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Original Article

Experimental study of miR-503 regulating the activity as well as the function of degenerated human nucleus pulposus cells of the intervertebral disc through inhibiting Wnt pathway

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Abstract

Objectives: To preliminarily explore miR-503 in human degenerative disc nucleus pulposus cell effects as well as mechanisms. **Methods**: We utilized bioinformatics analysis to determine the miRNA differential expression as well as key signal pathways existing in human nucleus pulposus cells of the degenerative intervertebral discs. Human degenerative disc nucleus pulposus cell model was cultured and established *in vitro*. miR-503 and TNIK-related genes are knocked down and overexpressed by lentiviral infection, then we added Wnt signaling pathway agonists; CCK-8, ELISA, RT-PCR, Western blot were used to detect proliferation, apoptosis, and activity of cells. **Results**: Bioinformatics results demonstrated that miR-503 was significantly down-regulated in human nucleus pulposus cells of the degenerated intervertebral discs. The targeted differentially expressed genes were mainly enriched in Wnt signaling pathway. However, after screening differential genes in the Wnt pathway, it was demonstrated that miR-503 mainly regulates TNIK to achieve Wnt pathway regulation. Cell experiments *in vitro* showed that cell activity and function were decreased while apoptosis was increased in the degenerative cell model. **Conclusions**: miR-503 can improve the function and activity of human nucleus pulposus cells of degenerated intervertebral disc by inhibiting Wnt expression. miR-503 mainly regulates the Wnt pathway through targeted binding with TNIK.

Keywords: Bioinformatics Analysis, Human Nucleus Pulposus Cells, Lumbar Disc Degeneration, miR-503, Wnt

Introduction

As a degenerative disease, the main pathological mechanism of intervertebral disc degeneration is the aging as well as degeneration of the human intervertebral disc, which leads to disorders such as intervertebral disc herniation, and low back pain in patients[1]. At present, the main concept of treatment for intervertebral disc degeneration (IDD) aimed at symptomatic relief. However, the treatment methods cannot provide an effective treatment for IDD at the mechanism level. Discovering ways to effectively prevent the progression of IDD is still a clinical challenge[2,3].

As basic research advances, an increasing number of studies show that the progression of IDD is closely related to the differential expression of miRNA[4]. As an endogenous non-coding RNA, miRNA mainly exerts its regulatory effect through targeted binding to the non-coding region of mRNA[5]. Previous studies have demonstrated that miRNA could target a variety of mRNAs, thereby exerting different regulatory effects on cell proliferation, apoptosis, aging, and autophagy. At the same time, miRNA can regulate the switching of signal pathways to achieve cell function regulation[6]. Therefore, our study hypothesis that miRNA may regulate the development of IDD by regulating the switch of the signal pathway in nucleus pulposus cells of intervertebral disc. In the early stage of this study, bioinformatics analysis demonstrated that there were

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8 differentially expressed miRNAs in degenerated human nucleus pulposus cells of the intervertebral disc. Five miRNAs were up-regulated, in the meantime, 3 were down-regulated. However, only miR-503 has been experimentally proved to be correlated with the degeneration of nucleus pulposus cells of human intervertebral disc[7]. Based on the above-mentioned preliminary research, we need to find out the miR-503 regulatory molecular mechanism in the cell. Through enrichment analysis of miR-503 targeted differentially expressed genes, it is found that differentially expressed genes are mainly enriched in Wnt signaling pathway.

As a signal pathway closely related to aging and apoptosis, Wnt signaling pathway also plays a decisive role in IDD[8,9]. Yun et al. demonstrated that the normal physiological function of the intervertebral disc was significantly improved through inhibiting Wnt signaling pathway[10]. Chen et al. demonstrated that cell proliferation was promoted by blocking the Wnt signaling pathway, resulting in regeneration of degenerated disc tissue[11]. The above research results show the importance of Wnt signaling pathway in IDD. However, there is no unformed conclusion on how alterations in the wnt signaling pathway are achieved in IDD. Zheng et al. used gene chip technology to detect the degenerated nucleus pulposus tissue, and the results demonstrated that in the degenerated nucleus pulposus tissue, the expression of miRNA-503 was significantly down-regulated, while the expression of apoptosis and proteins related to Wnt signaling pathway was significantly up-regulated[12]. This result is in line with the previous bioinformatics analysis studies. However, there is no research on whether miRNA-503 and Wnt signaling pathway share a mutual regulatory relationship. On this basis, this study aims to detect the expression of miR-503 in degenerated human nucleus pulposus cells as well as the regulation of Wnt pathway through cell experiments. Our study aims to provide a theoretical basis for clarifying specific molecular mechanisms of human lumbar intervertebral disc degeneration.

Materials and Methods

Bioinformatics analysis

By looking through the GEO database with the website link " https://www.ncbi.nlm.nih.gov/geo/ " with (("IDD") AND ("nucleus pulposus cell")) as the keyword, and utilize gene chip technology to acquire the miRNA expression data and mRNA expression data of normal nucleus pulposus cells of intervertebral disc and degenerated lumbar nucleus pulposus cells of intervertebral disc. We used P.val<0.01, |logFC|>1 to choose differentially expressed miRNAs as well as mRNAs found in Mirdb with website link " https://www.mirdb.org/cgi-bin/search.cgi " to obtain targeted mRNAs for differentially expressed miRNAs. We extracted the targeted differentially expressed mRNA of miR-503 for KEGG enrichment analysis, and draw a circle diagram in R.4.1.1 to clarify the signal pathway of targeted differentially expressed mRNA enrichment. We used Cytoscape 3.7.0 to establish the targeting relationship of miR-503-mRNA-pathway. KEGG enrichment pathway analysis was performed on the targeted mRNA of miR-503, and a chord diagram was drawn to show the significant difference in the enrichment pathway. We utilized TargetScan to predict the targeted mRNA of miR-503 and draw a Venn diagram together with KEGG enrichment analysis and MiRDB prediction results to specify how miR-503 regulates the Wnt signaling pathway.

Main materials and instruments

Low-sugar DMEM culture medium, fetal bovine serum (Hyclone, USA), PBS containing double antibodies, recombinant human interleukin-6 (Jiyuan Biotechnology Co., Ltd., Shanghai), type II collagenase (Sigma, USA), CCK-8 kit, ELISA apoptosis detection kit (Gibco, USA), RT-PCR kit (Dalian Bao Bioengineering Co., Ltd.), Trizol reagent (Gibco, USA), type II collagen antibody (Proteintech, China, 28459-1-AP), Wnt antibody (Abcam, USA, ab27794), GADPH antibody (Proteintech, China, 60004-1-Ig), goat anti-rabbit (Beijing Kangwei Century Biotech), reverse transcription kit (Takara, China), qPCR kit (Takara, China).

Acquisition and culture of human intervertebral discs nucleus pulposus cells

Human nucleus pulposus cells of the intervertebral disc were purchased from Procell, China. The obtained human nucleus pulposus cells of the intervertebral disc were cultured in DMEM medium. The medium contains 10% fetal bovine serum as well as 1% double-antibody. We chose an incubator with a temperature of 37°C and 5% CO2, and the medium is switched once a day. Cell growth density is observed under an inverted microscope. When the cells grow to 85% density, the cells are passaged.

The establishment of nucleus pulposus cell model of degenerative human intervertebral disc

Our study used human nucleus pulposus cells of the intervertebral disc for cell culture. After cell density grows to 80%~90%, liquid exchange treatments were performed. At the same time, TNF-α (20μg/L tumor necrosis factor-α) was added to the culture medium. After culturing at 37°C for 24 hours, the culture medium was discarded after changing the medium[13].

Establishment of knockdown/overexpression miR-503 cell line by lentivirus infection

Evaluation of lentivirus infection efficiency

We used human nucleus pulposus cells of degenerated intervertebral disc as the research object. When the cell growth density reaches 80%, the cells are trypsinized and resuspended. The obtained cells are resuspended at a density of 3x103/mL and then seeded in a 96-well plate. We placed them in a 37-degree constant temperature incubator for one day. After diluting 10uL lentivirus by 10, 100, and

1000 times respectively, we added 100uL diluted lentivirus-containing medium to each well and continue culturing them for 72 hours in a constant temperature incubator. We observed the infection efficiency of lentivirus under a fluorescence microscope.

Construction of miR-503 knockdown/overexpression cell line

We used human nucleus pulposus cells of degenerated intervertebral disc as the research object. When the cell growth density reached 80%, the cells were trypsinized and resuspended. The obtained cells were seeded in a 6-well plate. The plate was equipped with a density of 1x105/well. We placed them in an incubator at 37 degrees for one day to let them adhere to the wall. We diluted the purchased lentivirus with a DMEM solution containing 10% fetal bovine serum at a ratio of 1:10. After discarding the medium in the 6-well plate, we added 1 ml diluent containing lentivirus to each well. We then incubated them in an incubator at 37 degrees for one day, discarded the medium containing lentivirus, and added 2ml DMEM containing 10% fetal bovine serum Medium. We continued to cultivate them in a constant temperature incubator.

Double luciferase gene report experiment

We used Starbase 2.0 to predict the binding location of miR-503 and TNIK, and selected the appropriate mutation location. We constructed a dual fluorescein reporter gene of miR-503 containing the predicted location of TNIK. We used MIR-503-WT+ TNIK mimic, MIR-503-MUT+ inhibitor control, MIR-503-WT+ inhibitor control, MIR-503-WT+ mimic control, MIR-503-WT+ TNIK inhibitor, MIR-503-MUT+ mimic control, MIR-503-MUT+ TNIK mimic, and MIR-503-MUT+ TNIK inhibitor in order to transfect human nucleus pulposus cells of degenerated intervertebral disc. Luciferase activity was detected using the dual-luciferase reporter gene assay system right after 48 hours of transfection (Promega, USA).

CCK-8 experiment

The human degenerative nucleus pulposus cells of intervertebral disc were processed according to different grouping treatment conditions. They were inoculated into 96-well plates and then incubated in a constant-temperature incubator. We utilized CCK-8 technology in order to detect the proliferation of cells at 0, 24, and 72 hours after inoculation. We took out the 96-well plate from the constant temperature incubator, drew the medium from each well, added 100ul of CCK8 solution to each well, and placed them in the incubator for 2 hours. Finally, we measured the absorbance of each well at a wavelength of 450nm.

ELISA experiment

The changes in cellular function were detected by Bax ELISA kit, ELISA apoptosis kit and ELISA proliferation kit respectively. Bcl-2 ELISA kit was used to detect cell apoptosis. After the human nucleus pulposus cells of degenerated intervertebral disc were processed according to different grouping treatment conditions, the cells were first collected by centrifugation, and then 200ul cytolytic buffer was resuspended, and the cells were allowed to rest for 30 minutes. After centrifugation at 1000r/min for 10min, 20ul of supernatant was added to the ELISA plate. Then we added 80ul of immunoreaction reagent to each well plate and incubate for 2h at room temperature. We aspirated the supernatant and washed it three times. After adding 100ul buffer, we let it stand for 30min. The substrate buffer was used as the blank control group to measure the absorbance at a wavelength of 405nm.

Western blot

After treating the human nucleus pulposus cells of degenerated intervertebral disc according to different conditions, the cells were trypsinized, centrifuged, and collected 120 hours after the treatment. The cell content is obtained from the cell lysate, and the total protein in the cell is collected by centrifugation. Quantitative detection of protein concentration was carried out according to the BCA method. After the protein concentration was quantified, SDS-PAGE electrophoresis was performed for each tissue/cell. After the electrophoresis was over, the protein that separated by electrophoresis was transferred to PVDF membrane using a transfer device. After the transfer, the PVDF membrane was taken out and sealed with 5% skim milk for 1 hour, later rinsed with PBS. Then we added the primary antibody col-2 (1:500) and Wnt (1:500) overnight at 4°C. After that, we added a secondary antibody (goat anti-rabbit antibody 1:1000) and incubated it for 1h. Finally, a chemiluminescence imaging system was used for PVDF film exposure detection.

RT-PCR

After treating the human nucleus pulposus cells of degenerated intervertebral disc according to different grouping treatment conditions, the cells were trypsinized, centrifuged, and collected 120 hours after the treatment. The trizol method was adopted by our experiment to extract total cell RNA. We took 2uL of the dissolved liquid in a microspectrophotometer to determine the concentration and purity of RNA in the solution. The Takara reverse transcription kit was used to establish a reverse transcription reaction system to reverse RNA into cDNA. A cDNA reaction system was established using a qPCR kit, with a reaction at 95°C for 10 minutes, 95°C for 30 seconds, and 60°C for 30 seconds; 40 cycles. Dissolution curve analysis was performed, and the final data was adopted in order to statistically analyze Wnt and miR-503 expression levels in each sample with $2^{-\Delta\Delta CT}$.

Statistical Analysis

All analyses were performed using the statistical package SPSS 23.0. The data obtained were expressed in the form of mean ± standard deviation. The comparison between multiple

Table 1. Human nucleus pulposus cells miR-503 of degenerated intervertebral disc and targeted differentially expressed mRNA.

miRNA	logFC	P.Value	Genes
hsa-miR-503	-2.254	0.00362	TUSC1, MPZL1, PRKCI, TNIK, UBE3A, DAAM1, AXIN2, CCDC71L, RABL2A, EXPH5, RC3H1, SMTNL2, LPAR1, NR3C1, P LXNC1, I FITM1O, LPP, HNRNPU, PSMG2, ATF3, CBX5, SAMD5, GNB4, STAG1, NAGS, JAZF1, CHML, ZNF747

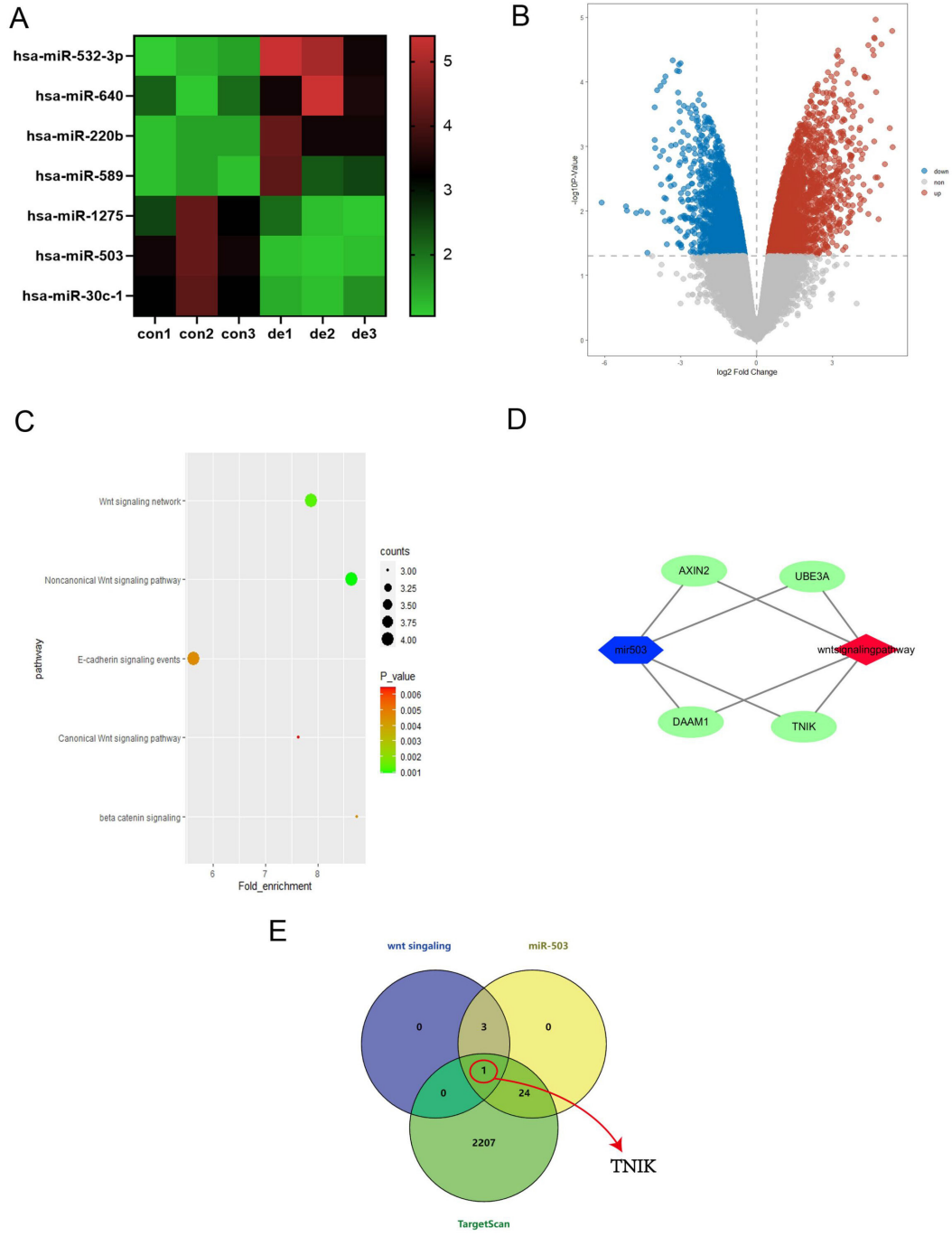

Figure 1. Bioinformatics analysis of degenerated human lumbar nucleus pulposus cells of intervertebral discs compared with normal nucleus pulposus cells. A: Heat map miRNA; B: Volcano map displays differentially expressed mRNA; C:Bubble map displays KEGG enrichment signal pathway analysis results; D:miR-503-gene-pathway network map construction; E: Venn map clarifies the common gene (TNIK) between TargetScan and Wnt signaling pathway and miR-503 target differential genes.

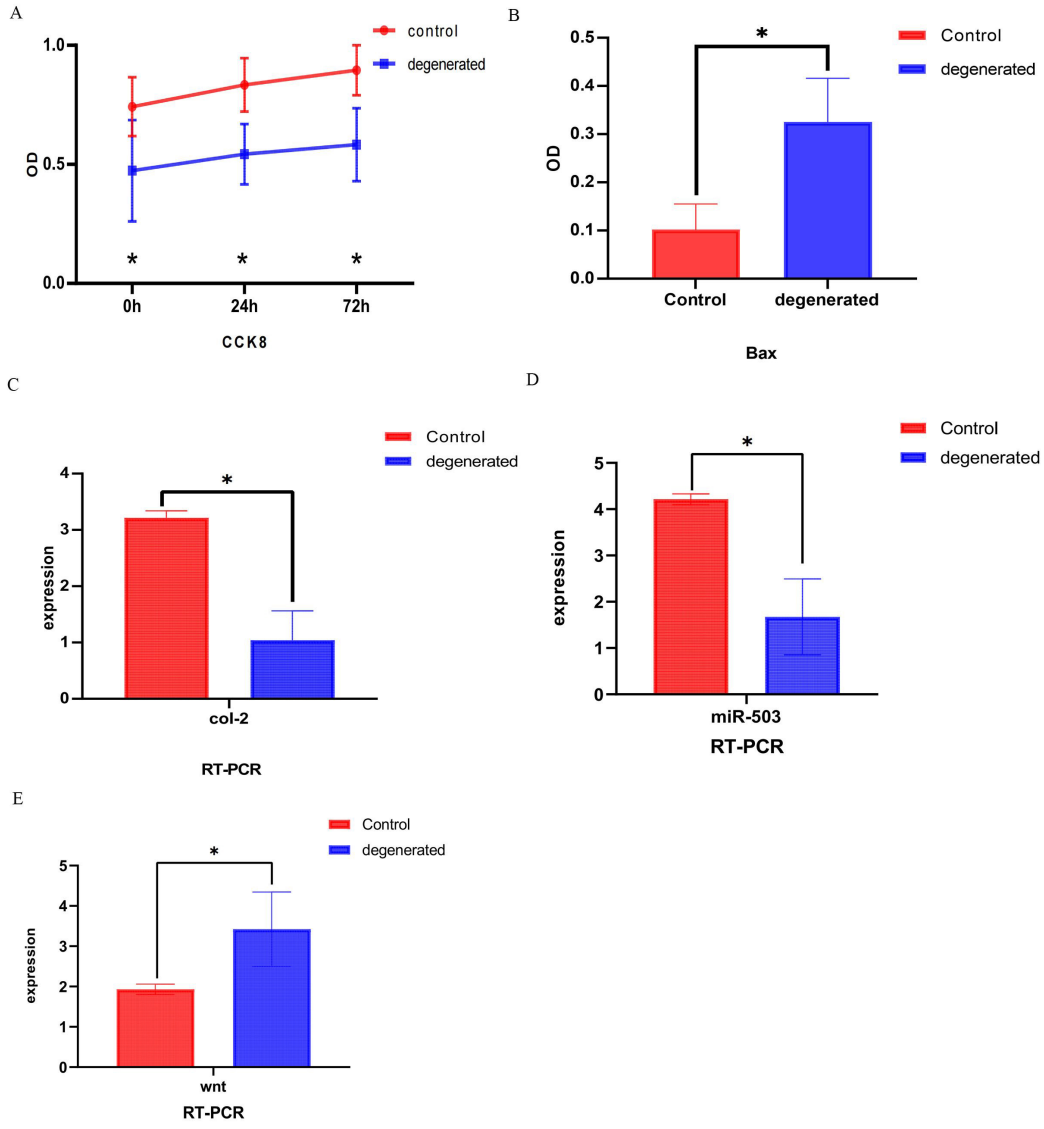

Figure 2. Changes in the activity and function human degenerated intervertebral discs nucleus pulposus cells. A:CCK8 detects the changes of cell viability; B: ELISA detects the expression of apoptosis-related protein Bax; C, D, E: RT-PCR detects the expression changes of col-2, wnt, miR-503 in the cells.*P<0.05.

groups was carried out by analysis of variance. Analysis of variance was statistically significant by the independent sample T-test between the two groups. Statistical significance was set at p<0.05.

Results

Bioinformatics analysis results

Through keyword search, the data sets GSE67567 and GSE147383 were finally obtained as the research objects. GSE67567 (Exiqon miRCURY LNA microRNA Array) was the differential expression of miRNA between degenerative nucleus pulposus cells and normal nucleus pulposus cells detected using gene chip technology. Affymetrix Human Genome U133 Plus 2.0 Array (GSE147383) was mRNA differential expression between degenerative nucleus pulposus cells as well as normal nucleus pulposus cells detected using gene chip technology. We set the differentially expressed mRNA and miRNA screening level to P.val<0.01, |logFC|>1 and finally obtained 7 differentially expressed miRNAs, of which 4 were up-regulated and 3 were down-regulated. The details of the differentially expressed miRNA are demonstrated in Figure 1A. The volcano map is demonstrated in Figure 1B. The results of the miR-503 targeted differentially expressed mRNA data are demonstrated in Table 1. The KEGG of the targeted

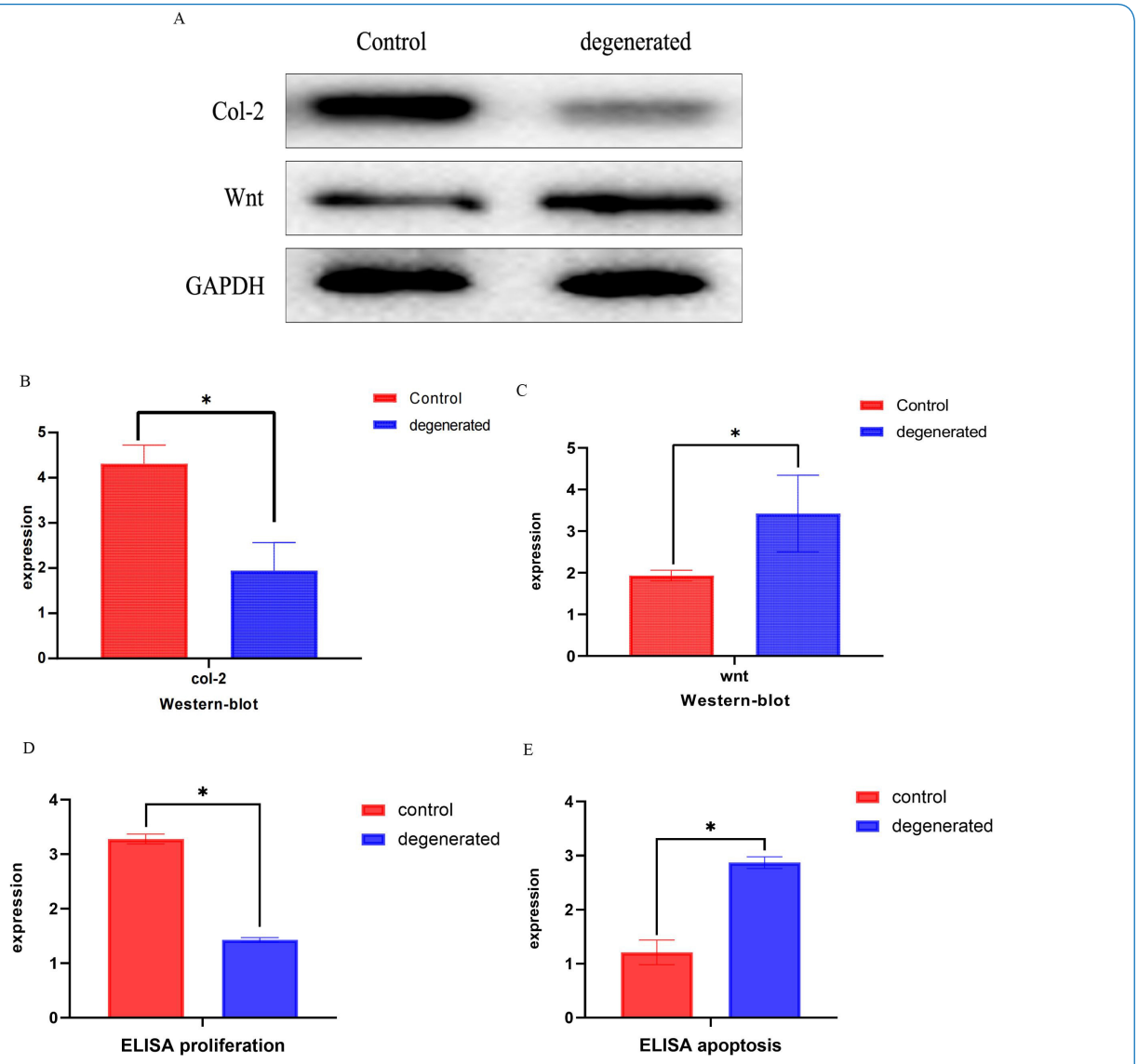

Figure 3. Changes in the activity and function human degenerated intervertebral discs nucleus pulposus cells. A: Western blot technology detects type II collagen expression as well as Wnt expression in the two groups of cells. B, C: The expression of col-2 and wnt in cells was analyzed by western blot; D, E: The proliferation ability and apoptosis of human degenerated intervertebral discs nucleus pulposus cells were detected by ELISA. * P<0.05.

differential genes of miR-503 is demonstrated in Figure 1A. Enrichment analysis, KEGG enrichment analysis results demonstrated that differential genes are mainly enriched in the Wnt signaling pathway (Figure 1C). According to the differential genes in the Wnt signaling pathway, the miR503-gene-Wnt network diagram can be drawn to show that the differential genes enriched in the Wnt signaling pathway are TNIK, UBE3AD, DAAM1, AXIN2 (Figure 1D). By drawing a Venn diagram, the results show that the TargetScan, Wnt signaling pathway, and miR-503 targeting differential genes are intersected to obtain TNIK as the target mRNA of miR-503 in the process of lumbar disc degeneration (Figure 1E).

Decreased activity as well as the function of human degenerated intervertebral discs nucleus pulposus cells

Compared with normal human nucleus pulposus cells of the intervertebral disc, CCK8 results demonstrated that the proliferation ability of human nucleus pulposus cells of degenerated intervertebral disc was inhibited (P<0.05, Figure 2A), and ELISA results demonstrated that the apoptosis-related protein Bcl-2 of human nucleus pulposus cells of degenerated intervertebral disc was increased (P<0.05, Figure 2B). RT-PCR demonstrated that Col-2 expression in nucleus pulposus cells of human degenerated intervertebral disc was significantly reduced, (P<0.05, Figure 2C). Wnt

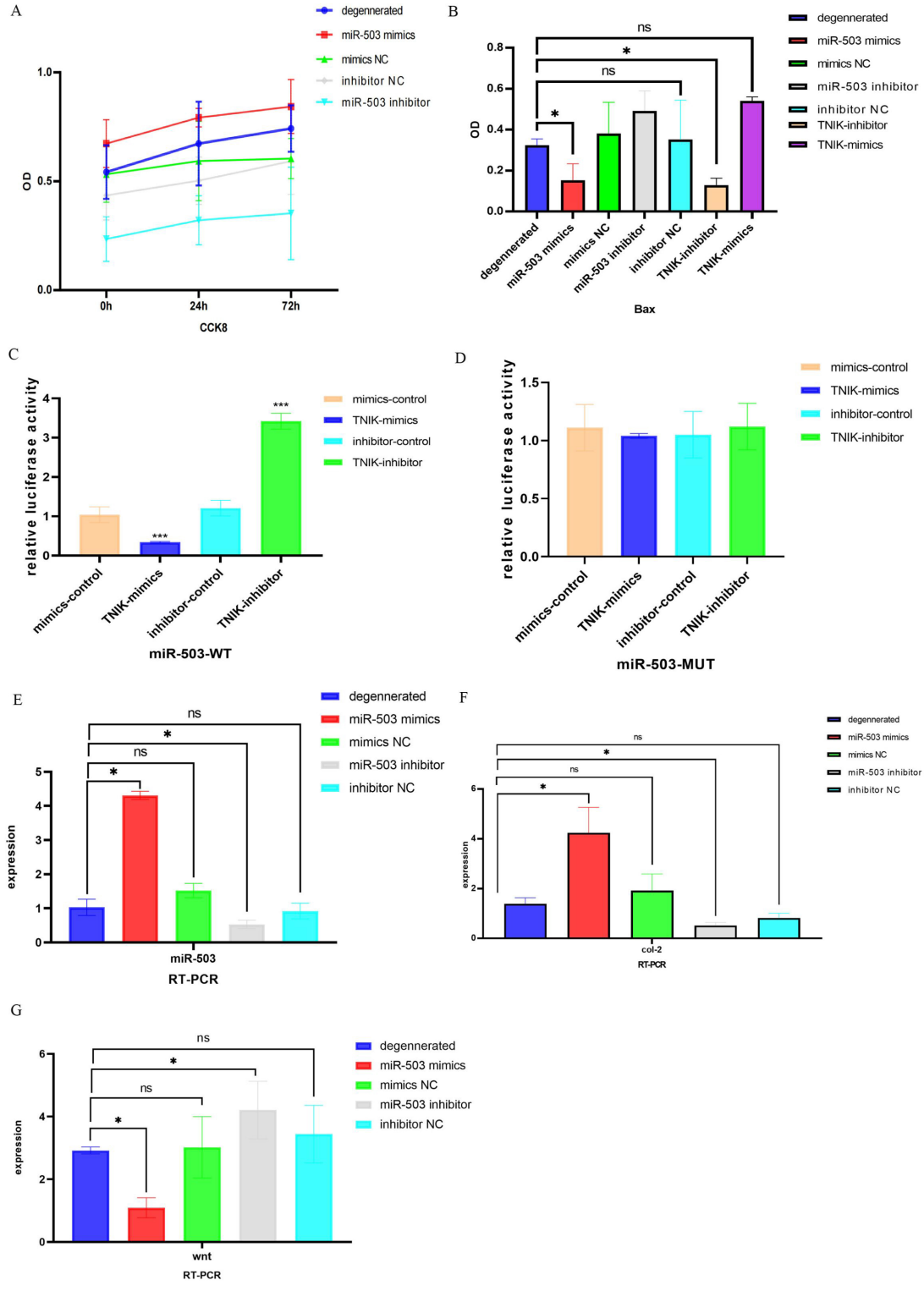

Figure 4. The effect of miR-503/TNIK on the function as well as activity of human nucleus pulposus cells of degenerated intervertebral discs. A: CCK8 detects cell activity; B: The expression of Bax was detected by ELISA; C, D: Dual luciferase gene reporter was used to detecte the targeted binding of miR-503 to TNIK; E, F, G: RT-PCR was used to detect the expression of miR-503, col-2 and wnt in cells. *P<0.05.

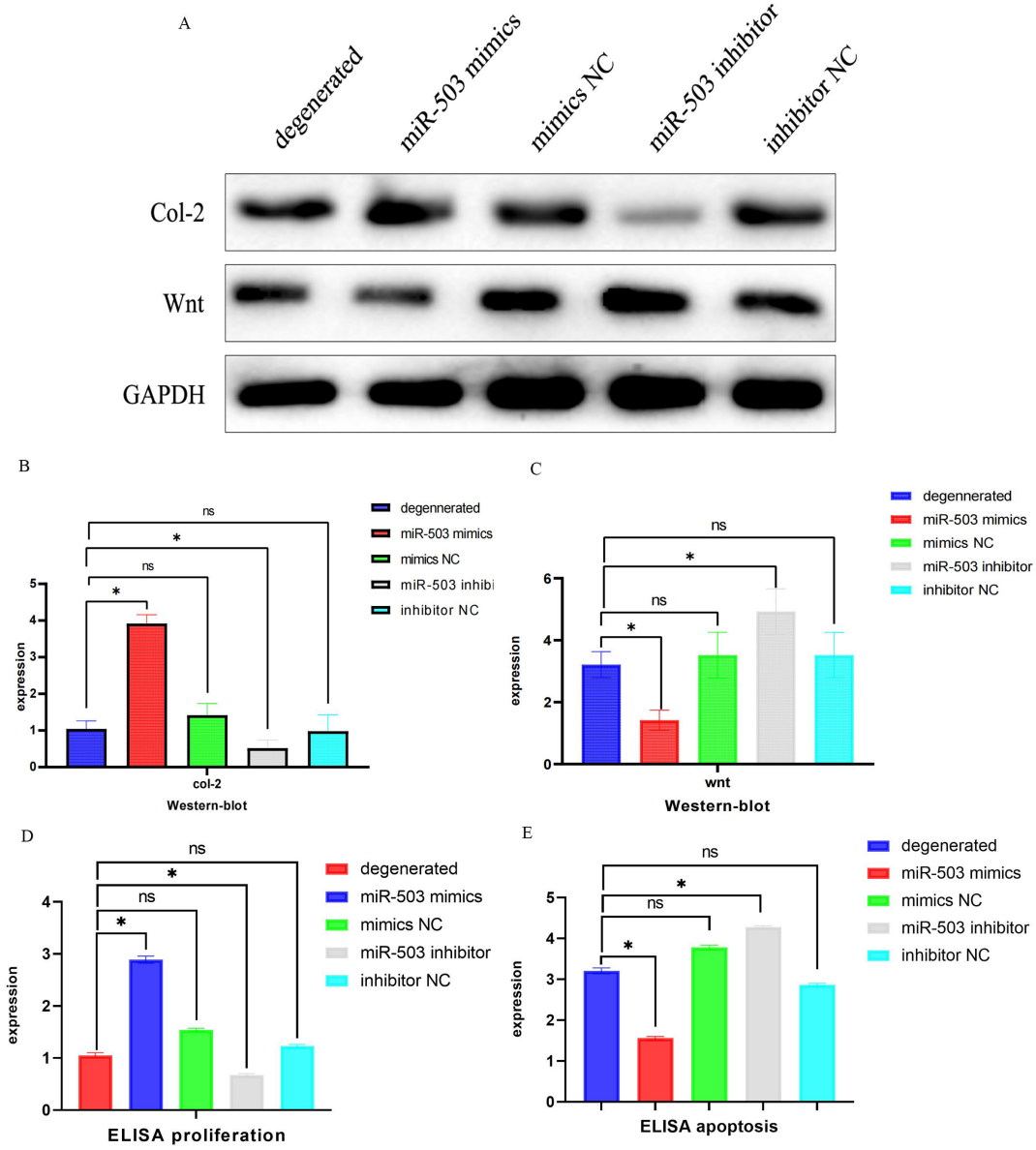

Figure 5. Effects of miR-503 on the function of human degenerative disc nucleus pulposus cells. A: Western blot detects the expression of col-2 and wnt in the cells of each group. B, C: Western blot results show the expression of col-2 and wnt in each group; D. ELISA detects the proliferation of each group; E. ELISA detects the apoptosis of each group. * P<0.05.

expression was `significantly increased (P<0.05, Figure 2D). miR-503 expression was detected by RT-PCR technology and it was found that miR-503 expression in the nucleus pulposus cells of human degenerated intervertebral disc was vastly reduced (P<0.05, Figure 2E). Western-blot showed that Col-2 expression in nucleus pulposus cells of human degenerated intervertebral disc was significantly reduced, and Wnt expression was increased (P<0.05, Figure 3A, B, C). Apoptosis experiments showed that human nucleus pulposus cells of the intervertebral disc were significantly increased compared with normal cells (P<0.05, Fig 3D), and cell proliferation experiments showed that human nucleus pulposus cells of the intervertebral disc were significantly decreased compared with normal cells (P<0.05, Figure 3E)

In human nucleus pulposus cells of degenerated intervertebral disc, miR-503 can target bind to TNIK and regulate Wnt pathway and cell function and activity

The CCK8 results demonstrated that in the degenerated human nucleus pulposus cells of the intervertebral disc, cell activity was significantly inhibited after inhibiting miR-503 or overexpressed TNIK, when miR-503 was overexpressed

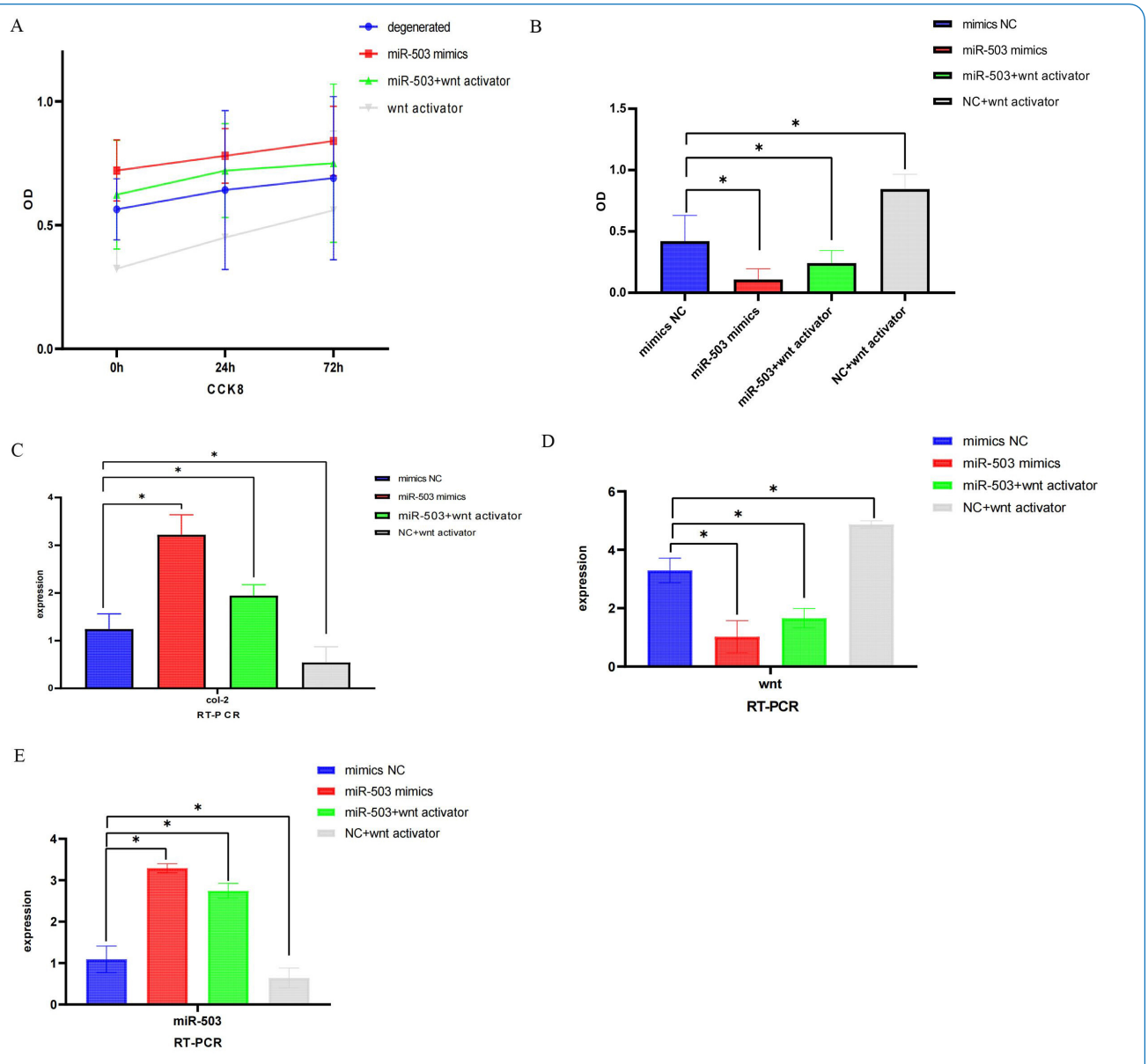

Figure 6. miR-503 regulates the function and activity of human nucleus pulposus cells of degenerated intervertebral disc through Wnt. A: CCK8 was used to detect cell activity; B: ELISA was used to detect apoptosis-related protein Bax expression level; C, D, E: RT-PCR was used to detecte the expression of Col-2, Wnt and miR-503 in each group of cells.* P<0.05.

or TNIK was inhibited, CCK8 demonstrated a significant increase in cell activity (P<0.05, Figure 4A); ELISA showed that the expression of Bax was significantly increased in cells by inhibiting miR-503 or overexpressing TNIK (P<0.05). After overexpressing miR-503 or inhibiting TNIK, the expression of Bax in cells was significantly inhibited (P<0.05, Figure 4B); Double luciferase gene report experiment showed that TNIK and miR-503 have targeted binding sites (Figure 4C, D). RP-PCR showed that the expression of miR-503 and col-2 in cells was significantly inhibited when miR-503 or overexpressed TNIK was inhibited (P<0.05), and the expression of wnt was significantly increased (P<0.05); After overexpressing miR-503 or inhibiting TNIK, the expressions of miR-503 and col-2 in cells were significantly increased (P<0.05), and the expression of Wnt was significantly inhibited (P<0.05, Figure 4E, F, G). Western blot showed that the expression of col-2 and Wnt was significantly inhibited after inhibiting miR-503 or overexpressing TNIK in cells (P<0.05). The expression of col-2 and Wnt was significantly increased after overexpressing miR-503 or inhibiting TNIK. (P<0.05, Figure 5A,B,C). The results of ELISA to detect cell proliferation and apoptosis showed that the cell proliferation ability was

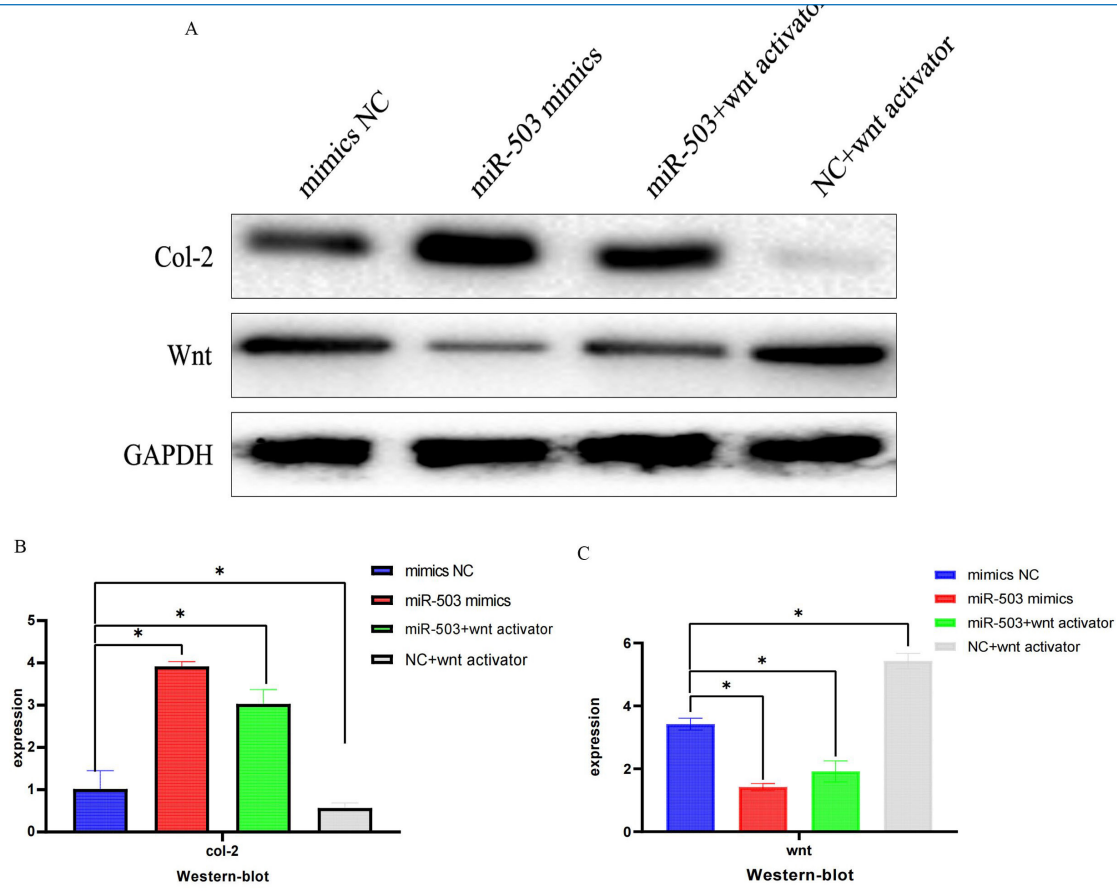

Figure 7. Western blot technology verifies that miR-503 regulates the function of human nucleus pulposus cells of degenerated intervertebral disc through Wnt. Western blot was used to detect the expression of col-2 and wnt in cells of each group. B, C. Western blot results showed the expression of col-2 and wnt after overexpression of miR-503, activation of Wnt, and overexpression of miR-503 combined with activation of wnt in degenerated human intervertebral disc nucleus pulposus cells. * P<0.05.

significantly decreased after the intracellular inhibition of miR-503 or the overexpression of TNIK, and the apoptosis was significantly increased (P<0.05). After overexpressing miR-503 or inhibiting TNIK, the proliferation ability was significantly increased, and the apoptosis was significantly decreased (P<0.05, Figure 5D,E). The results of the above studies showed that when miR-503 was inhibited or TNIK was overexpressed, the expressions of col-2 and Wnt in cells were decreased; the cell proliferation ability was decreased, and the apoptosis was increased.

In human nucleus pulposus cells of degenerated intervertebral disc, miR-503 regulates cell function and activity by regulating the Wnt pathway

miR-503 was overexpressed in degenerated human intervertebral disc nucleus pulposus cells, respectively, and the Wnt signaling pathway was activated to detect changes in cell function and activity. The results of CCK-8 showed that based on the overexpression of miR-503, the cell viability was significantly inhibited at 0, 24, and 72 hours after the addition of the Wnt activator (P<0.05, Figure 6A). ELISA was used to detect the expression of apoptosis-related protein Bax, which showed the apoptosis of Wnt activator was increased based on overexpression of miR-503,(P<0.05, Figure 6B); RT-PCR was used to detect the expressions of Col-2 and Wnt in each group of cells. The results showed that based on the overexpression of miR-503, the expression of Wnt increased (P<0.05, Figure 6C) and the expression of col-2 decreased (P<0.05, Figure 6D) after adding the Wnt activator. The expression of miR-503 had no significant change compared with the miR-503 overexpression group (P>0.05, Figure 6E). Western blot was used to detect the expression of Col-2 and Wnt in each group of cells. The results showed that based on overexpression of miR-503, the expression of Wnt increased (P<0.05) and the expression of col-2 expression decreased (P<0.05, Figure 7A,B,C) after adding the Wnt activator.

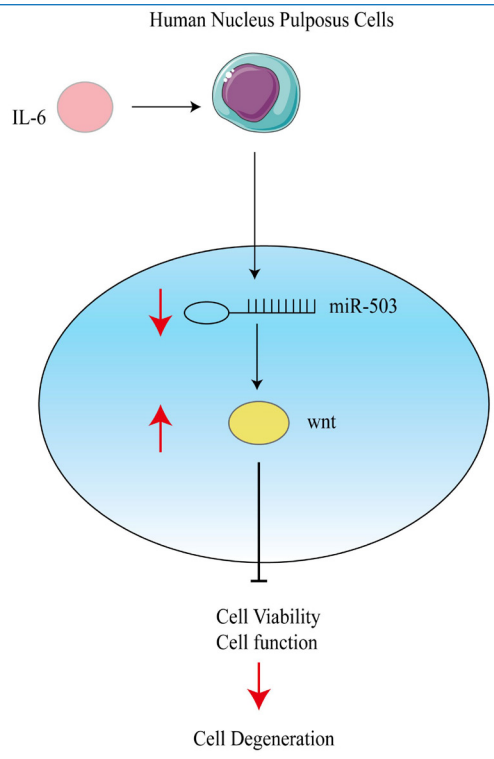

Figure 8. Research hypothesis. miR-503 regulates the activity and function of degenerated human lumbar nucleus pulposus cells of intervertebral disc through targeted binding with TNIK to inhibit the Wnt signaling pathway.

Discussion

In this study, the function and activity of the cells were tested by adding miR-503 mimics, miR-503 inhibitors, and Wnt signaling pathway activators into human degenerated intervertebral discs nucleus pulposus cells. The results show that in degenerated intervertebral disc nucleus pulposus cells, miR-503 regulates the function as well as activity of nucleus pulposus cells of intervertebral disc by inhibiting the Wnt signaling pathway. Increasing the expression of miR-503 can significantly inhibit Wnt signaling pathway and improve the activity as well as the function of human nucleus pulposus cells of degenerated intervertebral disc (Figure 8).

Previous studies have proved that the senescence of the human intervertebral disc nucleus pulposus cells is inseparable from IDD[14,15]. As one of the most important parts of the spine, the intervertebral disc has several main functions. The first intervertebral disc can effectively transmit and absorb the external force of the spine in the axial direction, thereby alleviating the stimulation to the spinal cord; the second intervertebral disc can be effective in maintaining the flexibility of the spine. The main parts of the intervertebral disc are the nucleus pulposus cells as well as the surrounding extracellular matrix. Driven by peripheral growth factors, the nucleus pulposus cells and the surrounding ECM can maintain a dynamic balance in normal intervertebral disc tissue. However, with the senescence of intervertebral disc nucleus pulposus cells, the secretory function declines. It is also accompanied by the degradation of ECM, which ultimately leads to IDD development. Previous studies have demonstrated that in intervertebral disc nucleus pulposus cells of IDD patients, apoptosis is significantly increased[16,17]. The cell proliferation is significantly correlated with intervertebral disc degeneration negatively[18,19]. It was found that the proliferation ability of degenerated human nucleus pulposus cells of the intervertebral disc treated with TNF-α was significantly worse than that of normal human nucleus pulposus cells of the intervertebral disc in this study with $P<0.05$. Previous studies also indicated that during the progression of IDD, the expression of extracellular matrix (ECM) is also significantly reduced, and the degradation rate of ECM is also significantly increased. In the early stage of this study, the western blot was performed on the cells after establishing the degenerated human disc nucleus pulposus cell model. It was found that the expression of type 2 collagen in the degenerated nucleus pulposus cells of human intervertebral disc was also significantly reduced with $P<0.05$.

In order to clarify the specific molecular mechanism that causes the changes in the function and activity of human degenerated intervertebral disc nucleus pulposus cells. In this study, bioinformatics analysis found that there are 5 up-regulated differentially expressed miRNAs and 3 down-regulated differentially expressed miRNAs in human nucleus pulposus cells of degenerated intervertebral disc. microRNA is a type of small RNA that exists in cells and is about[20-24] nucleotides in length. It is currently believed that microRNA is a very important post-transcriptional gene regulatory factor in organisms. Because it is a short RNA chain composed of nucleotides, it can exist in animal cells, plant cells, and viruses. In the human body, its content only accounts for 1-3% of all RNA, but miRNA regulates the expression of about 20% of protein-coding genes in the body[20]. As an endogenous non-coding RNA, miRNA's main regulatory mechanism for the expression of coding RNA is to organize the translation of mRNA by binding to the target gene's mRNA, thus further regulating the protein expression of the corresponding gene[21,22]. At present, various studies have demonstrated that miRNA has an important regulatory role in senescence, apoptosis, as well as functional degeneration of human intervertebral disc nucleus pulposus cells[23-25]. On this basis, this study conducted a literature search on the above 8 miRNAs and found that only miR-503 was experimentally verified to be closely related to the degeneration of human nucleus pulposus cells of the intervertebral disc[26,27]. As a result, our study aimed to further explain how miR-503 causes degeneration of human intervertebral disc nucleus pulposus cells. This study also aimed to knockdown/overexpress human miR-503 through lentivirus infection technology. The results demonstrated that after knocking down miR-503, CCK-8 technology demonstrated that the

cell proliferation ability was further inhibited, while western blot and PCR technology also demonstrated type 2 collagen expression was significantly reduced, and the expression of proteins related to the Wnt signaling pathway was significantly increased (P<0.05). After overexpression of miR-503, CCK-8 technology demonstrated that the proliferation ability of cells was significantly improved, and western blot and PCR technology demonstrated that the expression of type 2 collagen was significantly improved. The above experimental results show that the function and activity of human nucleus pulposus cells of degenerated intervertebral disc are significantly improved after miR-503 overexpression. How does the expression of miR-503 regulate cell function and activity in the human degenerated intervertebral disc nucleus pulposus cells? This question has not yet been fully elucidated. miRNA can affect the functional activity of cells in a variety of ways, such as miRNA targeted binding to mRNA, targeted binding to lncRNA, forming a ceRNA network, or regulating signal pathway switches[23]. And signal pathway switches have a direct impact on the function of cells. On this basis, this study conducted KEGG enrichment analysis on the differentially expressed mRNA targeted by miR-503. The results show that the differentially expressed targeted mRNA is mainly enriched in Wnt signaling pathway.

Wnt signaling pathway plays an important role in the regulation of cell differentiation as well as proliferation[28,29]. Wnt pathway, as a classic pathway that has been verified in cells, is mainly composed of Wnt protein, b-catenin, and glycogen synthase kinase. Each component is mainly located in the cytoplasm. When Wnt pathway is activated, the concentration and content of b-catenin in the cell increase sharply. At the same time, a large amount of b-catenin enters the cytoplasm through the nuclear membrane, which further affects the transcription and expression of cell genes, thereby affecting cell proliferation, aging, etc. In the progress of IDD, Chen showed that after blocking Wnt signaling pathway in the degenerative disc nucleus pulposus cells. And proliferation capability of the disc nucleus pulposus cells has also been significantly improved, and the cell function has also been restored[30]. At the same time, Hua discovered that proliferation-related gene expression was noticeably increased after inhibiting Wnt signaling pathway in normal nucleus pulposus cells of intervertebral disc[31]. In our study, the establishment of degenerated human nucleus pulposus cells of the intervertebral disc found that Wnt signaling pathway was significantly up-regulated (P<0.05). After overexpression of miR-503 in human nucleus pulposus cells of degenerated intervertebral disc, Wnt signaling pathway-related protein expression level was significantly inhibited with P<0.05. Research results demonstrated above also prove that miR-503 plays a vital role in the development of degeneration of the intervertebral disc nucleus pulposus. There is a certain correlation between miR-503 and Wnt signaling pathway. In order to clarify the interaction between miR-503 and the Wnt signaling pathway, in our study, a Wnt agonist was added to the miR-503 overexpression group. The results demonstrated that cell activity was significantly inhibited after the addition of Wnt agonist, and miR- The expression of 503 was also significantly reduced (P<0.05).

As miRNA, miR-503 mainly regulates protein expression and signal switching through targeted binding to mRNA. In the early stage of our study, bioinformatics analysis was used to preliminarily clarify that miR-503 may be targeted to bind to TNIK to regulate the switch of the Wnt pathway. As a member of the GCK IV family, TNIK is widely expressed in the body. Studies have demonstrated that TNIK is expressed in the heart, brain, nerves, and other tissues and organs. The main function of TNIK is closely linked to the cytoskeleton as well as development of cells and tissues. Studies have demonstrated that TNIK can regulate cell proliferation, apoptosis, and functional changes by participating in the formation of the Wnt transcription complex and the expression of the Wnt signaling pathway. On this basis, studies have demonstrated that TNIK can regulate the progression of various diseases such as schizophrenia, small bowel adenocarcinoma, and coagulopathy by regulating the Wnt signaling pathway. In our study, the dual-luciferase gene report experiment verified that miR-503 can target TNIK in degenerative lumbar nucleus pulposus cells of intervertebral disc.

Based on the above results, this study shows that in human nucleus pulposus cells of degenerated intervertebral disc, miR-503 can regulate the expression of Wnt-related pathway signaling proteins, thereby affecting the regulation of Wnt signaling pathways on cell function, and ultimately causing IDD. However, miR-503 mainly achieves the regulation of the Wnt signaling pathway through targeted binding with TNIK. Therefore, miR-503 is expected to become an effective target for the clinical treatment of IDD. Nonetheless, there are still some shortcomings in this study. This study is only limited to cell experiments. By constructing a model of nucleus pulposus cells of degenerative intervertebral discs, the effect of miR-503 in the degenerative disc cells is preliminarily proved, it bears uncertainty compared to specific pathological changes of clinical patients. Therefore, our study aims to verify the specific mechanism of miR-503 in the occurrence and development of IDD in animal experiments and human experiments, so as to conduct a more in-depth discussion on the pathogenesis of IDD.

Authors' contributions

Conception and design: Xiaoming Shi, Shaohua Tian; Administrative support: Shaohua Tian, Yinan Tian; Provision of study materials or patients: Xiaoming Shi, Shaohua Tian; Collection and assembly of data: Xiaoming Shi, Shaohua Tian, Yinan Tian; Data analysis and interpretation: Xiaoming Shi, Shaohua Tian; Manuscript writing: All authors; Final approval of manuscript: All authors.

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