

MiR-146 regulates the repair and regeneration of intervertebral nucleus pulposus cells *via* Notch1 pathway

R.-S. YANG¹, Y.-H. WANG², C. DING³, X.-H. SU¹, X.-B. GONG⁴

¹Department of Nuclear Medicine, Zhongshan Hospital Xiamen University, Xiamen, China

²Department of Pathology, The First Affiliated Hospital of Xiamen University, Xiamen, China

³Department of Anesthesiology, Jining First People's Hospital Affiliated to Jining Medical College, Jining, China

⁴Department of Spinal Surgery, Jining First People's Hospital Affiliated to Jining Medical College, Jining, China

Rongshui Yang and Yuhuan Wang contributed equally to this work

Abstract. – **OBJECTIVE:** The aim of this study was to investigate the effects of regulation of micro-ribonucleic acid (miR)-146 expression targeting the Notch1 pathway on the repair and regeneration of intervertebral nucleus pulposus cells, and to explore the possible underlying mechanism.

MATERIALS AND METHODS: Intervertebral nucleus pulposus cells were harvested from rats and cultured *in vitro*. All cells were randomly divided into 4 groups, including the normal group, the miR-146 inhibitor group, the miR-146 over-expression group and the negative control group. The cells in the normal group were cultured normally. Meanwhile, the cells in the miR-146 inhibitor group and miR-146 over-expression group were transfected with inhibitor-containing plasmid and miR-146-containing plasmid, respectively. However, the cells in the negative control group were transfected with empty plasmid. 24 h after transfection, the cells were collected for subsequent experiments. The differentiation of nucleus pulposus cells was detected via toluidine blue staining. The relative protein expression levels of Notch1, aggrecan (ACAN) and COL II were detected via Western blotting. Meanwhile, the mRNA expressions of miR-146 and Notch1 were detected via quantitative Polymerase Chain Reaction (qPCR). Furthermore, the apoptosis and proliferation of cells were detected via terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL) and Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) assay, respectively.

RESULTS: Compared with the normal group, toluidine blue positive staining was significantly increased in miR-146 overexpression group

($p < 0.05$), whereas was significantly decreased in the miR-146 inhibitor group ($p < 0.05$). The results of Western blotting revealed that compared with normal group, the protein expression of Notch1 was markedly decreased in miR-146 over-expression group ($p < 0.05$), whereas the expression levels of ACAN and COL II were notably increased ($p < 0.05$). However, the miR-146 inhibitor group exhibited significantly increased the protein expression level of Notch1 ($p < 0.05$), as well as markedly decreased the expressions of ACAN and COL II ($p < 0.05$). The results of qPCR showed that compared with the normal group, the expression level of miR-146 was significantly increased in the miR-146 over-expression group. However, the mRNA expression level of Notch1 was remarkably decreased ($p < 0.05$). Similarly, the miR-146 inhibitor group exhibited significantly decreased expression level of miR-146, as well as markedly increased mRNA expression level of Notch1 ($p < 0.05$). Compared with those in the normal group, the cell proliferation rate was markedly increased, whereas cell apoptosis was remarkably decreased ($p < 0.05$) in the miR-146 over-expression group. Furthermore, the cell proliferation rate was significantly decreased, while the cell apoptosis was remarkably increased ($p < 0.05$) in the miR-146 inhibitor group.

CONCLUSIONS: Regulating miR-146 expression can target the Notch1 signaling pathway, thereby exerting important influences on the repair and regeneration of intervertebral nucleus pulposus cells.

Key Words:

Intervertebral disc degeneration, MiR-146, Notch1 signaling pathway, Nucleus pulposus cells.

Introduction

Lumbar intervertebral disc disease is a common clinical disease in orthopedics. It is also a common cause of lumbago, leg pain, radioactive numbness of lower extremities and even gaitism and irreversible damage to lower limb nerves^{1,2}. Currently, it is believed that intervertebral disc degeneration (IDD) is one of the major causes of lumbar intervertebral disc disease. As an important cause of lumbago in patients, IDD frequently occurred in middle-aged and elderly people. Gradual declines in water content, collagen and proteoglycan in the nucleus pulposus, as well as a decrease in the elasticity may eventually lead to IDD^{3,4}. Jakoi et al⁵ have demonstrated that a series of pathological reactions occur in the nucleus pulposus during IDD. It is well known that nucleus pulposus is an important component of the intervertebral disc. In particular, nucleus pulposus cells inside the intervertebral disc are a kind of collagen-proteoglycan complex closely related to the pathological reactions of IDD.

In recent years, multiple studies on the intervertebral disc have focused on the signaling pathways. The Notch pathway, as an important intracellular signal transduction pathway, has been proved to be closely related to various orthopedic diseases. Meanwhile, it has been confirmed to be involved in the regulation of bone development and metabolism⁶. Currently, it is believed that the Notch pathway participates in regulating the proliferation and differentiation of intervertebral nucleus pulposus cells, thus playing an important role in IDD. Micro ribonucleic acids (miRNAs) are a kind of non-coding RNAs that have been considered to play an important regulatory role in the human body. Previous researches have demonstrated that miRNAs are closely related to the pathogenesis of various diseases. MiRNAs may degrade or inhibit downstream mRNAs by pairing with the corresponding untranslated region of downstream target genes, thereby playing an important role in transcriptional regulation. Furthermore, miRNAs regulate various pathological processes, such as cell proliferation, differentiation and apoptosis in the body^{7,8}. It has been found that miR-146 is specifically expressed in chondrocytes, which is also closely related to cell proliferation and differentiation. However, the exact role of miR-146 and the possible underlying mechanism remain unclear.

The aim of this study was to further clarify whether miR-146 regulated intervertebral nucleus

pulposus cells by targeting the Notch1 signaling pathway, thereby influencing their repair and regeneration.

Materials and Methods

Laboratory Animals

A total of 12 specific pathogen-free Sprague-Dawley (SD) rats aged 1 month were purchased from Shanghai SLAC Laboratory Animal Co., Ltd., [license No.: SCXK (Shanghai, China) 2014-0003]. All rats were fed in the Laboratory Animal Center, with normal food and sterile filtered water every day under 12/12 h light/dark cycle and regular room temperature and humidity. This study was approved by the Animal Ethics Committee of Xiamen University Animal Center.

Laboratory Reagents and Instruments

Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA), TRIzol lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA), Dual-Luciferase kit (Beyotime, Shanghai, China), primary antibodies: anti-Notch1 antibody (Abcam, Cambridge, MA, USA), anti-aggrecan (ACAN) antibody (Abcam, Cambridge, MA, USA), anti-COL II antibody (Abcam, Cambridge, MA, USA) and anti-Sox9 antibody (Abcam, Cambridge, MA, USA), AceQ quantitative Polymerase Chain Reaction (qPCR) SYBR Green Master Mix kit (Vazyme, Nanjing, China), HiScript II Q RT SuperMix For qPCR (+gDNA wiper) kit (Vazyme, Nanjing, China), Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) cell proliferation kit (Sigma-Aldrich, St. Louis, MO, USA), terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL) apoptosis kit (Sigma-Aldrich, St. Louis, MO, USA), optical microscope (Leica DMI 4000B/DFC425C, München, Germany) and fluorescence qPCR instrument (ABI 7500, Applied Biosystems, Foster City, CA, USA).

In Vitro Acquisition and Culture of Nucleus Pulposus Cells

SD rats fed normally were anesthetized *via* intraperitoneal injection of 7% chloral hydrate (5 mL/kg). After successful anesthesia, the skin was locally prepared and disinfected. Subsequently, the skin, subcutaneous tissue and deep fascia were cut along the longitudinal axis of the spine, followed by exposure of the lumbar intervertebral disc. Then, collected intervertebral disc tissues

were fully placed in normal saline. After removal of excess tissues and blood vessels, the intervertebral disc was cut into 1 mm³ tissue blocks. After centrifugation, the tissue blocks were collected and digested with 0.25% trypsin at room temperature for 15 min. The tissue blocks were mechanically blown and beaten to ensure that they were completely digested into a single-cell suspension. After centrifugation, the supernatant was discarded and the cells were resuspended with complete medium. The density of the cells was adjusted. The cells were inoculated into a culture flask (10⁶/mL), and cultured in an incubator with 4% CO₂ at 35°C. Cell passage was performed every 3 days. After passage until the third generation, the cells were collected for subsequent experiments.

Cell Grouping and Treatment

Nucleus pulposus cells cultured *in vitro* were randomly divided into 4 groups, including the normal group, miR-146 inhibitor group, miR-146 over-expression group and negative control group. The cells in the normal group were cultured normally without any treatment. The cells in the miR-146 inhibitor group were transfected with the plasmid containing miR-146 inhibitor, to inhibit the transcription of miR-146. The cells in the miR-146 over-expression group were transfected with the plasmid containing miR-146 mimics, to promote the transcription of miR-146. Meanwhile, the cells in the negative control group were transfected with empty plasmid as a negative control. After culturing for 48 h, transfected cells in each group were collected and detected.

Western Blotting

Collected cells were first added with lysis buffer, followed by an ice bath for 60 min and centrifugation at 1400 g for 5 min. The concentration of extracted protein was quantified using the bicinchoninic acid (BCA; Pierce, Waltham, MA, USA) method. Subsequently, the proteins were denatured *via* heating. After separation *via* gel electrophore-

sis, the protein samples were transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). After washing and sealing with blocking buffer for 1.5 h, the membranes were incubated with primary antibodies of anti-Notch1 (1:1000), anti-ACAN (1:1000) and anti-COL II (1:1000) at 4°C overnight. On the next day, the membranes were incubated with the corresponding secondary antibodies (1:1000). Then, the membranes were washed with Tris-Buffered Saline and Tween 20 (TBST). Finally, the image was developed in chemiluminescence reagent in the dark for 1 min, followed by analysis using the gel scanning imaging system.

Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was extracted from cells and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) according to the instructions of the reverse transcription kit. A 20 µL reaction system was set up. Specific reaction conditions were as follows: reaction at 51°C for 2 min, pre-denaturation at 96°C for 10 min, denaturation at 96°C for 10 s, annealing at 60°C for 30 s, for a total of 40 cycles. With glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference, the relative expression level of related mRNAs was calculated. Primer sequences used in this study were shown in Table I.

TUNEL Apoptosis Assay

Cells collected in each group were first fixed with 4% paraformaldehyde solution. TdT reaction solution was added dropwise for reaction in the dark for 1 h. Then, the reaction was terminated *via* incubation with deionized water for 15 min. Subsequently, endogenous peroxidase activity was blocked with hydrogen peroxide, and a working solution was added dropwise for 1 h of reaction. After washing, diaminobenzidine (DAB) solution (Solarbio, Beijing, China) was added dropwise for color development, followed by observation.

Table I. Primer sequences.

Name	Primer sequence
miR-146	Forward primer: 5'-TCCACCAAGAAGCTGAGCGAG-3' Reverse primer: 5'-GTCCAGCCCATGATGGTTCT-3'
Notch	Forward primer: 5'-TCCACCAAGAAGCTGAGCGAG-3' Reverse primer: 5'-GTCCAGCCCATGATGGTTCT-3'
GAPDH	Forward primer: 5'-ACGGCAAGTTCAACGGCACAG-3' Reverse primer: 5'-GAAGACGCCAGTAGACTCCACGAC-3'

CCK-8 Cell Proliferation Assay

Transfected cells were first inoculated into 96-well plates (100 μ L/well), followed by culturing in an incubator for 24 h. 3 replicates were set for each well. Briefly, 10 μ L of CCK-8 solution was added in each well, followed by reaction for 2 h in the dark. The absorbance of each well at 450 nm was detected using a microplate reader. Finally, the cell proliferation rate was calculated.

Toluidine Blue Staining

Cells collected in each group were first fixed with 4% paraformaldehyde solution. After washing and staining with toluidine blue dye for 2 h, the staining was observed. When the staining degree was moderate, the mixed liquid was removed. Then, the cells were washed and naturally air dried at room temperature. The mean optical density of positive expression of extracellular matrix was observed and calculated under an optical microscope.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Enumeration data were expressed as mean \pm standard deviation. *t*-test was used for comparison of data in line with normal distribution and

homogeneity of variance. Corrected *t*-test was applied for comparison of data in line with normal distribution and heterogeneity of variance. A non-parametric test was used for data not in line with normal distribution and homogeneity of variance. Rank sum test was adopted for ranked data, and the chi-square test was adopted for enumeration data. $p < 0.05$ was considered statistically significant.

Results

Toluidine Blue Staining

As shown in Figure 1, toluidine blue positive staining displayed purple-red color. Purple-red positive staining could be seen in some cells of the normal group and negative control group. No purple-red positive staining could be found in the miR-146 inhibitor group. Meanwhile, significant purple-red positive staining could be observed in the miR-146 overexpression group. According to statistical analysis results (Figure 2), no significant difference was found in purple-red positive expression between the negative control group and normal group ($p > 0.05$). However, it was markedly decreased in the miR-146 inhibitor group when compared with the normal group, showing a statistically significant difference ($p < 0.05$). Furthermore, it was remarkably increased in the miR-146 over-expression group ($p < 0.05$).

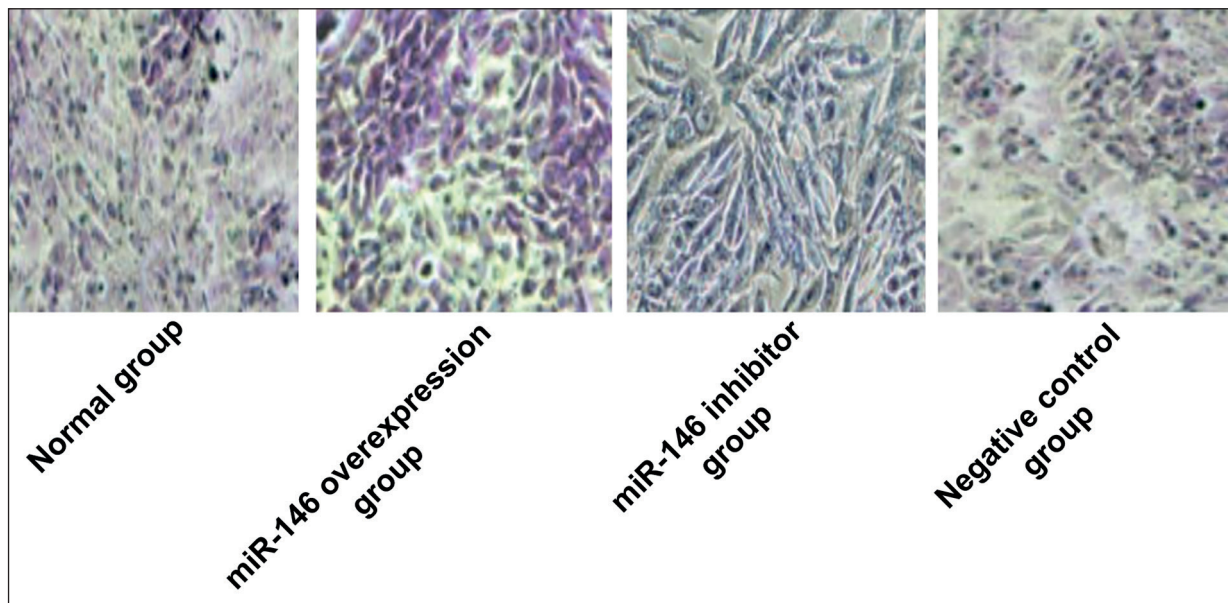


Figure 1. Toluidine blue staining (magnification $\times 40$).

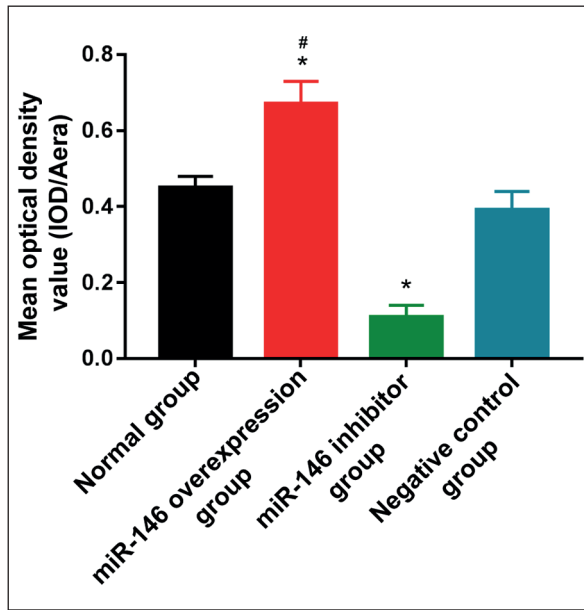


Figure 2. Mean optical density value of toluidine blue positive expression. Note: $p^* < 0.05$ vs. normal group, $p^{\#} < 0.05$ vs. miR-146 inhibitor group.

Relative Expression Levels of Proteins Detected Via Western Blotting

As shown in Figure 3 and 4, compared with the normal group, the miR-146 inhibitor group exhibited significantly increased the protein expression level of Notch1, as well as markedly decreased the expression levels of ACAN and COL II, displaying statistically significant differences ($p < 0.05$). However, miR-146 over-expression group showed significantly decreased protein expression level of Notch1, as well as remarkably increased protein expressions of ACAN and COL II, displaying statistically significant differences ($p < 0.05$). However, no significant differences were found in the protein expression

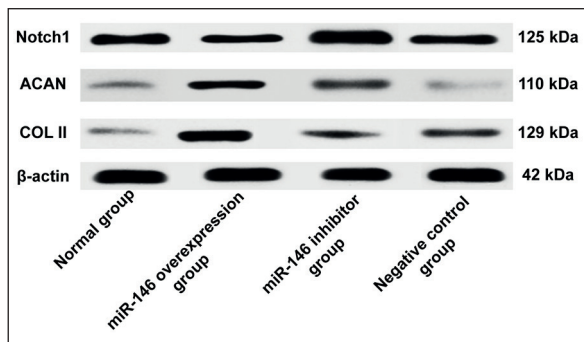


Figure 3. Protein expression detected via Western blotting.

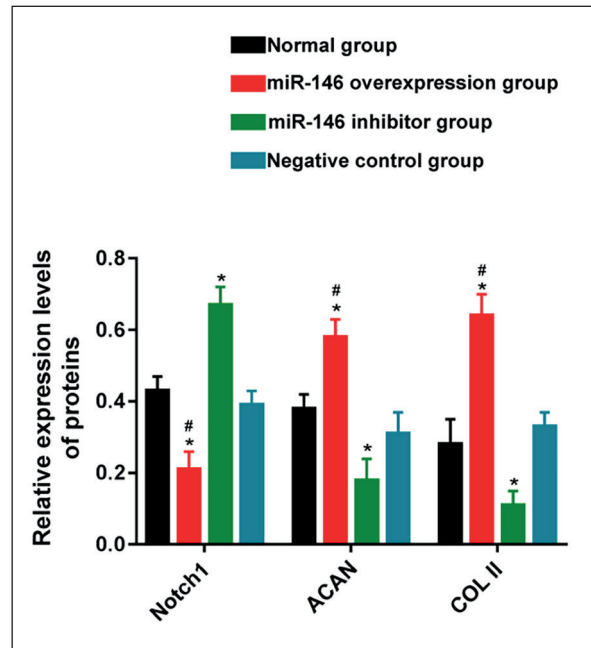


Figure 4. Relative expression levels of proteins in each group. Note: $p^* < 0.05$ vs. normal group, $p^{\#} < 0.05$ vs. miR-146 inhibitor group.

levels of Notch1, ACAN and COL II between the negative control group and normal group ($p > 0.05$).

Relative Expression Levels of mRNAs Detected Via qPCR

As shown in Figure 5, compared with the normal group, the miR-146 inhibitor group exhibited markedly decreased expression level of miR-146, while remarkably increased mRNA expression level of Notch1, showing statistically significant differences ($p < 0.05$). However, miR-146 over-expression group exhibited remarkably increased miR-146 expression level as well as decreased mRNA expression level of Notch1, showing statistically significant differences ($p < 0.05$). Furthermore, no significant differences were observed in the mRNA expression levels of miR-146 and Notch1 between the negative control group and normal group ($p > 0.05$).

Apoptosis Rate Detected Via TUNEL

Compared with the normal group, the apoptosis rate of cells was remarkably increased in the miR-146 inhibitor group, showing a statistically significant difference ($p < 0.05$). However, cell apoptosis was remarkably decreased in the

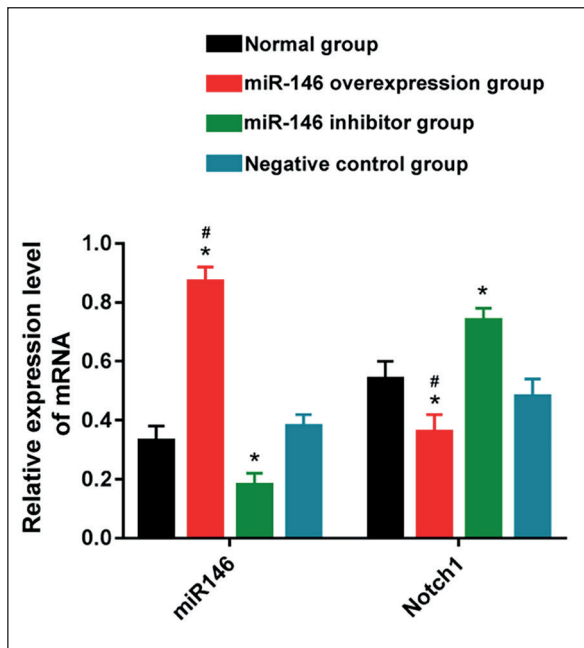


Figure 5. Relative expression levels of mRNAs in each group. Note: $p^* < 0.05$ vs. normal group, $p\# < 0.05$ vs. miR-146 inhibitor group.

miR-146 over-expression group ($p < 0.05$). No significant changes in cell apoptosis rate were found in the negative control group ($p > 0.05$; Figure 6).

Cell Proliferation Rate Detected Via CCK-8 Assay

Compared with the normal group, the cell proliferation rate was remarkably decreased in the miR-146 inhibitor group, showing a statistically significant difference ($p < 0.05$). However, cell proliferation was remarkably increased in the miR-146 over-expression group ($p < 0.05$). There were no significant changes found in the cell proliferation rate in the negative control group ($p > 0.05$; Figure 7).

Discussion

With an in-depth study of the intervertebral disc, researchers have paid increasingly more attention to the important pathological role of IDD in a variety of spine-original diseases, including lumbar disc herniation, lumbago and scoliosis. IDD is considered one of the important pathogenic factors of severe lumbago, limitation of lumbar motion, radioactive pain and numbness in both lower extremities in patients with spinal diseas-

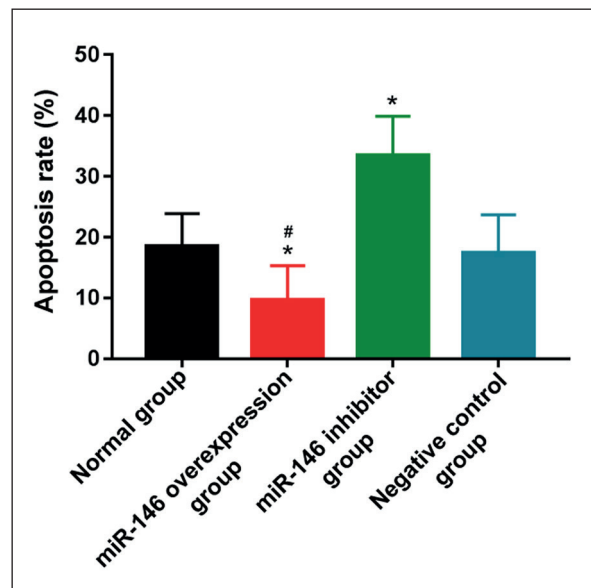


Figure 6. Cell apoptosis rate in each group. Note: $p^* < 0.05$ vs. normal group, $p\# < 0.05$ vs. miR-146 inhibitor group.

es. Nucleus pulposus is a vital component of the intervertebral disc. Meanwhile, nucleus pulposus cells are considered to play an important role during IDD. Studies^{9,10} have found that nucleus pulposus forms the complex with collagen and proteoglycan in a jelly-like form through the grid-like mode. This enables the intervertebral disc to bear the load and the spine to have important

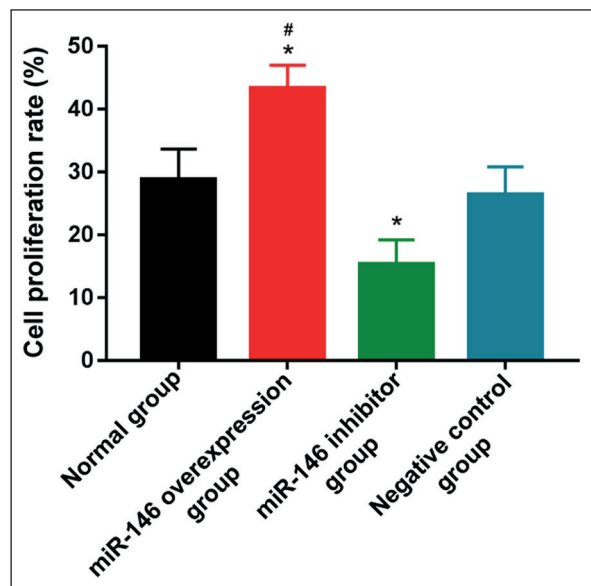


Figure 7. Cell proliferation rate in each group. Note: $p^* < 0.05$ vs. normal group, $p\# < 0.05$ vs. miR-146 inhibitor group.

physiological functions of damping and balance maintenance. Therefore, intervertebral nucleus pulposus cells have become a research hotspot currently.

As a highly-conserved signaling pathway, the Notch signaling pathway is closely related to the signal transduction of interaction between cells. Meanwhile, it exerts important physiological functions in the processes of bone development and metabolism. In particular, some scholars have pointed out that the Notch signaling pathway plays a crucial role in the proliferation and differentiation of nucleus pulposus cells^{11,12}. The Notch receptor is an important component of the Notch signaling pathway, including four family members (Notch1, Notch2, Notch3, and Notch4). As a kind of transmembrane receptor, it can bind to its ligands to exert important physiological functions. Researches^{13,14} have demonstrated that Notch1 receptor is highly and extensively expressed in nucleus pulposus cells. Meanwhile, the expression and signal transduction of Notch1 receptor are significantly activated in degenerated intervertebral disc tissues. Eventually, this inhibits the proliferation of nucleus pulposus cells and accelerates IDD. In addition, further reports have clarified that hypoxia in the intervertebral disc can activate the Notch signaling pathway to up-regulate the expression of the Notch1 receptor, thus regulating the self-repair and proliferation of damaged nucleus pulposus cells. When it is insufficient to maintain the normal number and differentiation of nucleus pulposus cells due to abnormal expression of Notch1 receptor in the body, the apoptosis of nucleus pulposus cells will be increased. Moreover, the reparability of cells will decline¹⁵. Both ACAN and COL II are specific surface antigens and characteristic markers of nucleus pulposus cells. They are closely related to the proliferation and repair of nucleus pulposus cells. Higher expression levels of ACAN and COL II corresponds to a significantly larger number of nucleus pulposus cells, as well as stronger proliferation and reparability. MiRNAs are a kind of non-coding RNAs that can bind to target genes. They may degrade target genes or inhibit target gene translation, eventually regulating the transcriptional process. Nowadays, miRNAs have become a research hotspot in the fields of cancer, cardiovascular diseases and bone diseases¹⁶⁻¹⁸. As an important non-coding RNA, miR-146 has been proved to be widely expressed in cartilages and nucleus

pulposus tissues and cells. MiR-146 overexpression *via* transfection into nucleus pulposus cells can significantly lower the degree of IDD and reduce inflammation. On the contrary, miR-146 knockout in nucleus pulposus cells increases the degree of IDD^{19,20}. These findings indicate that miR-146 plays an important role in regulating the physiological functions of nucleus pulposus cells. In this study, we confirmed that the protein and mRNA expression levels of Notch1 were significantly inhibited in the miR-146 over-expression group. However, they were markedly up-regulated in the miR-146 inhibitor group, suggesting that miR-146 played an important role in negatively regulating Notch1 receptor expression. According to further investigations, toluidine blue purple-red positive expression, cell proliferation rate and relative protein expression levels of ACAN and COL II in miR-146 over-expression group were remarkably higher than those of the normal group and miR-146 inhibitor group. However, the apoptosis rate of cells in miR-146 inhibitor group was significantly lower than that of the normal group and miR-146 inhibitor group. Our findings indicated that over-expression of miR-146 promoted the proliferation of nucleus pulposus cells and differentiation into chondrocytes. Moreover, miR-146 up-regulation could effectively inhibit the apoptosis of nucleus pulposus cells.

Conclusions

We showed that miR-146 expression can target the Notch1 signaling pathway, thereby exerting important influences on the repair and regeneration of intervertebral nucleus pulposus cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (No. 81571707,81071182), the Program for Training Young Talents of Fujian Health (No. 2014 ZQN ZD 35), the Natural Science Foundation of Fujian (No. 2015J01519), the Technology Foundation for Selected Overseas Chinese Scholar, the Ministry of Human Resources and Social Security of China (No. 2014 240) and the Scientific Research Foundation for the Returned Overseas Chinese Scholars, Ministry of Education of China (No. 2014 1685).

References

- 1) KIM SH, CHOI SS, LEE MK, KIN JE. Complex regional pain syndrome caused by lumbar herniated intervertebral disc disease. *Pain Physician* 2016; 19: E901-E904.
- 2) KIM CH, CHUNG CK, KIM MJ, CHOI Y, KIM MJ, HAHN S, SHIN S, JONG JM, LEE JH. Increased volume of lumbar surgeries for herniated intervertebral disc disease and cost-effectiveness analysis: a nationwide cohort study. *Spine (Phila Pa 1976)* 2018; 43: 585-593.
- 3) XU BS, LIU Y, XU HW, YANG Q, MA XL, HU YC. Intervertebral fusion with mobile microendoscopic discectomy for lumbar degenerative disc disease. *Orthop Surg* 2016; 8: 241-245.
- 4) WEBER KT, ALIPIUI DO, SISON CP, BLOOM O, QURAISHI S, OVERBY MC, LEVINE M, CHAHINE NO. Serum levels of the proinflammatory cytokine interleukin-6 vary based on diagnoses in individuals with lumbar intervertebral disc diseases. *Arthritis Res Ther* 2016; 18: 3.
- 5) JAKOI AM, PANNU G, D'ORO A, BUSER Z, PHAM MH, PATEL NN, HSIEH PC, LIU JC, ACOSTA FL, HAH R, WANG JC. The clinical correlations between diabetes, cigarette smoking and obesity on intervertebral degenerative disc disease of the lumbar spine. *Asian Spine J* 2017; 11: 337-347.
- 6) DEFTOS ML, HE YW, OJALA EW, BEVAN MJ. Correlating notch signaling with thymocyte maturation. *Immunity* 1998; 9: 777-786.
- 7) ZHAO Y, LIU X, LU YX. MicroRNA-143 regulates the proliferation and apoptosis of cervical cancer cells by targeting HIF-1alpha. *Eur Rev Med Pharmacol Sci* 2017; 21: 5580-5586.
- 8) LEE Y, AHN C, HAN J, CHOI H, KIM J, YIM J, LEE J, PROVOST P, RADMARK O, KIM S, KIM VN. The nuclear RNase III Drosha initiates microRNA processing. *Nature* 2003; 425: 415-419.
- 9) TANG X, JING L, RICHARDSON WJ, ISAACS RE, FITCH RD, BROWN CR, ERICKSON MM, SETTON LA, CHEN J. Identifying molecular phenotype of nucleus pulposus cells in human intervertebral disc with aging and degeneration. *J Orthop Res* 2016; 34: 1316-1326.
- 10) LI P, GAN Y, WANG H, ZHANG C, WANG L, XU Y, SONG L, LI S, LI S, OU Y, ZHOU Q. Dynamic compression effects on immature nucleus pulposus: a study using a novel intelligent and mechanically active bioreactor. *Int J Med Sci* 2016; 13: 225-234.
- 11) ROYBAL KT, WILLIAMS JZ, MORSUT L, RUPP LJ, KOLINKO I, CHOE JH, WALKER WJ, McNALLY KA, LIM WA. Engineering T cells with customized therapeutic response programs using synthetic notch receptors. *Cell* 2016; 167: 419-432.
- 12) ZHANG J, FUKUHARA S, SAKO K, TAKENOCHI T, KITANI H, KUME T, KOH GY, MOCHIZUKI N. Angiopoietin-1/Tie2 signal augments basal Notch signal controlling vascular quiescence by inducing delta-like 4 expression through AKT-mediated activation of beta-catenin. *J Biol Chem* 2011; 286: 8055-8066.
- 13) RODRIGUES-PINTO R, RICHARDSON SM, HOYLAND JA. An understanding of intervertebral disc development, maturation and cell phenotype provides clues to direct cell-based tissue regeneration therapies for disc degeneration. *Eur Spine J* 2014; 23: 1803-1814.
- 14) RISBUD MV, SCHIPANI E, SHAPIRO IM. Hypoxic regulation of nucleus pulposus cell survival: from niche to notch. *Am J Pathol* 2010; 176: 1577-1583.
- 15) LIU Z, LI C, MENG X, BAI Y, QI J, WANG J, ZHOU Q, ZHANG W, ZHANG X. Hypoxia-inducible factor-1alpha mediates aggrecan and collagen Pi expression via NOTCH1 signaling in nucleus pulposus cells during intervertebral disc degeneration. *Biochem Biophys Res Commun* 2017; 488: 554-561.
- 16) CAI B, PAN Z, LU Y. The roles of microRNAs in heart diseases: a novel important regulator. *Curr Med Chem* 2010; 17: 407-411.
- 17) CHIYOMARU T, ENOKIDA H, TATARANO S, KAWAHARA K, UCHIDA Y, NISHIYAMA K, FUJIMURA L, KIKKAWA N, SEKI N, NAKAGAWA M. miR-145 and miR-133a function as tumour suppressors and directly regulate FSCN1 expression in bladder cancer. *Br J Cancer* 2010; 102: 883-891.
- 18) SMALL EM, FROST RJ, OLSON EN. MicroRNAs add a new dimension to cardiovascular disease. *Circulation* 2010; 121: 1022-1032.
- 19) ZHANG B, XU L, ZHUO N, SHEN J. Resveratrol protects against mitochondrial dysfunction through autophagy activation in human nucleus pulposus cells. *Biochem Biophys Res Commun* 2017; 493: 373-381.
- 20) PRIYADARSHANI P, LI Y, YAO L. Advances in biological therapy for nucleus pulposus regeneration. *Osteoarthritis Cartilage* 2016; 24: 206-212.