mir-301b-5p regulates the Wnt/β-catenin pathway through TXNIP/NLRP3 to reduce the occurrence of anorectal malformations in rats

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Research Article

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Abstract

Background To detect the expression of NOD-like receptor family pyrin domain 3 (NLRP3) inflammasomes and the spatiotemporal changes in mir-301b-5p expression during the anorectal development in normal and ARM fetal rats, and their potential association with anorectal malformations (ARMs).

Methods Sixty pregnant Wistar rats were randomly divided into normal (administered normal saline) and ARM groups (administered ethylene thiourea). We used immunohistochemistry, western blotting, real-time quantitative polymerase chain reaction (PCR), and fluorescence in situ hybridization (FISH) to detect the expression of proteins and miRNAs on gestational day (GD)14–GD16. According to the TargetScan, TXNIP is the target of mir-301b-5p and the combination of the two is verified by dual luciferase assay. PCR, western blotting, CCK-8 assay, and ELISA to verify the possible regulatory mechanisms in IEC-6 cells.

Results Compared with those in the normal group, the expression of NLRP3 inflammasomes increased on GD15 and GD16 in ARM fetal mice, whereas the expression of mir-301b-5p decreased and was related to the activation of NLRP3 inflammasomes. Overexpression of mir-301b-5p inhibited the expression of NLRP3/caspase-1 and decreased the secretion of IL-1β and IL-18 compared with NC group. The dual luciferase test verified that TXNIP is the target of mir-301b-5p. si-TXNIP inhibits the activation of NLRP3 inflammasomes and reduces the expression of β-catenin.

Conclusion mir-301b-5p negatively regulates TXNIP/NLRP3 axis, which regulate the wnt/β-catenin pathway in ARMs.

Introduction

Anorectal malformation is a common congenital malformation in pediatric surgery. It is characterized by intestinal development disorders after 4–8 weeks of embryo conception. The global incidence of anorectal malformation is approximately 1:1500–1:5000, and it is more common in men. Approximately 30–50% of children may have developmental abnormalities in other systems such as the genitourinary system and respiratory system. The causes of ARM are diverse. It is considered that ARM is caused by the combined action of genetic and environmental factors, but the exact pathogenesis is still unclear [1–3].

The nucleotide-binding oligomerization domain-like protein 3 (NLRP3) inflammasomes have been extensively studied; they are recognized by pathogen-related molecular patterns and hazard-related molecular patterns. NLRP3 inflammasomes are widely present in immune cells and are also expressed in non-immune cells [4–6]. Inflammasomes are multi-molecular protein complexes, including NLRP3, apoptosis-related speckle-like protein (ASC), and procaspase-1. When NLRP3 is activated, its conformation changes. NLRP3 oligomerizes and recruits ASC and pro-caspase-1, thereby triggering pro-caspase-1 self-cleavage. The pro-inflammatory effect of caspase-1 results in the production and secretion of mature IL-1β and IL-18 [7, 8]. Thioredoxin-interacting protein (TXNIP) is a binding protein of

NLRP3, and the binding of the two is essential for the subsequent combination and activation of inflammasomes[9, 10]. NLRP3 inflammasomes are involved in intestinal pathological processes such as inflammatory bowel disease [7], but their mechanism in ARM has not been studied yet.

MicroRNAs (miRNAs) are a kind of endogenous short-stranded non-coding RNAs (20–22 nt) that participate in various physiological and pathological processes, including metabolism, cell proliferation, apoptosis, differentiation, and tumorigenesis [9, 11, 12]. The expression of abnormal miRNAs is involved in the pathological process of ARM [13,14]. TargetScan (www.targetscan.org) predicts that TXNIP is the target gene of mir-301b-5p, and it is expressed in the lipopolysaccharide (LPS)-induced ALI rat model. The downregulation of mir-301b-5p is related to the effect of bone marrow mesenchymal stem cells (BM-MSCs) [15], but its mechanism of action needs further research.

Embryogenesis is coordinated by the crosstalk of the WNT and FGF signaling pathways, and is a process involving multiple intracellular cascades [16]. The WNT pathway is involved in the activation of NLRP3, and β-catenin can promote the expression of NLRP3 inflammasomes [17]. Therefore, the Wnt pathway may be involved in mir-301b-5p regulation in NLRP3 activation in ARM.

In this experiment, we explored the regulatory relationship between mir-301b-5p, TXNIP and NLRP3 in the pathogenesis of ARM through in vivo and in vitro experiments, in order to provide new targets for clinical treatment.

Material And Methods

Animal model

This study was approved by the ethics committee of Shengjing Hospital of China Medical University (2020PS388K(X1)). The ARM rat model was referred to previous literatures[13]. Sixty pregnant Wistar rats were randomly divided into two groups: one group was given 125mg/ mL ETU and the other group was given equal dose of normal saline without ETU.Pregnancy was continued under the original conditions. The fetuses were obtained by cesarean section on GD14–GD16, and the anorectal part was sampled under a microscope.

Cell transfection and processing

We purchased rat intestinal epithelial cells (IEC-6) from iCell Bioscience Inc., Shanghai, China. The cells were cultured in DMEM (Gibco, NY, USA) supplemented with 10% fetal bovine serum (HyClone Laboratories Inc., Utah, USA), 1% double antibody, and 89% high glucose, as a complete medium. Lip3000 (Invitrogen,Grand Island, NY, USA) was used to transfect an mir-NC/mimic, si-NC/TXNIP (RiboBio, Guangzhou, China), in order to construct an inflammatory state in vitro, IEC-6 was incubated with 20μg/mL LPS (Sigma, USA) for 4 hours. After supplementing the medium, then added 5 mM ATP (Sigma, USA) to the cells and incubate for 30 min.

Immunohistochemistry (IHC)

After deparaffinization of 4-μm fetus tissue sections, the antigen was thermally repaired in citrate buffer (pH 6.0) at 98 ℃ for 2 minutes, and then naturally cooled to 25℃. PV-9000 (ZSJQB Co., Wuhan, China) two-step method was used for immunohistochemical staining, according to the manufacturer's instructions. The sections were incubated with primary antibody TXNIP (Affinity, Shanghai, China; 1:1000), NLRP3 (Abcam, USA; 1:1000), caspase-1 (Novus, USA; 1:1000), IL-1β (Affinity; 1:1000), and IL-18 (Affinity; 1:1000) at 4°C for 16 h. After adding the amplification enhancer, the enhanced enzyme-labeled goat anti-mouse/rabbit IgG polymer was added dropwise on the tissue. DAB chromogenic solution was added dropwise on the tissue, and then. hematoxylin staining was performed for 1 min. Finally, dehydration was performed, and the tissue was mounted with neutral resin and photographed under a microscope (Nikon Corp., Tokyo, Japan).

Western blotting

The protein lysate was prepared using RIPA buffer:PMSF (100:1). The protein sample (20/40 μg) was mixed with 6× buffer, and then placed at 95℃ in a metal bath for 5 min. The proteins were resolved by SDS-PAGE gel electrophoresis and transferred on to PVDF membranes (Milipore,MA, USA). The membranes were washed with TBST (TBS+Tween) and blocked with 5% skim milk (TBST configuration) at room temperature for 2 h. Thereafter, the membranes were incubated with the following antibodies for 14 h at 4℃ after washing: TXNIP (Affinity; 1:1000), NLRP3 (Abcam; 1:1000), caspase-1 (Novus; 1:1000), IL-1β (Affinity; 1:1000), IL-18 (Affinity; 1:1000), β-actin (Affinity; 1:5000), and Tublin (Affinity; 1:5000). After washing, the membrane was incubated with the secondary antibody (goat anti-rabbit, Affinity; 1:8000) for 2 h. Thereafter, the membrane was washed again, and incubated with ECL luminescent solution (Thermo, USA). The PVDF membrane was placed in the developing instrument for exposure and development (Azure Biosystems, Inc., CA, USA).

FISH

We used 4.0-μm tissue sections and 5'-CY3-AGTAGTGCA ACCTAGTCAGAGC-3' probe (ServiceBio, Wuhan, China) for FSH. The paraffin sections were deparaffinized, digested with proteinase K (ServiceBio, Wuhan, China) for 20 min, and incubated at 37°C for 1 h for pre-hybridization. Thereafter, the hybridization solution containing the probe was added dropwise, and hybridization was performed overnight at 42°C in a thermostat. After washing, rabbit serum was added to block the membrane for 30 min at 25℃, and then mouse anti-DIG-HRP was added, and the sections were incubated for 50 min at 37℃; CY3-TSA (Servicebio, Wuhan, China) was added after PBS washing the sections. Finally, DAPI was added for counterstaining and allowed to react for 5 min in the dark. After mounting, the sections were photographed (Nikon DS-U3,Tokyo,Japan).

CCK-8 assay

We inoculated 8 × 10^3 IEC-6 cells per well in a 96-well plate. According to the CCK-8 instructions, 10 μl of CCK-8 reagent was added to each well. After incubating the cells at 37°C for 2 h, a microplate reader was used to measure the optical density of the samples at 450 nm.

Dual luciferase assay

TXNIP wild-type (WT) and mutant (WUT) plasmids were constructed by Hanbio Biotechnology Co., Shanghai, China. According to the manufacturer's instructions (Promega, Wisconsin, USA), lip3000 was used to transfect mir-NC+WT TXNIP, mir-NC+MUT TXNIP, mimic+WT TXNIP, and mimic+MUT TXNIP into 293T cells. The Du Al-Luciferase® reporter system was used to detect the luciferase activity 48 h after transfection.

Real-time quantitative PCR

The total RNA was extracted using TRIzol (Invitrogen, Carlsbad, USA), according to the manufacturer's instructions. Follow the instructions of PrimeScript™RT regent Kit (Takara Biotechnology co., Ltd., Dalian, China) for reverse transcription and using a SYBR

Premix Ex Taq Kit(Takara Biotechnology co., China) for gene amplification.Applied Biosystems Fast 7500 Real Time PCR System was used to perform pre-denaturation and PCR. The $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression of the target gene. The primer sequences are as follows:

β-actin: F: TGCTATGTTGCCCTAGACTTCG, R: GTTGGCATAGAGGTCTTTACGG; TXNIP: F: AGTCAGAGGCAATCACAT, R: ATCAGCAAGGAGTATTCAAC; u6: F: CTCGCTTCGGCAGCACA, R: AACGCTTCACGAATTTGCGT; mir-301b-5p: F: ACACTCCAGCTGGGGCTCTGACTAGGTTGC, R: TGGTGTCGTGGAGTCG.

ELISA

The level of IL-1β (Elabscience, Wuhan, China) and IL-18 (Boster, Wuhan, China) in the cell supernatant was determined according to the manufacturers' instructions.

Data analysis

All data were statistically analyzed using SPSS19.0 and prism 8.0. The results are expressed as mean ± SD of three experiments. One-way ANOVA and Student's *t*-test were used to analyze the differences between groups. Results with $P < 0.05$ were considered statistically significant ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$).

Result

Abnormal activation of NLRP3 can cause ARM

The IHC staining results showed that the number of NLRP3-, caspase-1-, ASC-, IL-1β-, and IL-18-positive cells and the expression intensity of these proteins in the intestinal mucosa and submucosa on GD15 and GD16 in the ARM group were higher than those in the normal group (Figure 1). The WB results were consistent with the IHC results,which found that compared with normal group, NLRP3/cleaved-caspase-1 was significantly up-regulated on GD15 and GD16 in the ARM group.(Figure 2).

mir-301b-5p is downregulated in ARM animal and cell models

In the ARM animal model, GD14-GD16 was detected during the critical period of anal development. The PCR results showed that compared with the normal group, the expression of mir-301b-5p in the hindgut of fetal mice in the ARM group was significantly reduced on GD15 and GD16. (Figure 3A). We further performed FISH, and the results were consistent with the PCR results. The hindgut of the ARM fetuses showed weaker fluorescence than normal groups(Figure 3B). Subsequently, we performed LPS+ATP treatment on IEC-6, and the PCR results showed that compared with the control group, mir-301b-5p was significantly downregulated in the treatment group (Figure 3C).

TXNIP is the target gene of mir-301b-5p

TargetScan database predicts that mir-301b-5p may be combined with TXNIP. We transfected mir-301b-5p NC/mimic in IEC-6, and the PCR results showed that the transfection efficiency was the highest at 48 h (Figure 4A). Subsequently, the transfected IEC-6 cells were treated with LPS+ATP. The PCR and western blotting results showed that compared with the NC group, the overexpression of mir-301b-5p downregulated the expression of TXNIP (Figure 4B,4C). The dual luciferase test further verified the relationship between the mir-301b-5p and TXNIP. The mir-301b-5p mimic reduced the luciferase expression of wild-type TXNIP, whereas mutant TXNIP did not change significantly (Figure 4D). Therefore, TXNIP is a direct target of mir-301b-5p.

mir-301b-5p inhibits the expression of TXNIP/NLRP3

TXNIP immunohistochemical staining was performed on the anorectal tissue of the normal group and the ARM group. The results showed that the number of positive cells in the intestinal mucosa and submucosa of the ARM fetuses on GD15 and GD16 was more than that of the normal group. The western blotting results were consistent with the IHC results, which revealed that the expression of TXNIP in the ARM group was higher than normal group. (Figures 5A , 5B). Further verification was performed in IEC-6 cells. The PCR results showed that the knockdown efficiency of si-2 was the highest after 48 h of transfection (Figure 5C), and TXNIP expression analysis was performed after si-2 knockdown, and the results of further western blot showed the protein knockdown efficiency of si-2, which was consistent with the PCR results. (Figure 5D). Then, after 48h transfection, we treated the cells with LPS + ATP, the western blotting results showed that compared with the NC group, si-TXNIP inhibited NLRP3 activation, western blot showed that the expression of cleaved-caspase-1 was down-regulated, and ELISA results revealed that the secretion of IL-1β and IL-18 was decreased (Figure 5E, 5F). To further explore the relationship among mir-301b-5p, TXNIP, and NLRP3, we transfected NC, si-2 TXNIP, mir-301b-5p mimic+NC, and mir-

301b-5p+si-2 TXNIP into IEC-6 cells. The western blotting results showed that, compared with that in the NC group, NLRP3 activity in the si-2 TXNIP, mir-301b-5p mimic, and mir-301b-5p+si-2 TXNIP group was inhibited, the western blotting results showed that, compared with the NC group, NLRP3 activity in the si-2 TXNIP, mir-301b-5p mimic and mir-301b-5p+si-2 TXNIP group was inhibited, reducing the expression of the NLRP3 inflammasome. ELISA results revealed that overexpression of mir-301b-5p and knockdown of TXNIP led to a reduction in the secretion of IL-1β and IL-18 (Figure 6A,6B).We want to further understand the effect of mir-301b-5p on cell death,and the CCK-8 results showed that the overexpression of mir-301b-5p reduced cell death and increased cell viability compared with NC group (Figure 6C).

mir-301b-5p regulates the Wnt/β-catenin pathway through TXNIP/NLRP3

Studies have found that the Wnt/β-catenin pathway can participate in the regulation of NLRP3 inflammasomes [17], and the Wnt pathway also plays an important role in the pathogenesis of ARM [16]. Western blotting revealed that compared with that in the NC group, the expression of β-catenin in the si-2 TXNIP, mir-301b-5p mimic and mir-301b-5p+si-2 TXNIP groups was significantly reduced, especially after co-transfection with mir-301b-5p+si-2 TXNIP (Figure 7).

Discussion

In this study, we analyzed the regulatory mechanism of mir-301b-5p and its target gene TXNIP in the activation of NLRP3 inflammasomes. We found that compared with normal group, the expression of mir-301b-5p was downregulated in the terminal rectum of ARM fetuses, whereas TXNIP and NLRP3 inflammasomes were upregulated. The dual luciferase assay verified that TXNIP is the target of mir-301b-5p, and its specific regulatory relationship was further explored in IEC-6 cells. The research have found that mir-301b-5p regulates the Wnt/β-catenin pathway through TXNIP/NLRP3 and participates in the pathological process of ARM.

The activation of NLRP3 inflammasomes is involved in the pathological process of various diseases, such as neurodegenerative diseases, endocrine diseases, and intestinal diseases [7, 18, 19]. Clinical trials have shown that ARM is positively related to fever in the mothers in the first trimester of pregnancy. In addition, genitourinary tract infections and exposure to chemicals and drugs can also affect the development of the anorectum during pregnancy [20–23]. Therefore, the presence of inflammation may lead to anorectal malformations. We found that on GD15 and GD16, compared with that in normal rats, the expression of NLRP3 inflammasomes in ARM rats was significantly increased. The development of the anorectum involves a variety of factors. The tissue cells must differentiate into the correct target cells after receiving appropriate signals at a specific time and localize to a specific location [24, 25]. We speculate that the activation of NLRP3 inflammasomes during the critical period of anorectal development may lead to abnormal death of urethral and rectal epithelial cells, failure to differentiate and localize in time, and lead to ARM.

miRNAs regulate translation or degradation by binding to the 3'-noncoding region (3'-UTR) of target genes, and negatively regulate gene expression at the transcriptional level. Therefore, they play an

important role in the pathogenesis of various diseases [11, 12]. Studies have confirmed that miRNAs are key regulators of NLRP3 inflammasome activation in several diseases [27]. We attempted to identify miRNAs closely related to the pathogenesis of ARMs. Sequencing analysis has revealed that mir-301b-5p expression is different in LPS-induced rat ALI models [15], but its specific mechanism in the disease has not been studied. We predicted the potential target of mir-301b-5p through TargetScan, and verified it through RT-qPCR and dual luciferase reporter gene assay. We found that the expression of mir-301b-5p in GD15 and GD16 anus hindgut of ARM fetal rats was significantly reduced compared with normal group. In addition, the overexpression of mir-301b-5p also inhibited the activation of NLRP3, resulting in reduced cleaved-caspase-1 expression and IL-1β and IL-18 secretion. We speculate that mir-301b-5p may reduce cell death and thus antagonize the effect of NLRP3 inflammasomes. The CCK-8 assay showed that the overexpression of mir-301b-5p can enhance cell viability.

In order to better understand the regulatory effect of mir-301b-5p on NLRP3 inflammasomes, its target gene TXNIP should be further studies. TXNIP is an endogenous thioredoxin (TXN)-negative regulator and an important factor in the redox balance in cells. Studies have found that TXNIP can not only aggravate oxidative stress, but is also an important mediator of NLRP3 inflammasome activation, via the NLRP3/TXNIP axis, which has become a target in several diseases such as diabetic nephropathy, renal tubular injury, and sepsis. As the core protein, NLRP3, once activated, will recruit ASC and caspase-1, and secrete mature IL-1β and IL-18 outside the cells to participate in and expand the inflammatory response [27–29]. We tested the anorectal sites of GD14–GD16 ARM fetal mice and found that TXNIP was upregulated on GD15 and GD16. We then knocked down TXNIP in IEC-6 cells and found that NLRP3 activity was inhibited, and the IL-1β and IL18 levels in the cell supernatant were decreased compaed with NC group. We speculate that the TXNIP/NLRP3 axis is involved in the pathological process of ARM.

The Wnt/β-catenin pathway is involved in the activation process of NLRP3 inflammasomes [17]. The Wnt pathway affects the cell cycle and regulates the biological behavior of cells. It is essential for embryonic development and regeneration, and is used in many tissues. It plays a role in diseases, including neurodegenerative diseases, skeletal diseases, myocardial infarction, and liver damage [30]. It also plays an important role in the pathogenesis of ARM. The intestines follow a basic developmental mechanism in different species. When the epithelial–mesenchymal transition is disturbed, it causes an abnormal mesenchymal response, leading to cloaca malformations. The benign transcription cycle of Wnt, Hedhehog, and NOTCH pathways plays an important role in the normal development of the intestine [14, 16]. In this study, we found that knocking down TXNIP leads to a decrease in the expression of β-catenin, and at the same time, the activation of NLRP3 inflammasomes is suppressed. We speculate that TXNIP affects the proliferation of intestinal epithelial cells by regulating the Wnt/β-catenin pathway in ARM, leading to cloaca deformity.

This study had some limitations. First, we only focused on overexpression, which could not better reflect the effect of the interaction between mir-301b-5p and TXNIP on the activation of NLRP3; in addition to the Wnt/β-catenin pathway, it can also possible that other signal pathways such as PI3K/AKT regulate

the activation of NLRP3 inflammasomes. Second, we only studied animal models and did not analyze clinical samples. There may be deviations in expression.

In summary, we found that a high expression of mir-301b-5p can reduce the occurrence of ARM. TXNIP is its target gene. Inhibition of TXNIP can reduce the activation of NLRP3 inflammasomes and hinder the Wnt/β-catenin pathway. Therefore, mir-301b- 5p may regulate the Wnt/β-catenin pathway through TXNIP/NLRP3, thereby affecting the occurrence of ARM.

Declarations

Author contributions

MHJ: conceived of or designed study; MMS: performed research, analyzed data and wrote the paper; DL: review and editing; NZC:resources; WG:supervision; PL:resources and contributed models.

Conflict of interest

Author declares no conflict of interest.

Availability of Data and Materials

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

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Figures

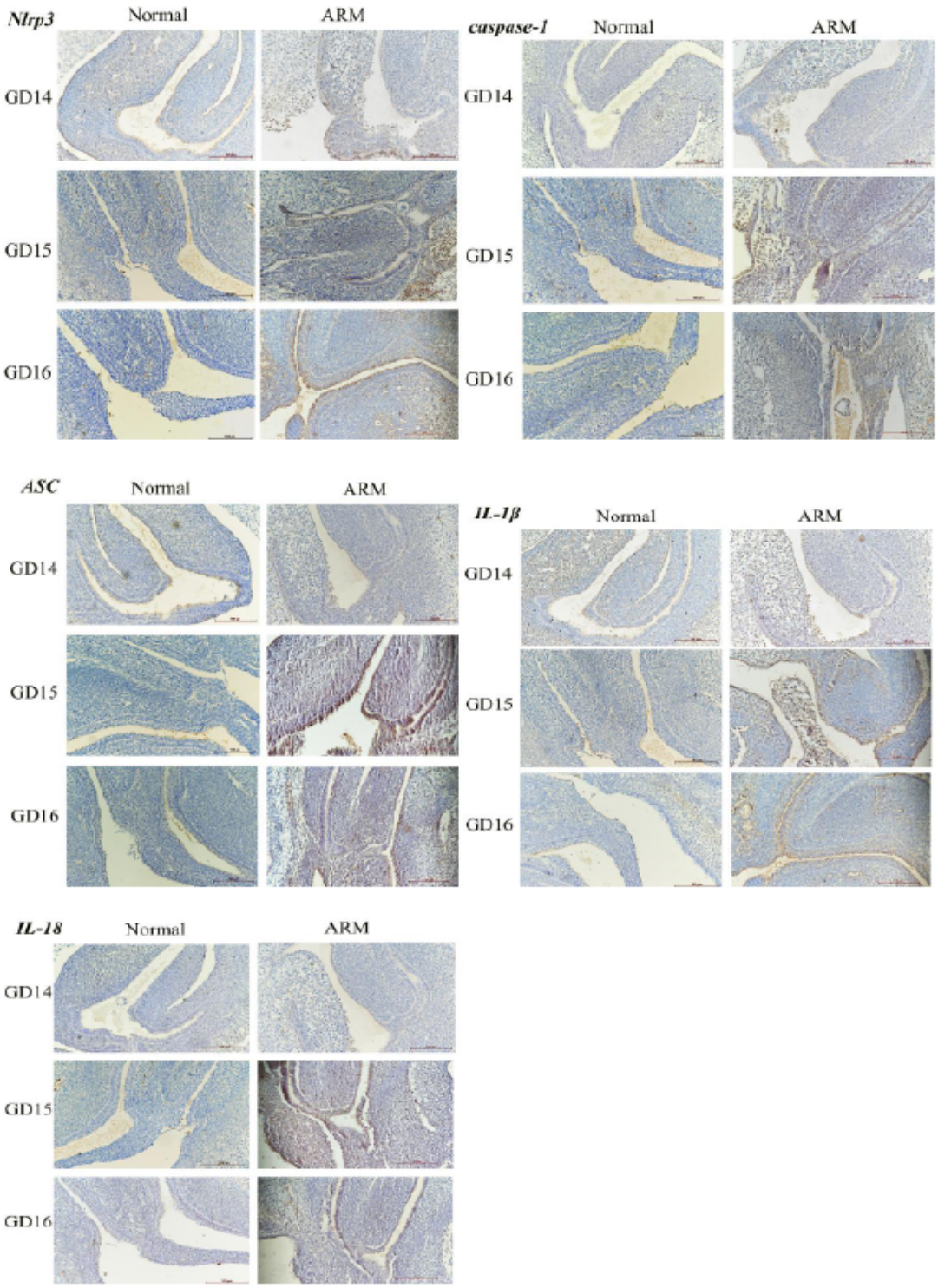

Figure 1

GD14–GD16 immunohistochemical results of NLRP3, ASC, caspase-1, IL-1β, and IL18 in the normal group and ARM group

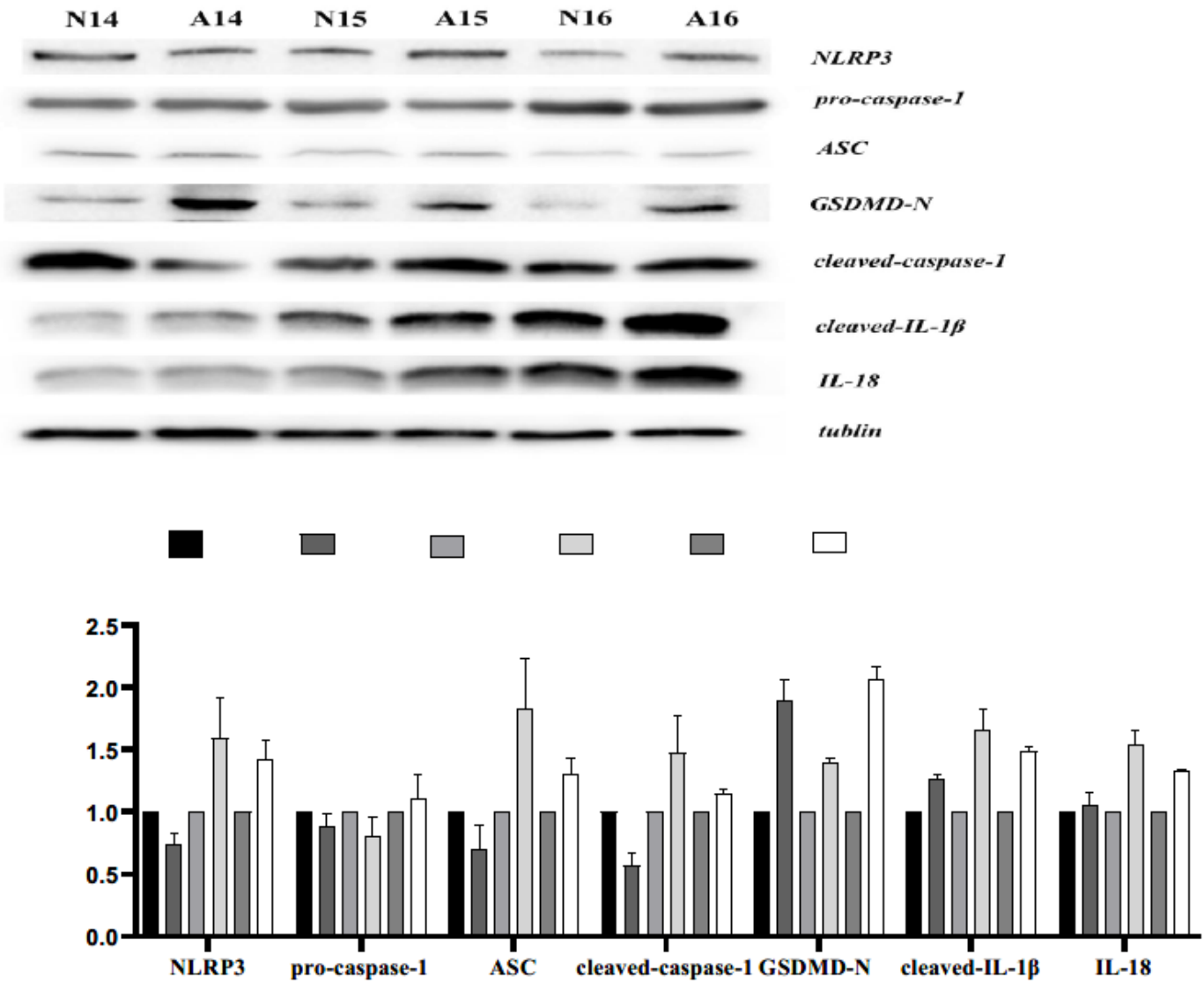

Figure 2

Western blotting and semi-quantitative analysis for NLRP3,pro-caspase-1,cleaved-caspase-1,GSDMD-N,ASC,cleaved-IL-1β,IL-18 on GD14–GD16 in the normal group and ARM group. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$)

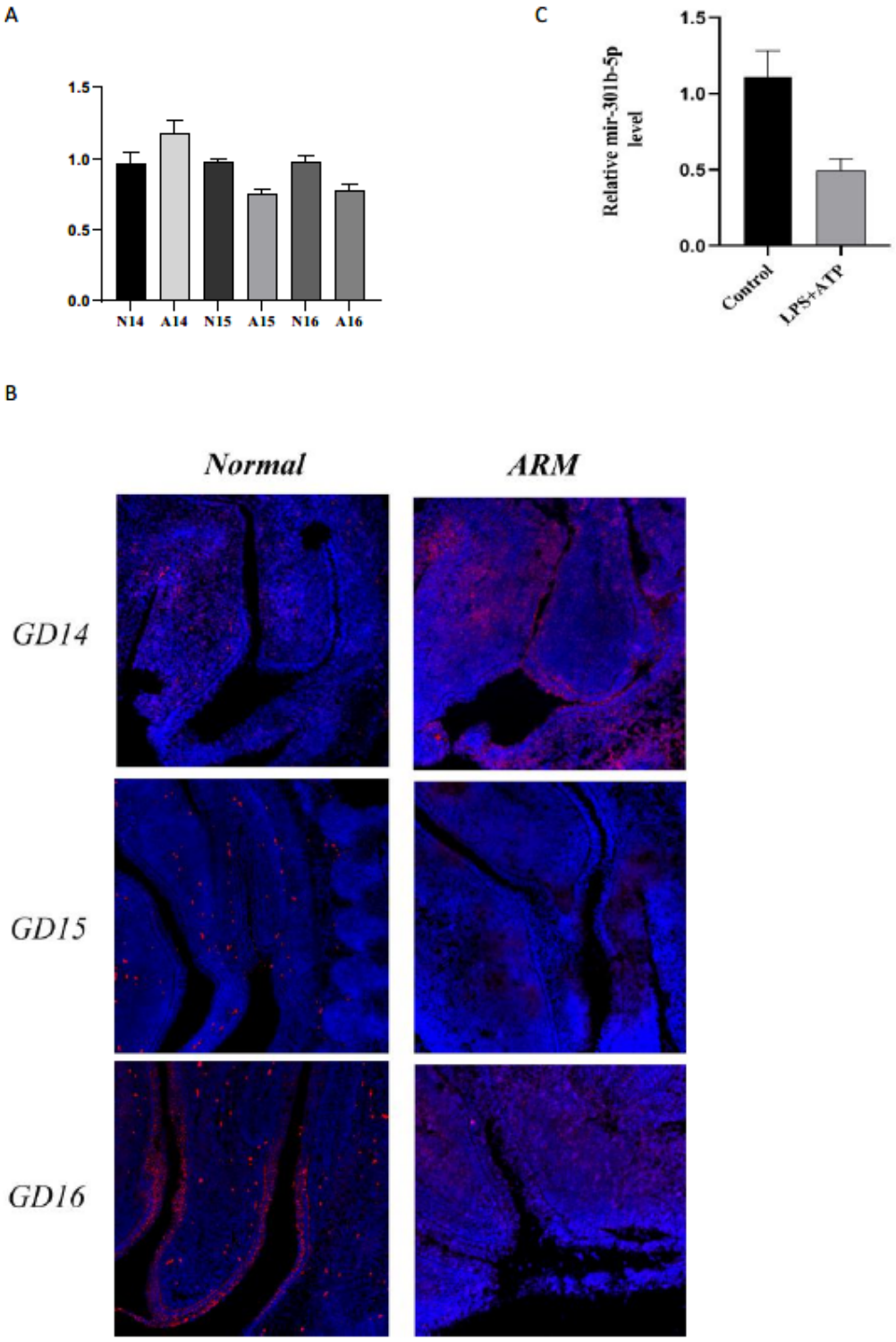

Figure 3

The expression of mir-301b-5p in ARM animal models and IEC-6. **A** qRT-PCR results of mir-301b-5p in the normal group and ARM group on GD14-GD16. **B** FISH localized the spatial expression of mir-301b-5p in the normal group and ARM group on GD14–GD16□10X□. **C** After LPS+ATP treatment of IEC-6 cells, the expression of mir-301b-5p changed. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$)

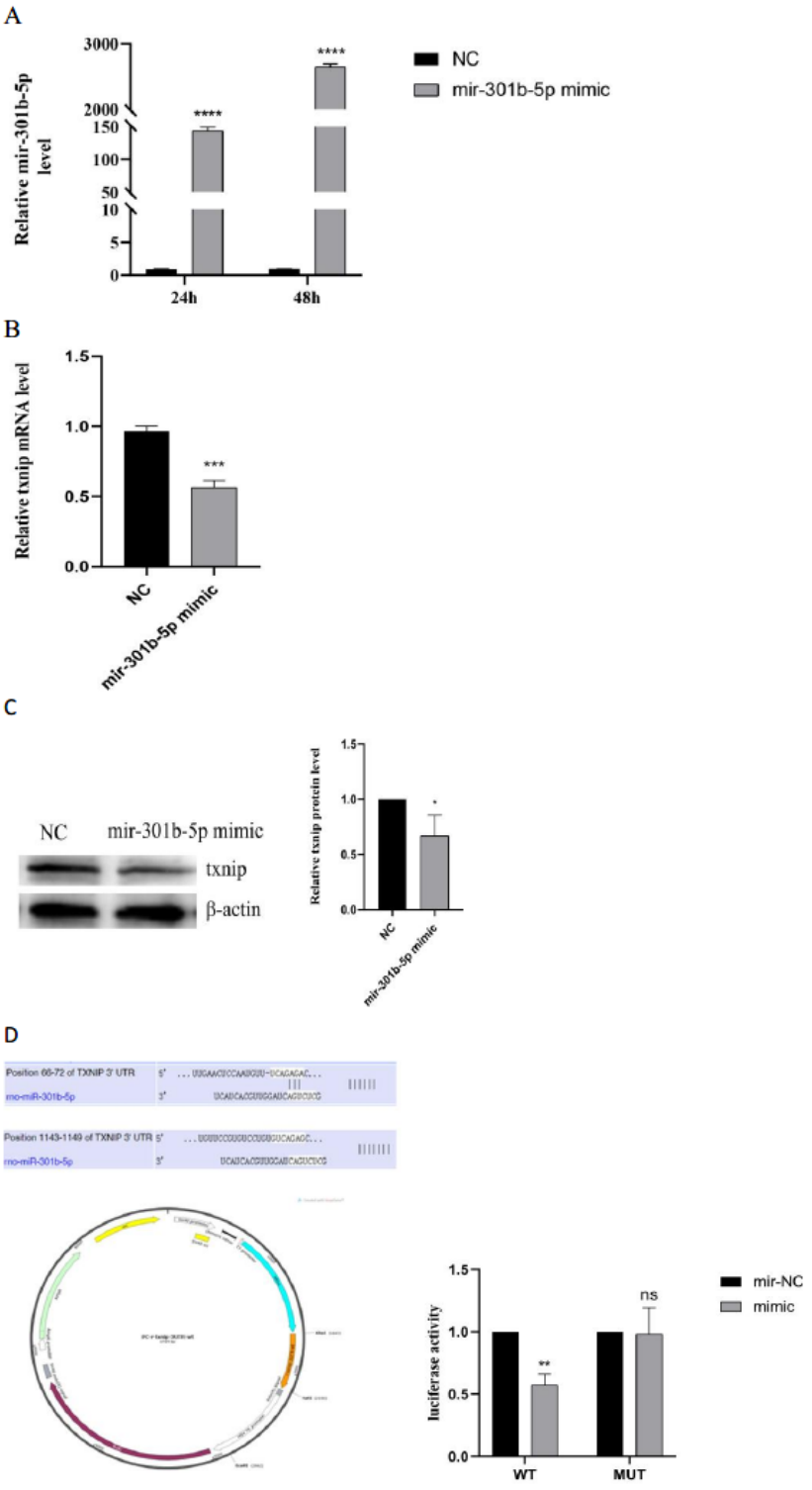

Figure 4

TXNIP is the target gene of mir-301b-5p. **A** The transfection efficiency of mir-301b-5p at 24 and 48 h. **B** The relative mRNA level of TXNIP was detected by RT qPCR after mir-301b-5p NC/mimic transfenction. **C** Western blotting of TXNIP in IEC-6 after mir-301b-5p NC/mimic transfection. **D** The binding site of mir-301b-5p on TXNIP 3 '- UTR was predicted. The direct targeting relationship between mir-301b-5p and

TXNIP 3' - UTR was verified by double luciferase report experiment.(*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

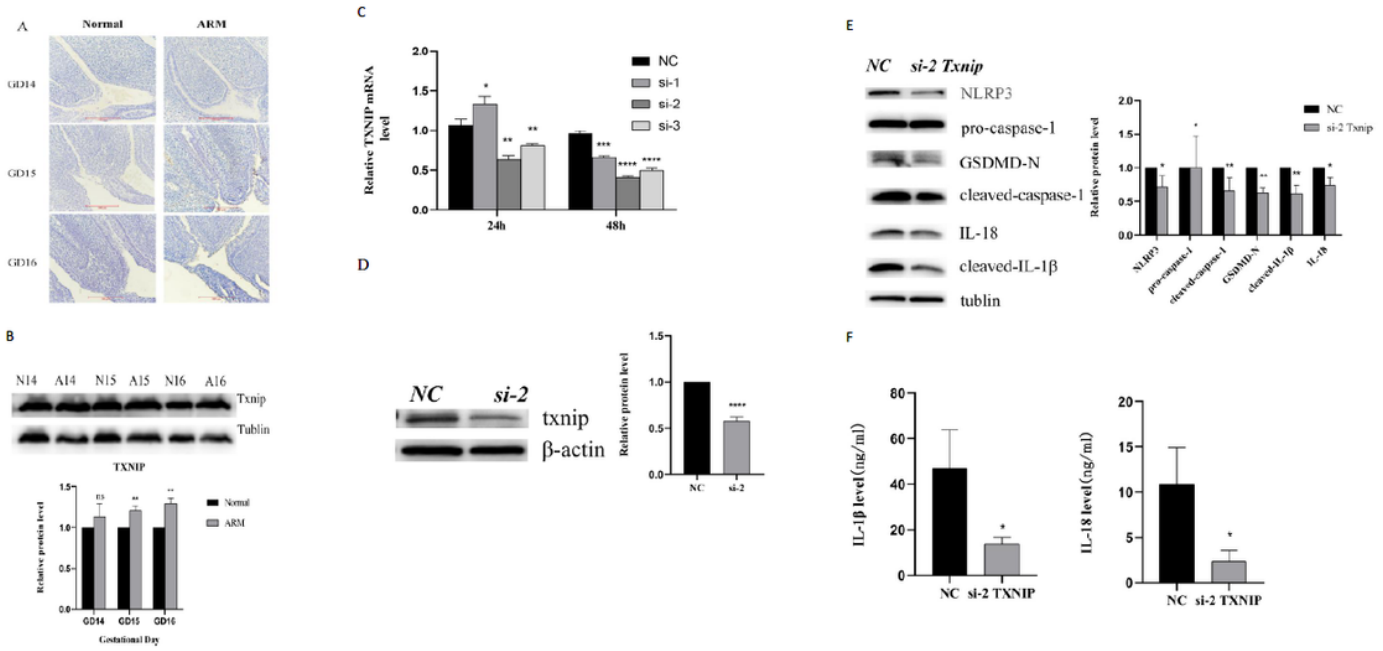

Figure 5

TXNIP expression in animals and transfection of si-TXNIP caused changes in NLRP3 inflammasome expression. **A** Immunohistochemical staining of TXNIP in the normal group and ARM group on GD14–GD16 (20X). **B** TXNIP protein expression and semi-quantitative changes in the GD14–GD16 anorectum of the normal group and ARM group. **C** The transfection efficiency of si-1, si-2 and si-3 at 24 and 48 h. **D** The knockdown of si-2 protein at 48 h. **E** The change in NLRP3 inflammasome expression caused by si-TXNIP. **F** The secretion of IL-1β and IL-18 in the cell supernatant after TXNIP knockdown. (*p < 0.05, **p < 0.01, ***p < 0.001, **** p < 0.0001)

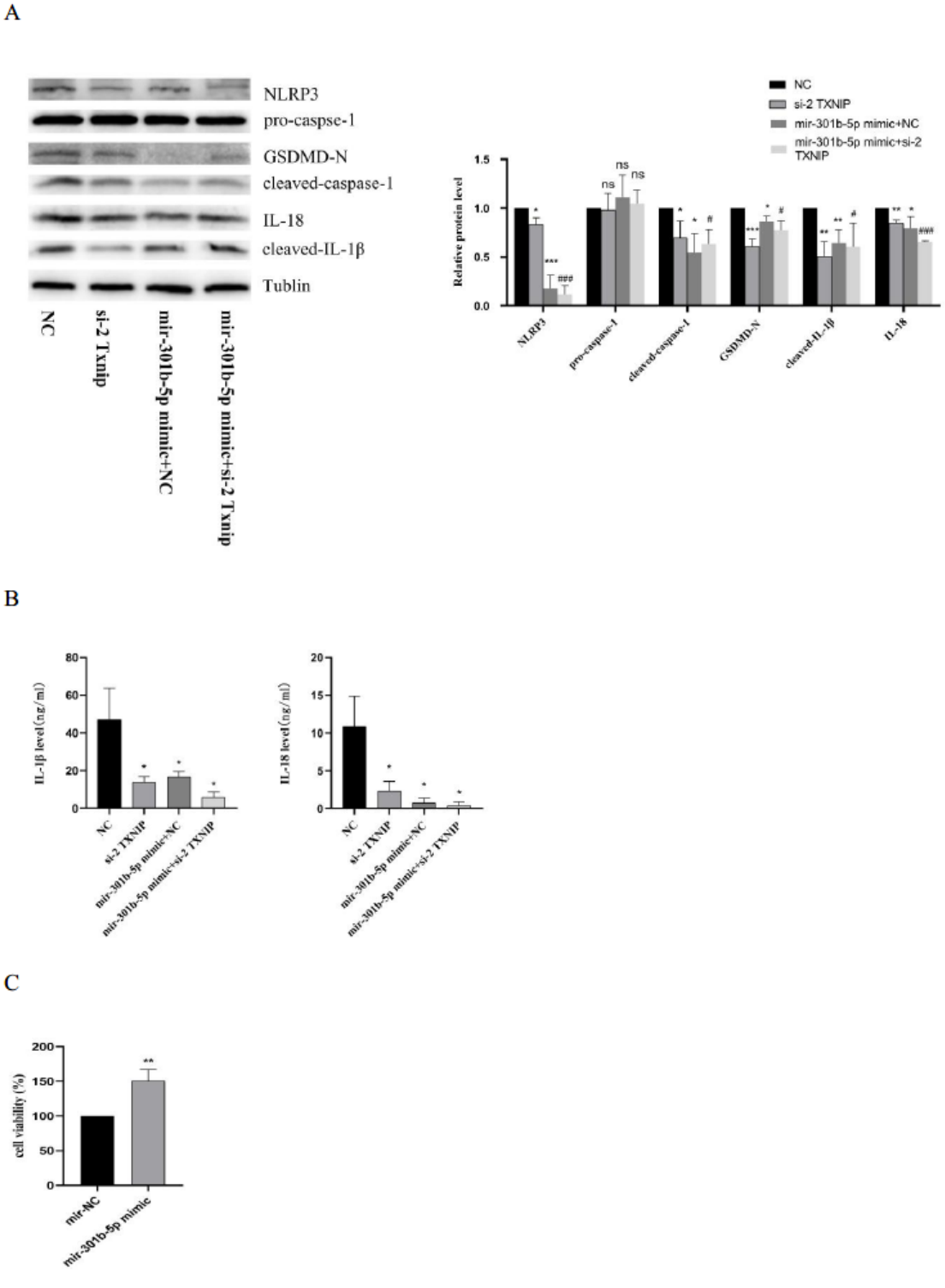

Figure 6

Effect of mir-301b-5p on NLRP3 inflammasomes. **A** The protein expression and semi-quantitative changes in NLRP3 inflammasomes after the overexpression of mir-301b-5p. **B** The changes in IL-1β and IL-18 secretion in the cell supernatant after the overexpression of mir-301b-5p. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$)

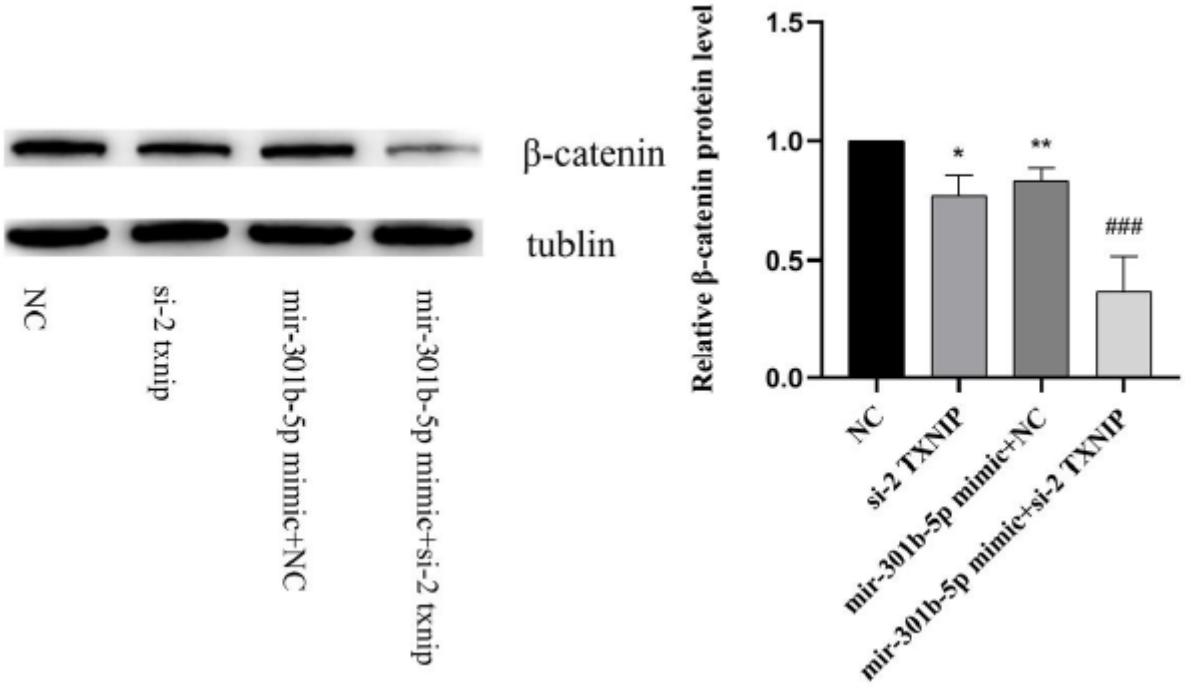

Figure 7

Knockdown of TXNIP causes protein expression change and semi-quantitative changes in β-catenin. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{###}p < 0.001$)