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Original Article

The ability to form cartilage of NPMSC and BMSC in SD rats

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Abstract: Objective: In this study, we observed the difference in the ability of cartilage differentiation between nucleus pulposus mesenchymal stem cells (NPMSC) and bone marrow mesenchymal stem cells (BMSC). Methods: NPMSC and BMSC were isolated from SD rats. Their proliferation abilities were detected by CCK-8 methods, their multilineage differentiation abilities were observed using Alizarin red staining, oil red O staining and Alcian Blue staining methods. The expression levels of osteogenic, adipogenic, chondrogenic genes were detected with RT-PCR and Western-blotting methods. Results: There was no obvious difference in the proliferation ability between NPMSC and BMSC cells. NPMSC and BMSC cells expressed stem cell genes and the surface markers and showed osteogenic, adipogenic and chondrogenic multi-directional differentiation capability of cartilage under induction in vitro. Conclusions: We confirmed that NPMSC with characteristics of stem cells can be isolated and cultured from nucleus pulposus tissues of intervertebral disc of SD rats, the chondrogenic ability of NPMSC and BMSC was similar under induction in vitro. This could provide a new seed cells for tissue engineering.

Keywords: Nucleus pulposus mesenchymal stem cells (NPMSC), bone marrow mesenchymal stem cells (BMSC), proliferation ability, multi-directional differentiation capability, RT-PCR, Western blotting

Introduction

The incidence of low back pain increased these years and the treatment of this disease has become a medical and social problem [1, 2]. Low back pain is caused by many reasons which including trauma, intervertebral disc degeneration, herniated disk and spinal canal stenosis [3-5], intervertebral disc degeneration is considered the main cause. Intervertebral disc degeneration showed reduction of nucleus pulposus cells and extracellular matrix and disc structure damage [6, 7]. Gene, bad habits, heavy manual labor and other physical disorders may lead to the degeneration of intervertebral disc [8].

Previous treatment methods of intervertebral disc degeneration included medication, steroid injection, physical therapy and operation. However, these methods only can relieve the clinical symptoms, but can not cure and prevent the process of intervertebral disc degeneration. At present, many new treatment methods and reversing intervertebral disc degeneration were developed such as molecular therapy and cell therapy [9-14]. Blanco [15] reported that some cells isolated from degenerative intervertebral disc tissues expressed stem cell markers CD44, CD29, CD90, CD73, CD105, CD166 and CD106 and did not express hematopoietic stem cell marker CD34, CD45, CD14 and CD19, which was not significantly different from that of bone marrow mesenchymal stem cells. They had osteogenic and chondrogenic differentiation ability but no adipogenic differentiation ability. These were in full compliance with the standards of multipotent mesenchymal stem cells defined by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy [16]. In this study, we observed the difference in the ability of cartilage differentiation between nucleus pulposus mesenchymal stem cells (NPMSC) and bone marrow mesenchymal stem cells (MSC).

Table 1. Primers used in the detection of gene expression

Primers	Sequences (5'-3')	Length of products
R-β-actin-F	CGTAAAGACCTCTATGCCAACA	163 bp
R-β-actin-R	AGCCACCAATCCACACAGAG	
R-NANOG-F	AGGACGAGACAGAAGGATCACC	218 bp
R-NANOG-R	ATAGAAGCCTCTTGGCGGAA	
R-OCT-4-F	GGTGGAGGAAGCTGACAACAA	396 bp
R-OCT-4-R	GGAAAAGGGACCGAGTAGAGTG	
R-SOX2-F	TGTTCAATCCCACCCTTTTCA	177 bp
R-SOX2-R	GGCAGCCTGGTTCCAATAA	
R-CD44-F	CAACGAAGATGCCTGGTACG	145 bp
R-CD44-R	GCAATGGTGCTGGAGATAAAAT	
R-CD105-F	GTCGGTTGTGATCTACAGCGTG	197 bp
R-CD105-R	CGAGTCTCAGTGCCATTTTGC	
R-CD73-F	CTCATCATCCTCAAGGCTCCA	314 bp
R-CD73-R	TCCTCCCTCCCAAGTTCTGT	
R-CD90-F	CCAAGCCACGGACTTCATT	297 bp
R-CD90-R	ATCGGGTCTCCAGGACAAAC	
R-CD45-Fn	ACCTGCTCCTGAAACTTCGAC	264 bp
R-CD45-Rn	CCCCAAATTGATTGTACTCCAC	
R-CD34-Fn	AACCACAGACTTACCCAACCG	313 bp
R-CD34-Rn	AGCTCTTCTCCCCTTTCCTTC	
R-CD14-F	TTGTCAGGAACTTTGGCTTTG	355 bp
R-CD14-R	TTGTGGGGTTGGGGATTTA	
R-HLA-DR-Fn	CCTTGACCTCAGTGAAAGCAGT	226 bp
R-HLA-DR-Rn	TGGGGCATTCCATAGCAGA	
R-ALP-F	GCCTGGACCTCATCAGCATT	420 bp
R-ALP-R	TGTAGCCACCAAACGTGAAAAC	
R-PPAR-2-F	TTTCTGGGTGGATTGAAGTGG	362 bp
R-PPAR-2-R	TTTGCATGGTTCTGAGTGCTAAG	
R-LPP-F	CGAGCCCTGCTACATCAATACG	288 bp
R-LPP-R	TCACGGTCCAGAGCCACAAT	
R-APP-F	ACCCATCAGGGACCAAAACC	221 bp
R-APP-R	GAGAAGGGCATCGCTTACAAA	
R-COL2A1-F	GGAATTTGGTGTGGACATAGGG	173 bp
R-COL2A1-R	GGACTGTGAGGTTAGGATAGTTGAA	
R-PG-Fn	CTTCTGACTCCAAAAGCCCACT	140 bp
R-PG-Rn	ACCTTCTTCCTCCTCTTCCTCC	
R-SOX9-F	AAACTTCAGTGGGAGCGACAA	110 bp
R-SOX9-R	AGGAGGGAGGGAAAACAGAGA	

Materials and methods

Experimental animals

6 healthy SD rats weight 250±10 g were supplied by Animal Laboratory Center of Shanxi Medical University. The rats were placed in a comfortable and quiet room for one week before the initiation of the experimental procedure. This study was audited and approved by Animal Ethics Committee of Shanxi Medical University. All experimental procedure and animal care were carried out under the guidance of the Ethics Committee in order to minimize the suffering of animals.

Isolation of NPMSC

The mice were killed and the mouse tails were collected. The nucleus pulposus tissues were separated from intervertebral disk in tail of each rat under sterile conditions with microscope. The nucleus pulposus tissues were flushed with PBS and cut into tissue fragments with size of 1×1×1. The tissue fragments were digested with 0.2% type II collagenase at 37°C for 2 h. PBS was added into them and they were centrifuged at 1000 rpm for 5 min and washed for 2 times. Then they were digested with 0.25% trypsase at 37°C for 10 min and PBS was added into them and they were centrifuged at 1000 rpm for 5 min and washed for 2 times. The isolated cells were cultured in DMEM/F12 medium containing 20% fetal bovine serum and penicillin and streptomycin at 37°C with 5% CO_2.

Isolation of BMSC

The long bone was taken from the hind legs of SD rats under sterile conditions and both ends of the long bones were cut. Bone marrow was flushed out with DMEM/Low glucose medium containing 10% fetal bovine serum and penicillin and streptomycin. They were mixed and filtrated with 200 mesh strainer to culture at 37°C with 5% CO_2.

Proliferation ability of NPMSC and BMSC

The proliferation ability of the third generation of NPMSC and BMSC cells was detected with CCK-8 method. The cell density was adjusted to 2×10^4 cells/ml and inoculated in 96 well plates with 100 μl/well. 10 μl CCK-8 reagents was added into each well and incubated at 37°C with 5% CO_2 for 1 h. The OD values were determined with Microplate Reader after culture for 0, 1, 3, 5, 7 and 9 days at wavelength of 450 nm.

Expression of stem cell gene and its surface markers, osteogenic gene, adipogenic gene and chondrogenic gene

Total RNA was extracted from NPMSC and BMSC cells using RNA extraction kit respectively. Primers were designed according to the sequences in GenBank using software prime 5.0. Sequences of primers were shown in **Table 1**. The PCR amplification conditions were 95°C 3 min; 94°C 30 s, 56°C 30 s, 72°C 90 s, 35 cycles and 72°C 8 min. The application products were detected using 1.5% agarose gel electrophoresis and photos were taken.

Multilineage differentiation ability of NPMSC and BMSC

Osteogenic induction: 1 ml 0.1% Gelatin Solution was added into each well of 6 well plates and shook gently to cover the bottom. They were placed at room temperature for 30 min and then Gelatin Solution was discarded and let the plates air-dry. The cells were cultured in the coated 6 well plates at 37°C with 5% CO_2 for 24 h. The medium was discarded gently and 2 ml OriCell TM Sprague-Dawley (SD) Rat Mesenchymal Stem Cell Osteogenic Differentiation Medium was added into them. The inducing medium was changed for every 3 days and they were induced for 21 days. After that the cells were fixed and stained with Alizarin red. The cells were washed with PBS and fixed with 4% formaldehyde for 30 min. Then they were washed with PBS for 2 times and stained with Alizarin red for 3-5 min at room temperature. They were observed with inverted microscope.

Adipogenic induction: The cells were cultured in the 6 well plates at 37°C with 5% CO_2. The medium was discarded and OriCellTM Sprague-Dawley (SD) Rat Mesenchymal Stem Cell Adipogenic Differentiation Medium A was added into each well and incubated for 3 days. Then the medium A was changed to medium B and incubated for 1 day and the medium B was changed to medium A again and the changes were done in the 3 cycles. When the lipid droplets appeared more but relatively small the cells can be maintained with medium B for 7 days and lipid droplets enlarged. After that the cells were fixed with 4% formaldehyde for 30 min. Then the cells were washed with PBS for 2 times and stained with oil red O at 37°C for 30 min. They were observed with inverted microscope.

Chondrogenic induction: The cells were cultured in the 6 well plates at 37°C with 5% CO_2. The medium was discarded and OriCellTM Sprague-Dawley (SD) Rat Mesenchymal Stem Cell Chondrogenic Differentiation Medium was added into each well and incubated for 21 days. The cells were embedded with paraffin and cut into slices. Then the slices were stained with Alcian Blue for 30 min and washed with PBS for 3 times. They were observed with inverted microscope.

RT-PCR detection

Total RNA was extracted from the cells using RNA extraction kit. The expression levels of type II collagen, proteoglycan and SOX9 were detected by RT-PCR using RT-PCR kit according to the manufacturer's instructions. The PCR amplification conditions were 95°C 15 s and 60°C 40 s for 40 cycles.

Western blotting detection

The cells were lysed in RIPA lysis buffer, and the lysates were harvested by centrifugation with 13,000 rpm at 4°C for 20 min. Protein samples were quantified with BCA kit. Approximately 50 μg proteins were separated by electrophoresis in a 10% sodium dodecyl sulfate polyacrylamide gel and were transferred onto PVDF membrane. After blocking the nonspecific binding sites for 120 min with 5% nonfat milk, the membranes were incubated with primary antibodies collagen-II (1:5000), SOX-9 (1:1000), proteoglycan (1:1000) and β-actin (1:2000) at 4°C overnight. The membranes were then washed three times with TBST buffer for 3 times and were probed with the horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (1:5,000) at room temperature for 1 h. The membranes were developed using an enhanced chemiluminescence system. The levels of proteins were normalized to the level of β-actin.

Statistical analysis

All statistical analyses were performed using SPSS version 11.0 statistical software. Data were expressed as means ± standard deviations (SD). Differences between the two groups of mice were analyzed using one-way ANOVA.

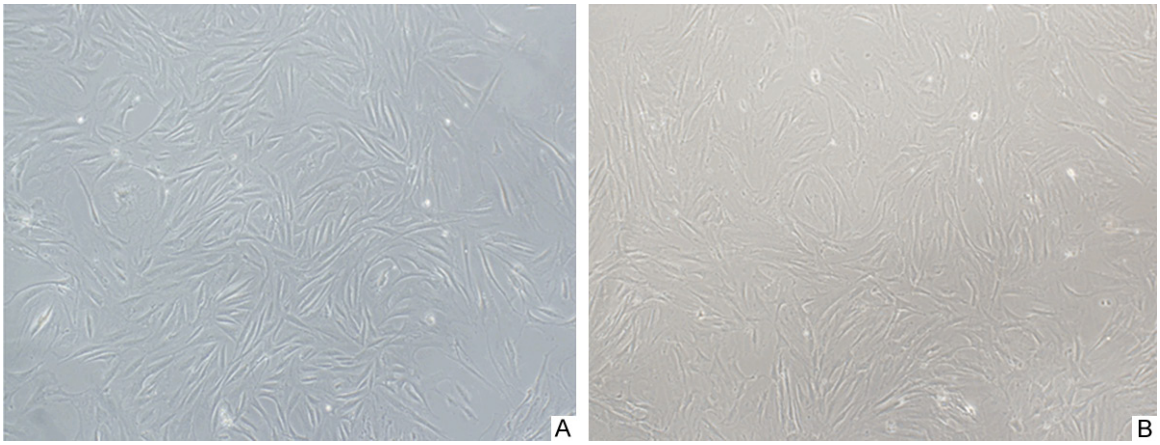

Figure 1. Morphology of primary NPMSC and BMSC. A: NPMSC; B: BMSC.

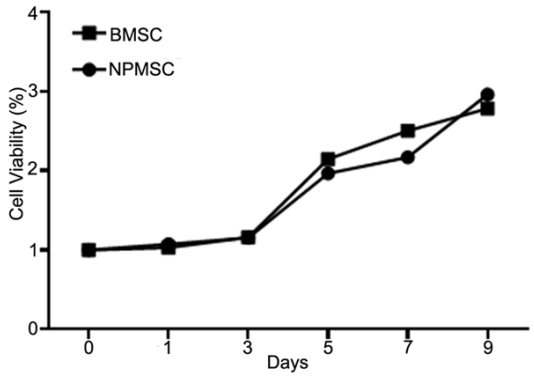

Figure 2. Proliferation ability of NPMSC and BMSC detected by CCK-8.

P<0.05 were considered statistically significant.

Results

Morphology of NPMSC and BMSC

Primary NPMSC cells can be adherent growth after isolation for 24 hours and most of them are spindle, containing a small amount of triangles and polygons. They reached 80%-90% fusion after culture for about 4 weeks. The growth of sub-cultured cells accelerated obviously and the morphology of the third generation almost remained spindle. Primary BMSC cells have similar morphology with that of NPMSC cells and like fibroblast morphology (**Figure 1**).

Proliferation ability of NPMSC and BMSC

Proliferation ability of NPMSC and BMSC was detected by CCK-8 methods. There was no obvious difference in the proliferation ability between NPMSC and BMSC cells. The ability was strongest on 3-5 days and decreased after culture for 7 days (**Figure 2**).

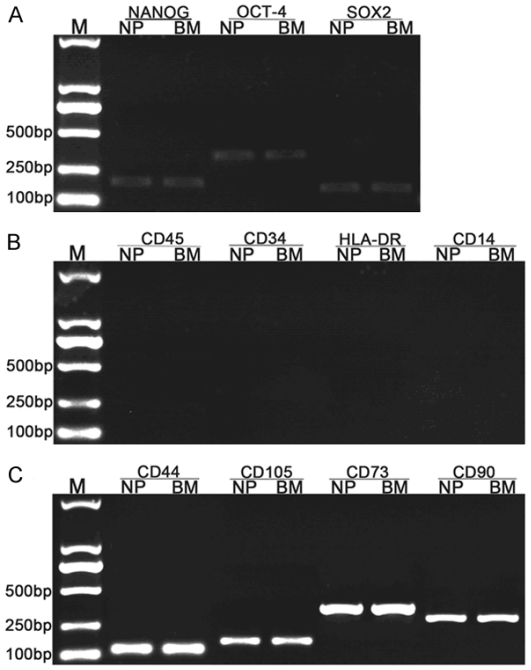

Figure 3. NPMSC and BMSC cells expressed stem cell genes and the surface markers of stem cell.

Gene expression

NPMSC and BMSC cells expressed stem cell genes NANOG, OCT-4 and SOX2, surface markers of stem cell CD44, CD105, CD73 and CD90, but they did not express hematopoietic stem cell markers CD45, CD34, CD14 and HLA-DR (**Figure 3**).

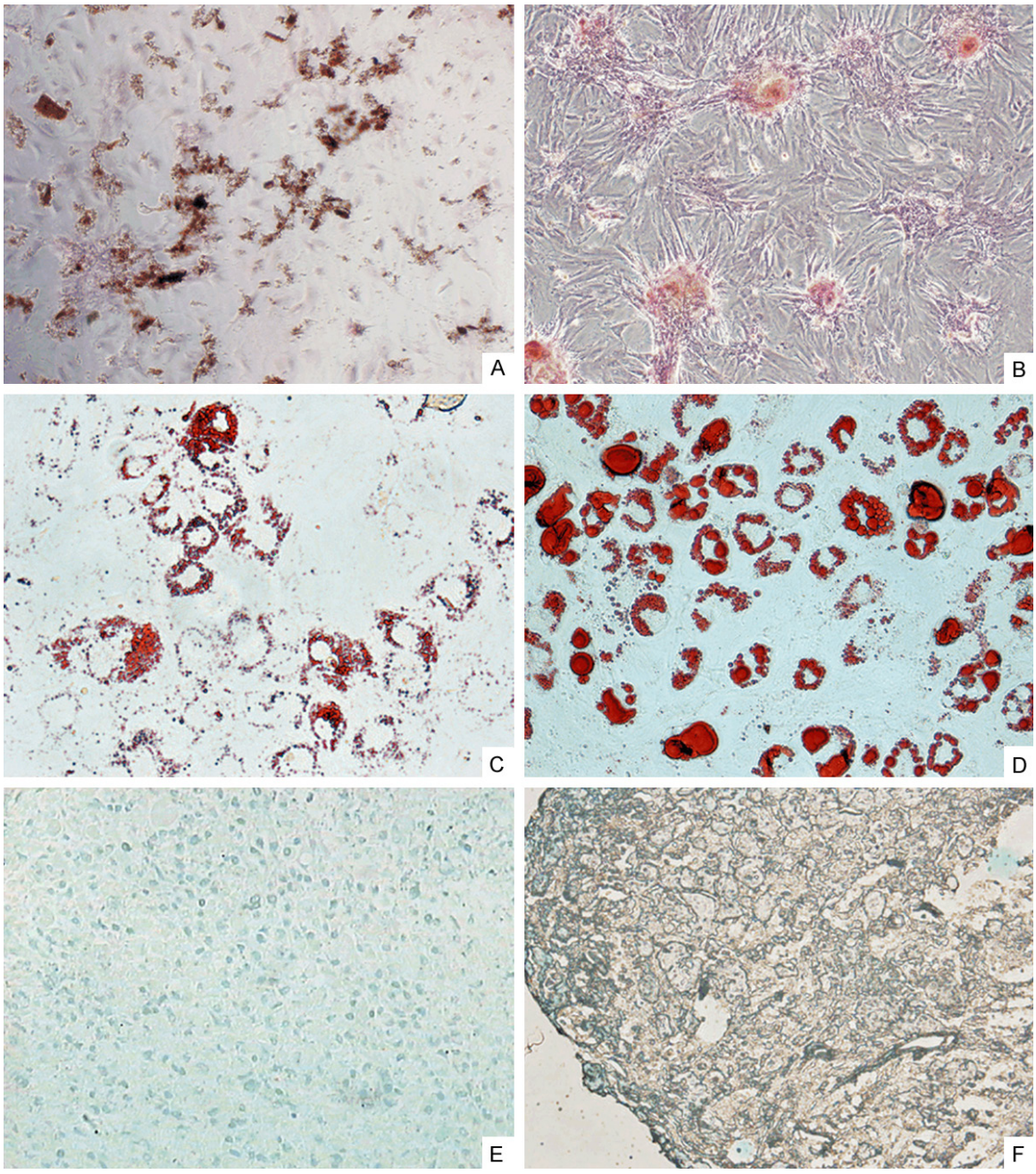

Figure 4. Osteogenic, adipogenic and chondrogenic multi-directional differentiation capability of NPMSC and BMSC. A: A large number of calcium nodules after osteogenic induction for 21 days in NPMSC (Alizarin red stain); B: A large number of calcium nodules after osteogenic induction for 21 days in BMSC (Alizarin red stain); C: The formation of red dye lipid droplet vacuoles after induction for 21 days in NPMSC (oil red O stain); D: The formation of red dye lipid droplet vacuoles after induction for 21 days in BMSC (oil red O stain); E: Aizen Proteoglycans presented after induction for 21 days in NPMSC (Alcian Blue stain); F: Aizen Proteoglycans presented after induction for 21 days in BMSC (Alcian Blue stain).

Multilineage differentiation ability of NPMSC and BMSC

NPMSC and BMSC cells showed osteogenic, adipogenic and chondrogenic multi-directional differentiation capability of cartilage under induction in vitro. The results of Alizarin red stain showed that there were a large number of calcium nodules after osteogenic induction for 21 days. The formation of Red dye lipid droplet

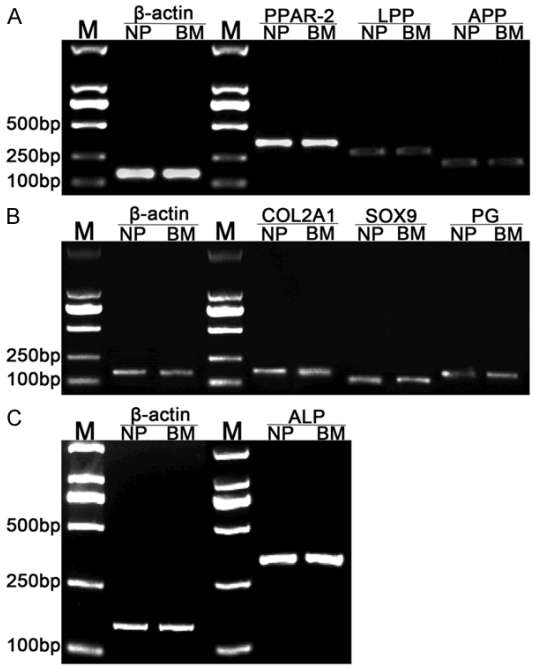

Figure 5. The expression of osteogenic, adipogenic, chondrogenic genes in NPMSC and BMSC. A: Adipogenic genes; B: Chondrogenic genes; C: Osteogenic gene.

vacuoles could be seen in oil red O staining results. Aizen proteoglycans could be seen in Alcian blue staining results (**Figure 4**).

Expression of osteogenic, adipogenic, chondrogenic genes

NPMSC and BMSC could express osteogenic gene ALP, adipogenic genes PPAR-2, LPP and APP, and chondrogenic genes type II collagen, proteoglycan and SOX9 after in vitro induction (**Figure 5**).

RT-PCR results

The expression levels of type II collagen, proteoglycan and SOX9 were higher in NPMSC and BMSC after in vitro induction than that before induction ($P<0.05$). There was no significant difference between NPMSC and BMSC ($P>0.05$, **Figure 6**).

Western blotting results

Western blotting results were consistent with that of RT-PCR results. The expression levels of type II collagen, proteoglycan and SOX9 were higher in NPMSC and BMSC after in vitro induction than that before induction. There was no significant difference between NPMSC and BMSC (**Figure 7**).

Discussion

The center of disc is nucleus pulposus cells rich in water and there is a solid flexible fiber ring surrounding the nucleus pulposus cells, the contact surface between the adjacent intervertebral discs formed by cartilage endplate [17]. The most prominent feature of intervertebral disc tissues was that they contained a large amount of extracellular matrix (ECM), which attracting and storing water by large molecular fine structure to maintain the survival of intervertebral disc cells, and the large molecular structure was composed of type II collagen and proteoglycan [18]. It relied on the balance of synthesis and degradation of extracellular matrix to keep intervertebral disc intact, the more the extracellular matrix in the intervertebral disc, the more the rich content. The reduced extracellular matrix caused the reduction of intradiscal moisture and lead to intervertebral disc degeneration [19]. Cell therapy provides a potential approach for the treatment of intervertebral disc degeneration [20-22]. Yoshikawa used autologous BMSC to cure protrusion of intervertebral disc and the low back pain symptoms in patients significantly reduced [23]. Orozco used autologous BMSC to cure lumbar intervertebral disc degeneration and the symptoms of 71% patients improved [24]. So cell therapy does have a reversing intervertebral disc degeneration effect in some extent.

In this study, we isolated and cultured NPMSC and BMSC cells. These two kinds of cells are very close in morphology and proliferation ability and both of them express stem cell genes NANOG, OCT-4 and SOX2. The expression of these genes confirmed the existence of stem cells in the nucleus pulposus tissues [25]. They expressed surface markers of stem cell CD44, CD105, CD73 and CD90, but they did not express hematopoietic stem cell markers CD45, CD34, CD14 and HLA-DR. They also have osteogenic, adipogenic and chondrogenic ability and accorded with international standard definition of stem cells (ISCT) [16]. The expression levels of type II collagen, proteoglycan and SOX9 increased in these two kinds of cells after induction in vitro. The micro environ-

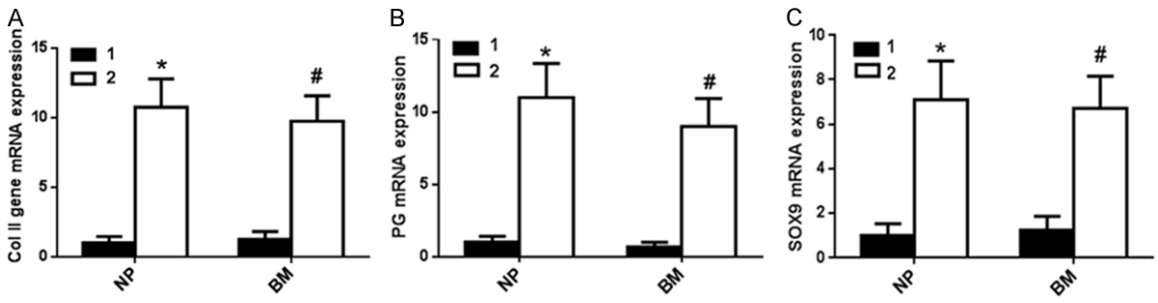

Figure 6. RT-PCR results of type II collagen, proteoglycan and SOX9 expression. A: Type II collagen; B: Proteoglycan; C: SOX9; 1: Before induction; 2: After induction; *P<0.05 vs. 1 in NPMSC; #P<0.05 vs. 1 in BMSC.

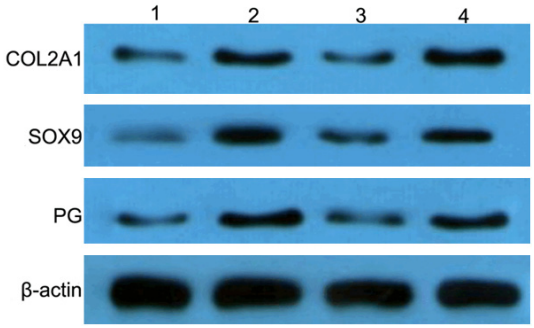

Figure 7. Western blotting results of type II collagen, proteoglycan and SOX9 expression. 1: Before induction in NPMSC; 2: After induction in NPMSC; 3: Before induction in BMSC; 4: After induction in BMSC.

ment in intervertebral disc is a hypertonic, hypoxia and low nutritional environment [26]. The NPMSC cells originated from nucleus pulposus of intervertebral disc, they may be more adapted to the environment in intervertebral disc than BMSC cell. Therefore, we thought that NPMSC may repair intervertebral disc degeneration better than BMSC, but these need further study.

Although cell therapy has a certain effect in the treatment of intervertebral disc degeneration, but it also has some risks. Vadala found that BMSC injection in degenerated intervertebral disc in rats could cause cell leakage and may induce osteophyte formation [27]. Therefore, how to control induction pathway of stem cells better, avoid unexpected differentiation and the risk of cancer need further study [28].

In a word, in this study we confirmed that NPMSC with characteristics of stem cells can be isolated and cultured form nucleus pulposus tissues of intervertebral disc of SD rats, the chondrogenic ability of NPMSC and BMSC was similar under induction in vitro. This could provide a new seed cells for tissue engineering.

Disclosure of conflict of interest

None.

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