miR-142-3p and HMGB1 Are Negatively Regulated in Proliferation, Apoptosis, Migration and Autophagy of Cartilage Endplate Cells

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Research Article

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Abstract

Background: cartilage endplate (CEP) degeneration plays a vital role in the pathological process of intervertebral disc degeneration. It has been previously reported that microRNAs may participate in the occurrence and development of intervertebral disc degeneration through regulating its' target genes directly. The regulatory roles of miR-142-3p /HMGB1 in some orthopedic diseases have been determined successively, but there was no report about the degeneration of CEP. Therefore, we aimed to determine the regulation of miR-142-3p /HMGB1or potential molecular mechanisms on proliferation, apoptosis, migration, and autophagy of CEP cells.

Methods: the target gene of miR-142-3p was determined by double luciferase assay. We selected ATDC5 cell lines. CCK-8 method was used to detect cell proliferation. Real-time fluorescence quantitative PCR was used to determine gene expression levels, and western blot analysis was used to determine protein expression levels. We chose the flow cytometry to measure cell apoptosis and cell cycle.

Results: the result of luciferase detection showed that the target gene of miR-142-3p in CEP cells was HMGB1. Knockdown the miR-142-3p inhibited the expression level of HMGB1, the proliferation and migration of CEP cells, but it promoted apoptosis of CEP cells. In addition, the detection results of the proteins related to apoptosis or autophagy showed that knockdown of miR-142-3p promoted apoptosis and autophagy.

Conclusion: The negative regulation of miR-142-3p /HMGB1 can affect the proliferation, apoptosis, migration, and autophagy of CEP cells. Our results provided a new idea for the targeted treatment of CEP degeneration by inhibiting the expression of HMGB1.

Introduction

Disc proscription disease (DDD) is the main cause and initiating factor of intervertebral Disc protrusion, degenerative vertebral canal stenosis, lumbar spondylolisthesis and other kinds of low back pain diseases (Neidlinger-Wilke and others 2014). Although the exact molecular mechanism of intervertebral disc degeneration is not clear, current studies suggested that apoptosis due to the various non-physiological reasons may be the main cause of intervertebral disc cell decline. For a long time, there have been many studies on disc degeneration caused by annulus fibrosus and nucleus pulposus. Factors such as IL1, IL-6, NO and MMPs may be involved in disc degeneration by causing inflammation, promoting apoptosis and other ways (Jimbo and others 2005). However, there are few studies on the effect of CEP on intervertebral disc degeneration. Studies have shown that intervertebral discs are the largest hypoxic tissue in the human body. However, degeneration of the cartilage endplate may result in decreased diffusion function, which will prevent the nucleus marrow from obtaining necessary nutrients and inhibit the excretion of metabolic wastes, thus degeneration of intervertebral disc is accelerated (Hwang and others 2004; Yuasa and others 2008). It is obvious that cartilage endplate cell degeneration may play a vital role in the pathological process of intervertebral disc degeneration, and prevention or

inhibition of cartilage endplate cell degeneration may be a potential target for intervertebral disc degeneration.

Micro RNA (miRNA) is a single stranded RNA molecule consisting of about 20~23 nucleotides in length. In recent years, miRNAs have attracted more and more attention in the study of disc degeneration. miRNAs are involved in the process of intervertebral disc cell proliferation, apoptosis and extracellular matrix synthesis by directly regulating the target genes, which may play an important role in occurrence and development of intervertebral disc degeneration (Li and others 2015; Wang and others 2015). Some microRNAs associated with various orthopedic diseases have been reported in previous studies, such as miR-34a (Krzeszinski and others 2014; Kurowska-Stolarska and others 2017), miR-181a (Shao and others 2015; Sun and others 2016) and miR-142-3p (Wang and others 2016). More importantly, Wang et al. found that miR-142-3p inhibited the inflammatory response and apoptosis of chondrocytes in osteoarthritis by inhibiting the NF-κβ pathway mediated by HMGB1 (Wang and others 2016).

High Mobility Group Protein (HMGB1) is a highly conserved nuclear protein that can be released into the extracellular environment to mediate inflammatory response after cell injury (Zhu and others 2013). Anderson et al. (Andersson and Harris 2010) found that the expression level of *HMGB1* was significantly increased in synovial membrane and serum of patients with rheumatoid arthritis, the expression level of *HMGB1* and its mRNA was most significant in the pannus area of articular cartilage invaded by synovial fluid. Studies have confirmed that the expression level of HMGB1 is significantly increased during the process of intervertebral disc degeneration (Gruber and others 2015a). More importantly, some scholars speculated that blocking the expression of HMGB1 to delay the CEP degeneration might become an important target for the treatment of intervertebral disc degeneration after relevant experiments (Harris and others 2012). These studies have all shown that HMGB1 plays an important role in the degeneration of cartilage endplate.

Subsequently, through literature review, we found that there were no reports on the regulation of miR-142-3p /HMGB1 in cartilage endplate cells. Therefore, in this study, we studied the expression regulation mechanism of miR-142-3p/HMGB1 in cartilage endplate cells to further understand the potential mechanism of action of miR-142-3p/HMGB1 in cartilage endplate degeneration.

Materials And Methods

Cell culture

Our study used ATDC5 cell line (BeNa Culture Collection: BNCC350793), which are derived from the mouse teratocarcinoma strain AT805.

After the primary cell attachment have reached 85%-90%, the cell passage begins. We add digestion solution containing 0.25% trypsin: 0.01% EDTA (1:1) for 1 min. Wait until the cells have shrunk and become rounded and the gap has increased before adding complete medium to stop the digestion. After the cells have shrunk, become rounded, and gaps have been increased, complete medium is added to

stop the digestion. We collected the cell suspension and centrifuged for 4 minutes, leaving the bottom cell cluster. Then add fresh complete medium and mix well by pipetting. The cells were inoculated into multiple petri dishes at 2×10^5/ mL, and cultured in a cell incubator with a constant temperature and saturated humidity (5% CO_2 at 37°C).

Cell transfection

Seed the cells in a 6-well plate. When the cells are about 80% confluent, Lipofectamine™ 2000 transfection reagent (thermofisher, 11668019) was used, and the experiment was carried out according to the kit instructions. After 48 hours of transfection, the cells were collected and used for further analysis.

Plasmid construction

The recovered and purified target fragment HMGB1-3UTR (XhoI/NotI) was linked with the pYr-MirTarget (XhoI/NotI) vector. The product was named pYr-MirTarget-HMGB1-3UTR.

Detection of gene expression level by RT-PCR method

Total RNA was extracted from the cells using TRIzol (Sigma, T9424-100ML). In our study, the expression level detection mainly included miRNA, *HMGB1*, *Bcl-2*, *Bax*, *P62* and *Beclin1*.

The real-time fluorescent quantitative PCR kits used in this study include Takara's TB Green^TM Premix ExTaq^TM II (Tli RNaseH Plus) and PrimeScriptTM RT Master Mix (Perfect Real Time), and TIANGEN's miRcute enhanced miRNA cDNA first-strand synthesis kit and miRcute enhanced miRNA fluorescence quantitative detection kit (SYBR Green). The specific experimental steps are carried out according to the instructions. All primers required for RT-PCR in this study were summarized in Table 1.

Protein detection (Western blot)

The main proteins tested include *HMGB1*, the apoptosis-related protein *Bcl-2/ Bax*, autophagy-related protein *P62* and *Beclin1*. All antibodies used in this study are from Proteintech

The cells were washed with pre-cooled PBS (phosphate buffer), then 50 μL cell lysate (RIPA) was add to each well. We transferred the cell-containing lysate to a 1.5 mL EP tube, sonicated it on ice for 1 h and then centrifuged at low temperature for 30 min. Finally, the supernatant (cell protein lysate) was transferred to a new EP tube.

Specific steps: polyacrylamide gel for electrophoresis, transfer membrane (wet transfer method: transfer protein on the gel to the nitrocellulose membrane), wash membrane (0.05%TBST), milk blocking (5% skimmed milk powder, room temperature 1h), incubate primary antibody, wash membrane again, incubate secondary antibody (HRP-labeled antibody), and color development (ClarityTM Westren ECL Substrate, Bio-RAD).

Dual luciferase detection report related to the regulation of HMGB1 and miRNA

We used bioinformatics related tools (TargetScan) to predict the target genes of miR-142-3p. In this study, primers were designed based on the predicted binding sites of miR-142-3p and target genes or mutations containing binding sites (target gene 3'UTR, Table 1). We amplified the 3'UTR sequence and constructed a 3'UTR luciferase reporter gene vector. Plasmid and miRNA were co-transfected into 293T cells, and the fluorescence intensity was measured by Promega GloMax 20/20 Luminometer.

Cell proliferation assay

CCK-8: Cells were inoculated to 96-well cell culture plates in the form of 1×10^5/mL. When the cells adhered to the wall and grew to about 90%, the cells were transfected according to different test groups. Change to complete medium after 6 hours. Discard the supernatant after 24 hours, then add the CCK-8 solution (dilute the medium and the CCK-8 stock solution at a ratio of 1:9), and react for 4 hours. Finally, the OD value was detected at 450nm in the microplate reader.

Apoptosis detection

FITC-Anexin-V/PI double staining method: cells are s inoculated to 6-well cell culture plate. When the cells adhere to the wall and grow to about 105-106, the cells are transfected according to different groups. Change to complete medium after 6 hours, digest with trypsin after 24 hours, and wash with PBS. Then discard the supernatant and add 500μL Binding Buffer to resuspend the cells.

Finally, according to the kit (Keygen BioTBCH: KGA108-2) instructions, add 5μL Annexin V-FITC and 5μL Propidium lodide staining solution respectively, and test on the flow cytometer.

Cell migration and cell cycle detection

The cell scratch method was used to detect the cell migration rate. The cell cycle is detected using a cell cycle detection kit (Keygen BioTBCH). The experimental operation was performed according to the kit instructions. Finally, use flow cytometry for detection and analysis. The data was sorted and analyzed using the cell cycle fitting software ModiFit.

Statistical analysis

All experimental data in our study were obtained through three repeated independent experiments, and the results are expressed as 'mean ± standard deviation'. According to the experimental conditions, t-test or ANOVA was selected to evaluate whether it is statistically significant ($p < 0.05$ indicates significant and statistically significant).

Results

Transfection efficiency

In this study, we conducted a series of experiments such as RNA extraction, fluorescent quantitative PCR (relevant primers are summarized in Table 1), western blot, etc. The results showed that after we added HMGB1 inhibitor (100 μM), the expression level of HMGB1 protein was significantly reduced (Figure 1A and 1B). After cell transfection, we tested the transfection efficiency. The results showed that the knockdown miR-142-3p group could significantly reduce the expression level compared with the control group, and the transfection efficiency reached 82% (Figure 1C).

HMGB1 is miR-142-3p target gene

Results of double luciferase assay: first, we found that miR-142-3p may target *HMGB1* through bioinformatics related tools (TargetScan) in the early stage. And mutants of mut-*HMGB1* appeared in the predicted binding region (miR-142-3p and *HMGB1* 3'UTR) in this study (Figure 2A). Then, we successfully constructed two vectors, which are wild-type wt-HMGB1 and mut-HMGB1 luciferase reporter vectors. The results (Figure 2B) showed that in the cells transfected with miR-142-3p mimic, the luciferase activity of wt-HMGB1 vector was significantly reduced ($p < 0.05$). However, miR-142-3p mimic has no significant effect on mut-HMGB1. In short, we can conclude that the target gene of miR-142-3p is *HMGB1*.

Cell proliferation and apoptosis

Cell proliferation: the results of protein detection (Figure. 3A) and RT-PCR (Figure. 3B) showed that the group of miR-142-3p inhibitor could significantly increase the gene and protein expression levels (*HMGB 1*). However, when we added *HMGB1* inhibitor, the expression level was significantly reduced. The above results strongly indicate that miR-142-3p has a negative regulatory effect on *HMGB 1*. As shown in Figure 3C, miR-142-3p inhibitor significantly inhibited the proliferation of cartilage end plate cells ($p < 0.01$), and the cell proliferation ability was restored after adding *HMGB 1* inhibitor.

Flow cytometry to detect apoptosis: the results showed (Figure 3D and 3E) that compared with the control group, knocking down miR-142-3p significantly promoted the apoptosis of cartilage endplate cells, and the apoptosis rate reached 8.10%. But when the *HMGB 1* inhibitor was added, it significantly improved the effect of knocking down miR-142-3p on CEP apoptosis, and the apoptosis rate will drop to 2.47%.

Detection results of proteins related apoptotic: the results of RT-PCR (Figure 4A) and western blot (Figure 4B and 4C) showed that knocking down miR-142-3p significantly increased the gene expression level of the apoptosis gene Bax ($p < 0.01$), and the protein expression also showed a significant increase ($p < 0.05$). However, gene and protein expression levels of Bax were significantly restored after adding the *HMGB1* inhibitor. Conversely, knocking down miR-142-3p will significantly reduce the gene expression level of the Bcl-2 related to anti-apoptotic ($p < 0.01$), and the protein expression also showed a significantly reduce ($p < 0.05$). However, Bcl-2 gene and protein expression levels were restored after adding *HMGB 1* inhibitor. The apoptotic protein test results also showed that knocking down miR-142-3p can promote the apoptosis of cartilage end plate cells, and the apoptosis is improved after adding

HMGB1 inhibitor. The above results also indicated that miR-142-3p knockdown promoted apoptosis of CEP cells, and the apoptosis may be improved after the addition of HMGB1 inhibitor.

Cell cycle and cell migration

Flow cytometry monitoring results showed (Figure 5A and 5B) that compared with the control group, knocking down miR-142-3p significantly increased the number of degenerative cartilage endplate cells in G0/G1 phase, but significantly reduced in S phase. Our results indicated that knocking down miR-142-3p may inhibit the cell cycle of CEP degeneration entering the S phase from G0/G1. And we found no other statistically significant results in other experimental groups.

The cell scratch test results showed (Figure 6A and 6B) that knocking down miR-142-3p significantly inhibited the migration of CEP cells, but when the *HMGB1* inhibitor was added, the cell migration ability was restored.

Autophagy

In our study, the autophagy-related proteins P62 and Beclin 1 were detected.

The results of RT-PCR showed that (Figure 7A): on the one hand, knocking down miR-142-3p significantly reduced the gene expression level of autophagy marker *P62*, while significantly increasing the gene expression level of *Beclin 1*. On the other hand, when the HMGB1 inhibitor was added, the gene expression levels of P62 and Beclin 1 were significantly increased and decreased, respectively.

Western blot obtained similar results to RT-PCR. (Figure 7B): knocking down miR-142-3p significantly reduced and increased the protein expression levels of P62 and Beclin 1, respectively. When HMGB1 inhibitor was added, the protein expression levels of P62 and Beclin 1 increased and decreased significantly. The above results indicated that the knockdown of miR-142-3p promotes autophagy of CEP cells, and this promotion effect may be restored after adding HMGB1 inhibitor.

Discussion

The mechanism of disc degeneration is complicated. Previous studies have found that the degeneration of CEP will cause a series of chain reactions to further increase the degree of degeneration (Buckwalter 1995). Therefore, cartilage endplate degeneration may be an important cause of intervertebral disc degeneration. Gruber et al. found that *HMGB1* gene expression levels have increased during the process of intervertebral disc degeneration. (Gruber and others 2015a). More importantly, previous studies have shown that the expression of *HMGB1* is regulated by microRNA in many diseases (Huang and others 2012). When we searched the literature, we found that miR-142-3p can inhibit the inflammatory response and apoptosis of chondrocytes in osteoarthritis by inhibiting the NF-κβ signaling pathway mediated by *HMGB1* (Wang and others 2016). These studies have suggested us that it is very meaningful to investigate the regulatory effects of miR-142-3p/HMGB1 in the degeneration of the intervertebral disc cartilage endplates and their regulatory mechanisms.

We have previously learned that *HMGB1* is highly expressed in cartilage endplate cells, and that miR-142-3p can target and regulate HMGB1 in cartilage end plate cells has been verified in our dual luciferase assay. We verified that miR-142-3p can target and regulate *HMGB1* in CEP cells in the results of dual luciferase detection. In this study, we discovered for the first time that miR-142-3p may have a negative regulatory effect on the expression of *HMGB1* in the proliferation, apoptosis, migration, autophagy and cell cycle of cartilage endplate degeneration cells. Specifically, we found that compared with control group (normal cells) or IN-miRNA-NC group (the negative control group that knocked down miRNA), knocking down miR-142-3p reduced the proliferation rate and migration rate of cartilage endplate cells, but the apoptosis rate was significantly increased. It also inhibits the cell cycle from G0/G1 phase into the S phase. However, when the *HMGB1* inhibitor was added, it can reduce the inhibitory effect of miR-142-3p knockdown on cell proliferation and migration to a certain extent, and improve the apoptosis rate of CEP cells. The results of our study were similar to those previous studies. Schwickert et al. (Schwickert and others 2015) and Lei et al. (Lei and others 2014) found that miR-142-3p can inhibit breast cancer and non-small cell lung cancer, respectively. and miR-142-3p was considered to be a microRNA that can inhibit tumors. Moreover, previous studies have also reported that miR-142-3p may inhibit chondrocyte apoptosis and inflammation in osteoarthritis by regulating *HMGB1* (Wang and others 2016). The results of above research and our study can suggest that the negative regulation between miR-142-3p and *HMGB1* affects the proliferation, apoptosis and migration of cartilage endplate cells.

In addition, we also found that miR-142-3p and *HMGB1* are negatively correlated in the expression of apoptosis-related proteins or autophagy-related proteins of CEP cells, and the results are statistically significant. The above suggests that the regulatory relationship between miR-142-3p and *HMGB1* may be involved in related apoptosis or autophagy pathways, but the specific molecular mechanism is still unclear. We need to do more necessary, rigorous and comprehensive experiments to verify our results.

What is more worthy of our attention is that studies have confirmed that autophagy is involved in various processes such as the regulation of biological development and metabolism, apoptosis and aging (Levine and Kroemer 2019). At the same time, autophagy was also considered to be involved in the occurrence and development of a variety of degenerative diseases (Leidal and others 2018), including the pathogenesis of intervertebral disc degeneration (Chen and Lv 2017). Gruber et al. (Gruber and others 2015b) found that the expression of autophagy-related Beclin 1 in degenerated annulus fibrosus tissue was more significantly up-regulated when compared with normal human annulus fibrosus tissue. Combined with our results, we speculated that the regulation of miR-142-3p/ HMGB1 in CEP cells may be involved in the process of cartilage endplate degeneration. Our results suggest that it may be possible to prevent cartilage endplate degeneration by promoting high expression of miR-142-3p to inhibit the expression level of HMGB 1. These results suggested that it may be possible to prevent cartilage endplate degeneration by promoting the high expression of miR-142-3p to inhibit the expression level of *HMGB 1*.

Conclusion

In summary, our study is the first to find a negative correlation between miR-142-3p and *HMGB1* in cartilage endplate cells. The regulation between them can affect the proliferation, apoptosis and migration of cartilage end plate cells. Although the specific molecular mechanism or the specific involved are not yet clear, our results provide a new idea for the targeted treatment of cartilage endplate degeneration, that is, it may be possible to inhibit the expression of *HMGB 1* through the regulation of miR-142-3p.

List Of Abbreviations

Cartilage endplate (CEP); disc proscription disease (DDD); High Mobility Group Protein (HMGB1).

Declarations

Ethics approval and consent to participate

This study was conducted under the standard approved by the Second Affiliated Hospital of Inner Mongolia Medical University.

Consent for publication

All the authors Known and agreed to publish the manuscript.

Availability of data and materials

We confirmed that the data of this study have been included in the manuscript.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contribution statement

Conceptualization, Xuejun Yang and Yong Zhu; methodology, Bo Wang and Demin Ji; software, Wenhua Xing, Feng Li and Zhi Huang; data curation, Wenhua Xing, Wenkai Zheng and Jianmin Xue; writing, review and editing, Bo Wang and Demin Ji.

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Table

Table 1

All primers used for RT-PCR.

Gene/miRNA	Primer sequence (5'-3')	
M-miR-142-3p	F	GCGCGTGTAGTGTTTCCTACTTTATGGA
BAX	F	AGACAGGGGCCTTTTTGCTAC
	R	AATTCGCCGGAGACACTCG
Bcl2	F	GTGGATGACTGAGTACCTGAACC
	R	AGCCAGGAGAAATCAAACAGAG
P62	F	GTGGGACAGCCAGAGGAACA
	R	GCCCTTCCGATTCTGGCAT
Beclin	F	GGGTCACCATCCAGGAACTCA
	R	CACCATCCTGGCGAGTTTCA
HMGB1	F	CAAGGACCCCAATGCACCCAAG
	R	AAGCCAGGATGCTCGCCTTTG
HMGB1 3' UTR-mut	F	ATAGTTAACAGAGTTCCGAATGTGTCTTTAGATAGC
	R	ACACATTCGGAACTCTGTTAACTATACAAAAAAAGA

F: forword; R: reverse.

Figures

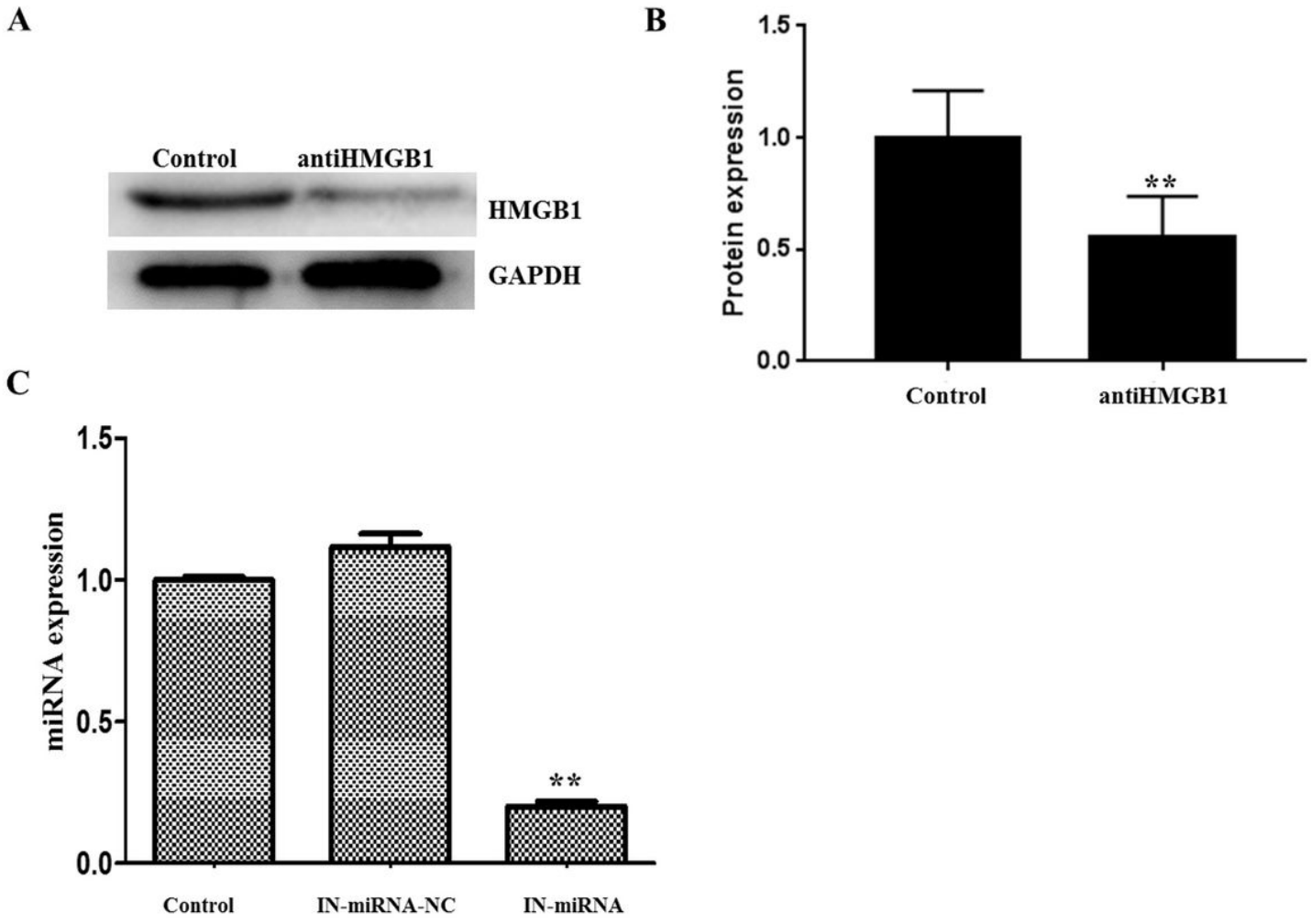

Figure 1

Selection of HMGB1 inhibitor (A: RT-PCR, B: western blot; 100 μM HMGB1 inhibitor can effectively inhibit the expression level of HMGB1) and verification of transfection efficiency (C: the transfection efficiency was 82%; '**' indicate that $p < 0.01$).

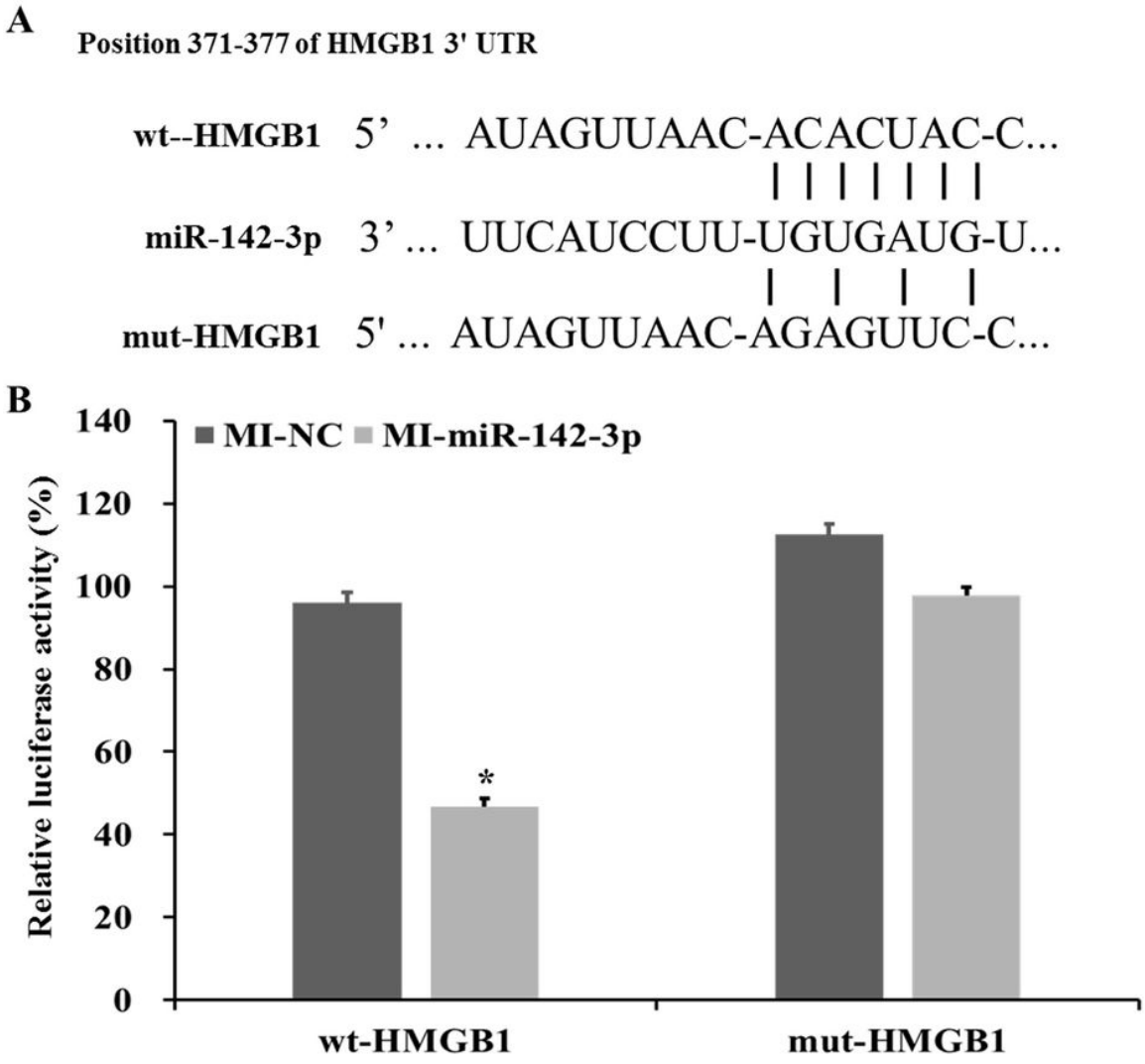

Figure 2

(A) predict the target gene of miR-142-3p through bioinformatics (TargetScan) and mut-HMGB1 set according to the binding site (mutation is in the predicted binding site of miR-142-3p and HMGB1); (B) the luciferase activities of wt-HMGB1 and mut-HMGB1 were monitored in different experimental groups.

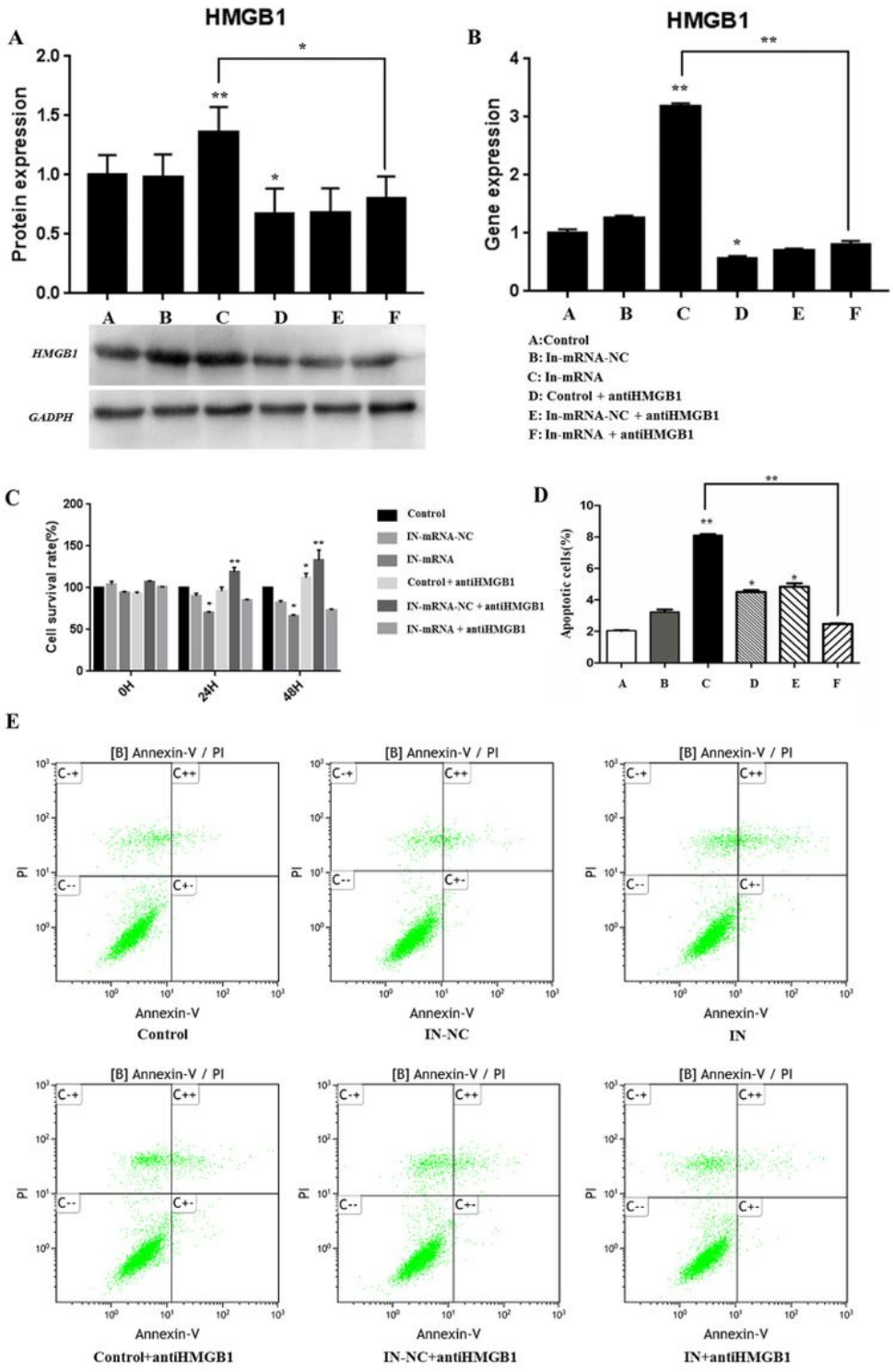

Figure 3

detect the regulatory effect of miR-142-3p on HMGB1 by western blot (A) and RT-PCR (B); (C) cell proliferation detection (monitoring at 0h, 24h and 48 h respectively); (D) determination of cell apoptosis rate; (E) flow cytometry to detect cell apoptosis rate.

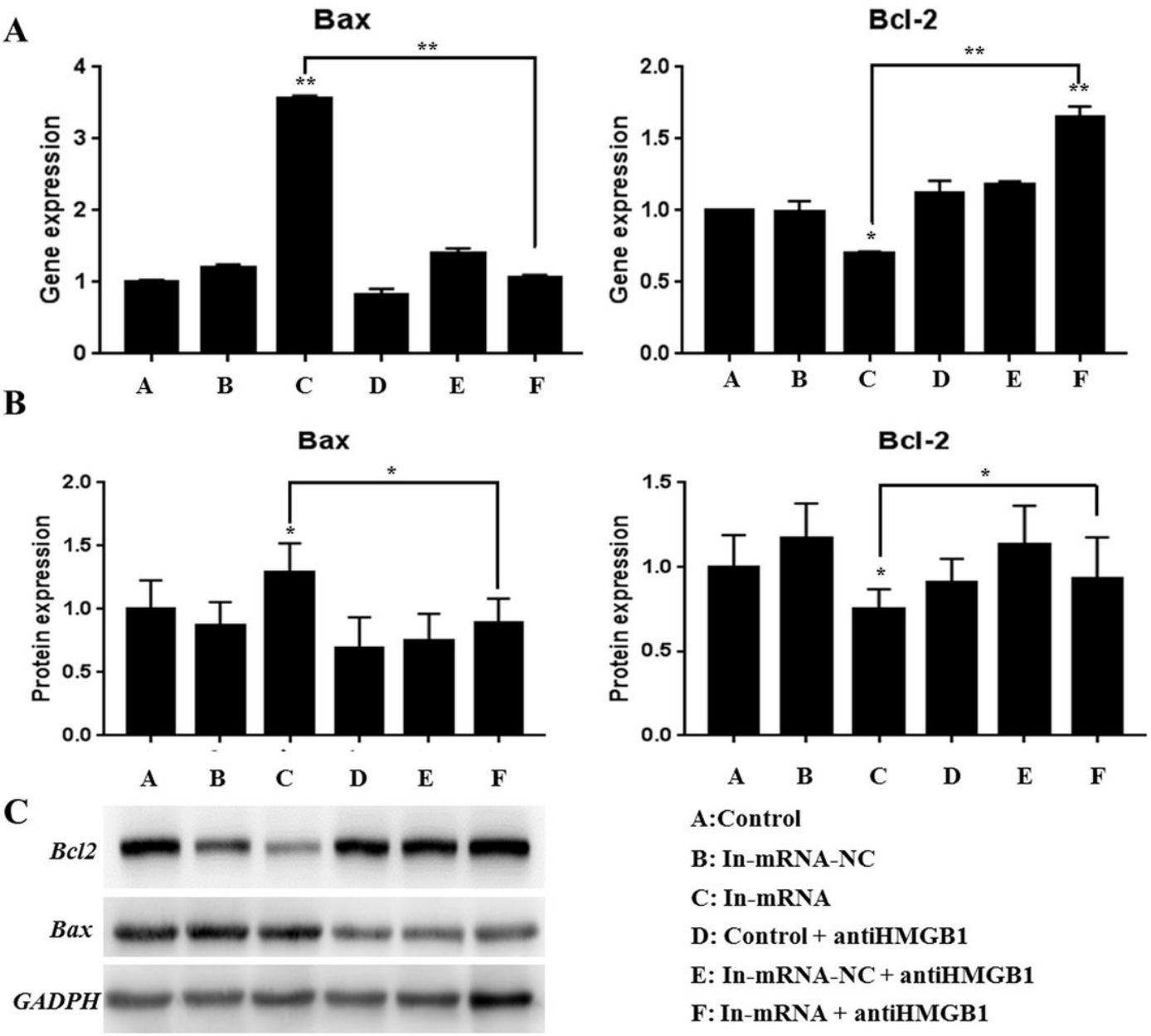

Figure 4

detection of apoptosis-related proteins (A: RT-PCR to detect gene expression level; B and C: western blot to detect protein expression level).

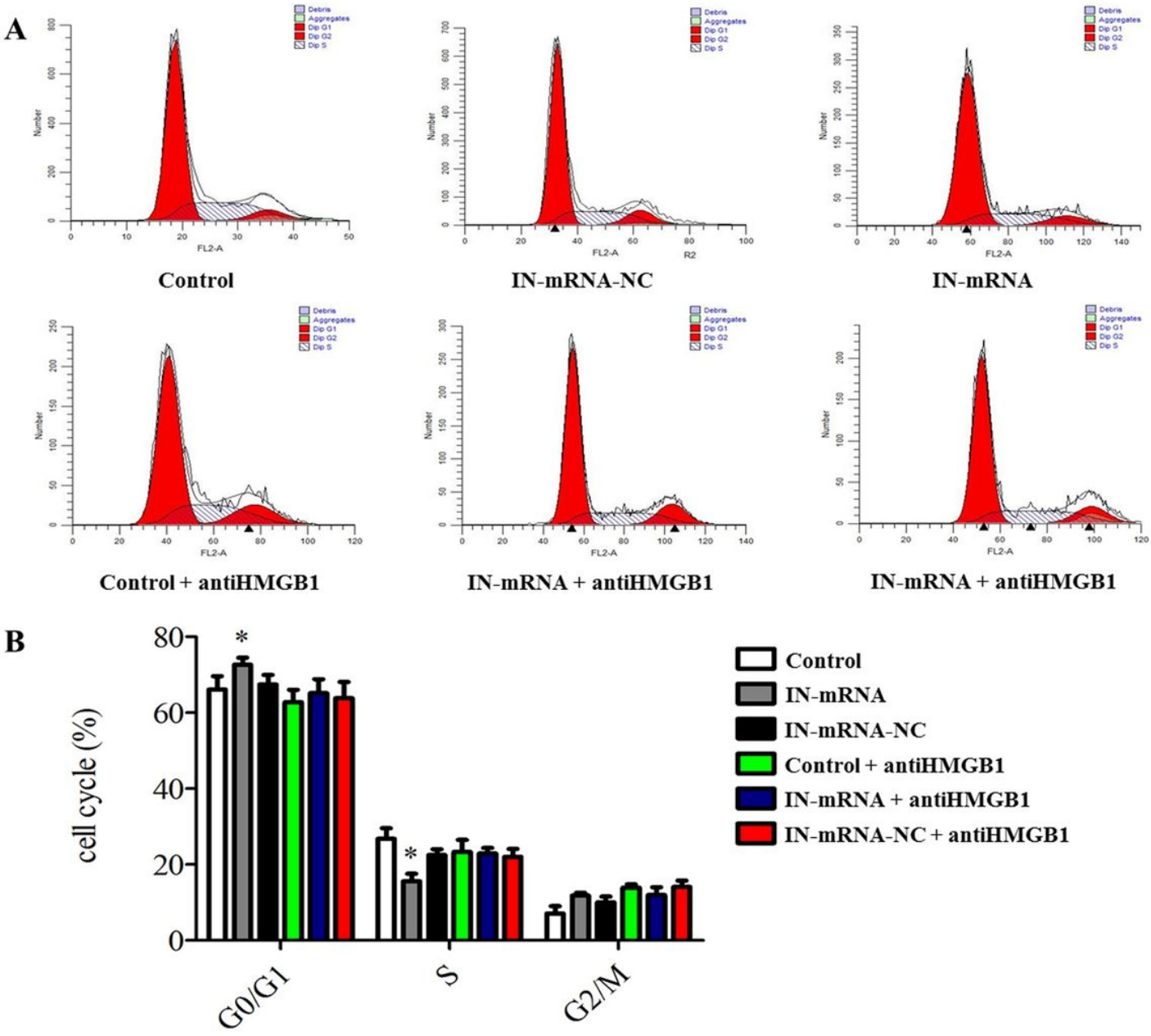

Figure 5

cell cycle detection (A: flow cytometry to detect cell cycle in different experimental groups; B: Knockdown of miR-142-3p can inhibit the degeneration of cartilage endplate cells from entering S phase from G0/G1 phase).

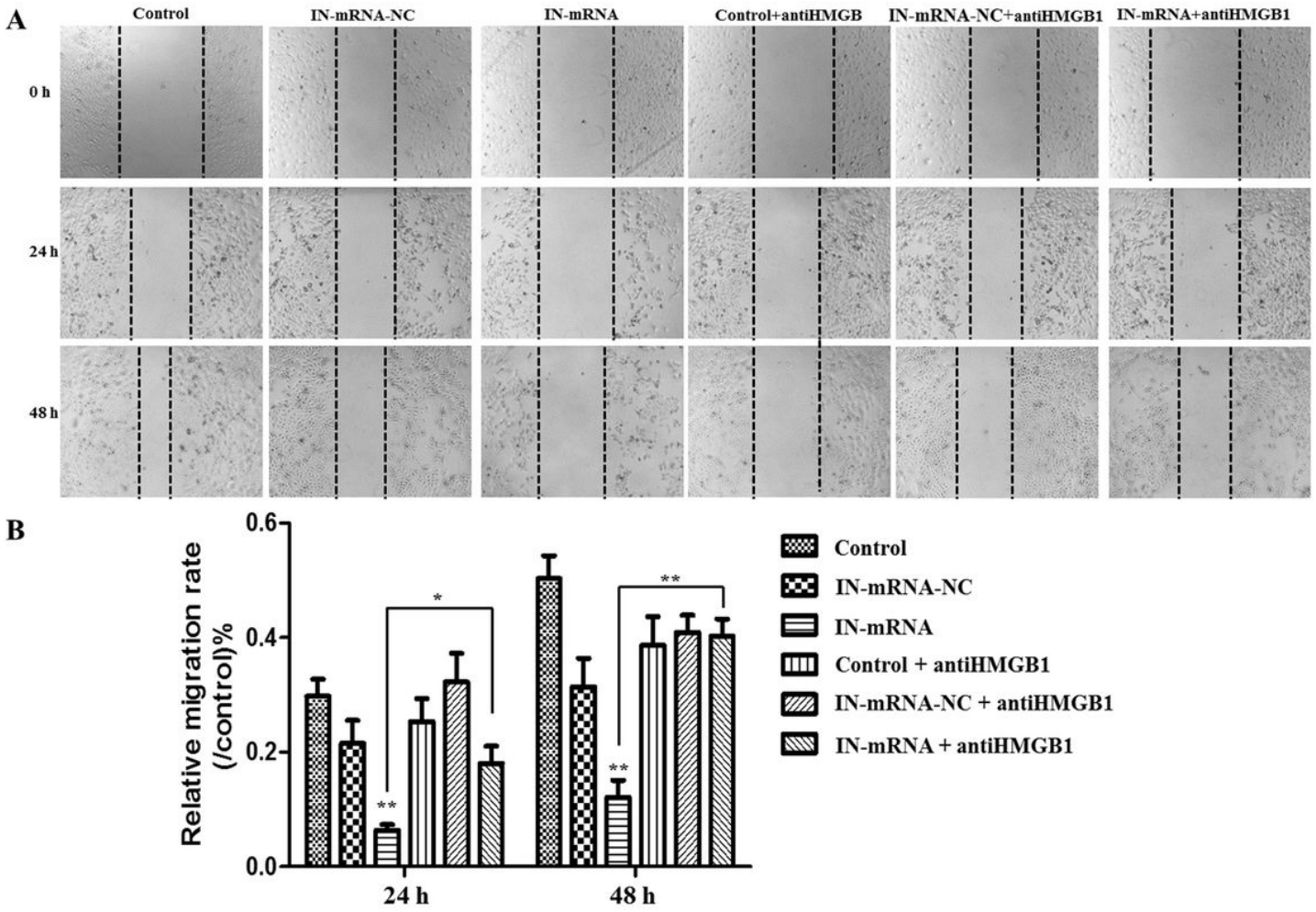

Figure 6

detection of cell migration (A: use cell scratch method to detect cell migration in different experimental groups; B: Knockdown of miR-142-3p significantly inhibits the migration of cartilage degenerative cells, after adding HMGB1 inhibitor, the cell migration ability can be restored).

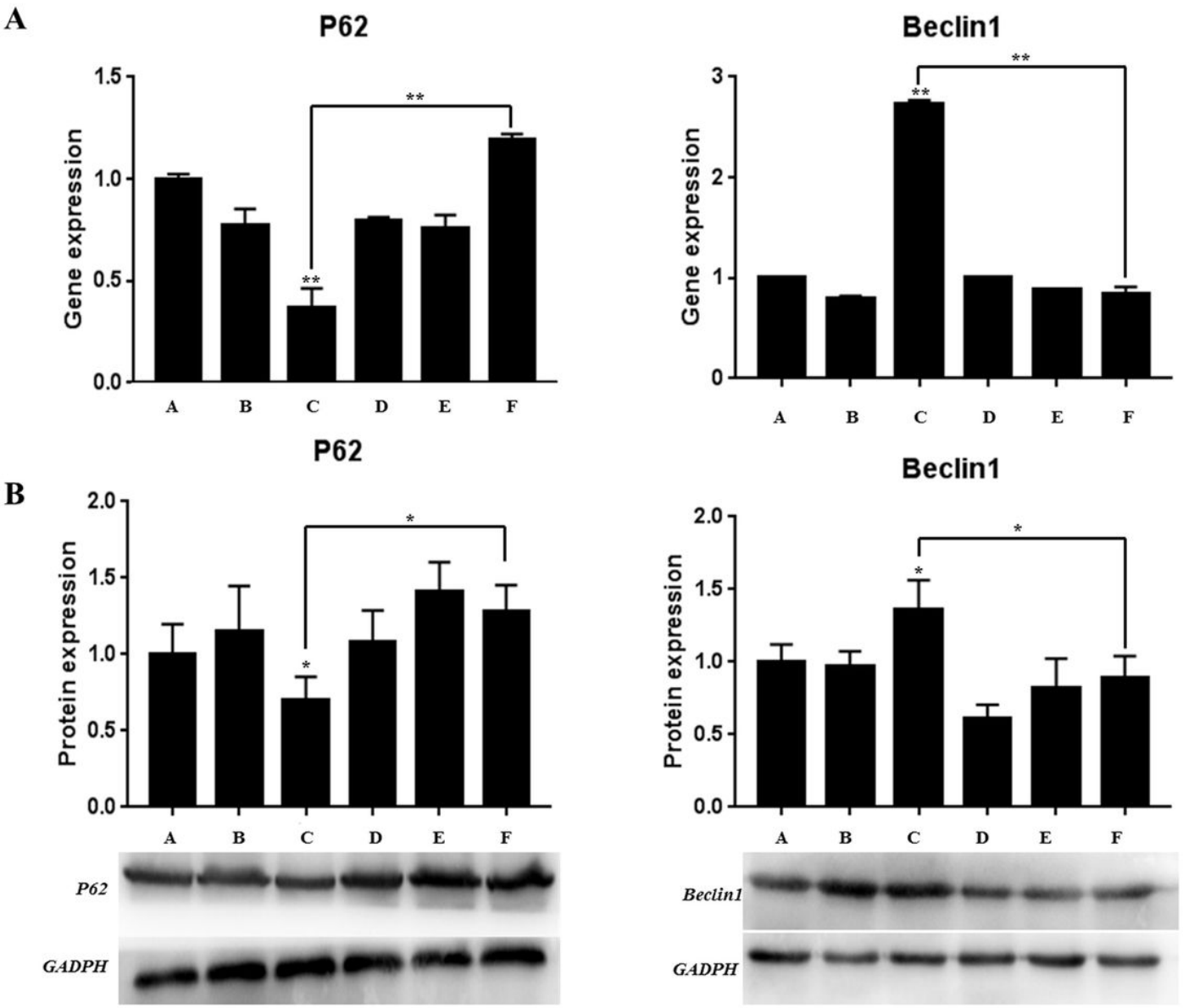

Figure 7

the detection of autophagy-related protein (A: detection of gene expression level by RT-PCR method; B: detection of protein expression level by western blot method).