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Original Article

Differentiation of immortalized human precartilaginous stem cells into nucleus pulposus-like cells

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Abstract: Objective: We aimed to explore the differentiation of immortalized human precartilaginous stem cells (IPSCs) into nucleus pulposus (NP)-like cells induced by transforming growth factor-β_1 (TGF-β_1) and examine its biological characteristics. Methods: The IPSCs were seeded onto chitosan/glycerophosphate (C/GP) scaffolds and induced into NP-like cells by adding TGF-β_1 under hypoxic conditions. The growth and differentiation of IPSCs were observed, and the formation of glycosaminoglycans (GAG) in the extracellular matrix of differentiating cells was detected by Alcian Blue staining. The expressions of type II collagen and aggrecan genes in NP-like cells were examined by reverse transcription-polymerase chain reaction (RT-PCR). The expressions of Sox9 and β-catenin were analyzed by Western blotting. Results: The IPSCs were observed to grow well on the C/GP scaffolds. After 7 days, Alcian Blue staining demonstrated the formation of GAG. The RT-PCR results showed that expression of type II collagen and aggrecan were upregulated compared with control group ($P < 0.05$, $P < 0.05$). Likewise, western blotting results showed that the expression of Sox9 and β-catenin was upregulated compared with control group ($P < 0.05$, $P < 0.05$). Conclusions: The findings demonstrate that IPSCs could be differentiated into NP-like cells following induction by TGF-β_1.

Keywords: Precartilaginous stem cells (IPSCs), nucleus pulposus-liked cells, transforming growth factor-β_1

Introduction

Intervertebral disc degeneration (IDD)-related intervertebral disc herniation is one of the most common clinical disorders, which causes the high incidence of pain in neck, shoulders, waist, or legs (a range of 60% to 80%). In United States, the number of patients with intervertebral disc herniation requiring operative treatment is about 300,000, and more attention has been paid on its increasing morbidity. However, both conservative and invasive treatments are not efficient to reconstruct the normal structure and recover the function of degenerated intervertebral discs.

The etiological factors of IDD are very complicated [1, 2]; however, a significant decrease in the quantity of nucleus pulposus (NP)-like cells is found as a common symptom of IDD, which will subsequently lead to the decrease of proteoglycan in the extracellular matrix (ECM) and the changes in structure and function of the intervertebral disc [3]. The reconstruction of the structure and physiological function of a degenerated intervertebral disc may depend on the increased NP cell numbers and synthesis of new ECM. Biological therapy, such as stem cell-mediated cell transplantation, is one of the most important approaches for the treatment of IDD-related intervertebral disc herniation [4]. Mesenchymal stem cells have received attention for their ability to differentiate into osteoblasts, cells that synthesize new bone [5]; however, the achievements are still far from successful bone regeneration.

Precartilaginous stem cells (PSCs) are a type of newly identified adult stem cells that exists in the most peripheral layer of the epiphyseal organ with a perichondrial mesenchyme in embryonic limbs. This is known as La Croix rings, and it plays a key role in cartilage growth, endochondral ossification and in the healing process of damaged articular cartilage [6]. Meanwhile, NP cells possess some characteristics similar to chondrocytes, and are also known as chondrocyte-like cells because of their

expression of chondrocyte-specific genes such as collagen II and aggrecan. As PSCs and NP cells possess some homology, we believe that PSCs-mediated cell transplantation, especially PSCs differentiated into NP-like cells, could be useful in the treatment of IDD. However, the biological properties of PSCs *in vitro* are unstable. Immortalized cells can possess relatively stable proliferative properties and functional status, and maintain the differentiated phenotype of primitive cells. Transfected cells possess the biological properties of primitive cells, and appear to serve as the standard cellular model for studying primitive cells *in vitro*, with a long-term and stable role in gene therapy without adverse effects [7]. Therefore, we established immortalized precartilaginous stem cells (IPSCs) as described in a previous study [8].

Currently, the accepted induction factor for differentiating stem cells into NP-like cells is transforming growth factor-β_1 (TGF-β_1). However, as far as we know, there have no reports on the possibility of differentiating IPSCs into nucleus pulposus (NP)-like cells using TGF-β_1 induction and the mechanism is far from understood. Therefore, in the present study, we aimed to explore the possibility of differentiating IPSCs into nucleus pulposus (NP)-like cells using TGF-β_1 and show the basis of IPSCs in the treatment of IDD-related diseases.

Material and methods

Materials

Reagents used in this study include Dulbecco's modified Eagle's medium-Ham's F12 (DMEM-F12), fetal bovine serum (FBS; Gibco, USA), Alcian blue 8 GX, β-sodium glycerophosphate, trypsin, ITS liquid Media Supplement (100 ×), dexamethasone (Sigma, USA), total RNA extraction kit, western blot kit (Tiangen, China), reverse transcription-polymerase chain reaction (RT-PCR) reagents (Toyobo, Japan), TGF-β_1 (R&D Systems, USA), chitosan (Protasan, NOR), mouse anti-human monoclonal antibodies against Sox9 and β-catenin (Ebioscience, USA).

Thawing and cultivation of IPSCs

Passage 3 IPSCs were removed from liquid nitrogen storage and thawed rapidly by swirling in a 40°C water bath. The supernatant was aspirated and discarded, and cells resuspended in DMEM-F12 with 20% FBS, rinsed three times, and then resuspended in DMEM-F12 with 20% FBS and cultured in a 37°C/5% CO_2 incubator (Forma Scientific).

Preparation of C/GP gels

Sterile chitosan and β-sodium glycerophosphate solutions were combined slowly, with a final volume ratio of β-sodium glycerophosphate: chitosan around 1:9. The C/GP gels were liquid at room temperature, transforming to semi-solid hydrogels at 37°C.

Differentiation of IPSCs to NP-liked cells

The passage 3 IPSCs were trypsinized and transferred to 6-well plates containing the C/GP gel at a concentration of 1×10^5 cells/mL. Experimental groups consisted of IPSCs on C/GP gels in normal medium consisting of 10% FBS in DMEM-F12 at 2% O_2 (control); IPSCs on C/GP gels in differentiating medium (DMEM-F12 supplemented with 10% FBS, 10 ng/mL TGF-β_1, 100 nM dexamethasone, 50 μg/mL L-ascorbic acid 2-phosphate, 100 μg/mL sodium pyruvate, 40 μg/mL proline and 100 × ITS-liquid media supplement) at 2% O_2 (induction group). After 7-day incubation, the biological characteristics of cells were examined.

Alcian blue staining analysis

At the end of the 7-day induction, cultures were washed three times, fixed with Kalhes's fixative (6 mL formalin, 15 mL 95% ethyl alcohol, 1 mL glacial acetic acid, 80 mL distilled water) for 20 min at room temperature, and then washed with 50% ethanol followed by 70% ethanol. Cultures were then stained with 1% (w/v) Alcian Blue solution (1 g Alcian Blue 8 GX, 100 mL 3% acetic acid) for 30 min and rinsed in distilled water. The formation of glycosaminoglycans (GAG) in differentiated IPSCs was observed using an inverted microscope (CK-24; Olympus), with digital micrographs captured.

RT-PCR analysis

Isolation of total RNA and reverse transcription were performed with the commercially available TRIZOL RNA and RT kits. The 175 bp type II collagen fragment was amplified using primers 5'-CAC TCA TCT GTT GTG ATG AGT TCT CC-3'and 5'-CAA CAC ACA CCA GCG CAG TTT-3'.

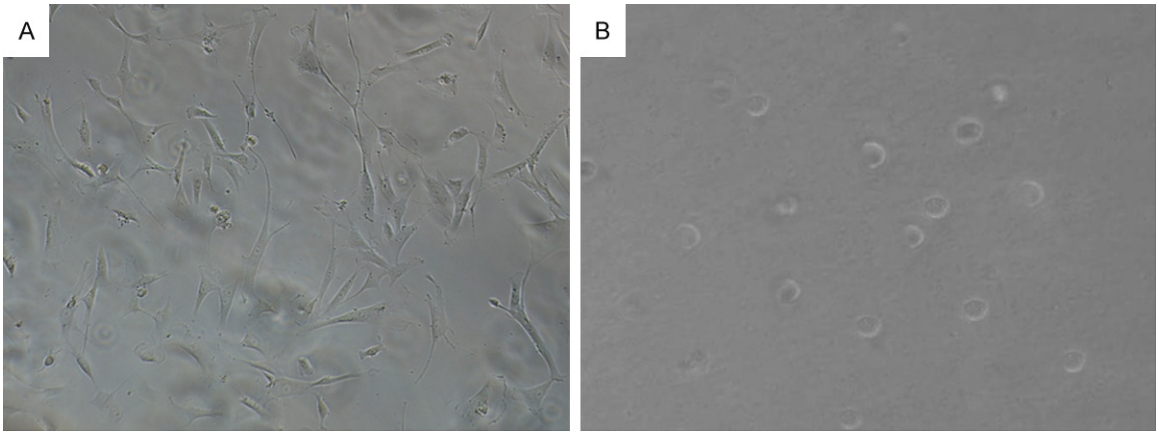

Figure 1. The shape of IPSCs before and after induction. (A) Represents IPSCs before induction (10 × magnification), (B) Represents induced IPSCs on C/GP gels (10 × magnification).

The PCR was performed with the following thermal cycling profile: denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, then a finial elongation step at 72°C for 10 min. The 276 bp aggrecan fragment was amplified using primers 5'-GGG TGA GGT CTT TTA TGC CA-3' and 5'-GCT TTG CAG TGA GGA TCA CA-3'. The PCR was performed according to the following thermal cycling profile: denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 53°C for 1 min and 72°C for 1 min, then a finial elongation step at 72°C for 10 min. A PTC-100 PCR system (MJ Research) was used for RT-PCT analysis. All PCR products were analyzed using 2.0% (w/v) agarose gel electrophoresis. Gels were stained with ethidium bromide and photographed under ultraviolet light. The bands corresponding to type II collagen and aggrecan were semi-quantified using a GIS gel image analysis system with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as an internal control. All assays were repeated six times.

Western blot analysis

At the end of the 7-day induction, the cultures were washed twice with phosphate-buffered saline (PBS), and treated with an extraction buffer. Protein samples (50 μg or 10-20 mL) were subject to electrophoresis on 10% SDS-polyacrylamide gels. Separated proteins were blotted onto PVDF membranes, and the blot was blocked for 1 h at room temperature with blocking buffer (0.1% Tris-buffered saline Tween-20 [TBST] with 5% fat-free dried milk powder). The blot was then incubated with primary antibodies (mouse anti-human monoclonal antibodies against β-catenin, 1:500; mouse anti-human monoclonal antibodies against Sox9 1:500; anti-GAPDH as an internal control, I: 80,000) at 4°C overnight. The blot was washed with 0.1% TBST, and incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000) at 37°C for 1 h. The blot was washed again and incubated with western blotting detection reagent, and then radiography was used to detect the expression of Sox9 and β-catenin protein in induced IPSCs. The bands corresponding to Sox9 and β-catenin protein were scanned and semi-quantified using Image Quant TL analysis software. All assays were repeated three times.

Statistical analysis

The experimental results were expressed as the means ± standard deviation and compared using the *t*-test following homogeneity test of variances. A *P*-value of less than 0.05 was considered significant.

Results

Differentiation of IPSCs to NP-liked cells

IPSCs exhibited a shuttle or triangle shape with a clear boundary, and were translucent in appearance after positive cell clones were picked and expanded (**Figure 1A**). In culture,

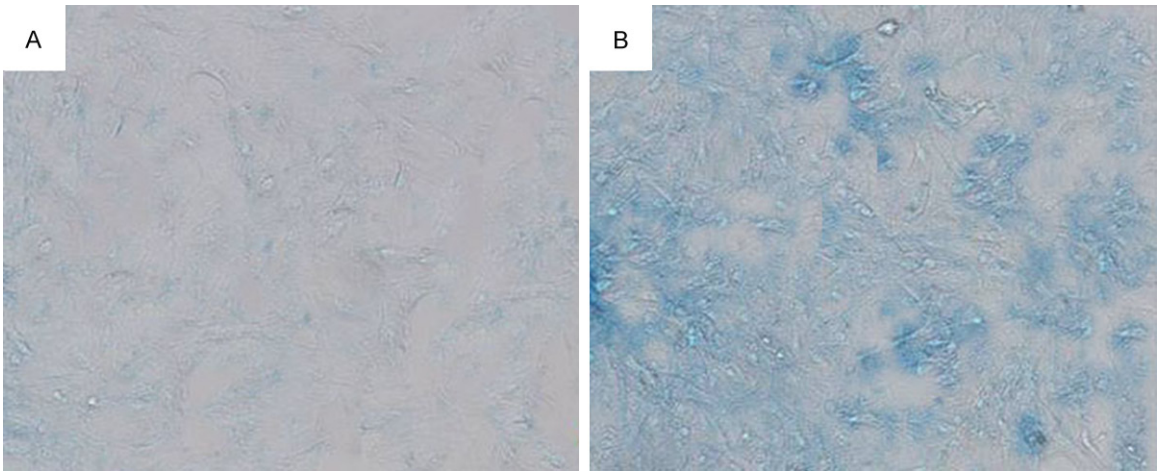

Figure 2. Alcian blue staining shows greater formation of GAG in the induction group (B) compared with that of the control group (A) (10 × magnification).

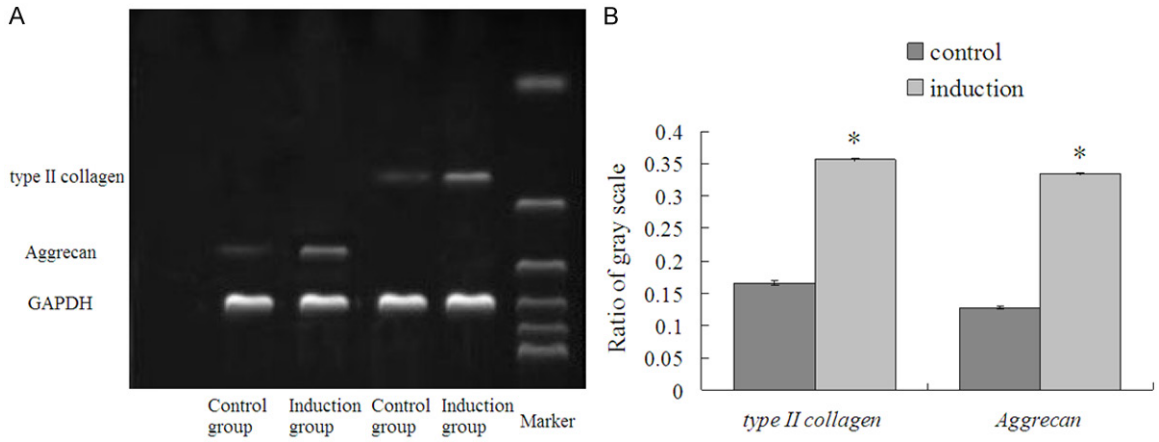

Figure 3. The mRNA expressions of type II collagen and aggrecan. (A) Represents RT-PCR analysis of type II collagen and aggrecan, and (B) Represents ratio of gray scale in groups (*$P < 0.05$, compared with control group).

IPSCs proliferated and expanded rapidly. Induced IPSCs on C/GP gels maintained a round morphology (**Figure 1B**), proliferating and expanding slowly.

Identification of biological characteristic in the induced IPSCs

Alcian Blue staining: At the end of the 7-day induction, Alcian Blue staining results showed a large degree of GAG formation in the extracellular matrix. The cultures in the induction group showed greater formation of GAG compared to the control group (**Figure 2**), which indicated that induced IPSCs had normal secretory function and that TGF-β_1 could promote the formation of extracellular matrix.

RT-PCR analysis

The expression of type II collagen and aggrecan mRNA was confirmed by RT-PCR in the induction and control groups. The 175 bp type II collagen fragment and 276 bp aggrecan fragment were amplified by PCR (**Figure 3A**), with the induction group showing higher expression of type II collagen and aggrecan compared to that in the control group ($P < 0.05$, **Figure 3B**), which indicated that expression of type II collagen and aggrecan mRNA was stable and existed in the induced IPSCs.

Western blot analysis

Following the 7-day induction, the induction group showed higher levels of protein expres-

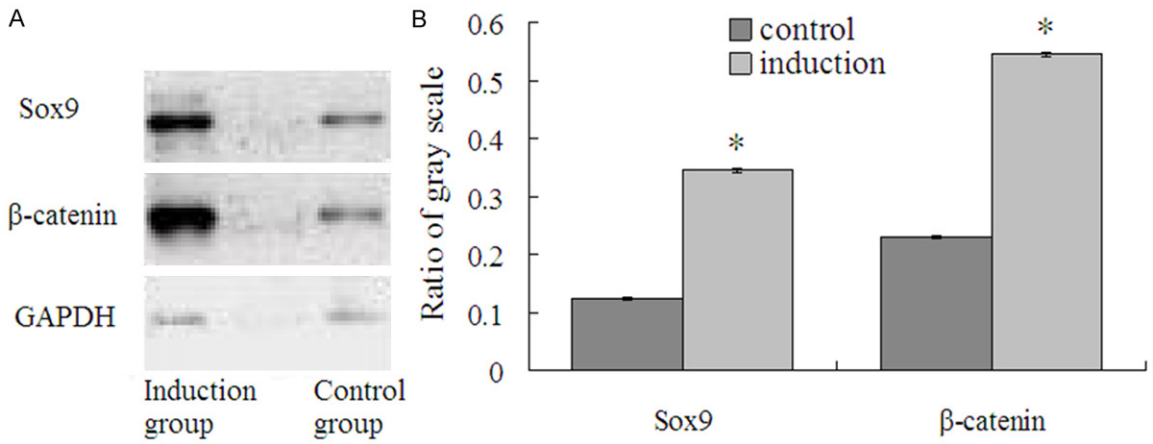

Figure 4. The protein expressions of Sox9 and β-catenin. (A) Represents western blot analysis of Sox9 and β-catenin, and (B) Represents ratio of gray scale in groups (*$P < 0.05$, compared with control group).

sion for Sox9 and β-catenin compared to the control group ($P < 0.05$; **Figure 4A**, **4B**), indicating that TGF-β_1 may upregulate the expression of Sox9 and β-catenin, thereby affecting the differentiation of IPSCs.

Discussion

The intervertebral disc is the largest avascular organ in the human body, hypoxia and acid three-dimensional (3D) environment. In order to study the regulatory mechanisms involved in differentiating IPSCs into NP-like cells, we investigated the mionectic environment, growth factor stimulation and 3D cultivation [9]. Currently, the accepted induction factor for differentiating stem cells into NP-like cells is TGF-β_1. TGF-β_1 is known to regulate cell proliferation and differentiation, extracellular matrix synthesis and apoptosis. Studies have revealed that mesenchymal stem cells can be differentiated into an NP-like phenotype by TGF-β_1 [10, 11]. Steck et al showed that by stimulation of TGF-β_1, dexamethasone and L-ascorbic acid, the induced mesenchymal stem cells could express chondrocyte-specific genes, such as type II collagen, aggrecan, decorin and fibromodulin [12]. Moreover, the levels of expression were similar to those observed in normal intervertebral disc tissues. Morigele et al demonstrated that treatment with TGF-β_1 combined with Notch1 knockdown was useful in the treatment of intervertebral disc degeneration by regulating the directional differentiation of mesenchymal stem cells (MSCs) [13]. Studies have shown that synovial stem cells could be differentiated into NP-like cells by TGF-β_1, with gene expression of type II collagen and aggrecan significantly increased [14].

In the present study, we evaluated the possibility of differentiating IPSCs into nucleus pulposus (NP)-like cells by using TGF-β_1 differentiating medium containing C/GP scaffolds under hypoxic conditions to. We found that IPSCs could be differentiated into NP-like cells with the following manifestations: the chondrocyte-specific gene expression of type II collagen and aggrecan was significantly elevated (**Figure 3**); protein expression of Sox9 and β-catenin was significantly elevated (**Figure 4**) indicating that TGF-β_1 may upregulate the expression of Sox9 and β-catenin, thereby affecting the differentiation of IPSCs; Alcian Blue staining analysis indicated that induced IPSCs exhibited normal secretory functions and TGF-β_1 could promote the formation of ECM.

Additionally, induced IPSCs showed a higher level of protein expression for β-catenin. While Wnt/β-catenin signaling is known to play an important role in proliferation and differentiation of stem cells, studies have shown that a low level of β-catenin is essential in maintaining the epidermal stem cell state. Upregulating the expression of β-catenin, which then translocates to the nucleus and modulates transcription of target genes, can activate epidermal stem cells and initiate differentiation. In contrast, blocking the expression of β-catenin in epidermal stem cells can main-

tain them in the pluripotent state [15]. Other research has shown that exogenous TGF-β_1 added to bone mesenchymal stem cells cultured *in vitro*, led to an upregulation in β-catenin expression, which was then translocated to the nucleus resulting in differentiation [16]. These results indicated that β-catenin is a key mediator in Wnt signaling, as a translocation mediator, and as an essential signal for regulation of proliferation and differentiation. It is postulated that TGF-β_1 may regulate the expression of β-catenin and affect differentiation of IPSCs; however, the mechanism by which this occurs is unclear and requires further research.

In conclusion, we have shown that IPSCs can be differentiated into NP-like cells following induction by TGF-β_1. This preliminary study provides a basis for trialing stem cell-mediated cell transplantation of IDD-related diseases in order to restore the structure and physiological function of degenerated intervertebral discs.

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Disclosure of conflict of interest

None.

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References

[1] Furukawa T, Ito K, Nuka S, Hashimoto J, Takei H, Takahara M, Ogino T, Young MF and Shinomura T. Absence of biglycan accelerates the degenerative process in mouse intervertebral disc. Spine 2009; 34: E911-917.

[2] Holm S, Mackiewicz Z, Holm AK, Konttinen YT, Kouri VP, Indahl A and Salo J. Pro-inflammatory, pleiotropic, and anti-inflammatory TNF-alpha, IL-6, and IL-10 in experimental porcine intervertebral disk degeneration. Vet Pathol 2009; 46: 1292-1300.

[3] Yang X and Li X. Nucleus pulposus tissue engineering: a brief review. Eur Spine 2009; 18: 1564-1572.

[4] Freimark D and Czermak P. Cell-based regeneration of intervertebral disc defects: review and concepts. Int J Artif Organs 2009; 32: 197-203.

[5] Neman J, Hambrecht A, Cadry C and Jandial R. Stem cell-mediated osteogenesis: therapeutic potential for bone tissue engineering. Biologics 2012; 6: 47-57.

[6] Robinson D, Hasharoni A, Cohen N, Yayon A, Moskowitz RM and Nevo Z. Fibroblast growth factor receptor-3 as a marker for precartilaginous stem cells. Clin Orthop Relat Res 1999; 367 Suppl: 167-175.

[7] Delgado JP, Parouchev A, Allain JE, Pennarun G, Gauthier LR, Dutrillaux AM, Dutrillaux B, Di Santo J, Capron F, Boussin FD and Weber A. Long-term controlled immortalization of a primate hepatic progenitor cell line after Simian virus 40 T-Antigen gene transfer. Onco-gene 2005; 24: 541-551.

[8] Wang J, Fang H, Xia R, Chen A and Cheng H. Immortalization of human precartilaginous stem cells by transfecting SV40Tag. J Huazhong Univ Sci Technolog Med Sci 2009; 29: 231-234.

[9] Hohaus C, Ganey TM, Minkus Y and Meisel HJ. Cell transplantation in lumbar spine disc degeneration disease. Eur Spine Suppl 2008; 4: 492-503.

[10] Risbud MV, Albert TJ, Guttapalli A, Vresilovic EJ, Hillibrand AS, Vaccaro AR and Shapiro IM. Differentiation of mesenchymal stem cells towards a nucleus pulposus-like phenotype in vitro: implications for cell-based transplantation therapy. Spine 2004; 29: 2627-2632.

[11] Kuh SU, Zhu Y, Li J, Tsai KJ, Fei Q, Hutton WC and Yoon ST. Can TGF-beta1 and rhBMP-2 act in synergy to transform bone marrow stem cells to discogenic-type cells? Acta Neurochir (Wien) 2008; 150: 1073-1079.

[12] Steck E, Bertram H, Abel R, Chen B, Winter A and Richter W. Induction of intervertebral disc-like cells from adult mesenchymal stem cells. Stem Cells 2005; 23: 403-411.

[13] Morigele M, Shao Z, Zhang Z, Kaige M, Zhang Y, Qiang W and Yang S. TGF-β_1 induces a nucleus pulposus-like phenotype in Notch 1 knockdown rabbit bone marrow mesenchymal stem cells. Cell Biol Int 2013; 37: 820-825.

[14] Chen S, Emery SE and Pei M. Coculture of synovium-derived stem cells and nucleus pulposus cells in serum-free defined medium with supplementation of transforming growth factor-beta1: a potential application of tissue-specific stem cells in disc regeneration. Spine (Phila Pa 1976) 2009; 34: 1272-1280.

[15] Huelsken J, Vogel R, Erdmann B, Cotsarelis G and Birchmeier W. β-Catenin controls hair fol-

licle morphogenesis and stem cell differentiation in the skin. Cell 2001; 105: 533-545.
[16] Jian H, Shen X, Liu I, He X and Wang XF. Smad3-dependent nuclear translocation of beta-catenin is required for TGF-beta1-induced proliferation of bone marrow-derived adult human mesenchymal stem cells. Genes Dev 2006; 20: 666-674.