

# MiR-25 Protects Against Apoptosis of Nucleus Pulposus Cells by Targeting SUMO2 in Intervertebral Disc Degeneration

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## Research article

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# Abstract

**Background** MiR-25 was reported to be down-regulated in patients with intervertebral disc degeneration (IDD). However, the potential role of miR-25 in IDD remained unclear. Therefore, the present study aimed to investigate the effects of miR-25 on human intervertebral disc nucleus pulposus cells (NPCs).

**Methods** We evaluated the expression of miR-25 and small ubiquitin-related modifier 2 (SUMO2) in human nucleus pulposus (NP) tissues by real-time PCR and western blotting. Then, the target relationship between miR-25 and SUMO2 was validated by luciferase reporter assay and biotin-coupled miRNA pulldown assay. The potential roles of miR-25 in NPC proliferation and apoptosis were confirmed using CCK-8 assay, EdU incorporation assay, and flow cytometry.

**Results** MiR-25 was lowly expressed in the patients with IDD. In addition, miR-25 facilitated the growth of NPCs by increasing cell proliferation and inhibiting apoptosis. Furthermore, we elucidated that SUMO2 was a target gene of miR-25, and was regulated by miR-25 through p53 signaling pathway. Restore of SUMO2 expression abrogated the effects of miR-25 on NPCs.

**Conclusion** MiR-25 promoted the proliferation, inhibited the apoptosis of NPCs, and suppressed the development of IDD via SUMO2-mediated p53 signaling axis.

## Highlights

1. MiR-25 is down-regulated in the patients with IDD.
2. MiR-25 promotes NPC proliferation and inhibits apoptosis.
3. SUMO2 is regulated by miR-25 through p53 signaling pathway.
4. MiR-25 increases NPC growth via targeting SUMO2 through p53 signaling pathway.

## Background

Intervertebral disc degeneration (IDD) is the leading cause of vertebral disc herniation, spondylolisthesis, spinal canal stenosis and other spinal degenerative diseases (1). Because of its high morbidity and disability rate, IDD has created a heavy burden to the society and the family (2). Although many patients with IDD have a response to the main treatment measures including nucleus pulposus allograft, spinal canal decompression and spinal fusion, poor long-term efficacy represents the major cause of treatment failure (3).

To the best of our knowledge, the apoptosis of NPCs, microfracture caused by excessive pressure, extracellular matrix degradation, and the abnormal expression of inflammatory factors can broke the dynamic balance of the anabolism and catabolism of the intervertebral disc matrix, leading to the occurrence and development of IDD (4, 5). However, the cellular and molecular mechanisms underlying IDD remain unclear. Therefore, more experiments are needed to explore the regulatory mechanism of NPCs in the pathophysiological process of IDD.

Non-coding RNAs, which can regulate gene expression, are involved in many pathophysiological processes in intervertebral disc cells (6, 7). MiRNA is a small, short-stranded fraction of lncRNA that binds to the 3'UTR of the target gene mRNA to inhibit mRNA translation or induce its degradation, thus regulating cell differentiation, proliferation, and survival (8). A variety of miRNAs are differentially expressed in intervertebral disc tissues with different degrees of degeneration, and are involved in the regulation of multiple physiological processes such as the apoptosis and proliferation of NPCs and the degradation of extracellular matrix (9). Further clarification of the key miRNA molecules regulating NPCs in IDD exploits new ideas for the diagnosis and treatment of IDD. It was recently reported that microarray analysis revealed that miR-25 was downregulated in the patients with IDD (10). However, the underlying role of miR-25 in IDD remains unclear. In this study, we found that miR-25-induced downregulation of the p53 signaling via targeting small ubiquitin-related modifier 2 (SUMO2) inhibited human intervertebral disc NPCs apoptosis.

## Methods

### Tissue collection

Nucleus pulposus (NP) tissues were collected from patients with IDD and spinal cord injury (n=30 per group). Our study was approved by the Ethic Committee of the First Affiliated Hospital of Jinan University. Written informed consent was obtained from each subject.

### Cell isolation and culture

These NP tissues were rinsed twice with PBS and sliced into 1 mm<sup>3</sup> pieces followed by digestion with trypsin. The isolated NPCs were grown in DMEM (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum, 100 mg/ml streptomycin, 100 U/ml penicillin, and 1% L-glutamine at 37°C.

### Cell transfection

Cell transfection was performed as mentioned previously (11) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions after the confluence reached 30-50%.

### Luciferase reporter assay

NPCs were transfected with the luciferase reporter vectors (Promega, Madison, WI, USA) containing SUMO2 3'UTR wild or mutant types and miR-25 mimic. The luciferase activities were measured after transfection.

### RNA pull down assay

Biotin-labeled RNAs were reversely transcribed and treated with RNase-free DNase I. The enrichment of SUMO2 mRNA in co-precipitated RNAs was determined by real-time PCR.

## Real-time PCR (RT-PCR)

To quantify the expression of miR-25, SUMO2, and p53, RT-PCR was performed using SYBR-Green Premix Ex Taq (Takara, Shiga, Japan). The thermocycling conditions: 95°C for 10 min; 40 cycles, 95°C for 15 sec; 60°C for 30 sec. The primers used in this study were as follows: miR-25, forward: 5'-ACTTTGTTTCGTTCCGGCTC-3', reverse: 5'-GAGCAGGGTCGGAGGT-3'; SUMO2, forward: 5'-GGCAACCAATCAACGAAACAG-3', reverse: 5'-TGCTGGAACACATCAATCGTATC-3'; p53, forward: 5'-GACGCTGCCCCACCATGAG-3', reverse: 5'-ACCACCACGCTGTGCCGAA A-3'; U6, forward: 5'-CTCGCTTCGGCAGCACA, reverse: 5'-AACGCTTCACGAA TTTGCGT-3'; GAPDH, forward: 5'-CCACGAAACTACCTTCAACTC-3', reverse: 5'-TCATACTCCTGCTGCTTGCTGATCC-3'.

## Western blot

Protein quantification was determined by the BCA method. The protein levels of indicated genes were detected by substrate coloration following electrophoresis separation.

## Cell proliferation assay

For CCK-8 assay, CCK-8 dyes (10 $\mu$ L) were added at 24, 48 and 72 h after transfection. Following incubation for 4 h, the number of surviving cells was assessed at 450 nm. For 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay, transfected cells were exposed to EdU solution (500  $\mu$ l; RiboBio, Guangzhou, China). Following incubation for 2 h, cells were fixed with 4% formaldehyde for 30 min, permeabilized with 0.5% Triton X-100 for 10 min and visualized by fluorescence microscopy.

## Cell apoptosis assay

Cells were incubated with Annexin V-FITC Apoptosis Detection kit (BD Biosciences, San Diego, CA, USA) in the dark for 15 min after digestion, centrifugation and PBS washing. The apoptotic rate was analyzed by flow cytometry.

## Statistical analysis

All data were expressed as mean  $\pm$  standard deviation (SD). Correlation analysis was performed using the Spearman's rank test. Statistical significance was compared by analysis of variance test.  $P < 0.05$  indicated a statistically significant difference.

# Results

## MiR-25 is lowly expressed in IDD patients

The expression of miR-25 was significantly decreased in the NP tissues from the patients with IDD compared with the controls (**Fig.1A**). The mRNA and protein levels of SUMO2, p53, and the p53 phosphorylation levels in the IDD group were markedly higher than those in the control group (**Fig.1B-1C**). Additionally, the expression of miR-25 was negatively correlated with SUMO2 mRNA levels in NP tissues of IDD patients (**Fig.1D**).

### **MiR-25 promotes proliferation and inhibits the apoptosis of human NPCs**

To further investigate the function of miR-25, we transfected miR-25 mimic or inhibitor into human NPCs to overexpress or knockdown miR-25 (**Fig.2A**). The cell viability was next assessed by CCK-8 assay, showing that the proliferation rate of NPCs was markedly increased by miR-25 overexpression, and was inhibited by miR-25 knockdown in a time-dependent manner (**Fig.2B**). Meanwhile, EdU assay further confirmed the pro-proliferative effects of miR-25 up-regulation and the anti-proliferative functions of miR-25 silencing (**Fig.2C**). Apoptosis was further evaluated by flow cytometry after transfection. Overexpression of miR-25 suppressed the apoptosis of NPCs, while the depletion of miR-25 induced apoptosis (**Fig.2D**). Then, Bax and Bcl-2 were detected by western blot. The pro-apoptotic protein Bax was markedly decreased in the miR-25 mimic-treated group, while anti-apoptotic protein Bcl-2 was increased. However, miR-25 suppression led to an opposite effect (**Fig.2E**).

### **SUMO2 is a direct target of miR-25 in human NPCs**

Based on bioinformatics tool, the complementary sequences of miR-25 was observed in the 3'-UTR of SUMO2 mRNA (**Fig.3A**). To determine the direct binding between miR-25 and SUMO2, we performed the luciferase reporter assay. MiR-25 mimic significantly reduced the luciferase activity of wild type but not the mutant SUMO2 3'-UTR in transfected NPCs, indicating a direct target relationship between miR-25 and SUMO2 (**Fig.3B**). In addition, we performed biotin-coupled miRNA pull down to verify the target relationship using NPCs transfected with biotinylated miR-25 or non-specific miRNA. After 48 h transfection, we found that SUMO2 mRNA was enriched in Bio-miR-25 pull down of NPCs compared to Bio-NC group (**Fig.3C**). As demonstrated in **Fig.3D-3E**, enforced expression of miR-25 observably repressed the mRNA and protein levels of SUMO2 in NPCs, whereas miR-25 inhibition increased the expression of SUMO2 at mRNA and protein levels.

### **MiR-25 exhibits its function in human NPCs via targeting SUMO2 through p53 signaling pathway**

To investigate whether the potential role of miR-25 in human NPCs was due to the direct targeting of SUMO2, the rescue experiment was performed. The addition of SUMO2 abrogated miR-25 overexpression-induced up-regulation of mRNA and protein levels of SUMO2 and p53, and p53 phosphorylation levels (**Fig.4A-4B**). Moreover ectopic expression of SUMO2 markedly reversed miR-25 up-regulation-induced NPC proliferation (**Fig.4C-4D**), as demonstrated by CCK-8 and EdU assay. In addition, flow cytometry results showed that miR-25-induced apoptosis inhibition in NPCs was attenuated by SUMO2 as well (**Fig.5A**). Moreover, we found that the restoration of SUMO2 expression prevented the up-

regulation of Bax protein expression and down-regulation of Bcl-2 in human NPCs in response to transfection with miR-25 mimic; this effect was confirmed by western blotting (**Fig.5B**).

## Discussion

In the current study, we proposed a protective role of miR-25 by coordinating SUMO2 expression in human NPCs. Our study initially demonstrated that miR-25 expression was significantly downregulated in patients with IDD. Moreover, miR-25 increased the proliferation of human NPCs and induced cell apoptosis. Furthermore, we identified that SUMO2 was the direct target of miR-25. Further investigation demonstrated that miR-25 regulated NPCs through p53 signaling pathway via targeting SUMO2 in IDD.

Several miRNAs are differentially expressed in IDD, including miRNA-222, miRNA-589, miR-574-3p, miR-199a-5p, and miR-483-5p (12, 13). In the present study, we have found a marked decrease in the expression of miR-25 in NP tissues of IDD patients, which was consistent with the previous research (10). Although the molecular mechanism underlying IDD is not fully illuminated, it is well documented that the aberrant loss of NPCs caused by apoptosis, which is attributed to the intervertebral disc degeneration, is the pathogenic process underlying IDD. Many miRNAs play vital roles in this pathogenetic process of IDD. For instance, miR-21 secreted by MSCs-derived exosomes protected human NPCs against apoptosis by targeting PTEN through PI3K-AKT signaling axis (14). MiR-532 contributed to the loss of NPCs via Bcl-9-mediated Wnt/ $\beta$ -catenin signaling, causing IDD development (15). Knockdown of miR-222 inhibited the apoptosis of human NPCs and inflammation via regulating TIMP3 in IDD (16). In the present study, our findings revealed that miR-25 overexpression accelerated the proliferation of human NPCs, and suppressed apoptosis, while inhibition of miR-25 prevented NPC cell growth. These findings suggest that the upregulation of miR-25 might be implicated in the development of IDD.

MiRNAs are recognized as regulators of target genes by inhibiting the translation or degradation of target mRNAs (17, 18). Herein, we applied bioinformatics prediction databases to predict putative miR-25 binding sites in the 3'-UTR of SUMO2. We also found that miR-25 level was negatively associated with SUMO2 expression in IDD patients. Consistently, the luciferase reporter assay combined with RNA pull down assay revealed that SUMO2 was indeed a direct target of miR-25, by which miR-25 exerted its functions in human NPCs. The regulatory effect of miR-25 on SUMO2 expression was further confirmed by real-time PCR and western blotting.

The small ubiquitin-related modifiers (SUMOs) is a group of ubiquitin-like proteins, which can be covalently connected to some substrate proteins such as I $\kappa$ B $\alpha$ , c-Jun, p53, etc. to participate in post-translational modification, regulate subcellular localization, and protein interactions and promote proteasome degradation (19, 20). SUMOylation is involved in the regulation of apoptosis-associated signaling pathways, such as p53, death-associated protein (Daxx) and dynamin-related protein 1 (Drp1) (21-23). As a member of the SUMOs superfamily, SUMO2 is crucial for the degradation and apoptosis of NPCs through the activation of p53 signaling pathway (24, 25). Based on our finding that SUMO2 was a direct target of miR-25 in human NPCs, we speculate that miR-25 controlled the growth of NPCs in IDD

via SUMO2. Indeed, we found that the upregulation of SUMO2 reversed the effects of miR-25 overexpression on NPC cell proliferation and apoptosis and the inhibition of p53 phosphorylation.

## Conclusion

In conclusion, miR-25 improved NPC cell viability, and inhibited apoptosis, partly via inhibition of SUMO2-mediated p53 signaling axis. Therefore, strategies to upregulate miR-25 have potential as therapeutic approaches in management of IDD.

## Abbreviations

Intervertebral disc degeneration (IDD); Small ubiquitin-related modifier 2 (SUMO2); Nucleus pulposus cells (NPCs); Nucleus pulposus (NP); Standard deviation (SD); Death-associated protein (Daxx); Dynamin-related protein 1 (Drp1)

## Declarations

### Ethics approval and consent to participate

The study was approved by the Ethic Committee of the First Affiliated Hospital of Jinan University (20180146). Written informed consent was obtained from each subject. **Consent for publication**

Not applicable.

### Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Competing interests

All authors declare that there is no competing interest.

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This study was supported by Science and Technology Project of Hunan Provincial Department of Education (No: 19C1619) which mainly contributed to carrying out the experiment and analyzing the results

### Authors' contributions

LS designed the experiments. LC and LJ were the major contributors in writing the manuscript. TG and WJ performed the experiments. All authors read and approved the final version of the manuscript.

## Acknowledgements

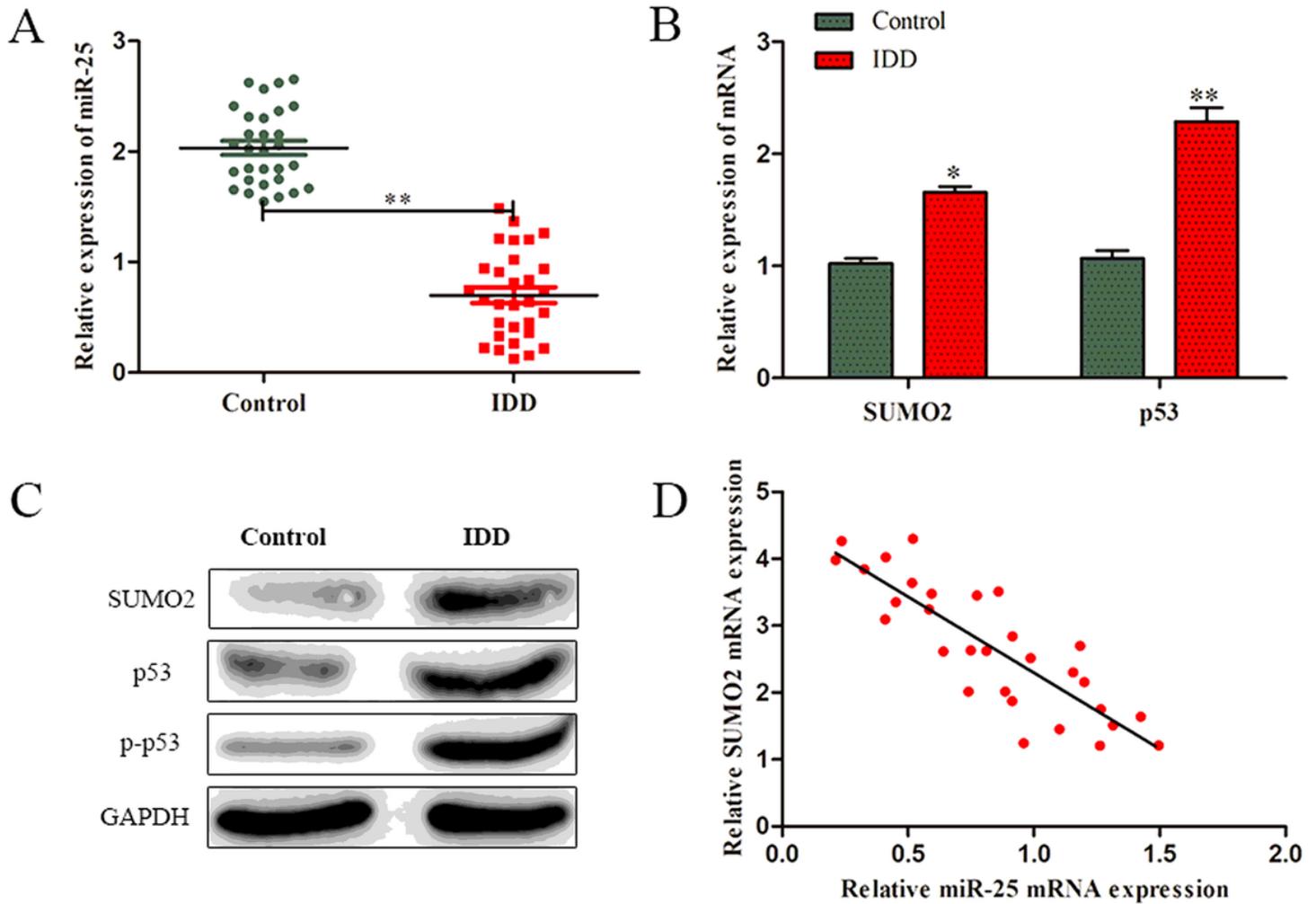
Not applicable.

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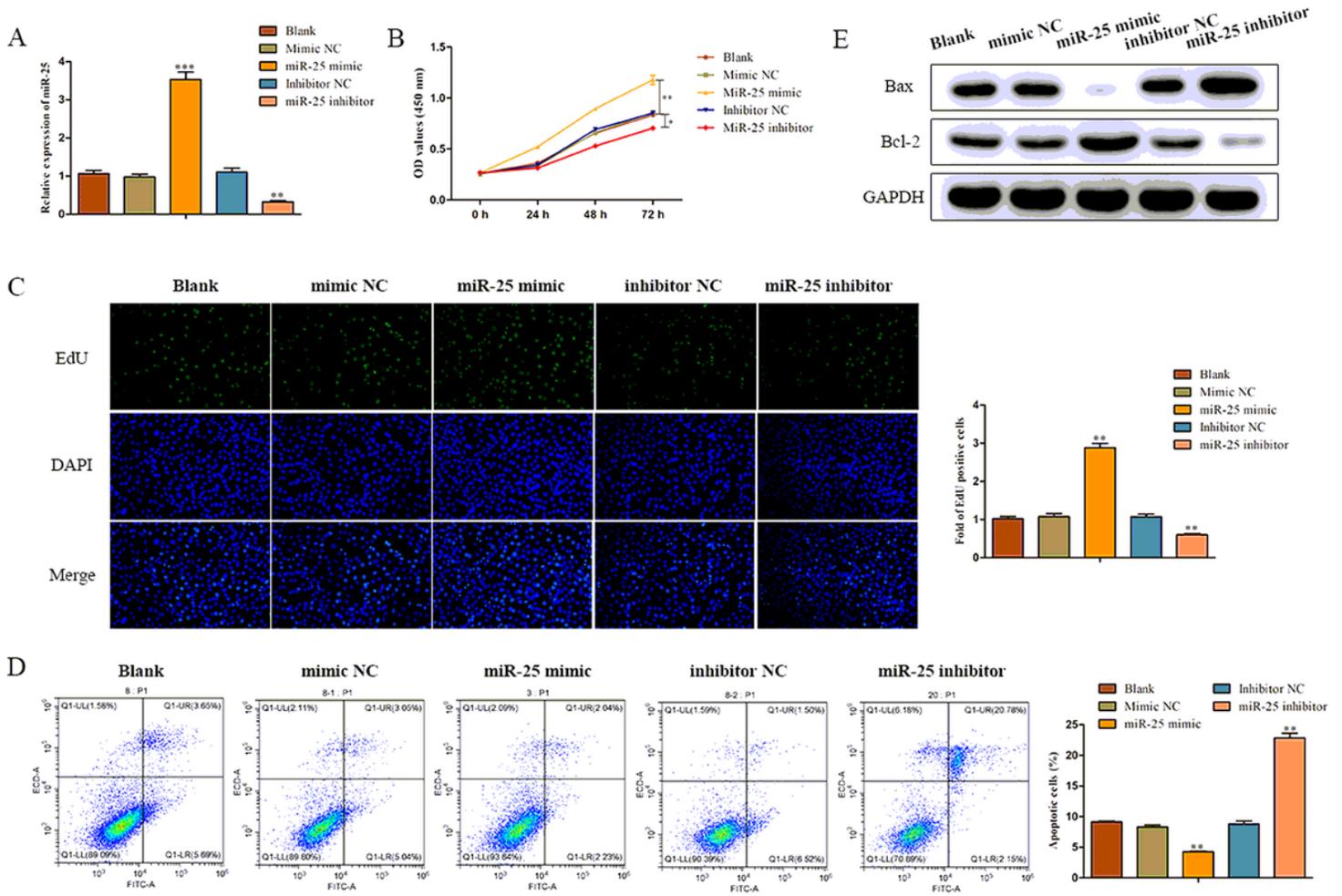
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## Figures



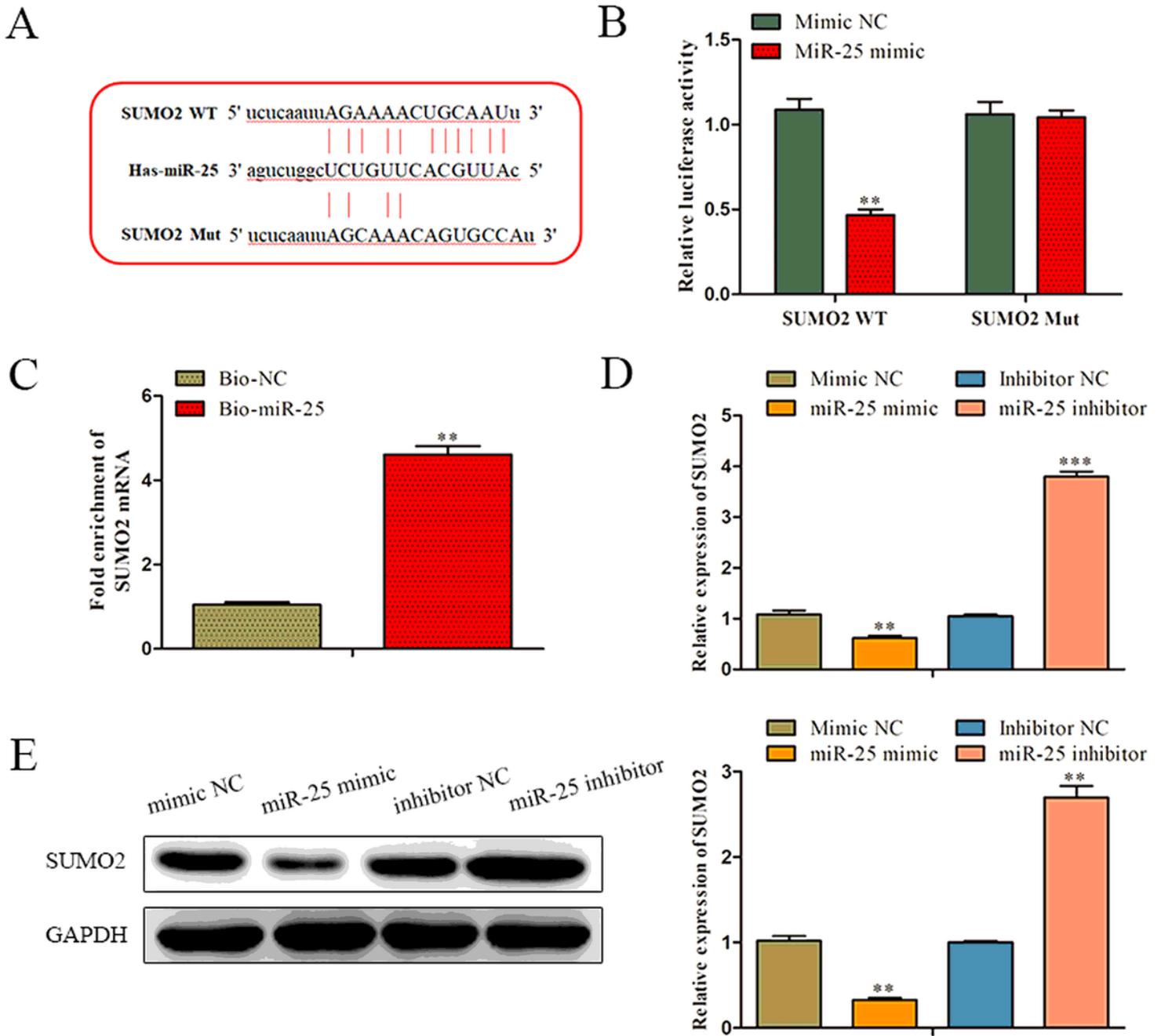
**Figure 1**

MiR-25 is down-regulated in IDD patients (A) The expression of miR-25 in the NP tissues of patients with IDD and control group (n=30) using RT-PCR assay. (B, C) The mRNA and protein levels of SUMO2, p53, and the p53 phosphorylation levels were explore using RT-PCR and western blotting analyses. (D) Expression correlation analysis between miR-25 and SUMO2 in NP tissues obtained from 30 IDD patients. Data were expressed as mean  $\pm$  SD. \*P < 0.05, \*\*P < 0.01 vs. control.



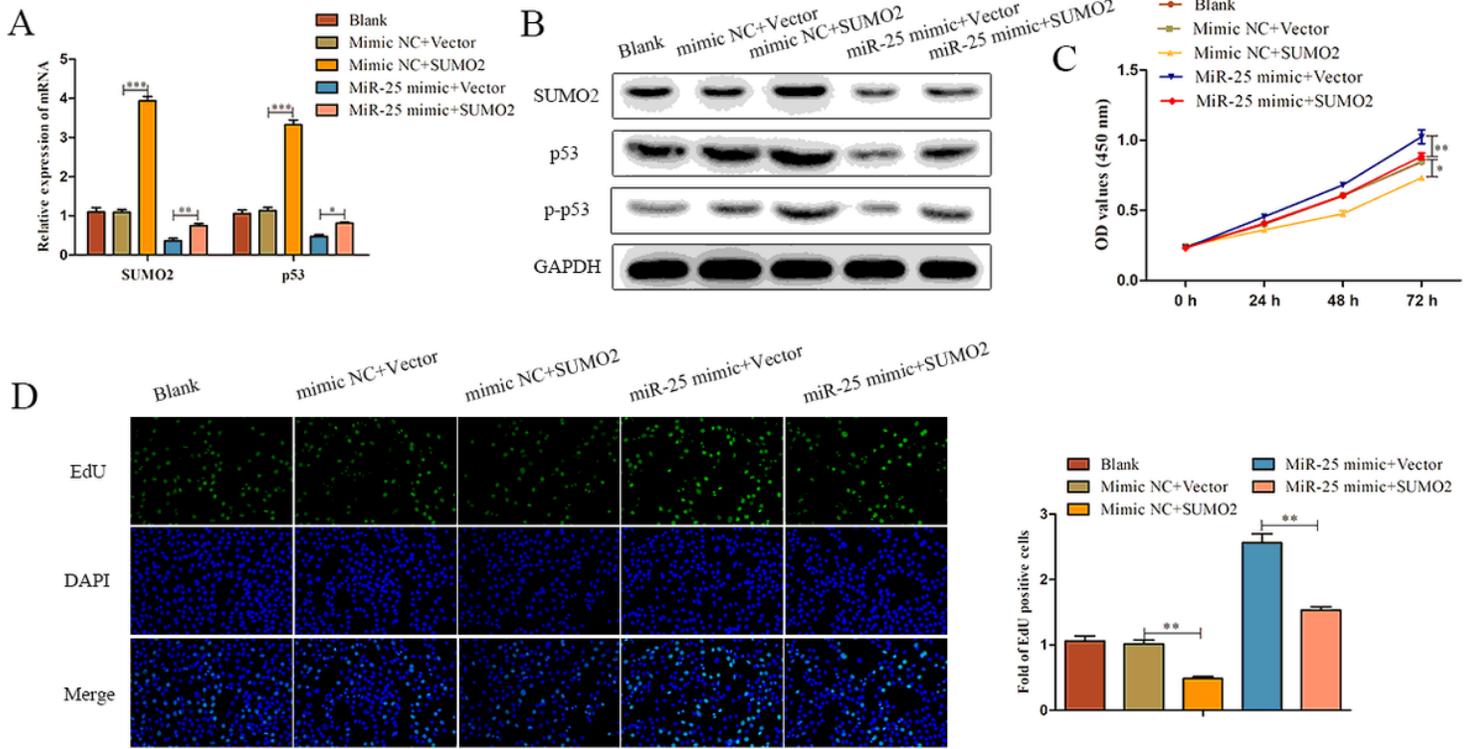
**Figure 2**

MiR-25 promotes proliferation and inhibits the apoptosis of human NPCs (A) The expression level of miR-25 was explored using RT-PCR assay. (B, C) CCK-8 and EdU analyses were performed to detected cell viability. (D) Apoptosis detection using flow cytometry. (E) Western blotting analysis for the protein levels of Bax and Bcl-2 in human NPCs after overexpression or knockdown of miR-25. Data were expressed as mean  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$  vs. blank.



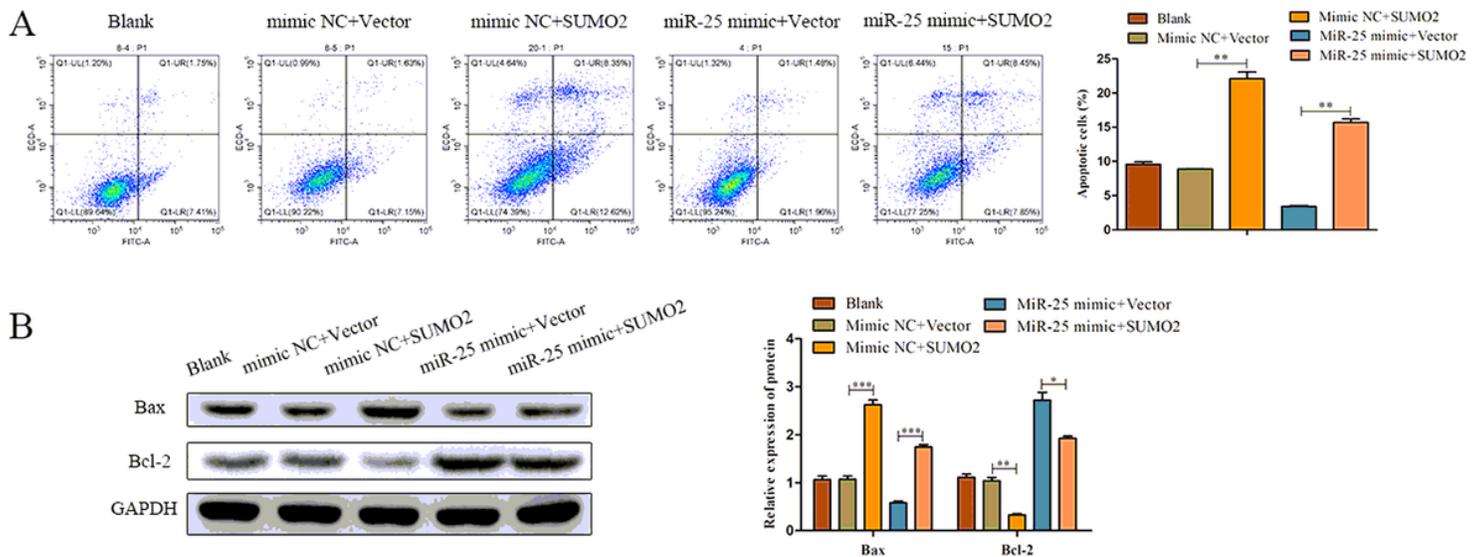
**Figure 3**

SUMO2 is a direct target of miR-25 in human NPCs (A) Sequence alignment of miR-25 with SUMO2. (B) Luciferase reporter assay in NPCs following transfection with miR-25 mimic, mimic NC, luciferase vector SUMO2, and the reporter construct. (C) The mRNA levels of SUMO2 were detected by biotin-coupled miR-130a-3p pull-down in NPCs. (D-E) The mRNA and protein levels of SUMO2 in NPCs following transfection with miR-25 mimic, inhibitor or negative controls. Data were expressed as mean  $\pm$  SD. \*\*P < 0.01, \*\*\*P < 0.001.



**Figure 4**

MiR-25 promotes human NPC proliferation via targeting SUMO2 through p53 signaling pathway (A, B). The mRNA and protein levels of SUMO2, p53, and the p53 phosphorylation were assessed using RT-PCR and western blot assays. (C, D), CCK-8 and EdU analyses were performed to detect cell viability in human NPCs after co-overexpression of miR-25 and SUMO2. Data were expressed as mean  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$  vs. blank.



**Figure 5**

MiR-25 inhibits the apoptosis of human NPCs via targeting SUMO2 (A) Apoptosis detection using flow cytometry. (B) The protein levels of Bax and Bcl-2 in human NPCs after co-overexpression of miR-25 and SUMO2. Data were expressed as mean  $\pm$  SD. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 vs. blank.