighboring cells, leading to synthesis of the extracellular matrix in a manner similar to that observed under *in vivo* conditions; the high density of cells in pellet culture allows easy cell-to-cell interaction [6,20,21]. The condensed culture condition is the initial stimulus required for inducing further autocrine/paracrine cytokine secretion on a cellular level. Because intervertebral discs are avascular tissues with low local oxygen tension, 2% O_2 was applied in our study. The differentiation-inducing medium containing TGF-β1 was used to ensure that MSCs differentiated along a chondrogenic direction to form NP-like cells. However, the differentiation mechanism is unclear; whether an associated change occurs at the protein level requires further research. According to Risbud et al., the MAPK signaling pathway plays an important role in the differentiation of MSCs towards NP-like cells [5]. Further studies are necessary to elucidate the underlying mechanism.

In summary, we have confirmed that genetic modification of MSCs with the anti-apoptotic Bcl-2 gene resulted in NP-like cell differentiation in response to hypoxic conditions. Genetically engineering cells to express Bcl-2 using a lentiviral vector could be an effective strategy for increasing cell survival while minimizing the risk of tumorigenesis. Finally, we demonstrated that a three-dimensional culture environment combined with hypoxia is able to induce differentiation of Bcl-2-MSCs towards an NP-like phenotype. These results suggest new avenues for cell-based tissue engineering treatments of intervertebral disc degeneration.

Acknowledgments

This work was supported by a grant from the National Natural Science Foundation of China (No. 30772206).

References

[1] G.J. Macfarlane, E. Thomas, P.R. Croft, A.C. Papageorgiou, M.I. Jayson, A.J. Silman, Predictors of early improvement in low back pain amongst consulters to general practice: the influence of pre-morbid and episode-related factors, Pain 80 (1999) 113–119.

[2] A. Colombini, G. Lombardi, M.M. Corsi, G. Banfi, Pathophysiology of the human intervertebral disc, Int. J. Biochem. Cell Biol. 40 (2008) 837–842.

[3] K. Luoma, H. Riihimaki, R. Luukkonen, R. Raininko, E. Viikari-Juntura, A. Lamminen, Low back pain in relation to lumbar disc degeneration, Spine 25 (2000) 487–492.

[4] H.E. Gruber, K. Leslie, J. Ingram, H.J. Norton, E.N. Hanley, Cell-based tissue engineering for the intervertebral disc: in vitro studies of human disc cell gene expression and matrix production within selected cell carriers, Spine J. 4 (2004) 44–55.

[5] M.V. Risbud, T.J. Albert, A. Guttapalli, E.J. Vresilovic, A.S. Hillibrand, A.R. Vaccaro, I.M. Shapiro, Differentiation of mesenchymal stem cells towards a nucleus pulposus-like phenotype in vitro: implications for cell-based transplantation therapy, Spine 29 (2004) 2627–2632.

[6] L.W. Xie, H. Fang, A.M. Chen, F. Li, Differentiation of rat adipose tissue-derived mesenchymal stem cells towards a nucleus pulposus-like phenotype in vitro, Chin. J. Traumatol. 12 (2009) 98–103.

[7] W. Zhu, J. Chen, X. Cong, S. Hu, X. Chen, Hypoxia and serum deprivation-induced apoptosis in mesenchymal stem cells, Stem Cells 24 (2006) 416–425.

[8] Y. Tsujimoto, S. Shimizu, VDAC regulation by the Bcl-2 family of proteins, Cell Death Differ. 7 (2000) 1174–1181.

[9] K.A. Webster, D.J. Discher, S. Kaiser, O. Hernandez, B. Sato, N.H. Bishopric, Hypoxia-activated apoptosis of cardiac myocytes requires reoxygenation or a pH shift and is independent of p53, J. Clin. Invest. 104 (1999) 239–252.

[10] W. Li, N. Ma, L.L. Ong, C. Nesselmann, C. Klopsch, Y. Ladilov, D. Furlani, C. Piechaczek, J.M. Moebius, K. Lutzow, A. Lendlein, C. Stamm, R.K. Li, G. Steinhoff, Bcl-2 engineered MSCs inhibited apoptosis and improved heart function, Stem Cells 25 (2007) 2118–2127.

[11] W. Luo, W. Xiong, M. Qiu, Y. Lv, Y. Li, F. Li, Differentiation of mesenchymal stem cells towards a nucleus pulposus-like phenotype utilizing simulated microgravity in vitro, J. Huazhong Univ. Sci. Technolog. Med. Sci. 31 (2011) 199–203.

[12] A. Limon, J. Briones, T. Puig, M. Carmona, O. Fornas, J.A. Cancelas, M. Nadal, J. Garcia, F. Rueda, J. Barquinero, High-titer retroviral vectors containing the enhanced green fluorescent protein gene for efficient expression in hematopoietic cells, Blood 90 (1997) 3316–3321.

[13] J.Y. Lee, R. Hall, D. Pelinkovic, E. Cassinelli, A. Usas, L. Gilbertson, J. Huard, J. Kang, New use of a three-dimensional pellet culture system for human intervertebral disc cells: initial characterization and potential use for tissue engineering, Spine 26 (2001) 2316–2322.

[14] J.H. Waterborg, H.R. Matthews, The Lowry method for protein quantitation, Methods Mol. Biol. 32 (1994) 1–4.

[15] H.E. Gruber, J. Ingram, E.N. Hanley Jr., An improved staining method for intervertebral disc tissue, Biotech. Histochem. 77 (2002) 81–83.

[16] M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, D.R. Marshak, Multilineage potential of adult human mesenchymal stem cells, Science 284 (1999) 143–147.

[17] Z.F. Lu, B. Zandieh Doulabi, P.I. Wuisman, R.A. Bank, M.N. Helder, Differentiation of adipose stem cells by nucleus pulposus cells: configuration effect, Biochem. Biophys. Res. Commun. 359 (2007) 991–996.

[18] B.A. Maldonado, T.R. Oegema Jr., Initial characterization of the metabolism of intervertebral disc cells encapsulated in microspheres, J. Orthop. Res. 10 (1992) 677–690.

[19] A. Colombini, P. Lanteri, G. Lombardi, D. Grasso, C. Recordati, A. Lovi, G. Banfi, R. Bassani, M. Brayda-Bruno, Metabolic effects of vitamin D active metabolites in monolayer and micromass cultures of nucleus pulposus and annulus fibrosus cells isolated from human intervertebral disc, Int. J. Biochem. Cell Biol. 44 (2012) 1019–1030.

[20] C. Le Visage, S.W. Kim, K. Tateno, A.N. Sieber, J.P. Kostuik, K.W. Leong, Interaction of human mesenchymal stem cells with disc cells: changes in extracellular matrix biosynthesis, Spine 31 (2006) 2036–2042.

[21] F. Tao, F. Li, G. Li, F. Pan, Differentiation of mesenchymal stem cells into nucleus pulposus cells in vitro, J. Huazhong Univ. Sci. Technolog. Med. Sci. 28 (2008) 156–158.

[22] F. Mwale, P. Roughley, J. Antoniou, Distinction between the extracellular matrix of the nucleus pulposus and hyaline cartilage: a requisite for tissue engineering of intervertebral disc, Eur. Cell Mater. 8 (2004) 58–63. discussion 63-54.

[23] A. Bertolo, M. Mehr, N. Aebli, M. Baur, S.J. Ferguson, J.V. Stoyanov, Influence of different commercial scaffolds on the in vitro differentiation of human mesenchymal stem cells to nucleus pulposus-like cells, Eur. Spine J. 21 (Suppl 6) (2012) S826–S838.