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

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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/HMGB1 or 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 polymerase chain reaction was used to determine gene expression levels, and western blot analysis was used to determine protein expression levels. We chose 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 of 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 provide a new idea for the targeted treatment of CEP degeneration by inhibiting the expression of HMGB1.

**Keywords**

endplate chondrocytes, miR-142-3p, HMGB1, proliferation, apoptosis, autophagy

## Introduction

Degenerative disc disease 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.[1] Although the exact molecular mechanism of intervertebral disc degeneration is not clear, current studies suggested that apoptosis due to the various nonphysiological reasons may be the main cause of intervertebral disc cell decline.[2-4] Studies have shown that oxidative stress-induced ferroptosis of annulus fibroblasts and nucleus pulposus cells is related to the pathogenesis of intervertebral disc degeneration.[5] In the process of intervertebral disc degeneration, fibrous annulus rupture, inflammation, or cartilage endplate (CEP) degradation will induce the production of reactive oxygen species and aggravate the

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oxidative stress of nucleus pulposus cells.[6] There have been many studies on the molecular mechanism of annulus fibrosus and nucleus pulposus degeneration affecting intervertebral disc degeneration for a long time.[7-9] However, there are relatively few studies on the molecular mechanism of CEP degeneration affecting intervertebral disc degeneration. Studies have shown that intervertebral discs are the largest hypoxic tissue in the human body.[10-12] However, the degeneration of the CEP at a certain level may lead to a decline in 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.[13,14] It is obvious that CEP cell degeneration may play a vital role in the pathological process of intervertebral disc degeneration, and prevention or inhibition of CEP cell degeneration may be a potential target for intervertebral disc degeneration. Therefore, it is necessary to explore the molecular mechanism of CEP degeneration.

MicroRNA (miRNA) is a single-stranded RNA molecule consisting of about 20 to 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.[15,16] Some miRNAs associated with various orthopedic diseases have been reported in previous studies, such as miR-34a,[17,18] miR-181a,[19,20] and miR-142-3p.[21] More important, 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.[21]

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.[22] Andersson and Harris[23] found that the expression level of *HMGB1* was significantly increased in synovial membrane and serum of patients with rheumatoid arthritis, and 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.[24] More important, 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.[25] These studies have all shown that HMGB1 plays an important role in the degeneration of CEP.

Subsequently, through literature review, we found that there were no reports on the regulation of miR-142-3p/HMGB1 in CEP cells. Therefore, in this study, we studied the expression regulation mechanism of miR-142-3p/HMGB1 in CEP cells to further understand the potential mechanism of action of miR-142-3p/HMGB1 in CEP 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% to 90%, the cell passage begins. We add digestion solution containing 0.25% trypsin and 0.01% EDTA (1:1) for 1 minute. 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 $2 \times 10^5$/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 manufacturer's 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 polymerase chain reaction (RT-PCR) kits used in this study include Takara's TB Green Premix ExTaq II (Tli RNaseH Plus) and PrimeScript RT Master Mix (Perfect Real Time), and TIANGEN's miRcute enhanced miRNA cDNA first-strand

**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 = forward; R = reverse; RT-PCR = real-time polymerase chain reaction.

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 are listed 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 precooled PBS (phosphate-buffered saline), 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 hour, and then centrifuged at low temperature for 30 minutes. 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 1 hour), incubate primary antibody, wash membrane again, incubate secondary antibody (HRP-labeled antibody), and color development (Clarity 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 $1 \times 10^5$/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 450 nm in the microplate reader.

## *Apoptosis Detection*

*FITC-Anexin-V/PI double-staining method*: Cells are inoculated to 6-well cell culture plates. When the cells adhere to the wall and grow to about 105 to 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 500 μL Binding Buffer to resuspend the cells.

Finally, according to the manufacturer's instructions (Keygen BioTBCH: KGA108-2), add 5 μL Annexin V-FITC and 5 μL propidium iodide 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 manufacturer's instructions. Finally,

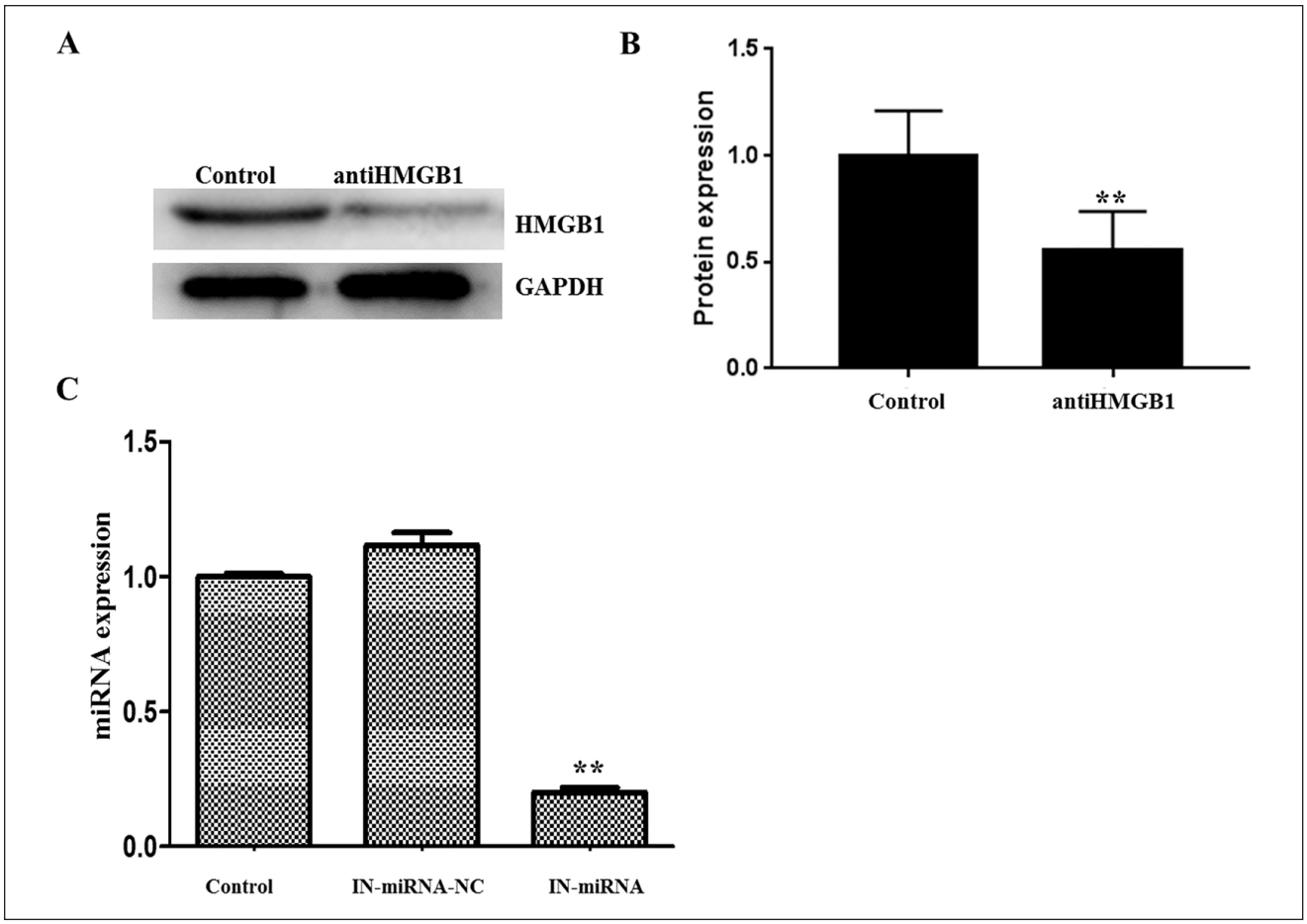


**Figure 1.** Selection of HMGB1 inhibitor (**A**: RT-PCR, **B**: western blot; 100 μM HMGB1 inhibitor can effectively inhibit the expression level of HMGB1) and verification of transfection efficiency (**C**: the transfection efficiency was 82%; "**" indicates that $P < 0.01$).

use flow cytometry for detection and analysis. The data were sorted and analyzed using the cell cycle fitting software ModiFit.

### *Statistical Analysis*

All experimental data in our study were obtained through 3 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 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, and so on. The results showed that after we added HMGB1 inhibitor (100 μM), the expression level of HMGB1 protein was significantly reduced (**Fig. 1A** and **B**). 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% (**Fig. 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 (**Fig. 2A**). Then, we successfully constructed 2 vectors, which are wild-type wt-HMGB1 and mut-HMGB1 luciferase reporter vectors. The results (**Fig. 2B**) showed that in the cells transfected with miR-142-3p mimic, the luciferase activity of

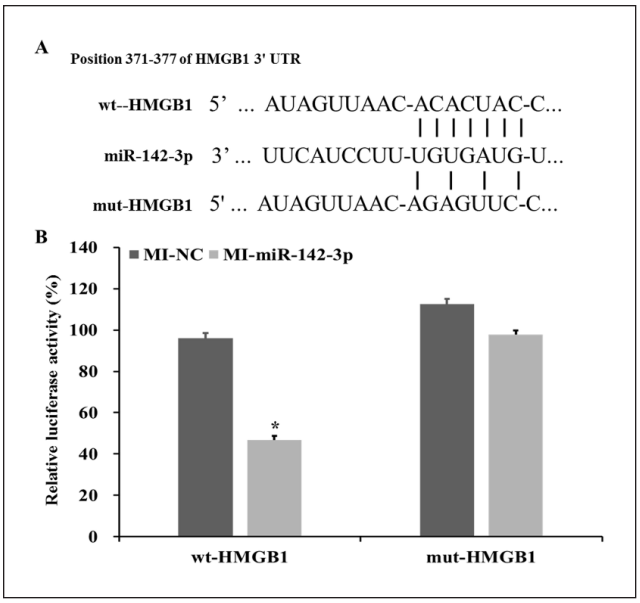


**Figure 2.** (**A**) Predict the target gene of miR-142-3p through bioinformatics (TargetScan) and mut-HMGB1 set according to the binding site (mutation is in the predicted binding site of miR-142-3p and HMGB1). (**B**) The luciferase activities of wt-HMGB1 and mut-HMGB1 were monitored in different experimental groups.

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 (**Fig. 3A**) and RT-PCR (**Fig. 3B**) showed that the group of miR-142-3p inhibitor could significantly increase the gene and protein expression levels (*HMGB1*). 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 *HMGB1*. As shown in **Fig. 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 *HMGB1* inhibitor.

*Flow Cytometry to Detect Apoptosis.* The results showed (**Fig. 3D** and **E**) that compared with the control group, knocking down of miR-142-3p significantly promoted the apoptosis of CEP cells, and the apoptosis rate reached 8.10%. But when the *HMGB1* inhibitor was added, it significantly improved the effect of knocking down miR-142-3p on CEP apoptosis, and the apoptosis rate dropped to 2.47%.

*Detection Results of Proteins Related Apoptotic.* The results of RT-PCR (**Fig. 4A**) and western blot (**Fig. 4B** and **C**) showed that knocking down of 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 reduction ($P < 0.05$). However, Bcl-2 gene and protein expression levels were restored after adding *HMGB1* 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 (**Fig. 5A** and **B**) that compared with the control group, knocking down miR-142-3p significantly increased the number of degenerative CEP 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 (**Fig. 6A** and **B**) 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 (**Fig. 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 (**Fig. 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

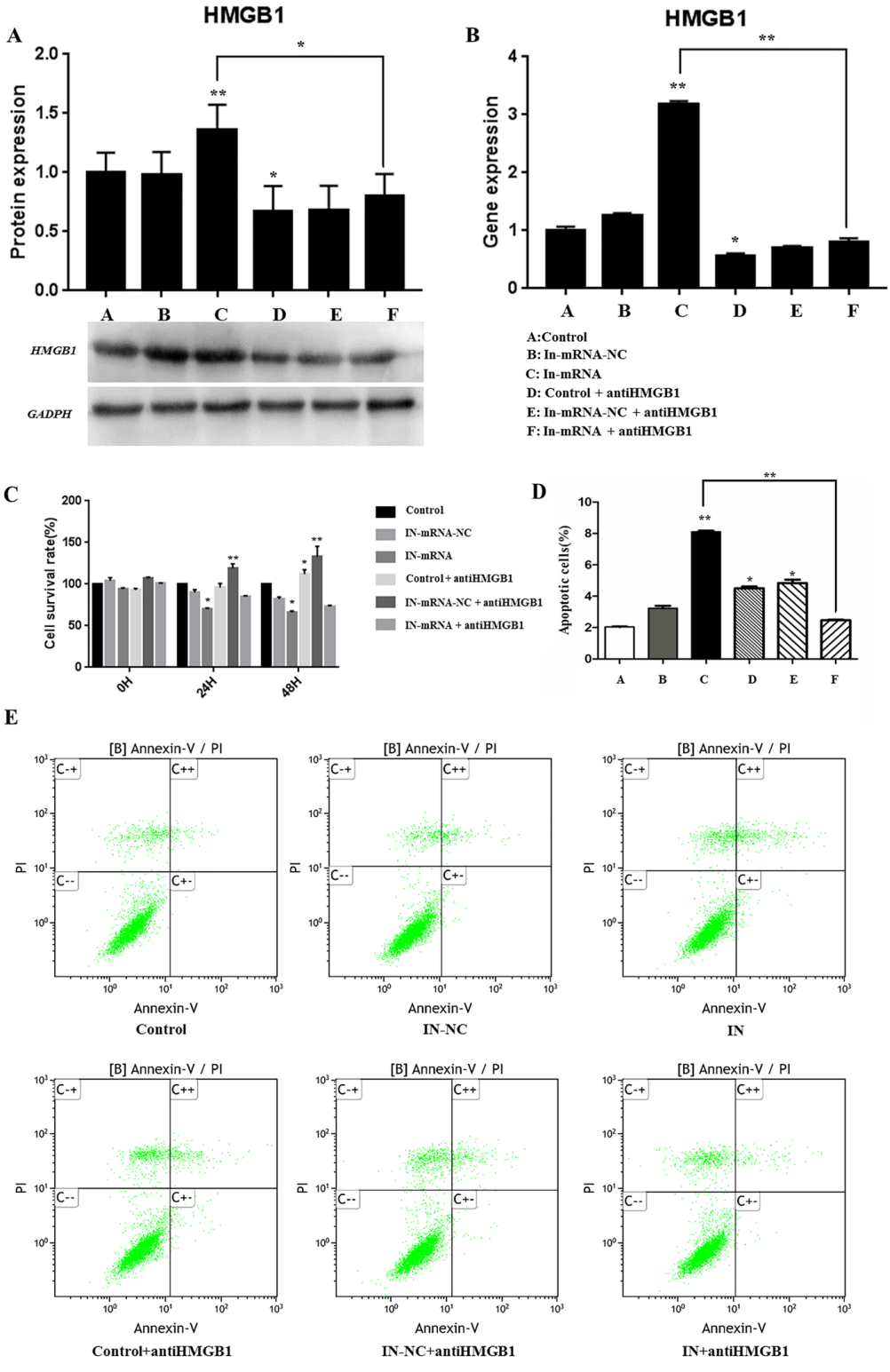


**Figure 3.** Detection of the regulatory effect of miR-142-3p on HMGB1 by western blot (**A**) and RT-PCR (**B**). (**C**) Cell proliferation detection (monitoring at 0 hours, 24 hours, and 48 hours, respectively). (**D**) Determination of cell apoptosis rate. (**E**) Flow cytometry to detect cell apoptosis rate.

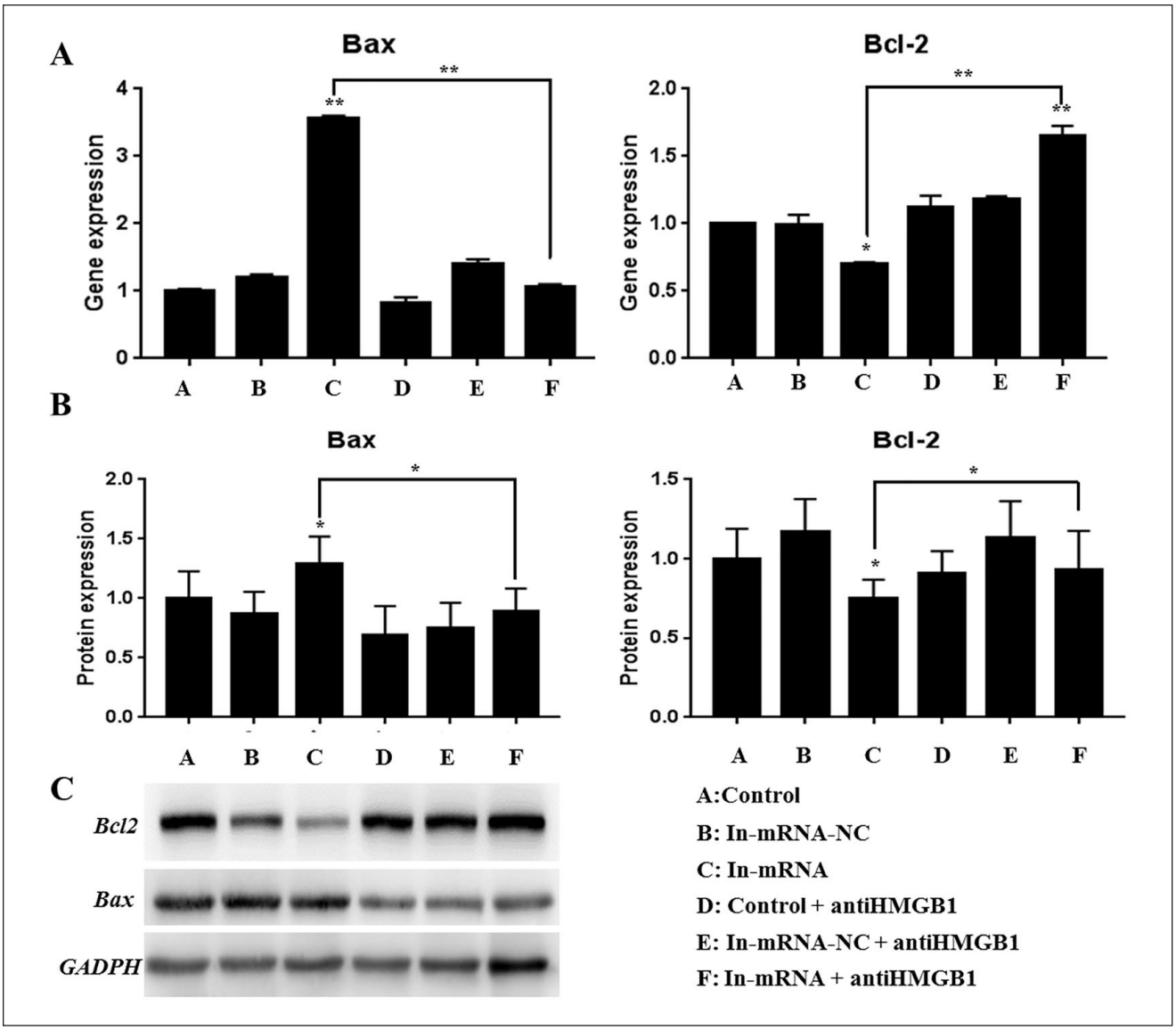


**Figure 4.** Detection of apoptosis-related proteins (**A**: RT-PCR to detect gene expression level; **B** and **C**: western blot to detect protein expression level).

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.[26] Therefore, CEP 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.[24] More important, previous studies have shown that the expression of *HMGB1* is regulated by microRNA in many diseases.[27] 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*.[21] These studies have suggested that it is very meaningful to investigate the regulatory effects of miR-142-3p/HMGB1 in the degeneration of the intervertebral disc CEPs and their regulatory mechanisms.

We have previously learned that *HMGB1* is highly expressed in CEP 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,

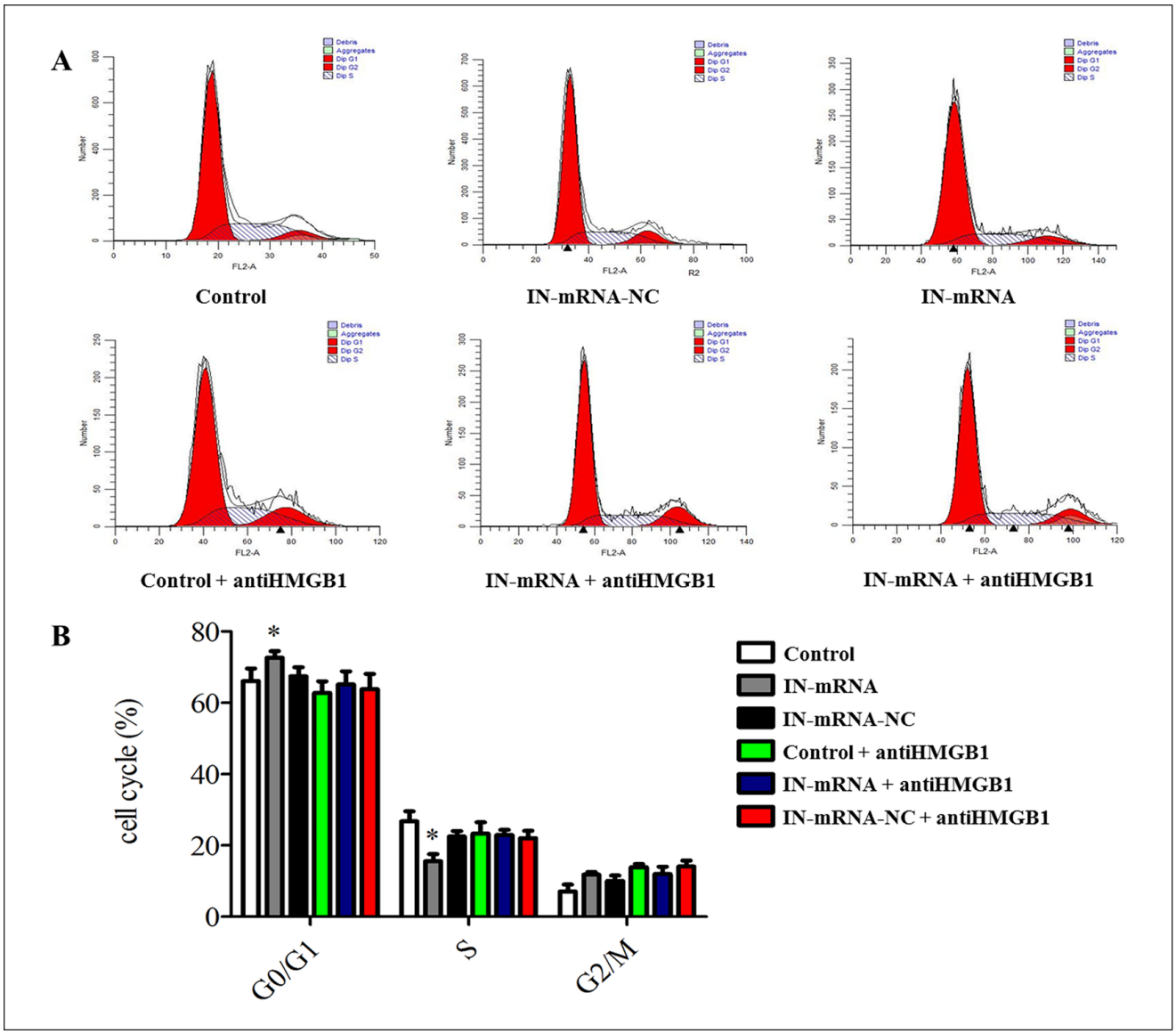


**Figure 5.** Cell cycle detection (**A**: flow cytometry to detect cell cycle in different experimental groups; **B**: knockdown of miR-142-3p can inhibit the degeneration of cartilage endplate cells from entering S phase from G0/G1 phase).

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 CEP 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 CEP 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*.[28] and Lei *et al*.[29] 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*.[21] 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 CEP cells.

In addition, we also found that miR-142-3p and *HMGB1* are negatively correlated in the expression of

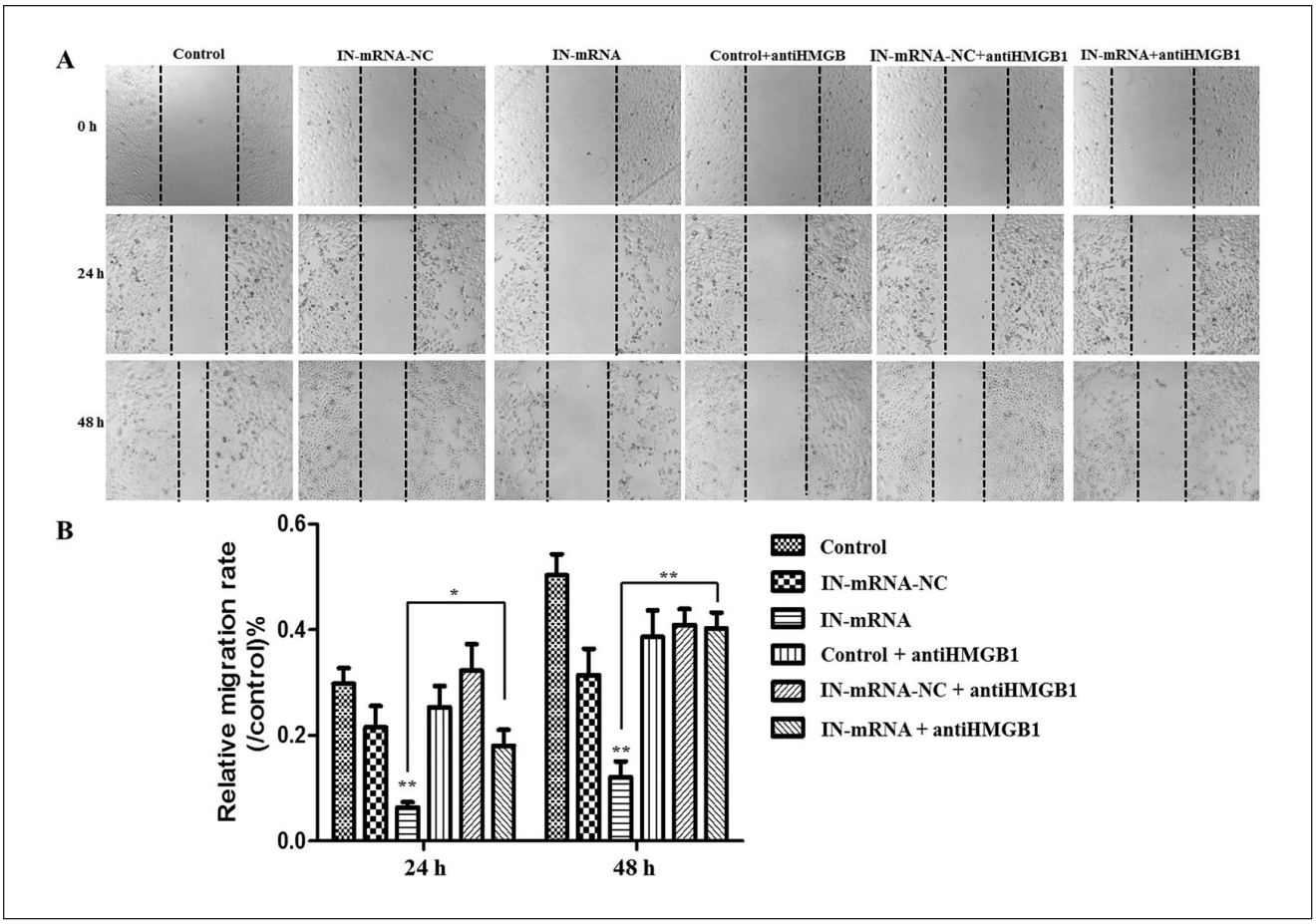


**Figure 6.** Detection of cell migration (**A**: use cell scratch method to detect cell migration in different experimental groups; **B**: Knockdown of miR-142-3p significantly inhibits the migration of cartilage degenerative cells, after adding HMGB1 inhibitor, the cell migration ability can be restored).

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.[30] At the same time, autophagy was also considered to be involved in the occurrence and development of a variety of degenerative diseases,[31] including the pathogenesis of intervertebral disc degeneration.[32] Gruber *et al*.[33] found that the expression of autophagy-related Beclin 1 in degenerated annulus fibrosus tissue was more significantly upregulated 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 CEP degeneration. Our results suggest that it may be possible to prevent CEP degeneration by promoting high expression of miR-142-3p to inhibit the expression level of HMGB1. These results suggested that it may be possible to prevent CEP degeneration by promoting the high expression of miR-142-3p to inhibit the expression level of *HMGB1*.

## Conclusion

In summary, our study is the first to find a negative correlation between miR-142-3p and *HMGB1* in CEP 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 CEP degeneration, that is, it may be possible to inhibit the expression of *HMGB1* through the regulation of miR-142-3p.

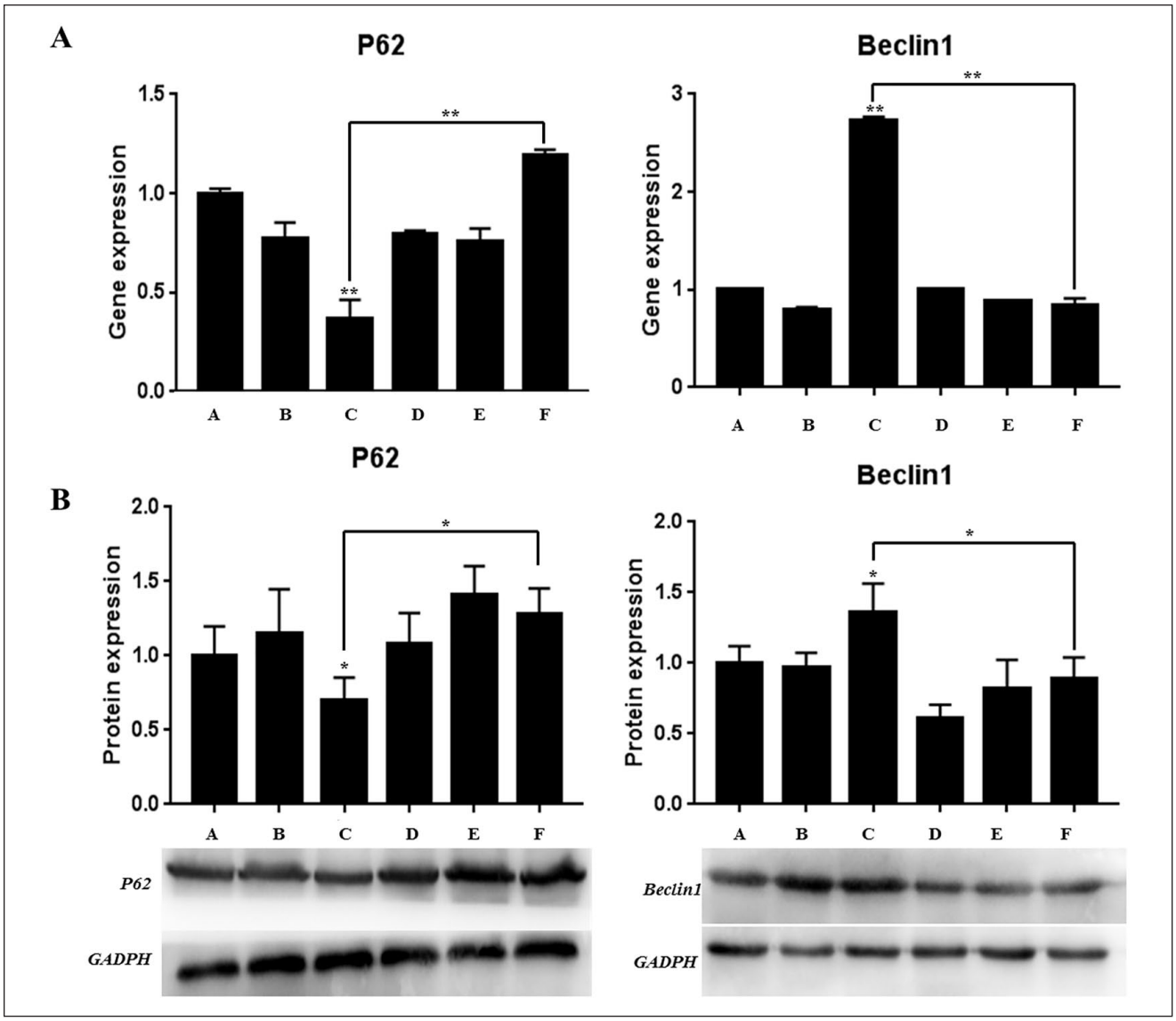


**Figure 7.** The detection of autophagy-related protein (**A**: detection of gene expression level by RT-PCR method; **B**: detection of protein expression level by western blot method).

## Author Contributions

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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## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Ethical Approval

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

## Informed Consent

Not applicable

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