European Review for Medical and Pharmacological Sciences 2017; 21: 1462-1470

Wnt11 overexpression promote adipose-derived stem cells differentiating to the nucleus pulposus-like phenotype

H.-T. CHEN[1,2], A.-B. HUANG[1], Y.-L. HE[1], J. BIAN[1], H.-J. LI[1]

[1]Department of Orthopedics, Taizhou People's Hospital Affiliated to Nantong University, Taizhou, Jiangsu, China
[2]Department of Orthopedics, the First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, China

Hongtao Chen and *Haijun Li* contributed equally

Abstract. – OBJECTIVE: Our present study aimed to evaluate the effects of Wnt11 overexpression on the adipose-derived stem (ADSCs) cells differentiation to the nucleus pulposus (NP) cells and its function in the ADSCs cells growth, proliferation and induction of the NP cells markers.

MATERIALS AND METHODS: The cell growth was detected using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) assay and the cell cycle was assessed by the flow cytometry. The cells morphology was evaluated using the transmission electron microscopy. The transfection efficiencies of Wnt11 lentivirus were observed under fluorescence microscope. Besides, Quantitative Real-time PCR and Western blot analysis were applied to detect the relative mRNA and protein levels.

RESULTS: Wnt11 lentivirus treatment could inhibit the ADSCs cells growth and arrest the cell cycle progression at the G0/G1 phase. Besides, the overexpression of Wnt11in ADSCs cells could induce the expression of the NP cells markers. Levels of SOX-9, aggrecan, and collagen type II were significantly increased in the ADSCs cells transfected with the Wnt11 lentivirus, in comparison with the untreated cells or the vector controls.

CONCLUSIONS: The Wnt11 overexpression may provide some experimental evidence for the possible opportunity of the Wnt11 to promote the ADSCs cells differentiating to the NP cells. Therefore, the Wnt11 overexpression may have a potential utility for the treatment of the intervertebral disc degeneration.

Key Words:
Intervertebral disc degeneration, Wnt11, Adipose-derived stem cells, Nucleus pulposus cells.

Introduction

Low back pain is a kind of common diseases[1], and the major cause of it is the intervertebral disc degeneration (IDD)[2]. Intervertebral disc (IVD) is mainly consists of three following components: the outer *annulus fibrosus*, the central hyperhydrated nucleus pulposus (NP), and the lower and upper cartilaginous endplates[3]. Though the causes responsible for IDD remain unknown, the decrease of the number and function of NP cells are believed to be a central trigger of IDD[4,5]. Over the last ten years, cell transplantation has turned into a major biological method to treat IDD[6-8]. Stem cell is a kind of fascinating cell sources for the IDD treatment, so as for their ability for multiline age differentiation, the high potential in culture, and the capacity of self-renewing[9]. Varieties of stem cells have been widely used in the cell transplantation for the clinical IDD treatment, for example: the adipose derived stem cells (ADSCs)[10]; the human umbilical tissue derived cells[11]; the synovium derived stem cells[12]; the bone marrow derived stem cells (BADSCs)[13]. Wnt family is a kind of highly conserved and secreted glycoproteins that participates in the regulation of many biological processes, for example, cellular development, growth, apoptosis, and proliferation[14,15]. Wnt genes can promote some signal transduction via many signaling pathways, as well as the Wnt noncanonical pathway[16,17]. Some studies have suggested that Wnt signaling may participate in the regulation of MSC (mesenchymal stem cells) and chondrogenesis differentiation. Witte et al[18] once reported that total 19 Wnt genes were found

Corresponding Author: Haijun Li, MD; e-mail: handsome_lhj@139.com

during the process of cartilage differentiation and mouse limb development. Besides, it was shown that Wnt3a could suppress the chondrogenic differentiation whereas promotes the proliferation of the ADSCs[19,20]. Moreover, the expression of either Wnt7a or Wnt1 can cause a distinct block in the chondrogenesis[21]. Ryu and Chun[22] reported that Wnt11 can stimulate the Col-II accumulation in the articular chondrocytes. In addition, gene expression peak of Wnt11 was found at the last stage of the human ADSCs differentiation, suggesting that Wnt11 may be involved in the regulation of the ADSCs differentiation[23]. In the present study we explored the potential effects of Wnt11 overexpression on the ADSCs cells differentiation to the NP cells. The function of Wnt11in the growth and proliferation of the ADSCs cells was also investigated. Furthermore, the expression of NP cells markers such as aggrecan, collagen type II and SOX-9 were also detected in the ADSCs cells after Wnt11 lentivirus transfection.

Materials and Methods

Cell Culture

Adult Sprague Dawley (SD) rats weighing 250-300 g were sacrificed for the following experiments. Rat adipose tissues were simply obtained by the liposuction from the abdominal subcutaneous fat. Then the harvested adipose tissues were orderly digested, filtered, and centrifuged. All detailed procedures were carried out according to the previous study[24]. The process for the rat ADSCs preparation was carried out under the GMP conditions in Stem Cell Research Center of Nanjing Medical University.

Wnt 11 Lentivirus Preparation

The rat Wnt11 lentivirus and its lentiviral vector green fluorescent protein (Lv-GFP) were pursued from the Genechem (Shanghai, China). To verify the lentiviral particles, the rats Wnt11 lentivirus and their lentiviral vector were transfected into the 293T cells following the manufacturer's protocol. Transduction of the Lv-GFP lentivirus was applied for the control. After 96 h transfection to ADSCs, cells were observed under fluorescence microscope to verify the transfection efficiency.

Cell Analysis by Flow Cytometry

Flow cytometry analysis was carried out to detect the characteristics of the isolated ADSC cells according to the previous study[25]. Anti-Sca-1 (Abcam, Cambridge, MA, USA) and CD44 antibodies (Abcam, Cambridge, MA, USA) were specifically employed for markers of adipose-derived stromal cells while CD45 antibodies (Abcam, Cambridge, MA, USA) and CD11b antibodies (Abcam, Cambridge, MA, USA) were used as negative control. Cell cycle analysis was performed on a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

MTT Assay

Cells were plated in the 24 well plates ($5*10^3$ cells per well) (Genechem, Shanghai, China). Lv-Wnt11 and the same volume Lv-GFP were added after cells attachment to the plates. Then, after the cells were cultured for 96 h, baseline values were obtained by an MTT assay (Life Technologies, Woburn, MA, USA). It is a colorimetric assay to test viable cells by measuring formazan reduced from dimethyl sulfoxide (DMSO). After 20 min of incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), we extracted formazan by dimethyl sulfoxide (DMSO) (Genechem, Shanghai, China) and tested the optical density at 540 nm. Experiments were triplicated (n=3).

Quantitative Real-time PCR

After treatment, the cells were collected to determine the expression of relative mRNA levels by qRT-PCR. Total RNA was extracted by the Trizol RNA extraction reagent (Invitrogen, Carlsbad, CA, USA) following the protocols and quantified by spectrophotometer method. Purified RNA with equal volume was reverse transcribed (RevertAid Fist Strand cDNA Synthesis Kit, Thermo Scientific, Waltham, MA, USA). The cDNA products were subjected to Real-time quantification on an ABI PRISM 7000 Sequence Detection System (TaKaRa Biotechnology, Dalian, China).

Western Blot

After treatment, the cells were collected and the protein extracted with radioimmunoprecipitation assay (RIPA) lysis buffer. The protein concentrations were quantified by the bicinchoninic acid assay (BCA) method. 30 mg protein samples were run on 10% gels, and then transferred to the polyvinylidene fluoride (PVDF) membrane. After 1 h of blocking with the 5% non-fat milk, the membranes were incubated with the primary

rabbit anti-Wnt11 (1:1000, Abcam, Cambridge, MA, USA), rabbit anti-aggrecan, anti-collagen type II and SOX-9 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and the rabbit anti GADPH (1:1000, Abcam, Cambridge, MA, USA) at 4°C over night. Washing in tris buffered saline-tween (TBST) for three times, the membranes were then incubated with a peroxidase (HRP) labeled secondary antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h. The bands were washed again, enhanced with chemiluminescence reagents and visualized with the ChemiDocTM MP Imaging System (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

All data were expressed as the mean ± standard error of the mean (SEM), and were analyzed using a one-way analysis of variance followed by Bonferroni-Dunn correction. Statistical analysis was performed using the SPSS software (SPSS Inc. Chicago, IL, USA) version 20.0.

Results

Characterization of Rat Adipose-Derived Stromal Cells

To confirm the isolated ADSCs validity, we performed the flow cytometry analysis. Cultivation of primary ADSCs for 5 days, the cells proliferated (Figure 1A). The results of flow cytometry analysis suggested that the isolated cells were positive for Sca-1 and CD44 (Figure 1B), which were recognized as ADSCs cells markers. Meanwhile, the cells showed strong negative signals of the stem non-specific markers CD45 and CD11b (Figure 1B).

Wnt11 Transfection in ADSCs

Inverted fluorescence microscopy was used to observe the effect of 96 h transfection of ADSCs with different multiplicity of infection (MOI). As shown in Figure 2, when transfected with Lv-GFP, ADSCs almost had no fluorescence expression. When transfected with Lv-Wnt11 MOI = 20, the ADSCs and fluorescence intensity was weak. When MOI = 40, light intensity and positive cells increased significantly, and positive cells remained spindle-shaped or short spindle shape. When MOI = 60, the fluorescence intensity and the number of positive cells were less than that MOI = 40.

Wnt11 Levels in ADSCs After Transfection

Western blot analysis and qRT-PCR experiment were applied to assess the levels of Wnt11 to further confirm the effectiveness of Wnt11 lentivirus transfection (MOI = 40). As shown in Figure 3A, Wnt11 mRNA level increased significantly in the Lv-Wnt11 group after 96 h transfection in comparison with the control group and the Lv-GFP group. Moreover, the consistent results acquired that the Wnt11 protein level increased markedly in the Lv-Wnt11 group after 96 h transfection in comparison with the control group and the Lv-GFP group (Figure 3B).

Wnt11 Overexpression Inhibited the ADSCs Proliferation

In comparison with the control group and the Lv-GFP group, the Wnt11 overexpression (MOI = 40) significantly inhibited the ADSCs proliferation (Figure 4A). Besides, whether the Wnt11 overexpression in the ADSCs could change, the cell cycle was investigated by the flow cytometry. As showed in Figure 4B, the cell cycle was obviously arrested at the G0/G1 phase in the ADSCs cells after transfected with the Lv-Wnt11. Meanwhile, the ADSCs number in S stage was significantly reduced (Figure 4B). These results suggested that the Wnt11overexpression could inhibit the ADSCs proliferation by resulting in a G0/G1 cell cycle arrest.

Wnt11 Overexpression Induced the Expression of NP Cells Markers

To test the hypothesis that overexpression of Wnt11 (MOI = 40) could induce the expression of NPs markers, SOX-9, aggrecan, and collagen type II levels were determined by the qRT-PCR and Western blot. As shown in Figure 5A-C, levels of aggrecan, collagen type II and SOX-9 were markedly increased in the ADSCs transfected with the Lv-Wnt11 in comparison with the untreated cells and the Lv-GFP vector.

Discussion

In our present research, the potential effects of Wnt11 overexpression on the ADSCs cells differentiation to NP cells were evaluated. Our results suggested that the Lv-Wnt11 treatment could inhibit the ADSCs cells growth and arrest the cell cycle progression at the G0/G1 phase. Besides, the overexpression of Wnt11in ADSCs cells could induce the expression of NPs mark-

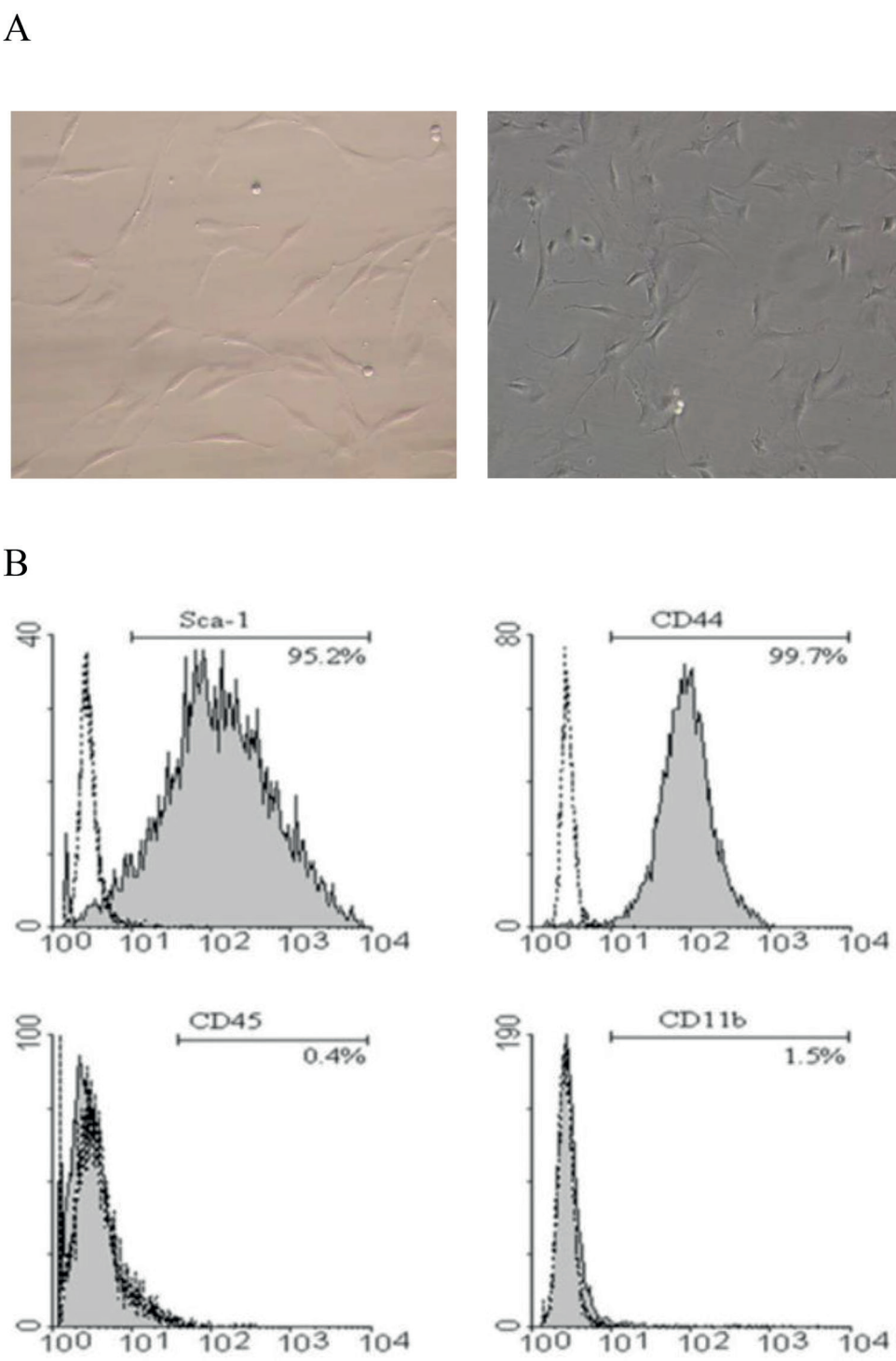

Figure 1. Characterization of rat adipose-derived stromal. ***(A)*** The ADSCs cells morphology was evaluated using the transmission electron microscopy. ***(B)*** Flow cytometry analysis was carried out to verify the validity of the isolated ADSCs. Positive cells markers include Sca-1 and CD44 and the negative ones include CD45 and CD11b.

ers. As shown, the levels of aggrecan, collagen type II and SOX-9 were markedly increased in the ADSCs transfected with the Lv-Wnt11 in comparison with the untreated cells and the Lv-GFP vector. The central NP consists of ample extracellular matrix and a small number of 1% of the volume NP cells in human normal disc[26]. In the process of IDD, water content together

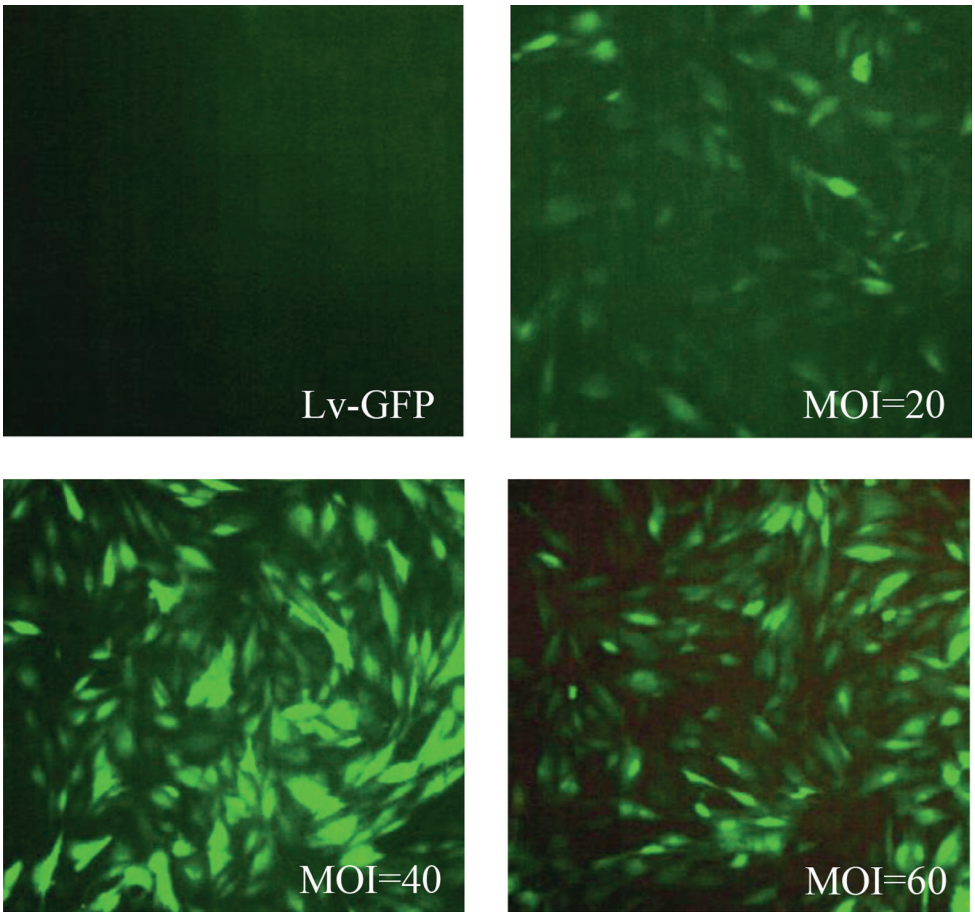

Figure 2. Wnt11 transfection in ADSCs. Inverted fluorescence microscopy was used to observe the effect of 96h transfection of ADSCs with different multiplicity of infection (MOI). Fluorescence expression of ADSCs after transfected with Lv-GFP, Lv-Wnt11 MOI = 20, Lv-Wnt11 MOI = 40, and Lv-Wnt11 MOI = 60.

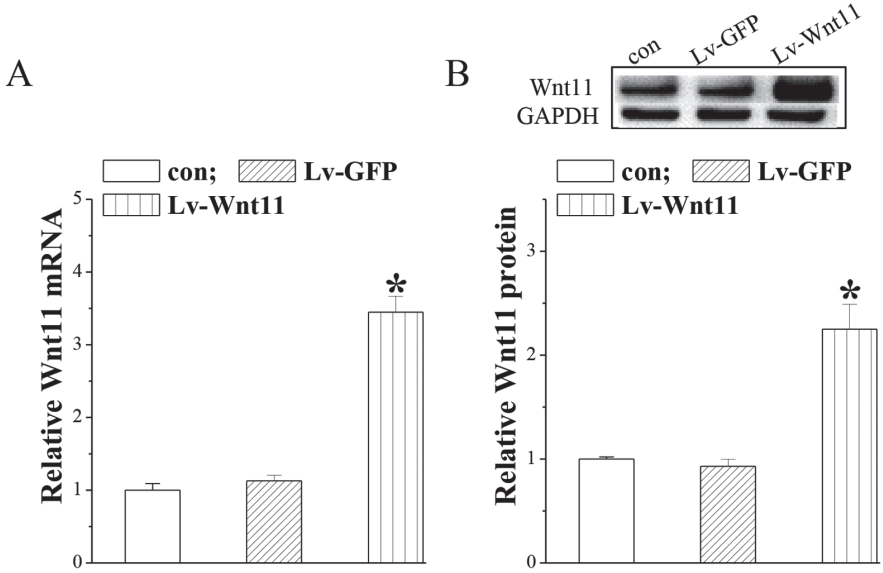

Figure 3. Wnt11 levels in ADSCs after transfection. RT-qPCR experiment ***(A)*** and Western blot analysis ***(B)*** was applied to assess the levels of Wnt11 to further confirm the effectiveness of Wnt11 lentivirus transfection. *Statistically significant difference ($p < 0.01$) between the treated groups and the control group.

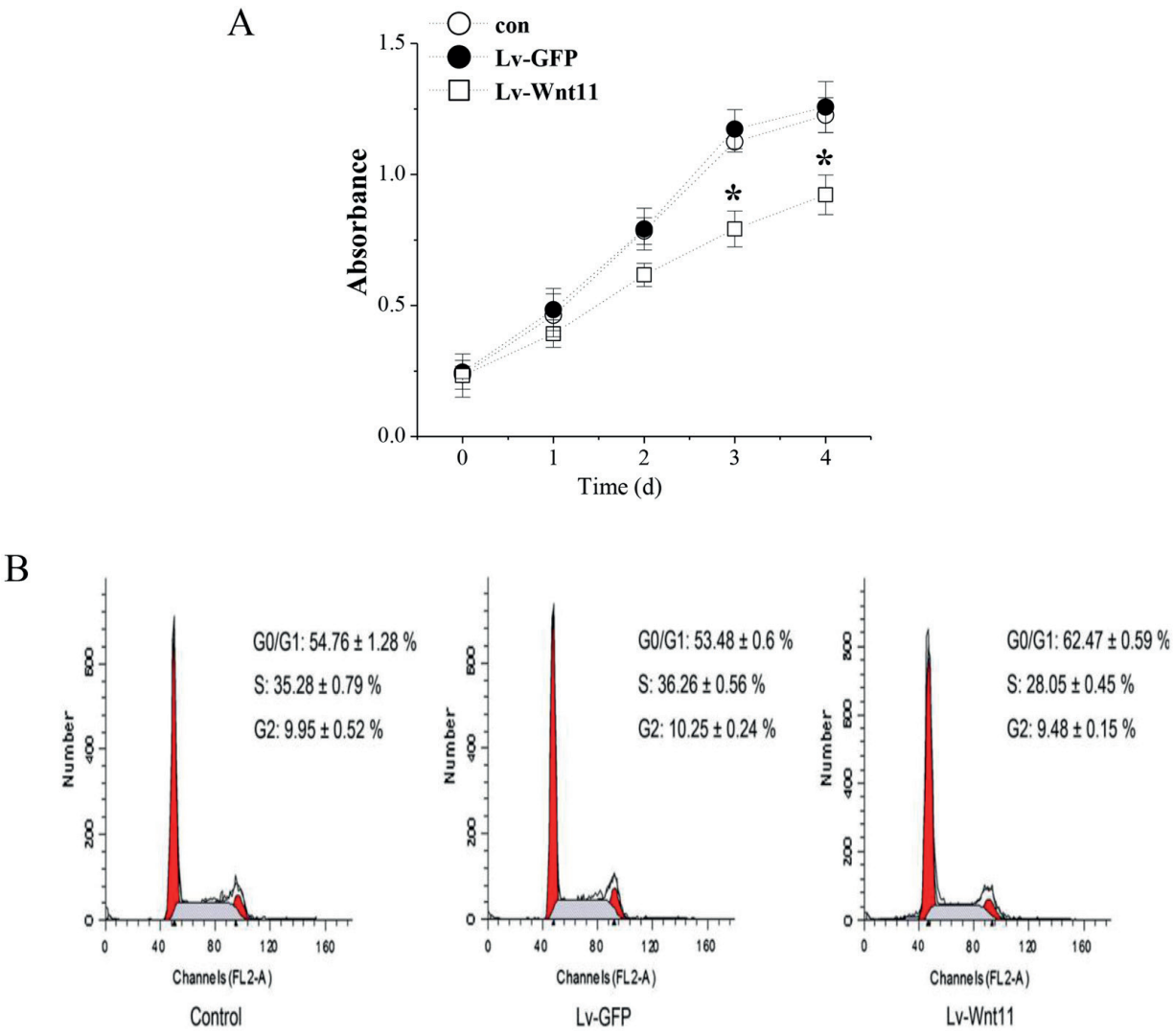

Figure 4. Wnt11 overexpression on the ADSCs proliferation. ***(A)*** The ADSCs cells proliferation after the Wnt11 lentivirus transfection. Cell proliferation was measured by MTT assay. ***(B)*** The cell cycle of ADSCs was evaluated by flow cytometry analysis. Significant differences in protein levels were observed between the treated and control groups (all $p < 0.01$).

with the proteoglycan in NP cells decreases. NP cells become more fibrous and less gelatinous for the formation of fissures and cracks in the *anulus fibrosus*, that finally results in the development of IDD[26]. In recent years, the stem cells application as a cellar treatment method of IDD, has brought a revolutionary chance with the tissue engineering development. More important, ADSC cells have obtained intensive attention as a kind of cell sources in the organ repairs, due to its abundance in nature and convenient availability[27]. Concerning the IDD, previous studies have found that the specific culture conditions, growth factors and the soluble factors released by the NP cells can promote the ADSCs differentiating to the NP cells phenotype. Tapp et al[28] once noted that the ADSCs produced a kind of collagen I rich ECM and proteoglycan after the TGF-b treatment in the 3D cultures. With the method of transwell co-cultures, Lu et al[29] found that the ADSC cells gained the ability of differentiating to the NP cells phenotype *in vitro*. Moreover, in a rabbit model studying the ADSCs implantation, Chun et al[10] found that the ADSCs injection into the injured lumbar discs could promote the cartilage regeneration, which turned out to be an effective treatment method of IDD. Nevertheless, the interaction network between the NP cells and the ADSC cells needs to be further implored. The new results in our

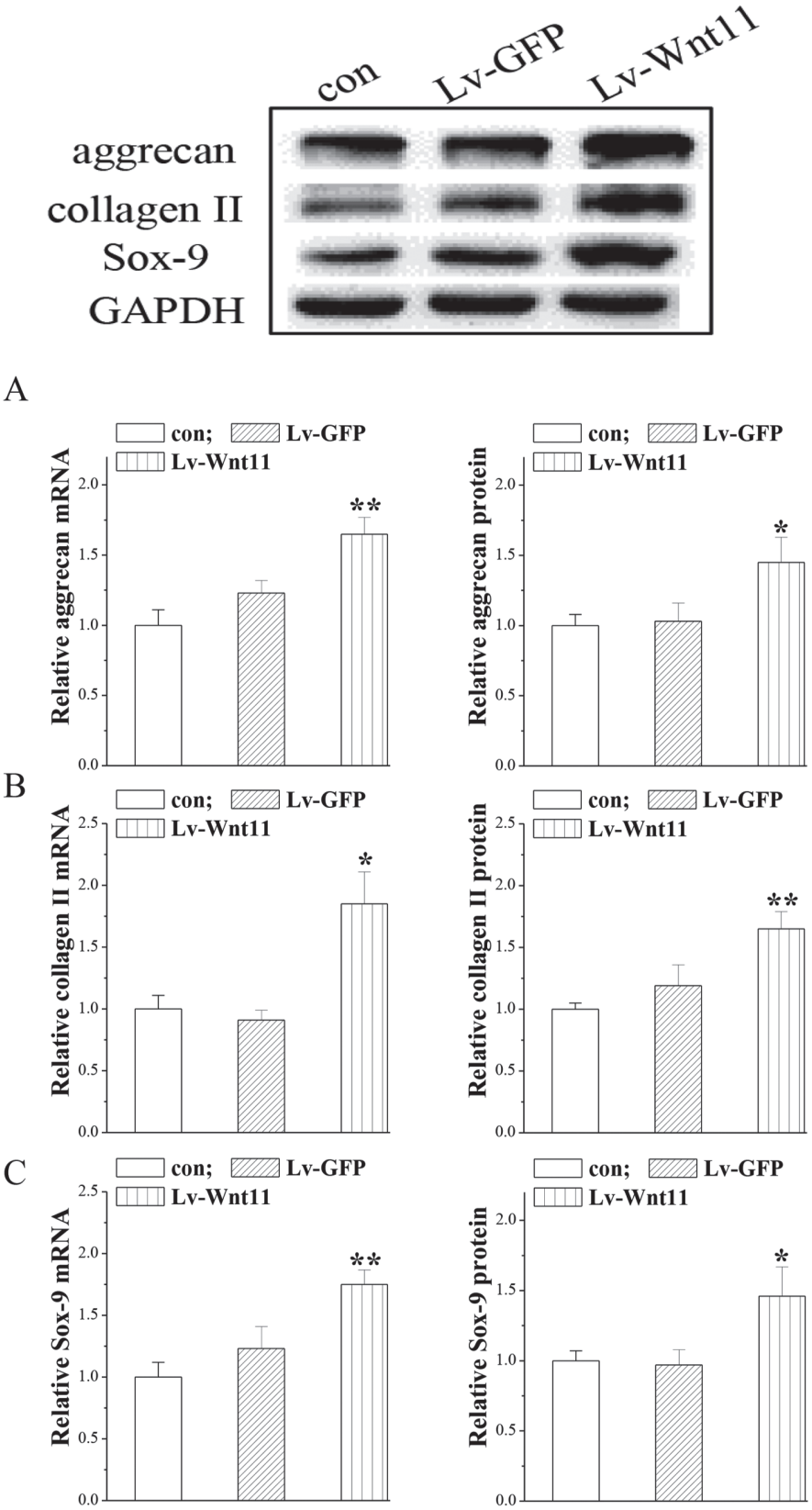

Figure 5. Wnt11 Overexpression on the levels of NP cells markers. RT-qPCR experiment and Western blot analysis was applied to assess the levels of aggrecan, collagen type II and SOX-9 ***(A-C)***. Statistically significant differences were between the treated groups and the control group (*$p < 0.05$, **$p < 0.01$).

present study were that the Lv-Wnt11 treatment could inhibit the ADSCs cells growth, arresting the cell cycle progression at the G0/G1 phase. Besides, the overexpression of Wnt11in ADSCs cells could induce the expression of NPs markers, as so for levels of aggrecan, collagen type II and SOX-9, which were markedly increased in the ADSCs after the infection of Lv-Wnt11.

Conclusions

This study was supported by Taizhou Science and Technology Support Plan (Social Development) (Grant No: TS201528) and 'Six Talent peak' of Jiangsu Province (High-level personnel) (Grant No: 2016-WSW-146).

Acknowledgements

This study was supported by Taizhou Science and Technology Support Plan (Social Development) (Grant NO: TS201528) and 'Six Talent peak' of Jiangsu Province(High-level personnel) (Grant NO: 2016-WSW-146).

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Balague F, Mannion AF, Pellisé F, Cedraschi C. Non-specific low back pain. Lancet 2012; 379: 482-491.
2) Castro-Mateos I, Hua R, Pozo JM, Lazary A, Frangi AF. Intervertebral disc classification by its degree of degeneration from T2-weighted magnetic resonance images. Eur Spine J 2016; 25: 2721-2727.
3) Grunhagen T, Wilde G, Soukane DM, Shirazi-Adl SA, Urban JP. Nutrient supply and intervertebral disc metabolism. J Bone Joint Surg Am 2006; 88 Suppl 2: 30-35.
4) Zhang DY, Wang ZJ, Yu YB, Zhang Y, Zhang XX. Role of microRNA-210 in human intervertebral disc degeneration. Exp Ther Med 2016; 11: 2349-2354.
5) Zhao H, Ni CF, Huang J, Zhao SM, Gu WW, Jiang H, Chen L, Tan TS. Effects of bone cement on intervertebral disc degeneration. Exp Ther Med 2014; 7: 963-969.
6) Hohaus C, Ganey TM, Minkus Y, Meisel HJ. Cell transplantation in lumbar spine disc degeneration disease. Eur Spine J 2008; 17 Suppl 4: 492-503.
7) Cui YZ, Yang XH, Liu PF, Wang B, Chen WJ. Preliminary study on diagnosis of lumbar disc degeneration with magnetic resonance T1p, T2 mapping and DWI quantitative detection technologies. Eur Rev Med Pharmacol Sci 2016; 20: 3344-3350.
8) Kregar VN, Urban J, Frohlich M, Neidlinger-Wilke C, Kletsas D, Potocar U, Turner S, Roberts S. Cell sources for nucleus pulposus regeneration. Eur Spine J 2014; 23 Suppl 3: S364-374.
9) Ma X, Lin Y, Yang K, Yue B, Xiang H, Chen B. Effect of lentivirus-mediated survivin transfection on the morphology and apoptosis of nucleus pulposus cells derived from degenerative human disc in vitro. Int J Mol Med 2015; 36: 186-194.
10) Chun HJ, Kim YS, Kim BK, Kim EH, Kim JH, Do BR, Hwang SJ, Hwang JY, Lee YK. Transplantation of human adipose-derived stem cells in a rabbit model of traumatic degeneration of lumbar discs. World Neurosurg 2012; 78: 364-371.
11) Leckie SK, Sowa GA, Bechara BP, Hartman RA, Coelho JP, Witt WT, Dong QD, Bowman BW, Bell KM, Vo NV, Kramer BC, Kang JD. Injection of human umbilical tissue-derived cells into the nucleus pulposus alters the course of intervertebral disc degeneration in vivo. Spine J 2013; 13: 263-272.
12) Miyamoto T, Muneta T, Tabuchi T, Matsumoto K, Saito H, Tsuji K, Sekiya I. Intradiscal transplantation of synovial mesenchymal stem cells prevents intervertebral disc degeneration through suppression of matrix metalloproteinase-related genes in nucleus pulposus cells in rabbits. Arthritis Res Ther 2010; 12: R206.
13) Wei A, Tao H, Chung SA, Brisby H, Ma DD, Diwan AD. The fate of transplanted xenogeneic bone marrow-derived stem cells in rat intervertebral discs. J Orthop Res 2009; 27: 374-379.
14) Cohen ED, Tian Y, Morrisey EE. Wnt signaling: an essential regulator of cardiovascular differentiation, morphogenesis and progenitor self-renewal. Development 2008; 135: 789-798.
15) Klaus A, Birchmeier W. Wnt signalling and its impact on development and cancer. Nat Rev Cancer 2008; 8: 387-398.
16) Luo T, Dunphy PS, Lina TT, McBride JW. Ehrlichia chaffeensis exploits canonical and noncanonical host wnt signaling pathways to stimulate phagocytosis and promote intracellular survival. Infect Immun 2015; 84: 686-700.
17) Weivoda MM, Ruan M, Hachfeld CM, Pederson L, Howe A, Davey RA, Zajac JD, Kobayashi Y, Williams BO, Westendorf JJ, Khosla S, Oursler MJ. Wnt signaling inhibits osteoclast differentiation by activating canonical and noncanonical cAMP/PKA pathways. J Bone Miner Res 2016; 31: 65-75.
18) Witte F, Dokas J, Neuendorf F, Mundlos S, Stricker S. Comprehensive expression analysis of all Wnt genes and their major secreted antagonists during mouse limb development and cartilage differentiation. Gene Expr Patterns 2009; 9: 215-223.
19) Boland GM, Perkins G, Hall DJ, Tuan RS. Wnt 3a promotes proliferation and suppresses osteo-

genic differentiation of adult human mesenchymal stem cells. J Cell Biochem 2004; 93: 1210-1230.

20) Qu F, Wang J, Xu N, Liu C, Li S, Wang N, Qi W, Li H, Li C, Geng Z, Liu Y. WNT3A modulates chondrogenesis via canonical and non-canonical Wnt pathways in MSCs. Front Biosci (Landmark Ed) 2013; 18: 493-503.

21) Rudnicki JA, Brown AM. Inhibition of chondrogenesis by Wnt gene expression in vivo and in vitro. Dev Biol 1997; 185: 104-118.

22) Ryu JH, Chun JS. Opposing roles of WNT-5A and WNT-11 in interleukin-1beta regulation of type II collagen expression in articular chondrocytes. J Biol Chem 2006; 281: 22039-22047.

23) Xu J, Wang W, Ludeman M, Cheng K, Hayami T, Lotz JC, Kapila S. Chondrogenic differentiation of human mesenchymal stem cells in three-dimensional alginate gels. Tissue Eng Part A 2008; 14: 667-680.

24) Huang YC, Kuo YH, Huang YH, Chen CS, Ho DR, Shi CS. The effects of adipose-derived stem cells in a rat model of tobacco-associated erectile dysfunction. PLoS One 2016; 11: e156725.

25) Wu B, Ma X, Zhu D, Liu Y, Sun Z, Liu S, Xue B, Du M, Yin X. Lentiviral delivery of biglycan promotes proliferation and increases osteogenic potential of bone marrow-derived mesenchymal stem cells in vitro. J Mol Histol 2013; 44: 423-431.

26) Chelberg MK, Banks GM, Geiger DF, Oegema TJ. Identification of heterogeneous cell populations in normal human intervertebral disc. J Anat 1995; 186 (Pt 1): 43-53.

27) Tapp H, Hanley EJ, Patt JC, Gruber HE. Adipose-derived stem cells: Characterization and current application in orthopaedic tissue repair. Exp Biol Med (Maywood) 2009; 234: 1-9.

28) Tapp H, Deepe R, Ingram JA, Kuremsky M, Hanley EJ, Gruber HE. Adipose-derived mesenchymal stem cells from the sand rat: transforming growth factor beta and 3D co-culture with human disc cells stimulate proteoglycan and collagen type I rich extracellular matrix. Arthritis Res Ther 2008; 10: R89.

29) Lu ZF, Zandieh DB, Wuisman PI, Bank RA, Helder MN. Differentiation of adipose stem cells by nucleus pulposus cells: configuration effect. Biochem Biophys Res Commun 2007; 359: 991-996.