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

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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 posus cells, and to explore the possible u lying mechanism.

MATERIALS AND METHODS: Intervertel nucleus pulposus cells were harvested fr rats and cultured *in vitro*. All ere ra domly divided into 4 groups udi he nor mal group, the miR-146 bitor up, the miR-146 over-expressio up a neg ative control group. The group were culture ormal anwhile, the cells in the miR-14 nhibitor gr nd miR-146 over-expressio p were tra ted with inhibitor-con ing mid and mi 146-containing plasmid, res ively. However, the cells in negative co group were transfected h empty plasmid h after transfection e cell ere collected for subsequent e ment e differentiation of nucleus pulpo c was de ted via toluidine blue stain e relati protein expression levels Noto ggr n (ACAN) and COL II were ected tern blotting. Meanwhile, the NA expre ons of miR-146 and Notch1 were ted via quantitative Polymerase Chain Re- CR). Furthermore, the apoptosis and proliferation of cells were detected via terminal deoxynucleotidyl transferase-mediated dUTP ick End Labeling (TUNEL) and Cell Counting t-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 05), whereas was significantly decreased in miR-146 i itor group (p<0.05). The results of Wester lotting revealed that compar ith nor group, the protein expression o as markedly decreased in miR-146 over-expression group (p<0.05), whereas xpression levels of ACAN and COL II were creased (p<0.05). However, the miR-6 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.

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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 gatism 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 al[5] 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 ways. The Notch pathway, as an important cellular signal transduction pathway, has proved to be closely related to various orthope diseases. Meanwhile, it has been med to involved in the regulation of opmen and metabolism[6]. Current is be ed that the Notch pathway partic in r the proliferation and diffe ntiat terver nucleus pulposus ce thus pla an important role in IDD. Mi bonucleic a (miRNAs) are a kind of g RNAs tha ve been considered to play an ortant regulatory role in the h an body. Pr s researches have demo ated that miRNAs closely related to the nogene of various diseases. MiRNAs n egrad inhibit downstream mRNAs by pair the cor ponding untranslated re- ion o nstrea rget genes, thereby playing impor in transcriptional regulation. thermor RNAs regulate various pathol- g processes, such as cell proliferation, differ- nd 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 liferation 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 pathog ree Sprague-Dawley (SD) rats aged onth were chased from Shanghai SLA aborato Anim Co., Ltd., [license No.: S (Sha ai, China) 2014-0003]. All rats were in Labora ry Animal Center, wit ormal and ste il- tered water every under 12/ igh k cycle and regula temperature umidity. This study app d by the Animal Ethics Committee of Xiamen ersity Animal Center.

Laboratory Reagents and Instruments

Dulbecco's Modified Eagle's Medium (DMEM; T mo Fisher S ntific, Waltham, MA, USA), TR lysis bu (Thermo Fisher Scientific, Wal MA A), Dual-Luciferase kit (Beyotime, ai, China), primary antibodies: ti-Notch1 antibody (Abcam, Cambridge, MA, USA), anti-aggrecan (ACAN) antibody (Abcam, ambr ge, 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 SμperMix 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 mm^3 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 (10^6/mL), and cultured in an incubator with 4% CO_2 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 tran ed with the plasmid containing miR-146 i tor, to inhibit the transcription of miR-146. cells in the miR-146 over-expression group w transfected with the plasmid con miR-14 mimics, to promote the transc iR-146 Meanwhile, the cells in the ive co l group were transfected with em lasmi ga- tive control. After cul ing cells in each group collect d detected.

Western Bl g

Collected c s were dded with lysis buffer, followed an ice bath 0 min and centrifugation 1400 g for 5 min. concentration of ext d prot was quantified using the bicinc ic ac BCA; Pierce, Waltham, MA, USA) met sequentl e proteins were denatured *via* hea After uration *via* gel electrophoresis, 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-N (1:1000), anti-ACAN (1:1000) and anti- II (1:1000) at 4°C overnight. On the ne ay, the membranes were incubated with the c ponding secondary antibodies (1:1000). hen, t m- branes were washed with Tris-B ered Salin Tween 20 (TBST). Finally, image as dev oped in chemiluminescen agent e dark fo 1 min, followed by anal sis gel scan ng imaging system.

Quantitativ erase Cha action (qPCR)

Total RNA was ext d from cells and reversel cribed into lementary deoxyrib nucleic acid (cDNA) ccording to the inst tions of the reverse transcription kit. A 20 µ action syst was set up. Specific reaction co ons were a llows: reaction at 51°C for 2 min, denat ion at 96°C for 10 min, denaturation for 10 s, annealing at 60°C for s, for a total of 40 cycles. With glyceraldehyde ate dehydrogenase (GAPDH) as an inrnal ference, 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.

ble 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 μL/well), followed by culturing in an incubator for 24 h. 3 replicates were set for each well. Briefly, 10 μL 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 Solu[tions] (SPSS) 20.0 software (IBM, Armonk, USA) was used for all statistical analysis. E[numeration] data were expressed a[s] n ± sta[ndard] dard deviation. *t*-test was us[ed] for c[omparison] of data in line with nor[mal] distrib[ution] 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 homo[geneity] ity of variance. Rank sum test was ado[pted] for ranked data, and the chi-square test [was] adopted for enumeration data. $p<0.05$ was [consi]dered statistically significant.

Res[ults]

Toluidine Blue S[taining]

As shown in F[igure] re 1, toluid[ine] blu[e] positive staining displ[ayed] rple-red co[lor] urple-red positive sta[ining] g co[lor] e seen in so[me] cells of the normal group and [negative] ive control group. No purpl[e] positive stainin[g] uld be found in the mi[R-146] o inhibitor group. M[eanw]hile, significant pu[rple]-red positive staining could be observed in th[e] iR-146 ove[r]pression group. According to sta[tistic]al analysi[s] esults (Figure 2), no significant [di]rence[s] found in purple-red positive expressi[on] en the negative control group and [nor]mal group ($p>0.05$). However, it was markedsed in the miR-146 inhibitor group when mpa[red] d with the normal group, showing a statistically significant difference ($p<0.05$). Furthermore, it was remarkably increased in the miR-146 over-expression group ($p<0.05$).

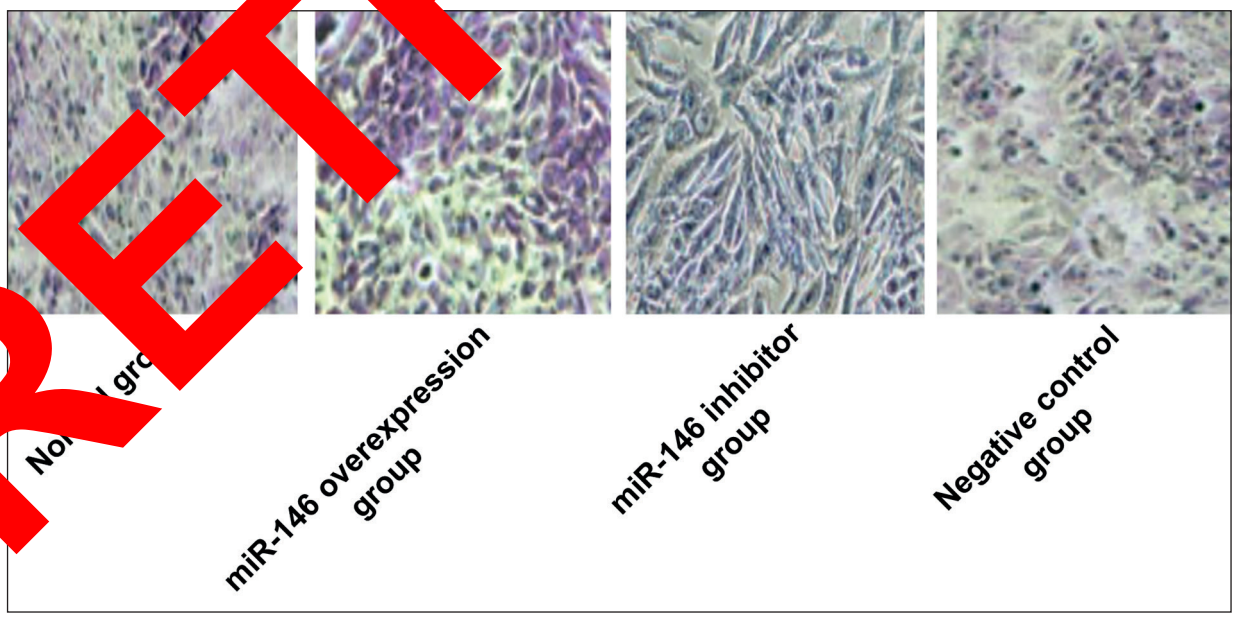

Figure 1. Toluidine blue staining (magnification × 40).

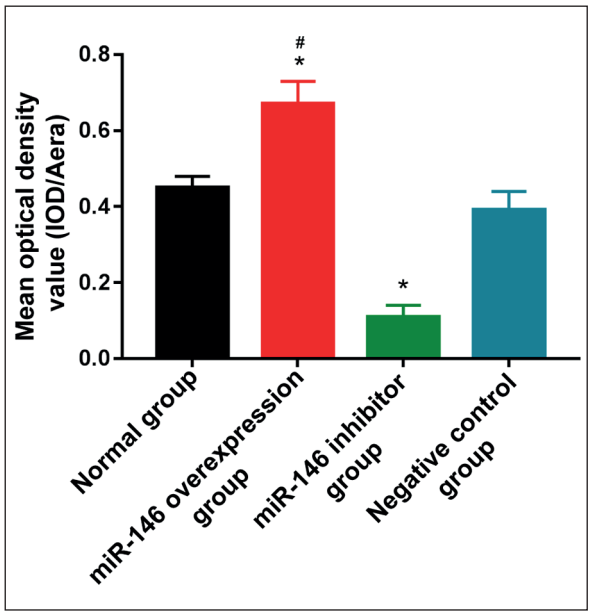

Figure 2. Mean optical density value of toluidine blue positive expression. Note: p*<0.05 *vs.* normal group, p#<0.05 *vs.* miR-146 inhibitor group.

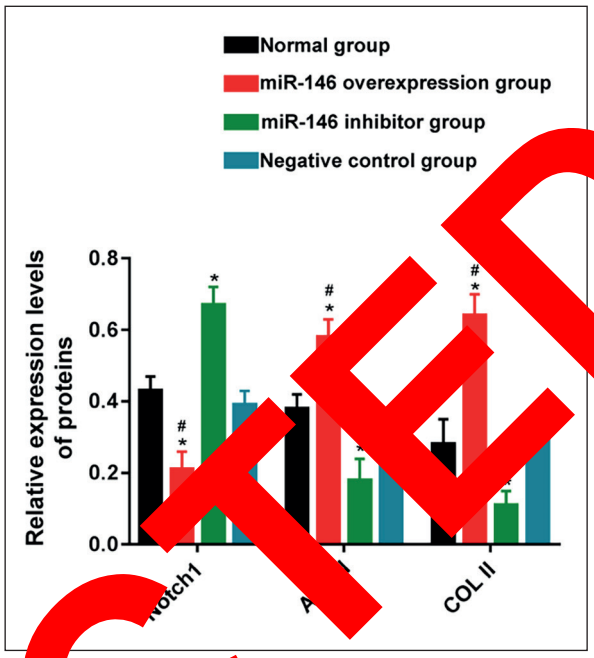

Figure 4. Relative expression levels of proteins in each group. Note: p*<0.05 *vs.* normal group, p#<0.05 *vs.* miR-146 inhibitor group.

Relative Expression Levels of Protein Detected Via Western Blotting

As shown in Figure 3 and 4, compared with the normal group, the miR-146 inhibitor group exhibited significantly increased 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 of Notch1, ACAN and COL II between the negative control group and normal group (p>0.05).

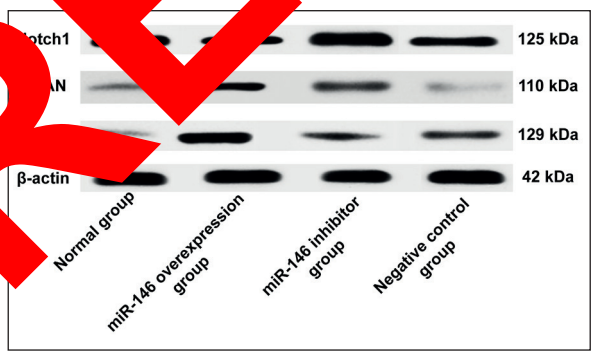

Figure 3. Protein expression detected via Western blotting.

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

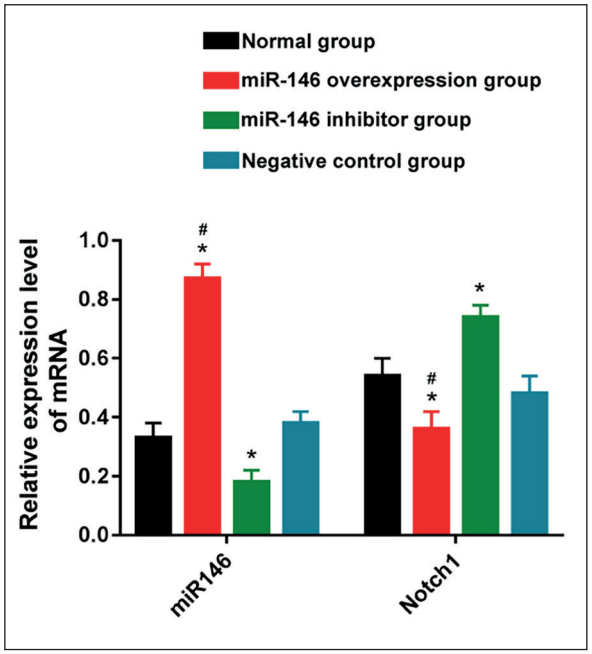

Figure 5. Relative expression levels of mRNAs in each group. Note: p*<0.05 *vs.* normal group, p#<0.05 *vs.* miR-146 inhibitor group.

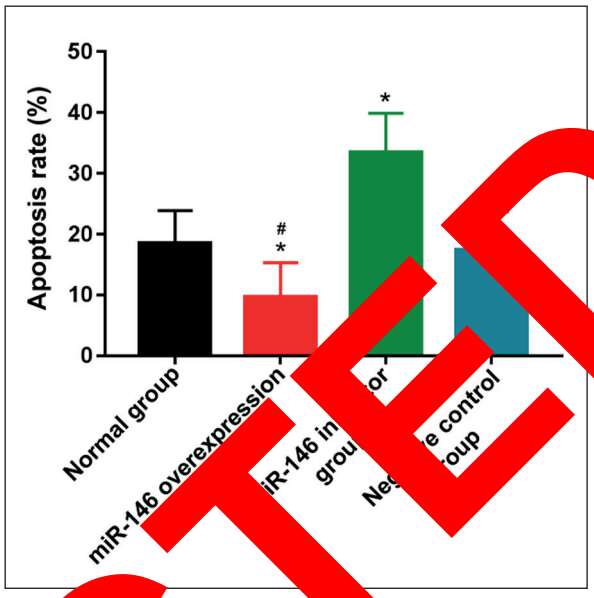

Fig. 6. Cell apoptosis rate in each group. Note: p*<0.05 *vs.* normal group, p#<0.05 *vs.* miR-146 inhibitor group.

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, cell proliferation rate was remarkably increased in the miR-146 inhibitor group, showing 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 diseases. Nucleus pulposus is a vital component of the intervertebral disc. Meanwhile, nucleus pulposus cells are considered to play an important role in 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

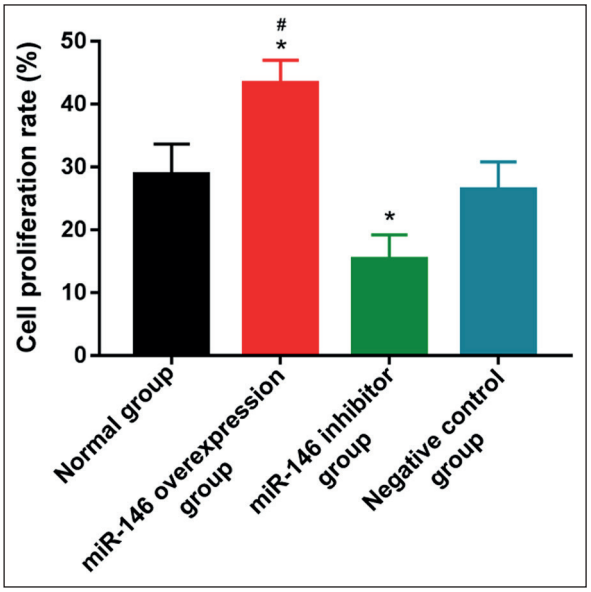

Figure 7. Cell proliferation rate in each group. Note: p*<0.05 *vs.* normal group, p#<0.05 *vs.* miR-146 inhibitor group.

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 prolifer of nucleus pulposus cells and accelerates . In addition, further reports have clarified hypoxia in the intervertebral disc can activ the Notch signaling pathway to ulate th expression of the Notch1 rece , t regulat ing the self-repair and prol ation o amaged nucleus pulposus cells. it is ient to maintain the norm nu ation of nucleus pu us cells to abnormal expression of N receptor i body, the apoptosis of pulposus cells d be increased. More ver, th airability of cells will decline[15] th ACAN a OL II are specific surfa ntigens and cha istic markers of nu pulpo cells. They are closely related t prol ation and repair of nucleus pulposus. Higher ression levels of ACAN nd C corre nds to a significantly larg- numbe us pulposus cells, as well as onger pr ration and repairability. MiR- are a kind of non-coding RNAs that can get genes. They may degrade target genes or inhibit target gene translation, eventually regulating the transcriptional process. wadays, miRNAs have become a research hotspot in the fields of cancer, cardiovascular diseases and bone diseases[16-18]. 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 incr the degree of IDD[19,20]. These findings i cate that miR-146 plays an important ro regulating the physiological functions ucleus pulposus cells. In this stud e co ed that the protein and mRNA e ssion lev f Notch1 were significantly i bited i the mi 146 over-expression gro lowe ey were markedly up-regulated i -146 in b- itor group, sugges that 46 pla an important role i egatively re tin otch1 receptor exp According ther investigations, luid lue purple-red positive expression, cell proli on rate and relative protei ssion levels ACAN and COL II in -146 over-expression roup were remarkab higher than those of the normal group and m 146 inhibit roup. However, the apoptosis of cells i iR-146 inhibitor group was sign tly lo than that of the normal group and mi ibitor group. Our findings indi- ed that over-expression of miR-146 promoted iferation 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

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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, Alipui 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. Co[r]ing notch signaling with thymocyte matura[t]. Immunity 1998; 9: 777-786.

7) Zhao Y, Liu X, Lu YX. MicroRNA-143 regula[tes] the proliferation and apoptosis[s] [o]al cance[r] cells by targeting HIF-1alpha[.] [R]ed Phar[m]acol Sci 2017; 21: 5580[-]6.

8) Lee Y, Ahn C, Han J, C[h]o, Kim [H], Provost P, Radmark [O], Kim [V]. RNase III Drosha [i]tates mi[R]NA processing. Nature 2003; 4[2]5-419.

9) Tang X, Jing [J], [W]dson WJ, Isaac[s] [C], Fitch RD, Brown CR, [J]ackso[n] [D], Setton LA, [C]hen J. Identifying molecular p[h]ype of nucleus pulposus [c]in human inte[r]ebral disc with aging a[n]egeneration. J Orth[o]es 2016; 34: 1316-[1]6.

10) [L]P, Ga[o] [Y], Wang H, Zhang C, Wang L, Xu Y, Song [S], Ou Y, [Z]ou Q. Dynamic compression e[f]on imma[t]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 re[s]ponse programs using synthetic notch [r]tors. Cell 2016; 167: 419-432.

12) Zhang J, Fukuhara S, Sako K, Takenouc[h] [M], Kitani H, Kume T, Koh GY, Mochizuki N. Angiop[o]1/Tie2 signal augments basal Notch [s]gnal c[o]lling vascular quiescence by indu[c] delta-like [l]-pression through AKT-me[d]d activation o[f] ta-catenin. J Biol Chem [2]011; 286: 8[0]-8066.

13) Rodrigues-Pinto R, Richa[r] SM[i]land JA. An understanding of in[v]ver[t] develop[m]t, maturation and [n]phenoty[p]rovides [i]s to direct cell-bas[e]ssue regene[r]n th[e]ies for disc degen[e] Eur Spine J [2]3: 1803-1814.

14) Risbud MV, Schipani [E], Shapiro IM. Hypoxic regulation of nucleus pulpos[u]ll survival: from niche t[o] Am J Pathol 20[1]76: 1577-1583.

15) [L]u Z, Li C, Meng X, Bai Y, Qi J, Wang J, Zhou Q, Zhang W, Zha[o] X. Hypoxia-inducible factor-1al[p]ha mediates [a]grecan and collagen Pi expres[s]n via NOT[C] signaling in nucleus pulposus [c]during i[n]vertebral disc degeneration. Bio[c]h[e]Res Commun 2017; 488: 554-561.

16) Cai B, Pan Z, Lu Y. The roles of microRNAs in heart [d]iseases: a novel important regulator. Curr Med [C]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.