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# The significant role of ATG5 in the maintenance of normal functions of Mc3T3-E1 osteoblast

Y.-M. WENG[1,2], C.-R. KE[2], J.-Z. KONG[2], H. CHEN[2], J.-J. HONG[2], D.-S. ZHOU[1]

[1]Department of Traumatic Orthopedics, Shandong Provincial Hospital, Shandong University, Jinan, China
[2]Department of Orthopedics, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, China

**Abstract. – OBJECTIVE: To observe the effects of autophagy-related gene 5 (ATG5) on the proliferation, differentiation, and apoptosis of Mc3T3-E1 osteoblast as well as the effects of ATG5 on apoptosis of osteoblasts under the conditions of non-oxidative stress and oxidative stress.**

**MATERIALS AND METHODS: ATG5 overexpressing and silencing cell lines were established in this experiment with lentiviral vector and transcription activator-like effect or nuclease (Talen) technique, respectively, using Mc3T3-E1 cells. Cell counting kit-8 (CCK-8) was used to detect the proliferation rate of osteoblasts, and flow cytometry was applied to detect the impacts of overexpressed and silenced ATG5 on the cell cycle. Alizarin red staining was used to detect the mineralization capacity of osteoblasts after 4-week osteoinduction differentiation. Quantitative Real-time polymerase chain reaction (qRT-PCR) and Western blot methods were adopted to detect the levels of gene and protein expressions of runt-related transcription factor 2 (Runx2), osteocalcin (OCN) and collagen I (COL-I) correlated with osteoblast differentiation after 48 h of osteoinduction differentiation. The staining with Annexin V-phycoerythrin/7-amino-actinomycin D (Annexin V-PE/7AAD) and flow cytometry were performed to detect the influence of ATG5 on osteoblast apoptosis.**

**RESULTS: Stable ATG5 overexpressing and silencing Mc3T3-E1 cell lines were established successfully. CCK-8 test results showed that ATG5 silence inhibited cell proliferation, but the overexpression of ATG5 did not result in an obvious change in cell proliferation. Cell cycle did not change when ATG5 was overexpressed, while was stagnated in S-phase when silenced. The number of mineralized nodules of cells was reduced notably when ATG5 was silenced, while the overexpression of ATG5 did not have an impact on mineralization capacity of the cell after 4-week of osteoinduction differentiation. The test results of qRT-PCR and Western blotting suggested that ATG5 silence inhibited the gene and protein expressions of Runx2, OCN, and COL-I, while the influence of overexpressed ATG5 on the expressions of genes related to osteoblastic differentiation was not obvious after 48 h of osteoinduction differentiation. ATG5 silence made the cells easier to be damaged by hydrogen peroxide, which resulted in the rise of apoptosis rate of osteoblasts, while the overexpressed ATG5 inhibited osteoblast apoptosis after treatment with hydrogen peroxide for 12 h.**

**CONCLUSIONS: ATG5 silence can lead to inhibition of osteoblast proliferation and differentiation. Moreover, it makes the cells easier to be damaged by oxidative stress, and it causes an increase in apoptosis. However, the overexpression of ATG5 strengthens the anti-oxidative capacity of osteoblasts and reduces apoptosis. ATG5 may be an effective target of anti-oxidative therapy for osteoporosis, which brings a new direction for the treatment of osteoporosis.**

*Key Words:*
Osteoporosis, ATG5, Autophagy, Proliferation, Apoptosis, Differentiation.

## Introduction

With the aging of the population, osteoporosis has become one of the most serious public health problems[1-3]. It is characterized by reduced bone mass and destruction of bone microstructure, which in turn lead to a decrease in bone strength and an increase in fragility, resulting in a series of clinical signs and symptoms such as pain and fractures. The maintenance of bone mass and bone microstructure depends on the dynamic balance of functions of osteoblasts and osteoclasts[4,5]. Various systemic and local hormones, growth factors and bioactive substances are involved in the maintenance of homeostasis


*Corresponding Author:* Dongsheng Zhou, MD; e-mail: yexia462909@163.com

of bone microenvironment. In addition, the effects of heredity, nutrition, mechanical stress, neural regulation, apoptosis, autophagy, oxidative stress response and other factors on the homeostasis of bone microenvironment have also been studied as hotspots. The direction of these researches ultimately comes down to the regulation of various factors on bone reconstruction. Autophagy is the main modulated way for cells to adapt themselves to the stress environment. Autophagy-related gene 5 (ATG5) is an important gene involved in autophagy. Mizushima et al[6] first identified that ATG5 of mammals is located in human chromosome 6q21, which contains 384 single nucleotide polymorphisms (SNP), encodes 276 amino acids, and participates in the occurrence and development of a variety of tumors[7-9]. Scholars have shown that ATG5, as a switch of autophagy and apoptosis, plays an important regulatory function in the occurrence and development of autophagy[10]. In the early stage of the formation of autophagic vacuoles, a complex formed by ATG12-ATG5-ATG16 binds to the external membranes of autophagic vacuoles, promoting the expansion of autophagic vacuoles as well as the movement and concentration of microtubule-associated protein light chain 3 (LC3) towards autophagic vacuoles. Sanjuan et al[11] have shown that lysosomes fail to fuse with phagosomes when ATG5 is deficient in macrophages. Moreover, Yousetl et al[12] found that the ATG5 protein specifically cleaved by the calcium-dependent neutral protease can be transferred from cytoplasm to mitochondria to regulate the release of cytochrome C and the activation of caspase, significantly promoting apoptosis. Recently, Li et al[13] have suggested that autophagy can reduce injury of oxidative stress on cells. However, there are many regulatory sites with various cellular cascade reactions in the downstream of autophagy signaling pathway. Excessive autophagy can lead to cell dysfunction, and even cell death[14,15]. Thus, we aimed to find key genes that could promote proliferation and differentiation of osteoblasts in the downstream genes of autophagy, thus accelerating proliferation and differentiation of osteoblasts, enhancing the anti-apoptotic ability of osteoblasts and improving the bone loss of osteoporosis. Given that ATG5 plays a key role in autophagy and is associated with mineralization of osteoblasts, gene knock-out and knock-in techniques were used to observe the effects of ATG5 on the proliferation, differentiation and apoptosis of Mc3T3-E1 osteoblast as well as the effects of ATG5 on apoptosis of osteoblasts under the conditions of oxidative and non-oxidative stress in this study.

## Materials and Methods

### Experimental Cells and Culture

Mouse pre-osteoblast cell line (Mc3T3-E1) was purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The cell culture medium was α-minimal essential medium (α-MEM), to which fetal bovine serum (FBS) concentration of 10% was added. The culture solution contained double antibodies (100 IU/mL penicillin G and 100 μg/mL streptomycin). The cells were inoculated in 10 cm culture plates, and placed in incubators containing 5% carbon dioxide ($CO_2$) at 37°C for culturing. The culture medium was replaced every other day, and the cells were passaged in the proportion of 1:6 after they were digested with 0.25% tyrosine.

### Experimental Cell Processing

Transcription activator-like effect or nuclease (TALENT) vector and lentiviral vector were used in this experiment to construct ATG5-silencing and overexpressing cell lines of Mc3T3-E1, respectively. 0.5 mM hydrogen peroxide-induced oxidative stress model of osteoblasts was adopted to observe the effects of ATG5 on the apoptosis rate of osteoblasts under the conditions of oxidative and non-oxidative stress, separately.

### Detection of Cell Survival Rate With Cell Counting kit-8 (CCK-8)

The cells in ATG5 group, green fluorescent protein (GFP) group, control (CON) group, silenced autophagy-related gene 5 (siATG5) group and silenced control (siCON) group, were inoculated in 96-well plates at a density of $2\times10^3$ cells/well. The proliferation activity of cells in each group was detected at 12 h, 24 h, 36 h, 72 h and 96 h, respectively. Three repeated wells were set up in each group. 10 μL CCK-8 solution were added to each well, and they were mixed well slightly. Optical density (OD) value at the wavelength of 450 nm was measured after the cells were cultured continuously for 1 h. The experiment was repeated for 3 times.

### Detection of Cell Cycle

Cells in logarithmic growth phase were taken from ATG5 group, GFP group, CON group, siA-

TG5 group and siCON group after 48-h stable cell transfection, and they were made into single cell suspension through conventional digestion. The suspension was inoculated in 6-well plates at a density of $1\times10^6$/mL. All the cells were collected 24 h later. About $1\times10^6$ cells were transferred to centrifugal tubes. They were centrifuged at 1,000 rpm for 65 min, followed by washing with phosphate-buffered solution (PBS) after the supernatant was discarded. About 0.5 mL PBS was reserved in the centrifugal tubes, and 5 mL 70% ice ethanol were added to the tubes and mixed well for fixation. Next, tubes were placed at 4°C for 48 h overnight; the ethanol was removed through centrifuging, and tubes were washed with PBS again. 1 mL PBS was left in the centrifugal tubes, and the cell mass was dispersed. 50 μg/mL propidium iodide (PI), 100 μg/mL ribonuclease A (RNaseA) and 0.2% polyethylene glycol octylphenol ether (Triton X-100) were added to the tubes, which were stained for 30 min at room temperature away from light. 30,000 cells were counted with a flow cytometer to analyze the phase of cell cycle and apoptosis.

### Detection of Differentiation Capacity of Osteoblasts With Alizarin Red Staining

Cells in each group were inoculated in 6-well plates in a density of $1.5\times10^6$ cells/well, and osteoblast-induced fluid (50 μg/mL ascorbic acid, 10 mmol/L glycerol-3-phosphate and 10 nmol/L dexamethasone) was added to the plates. After cells in each group were induced and cultured for 4 weeks, alizarin red staining was conducted, and the number of mineralized nodules was counted to compare the change in mineralization capacity of Mc3T3-E1 osteoblast between overexpressed ATG5 and silenced ATG5.

### Detection of Apoptosis Rate With Annexin V-Phycoerythrin/7-Amino-Actinomycin D (Annexin V-PE/7AAD) Double-Staining Method

After 48-h cell transfection, Mc3T3-E1 cells in ATG5 group, GFP group and CON group were processed for 12 h under oxidative and non-oxidative stress condition with 0.5 mM hydrogen peroxide, respectively, to detect the apoptosis rate. Detection method: after the cells in each group were processed, they were collected for counting. 100 μL buffer A, 5 μL Annexin V-PE and 5 μL 7-AAD were added to $10^5$ cells and incubated for 15 min at 37°C away from light. Another 400 μL buffer A were added, and a flow cytometer was used to carry out detection. Flow cytometry was adopted to count the apoptosis rate in the early and late stages, and that of dead cells in the late stage.

### Ribonucleic Acid (RNA) Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

TRIzol (Invitrogen, Carlsbad, CA, USA) was added to an appropriate amount of collected cells, and RNA of the cells was extracted according to the kit instructions. TaKaRa Primescript™ RT Reagent Kit (TaKaRa, Otsu, Shiga, Japan) was used to prepare a 10 μL reaction system. Reverse transcription was conducted to synthesize complementary DNA (cDNA). SYBR Premix Ex Taq reagent kit (TaKaRa, Otsu, Shiga, Japan) was used for amplification according to the instructions of the reagent kit during qRT-PCR.

### Western Blotting Analysis

The reagent kit for cell lysis solution was used to extract total protein after the cells were collected. Protein electrophoresis was conducted according to the standard procedures for Western-blot analysis after protein quantification with bicinchoninic acid (BCA). Corresponding antibodies of collagen I (COL-I), osteocalcin (OCN) and runt-related transcription factor 2 (Runx2) were used to detect the expression levels of protein in the cells of each group.

### Statistical Analysis

All the tests were repeated for at least three times. The data were expressed as mean ± standard errors of the mean (SEM). Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA) software was used for data analyses. Independent samples *t*-test was performed to compare each parameter between ATG5 group or siATG5 group and the control (CON) group. $p < 0.05$ suggested statistical differences.

## Results

### Impacts of ATG5 on Osteoblast Proliferation

Osteoblasts of the stably overexpressed ATG5 (ATG5 group), osteoblasts of empty vector (GFP group) and cells in the blank control group (CON group) were cultured for 0-96 h, respectively. CCK-8 reagent kits were used to detect cell growth condition every day. The curve of cell

proliferation and growth was drawn, and analysis of variance was conducted for the data. The results showed that there was no significant difference in cell growth rate between ATG5 group and the control group. Meanwhile, silenced cells in ATG5 group (siATG5), control group (siCON) and blank control group (CON group) were also observed. The results indicated that cell proliferation was inhibited due to ATG5 knockout, and the cells grew slower than those in the control group at 48 h, 72 h, and 96 h after culturing. These results preliminarily illustrated that the overexpression of ATG5 failed to promote cell growth and proliferation, but ATG5 knockout caused restriction of cell proliferation and decreased viability, suggesting that ATG5 plays an important role in maintaining normal cell functions (Figure 1).

### Impacts of ATG5 on Osteoblast Cycle

The results of ATG5 group, GFP group and CON group obtained by flow cytometry indicated that cell ratios in G1 phase, G2/M phase, and S-phase had no significant differences. When ATG5 was removed, the results of overall trend obtained by flow cytometry showed that after the plasmid of ATG5 was transfected to Mc3T3-E1 cell by TALENT, the cell ratio took up 34.3% in G1 phase, 14.3% in G2 phase and 51.4% in S-phase. Compared with the ratio of Mc3T3-E1 cell in the blank control group (60.8% in G1 phase and 22.5% in S-phase), the cell ratio in G1 phase in the ATG5 group was reduced, and that in S-phase was increased, suggesting that ATG5 is of great importance in the proliferation and cycle regulation of Mc3T3-E1 cell (Figure 2).

### Impacts of ATG5 on Osteoblast Differentiation

After 4-week osteoinduction culturing, the results of alizarin red staining showed that a wide range of mineralized nodules were observed in each group of cells. The mineralized nodules in ATG5 group were distributed denser than those in GFP group and CON group. The results of semi-quantitative analysis under the microscope revealed that the number of mineralized nodules in ATG5 group was larger than that in the control group, but the comparison had no statistical difference. After 48-h osteoinduction culturing, the results of alizarin red staining showed that a wide range of mineralized nodules were observed in siCON group and CON group, while there were less mineralized nodules in ATG5 group. The results of semi-quantitative analysis under the microscope indicated that the number of mineralized nodules in ATG5 group was less than that in the control group. The difference had statistical significance (Figure 3).

To further clarify the effects of ATG5 on osteogenic differentiation, changes in the gene and protein expressions of Runx2, OCN and COL-I were detected by qRT-PCR and Western blotting methods, respectively. The results showed that

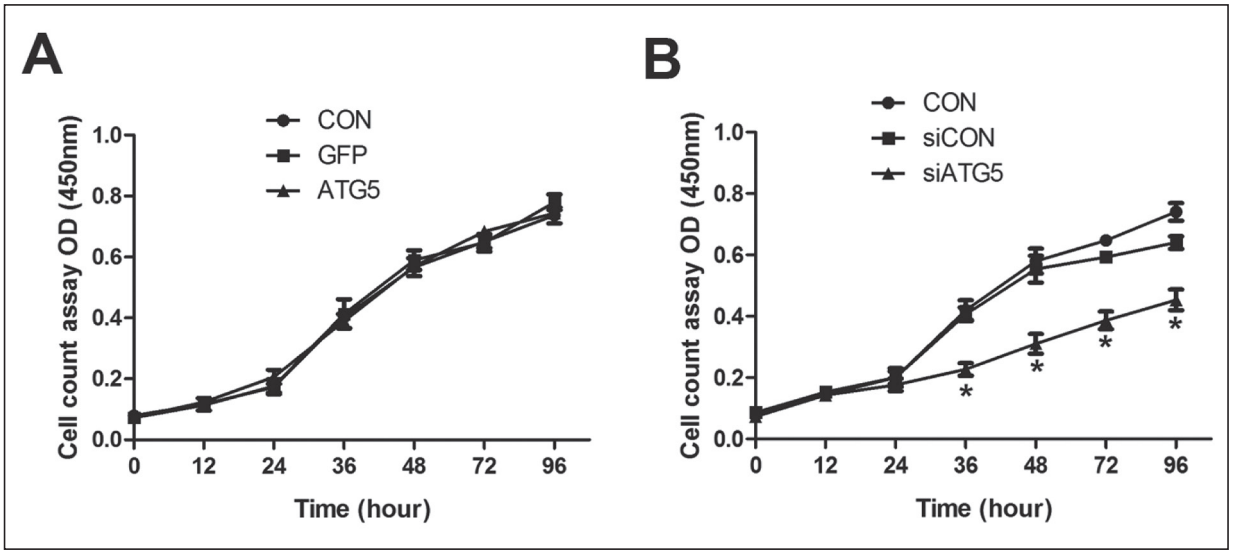


**Figure 1.** Impacts of ATG5 onMc3T3-E1 osteoblast proliferation were detected by CCK-8. ***A,*** The analysis of proliferative ability of Mc3T3-E1 osteoblast in CON, GFP, ATG5 group at different time point. ***B,*** The analysis of proliferative ability of Mc3T3-E1 osteoblast in CON, siCON, siATG5 group at different time point. *$p < 0.05$ *vs.* siCON group.

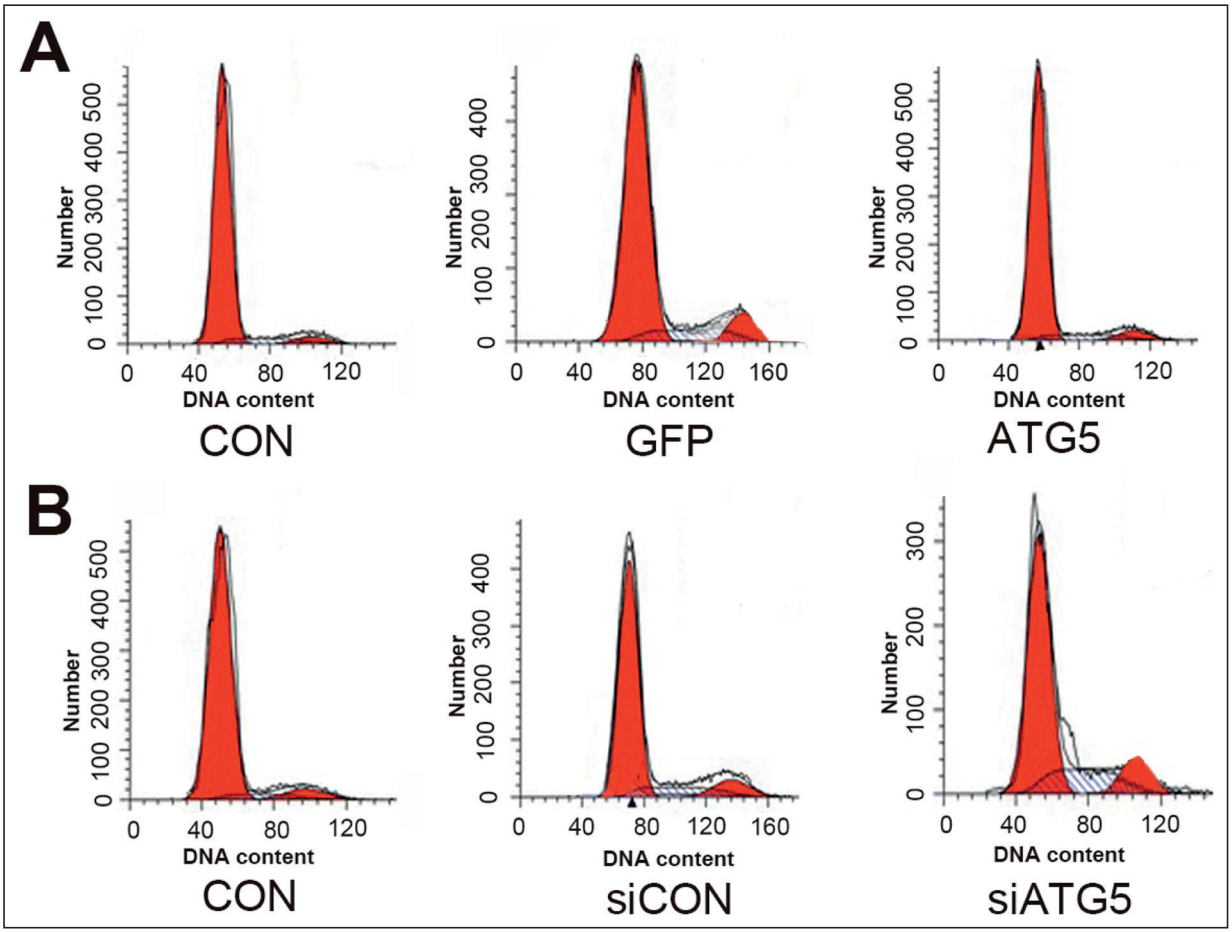


**Figure 2.** Impacts of ATG5 on Mc3T3-E1 osteoblast cycle were detected by flow cytometry. ***A,*** Cell cycle distribution was analyzed after transfection for 48 h with ATG5 lentivirus. ***B,*** Cell cycle distribution was analyzed after transfection for 48 h with TALENT ATG5 RNA vector.

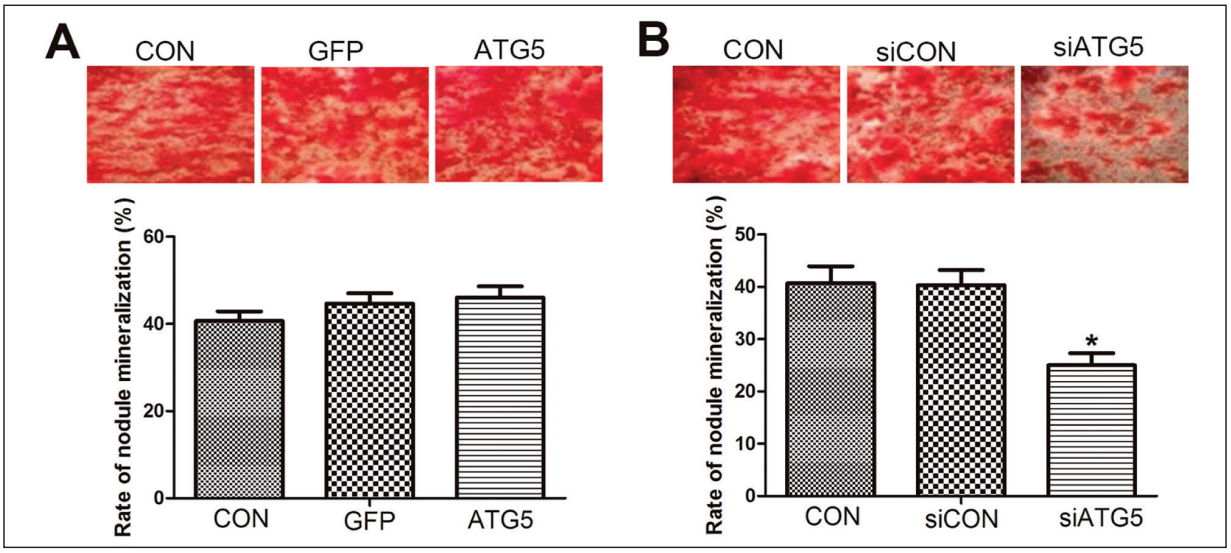


**Figure 3.** Impacts of ATG5 on Mc3T3-E1 osteoblast differentiation were detected by Alizarin red staining. ***A,*** Mineralized nodules stained by Alizarin red in CON, GFP, ATG5 group 4 weeks after osteoinduction culture and semi-quantitative analysis. ***B,*** Mineralized nodules stained by Alizarin red in CON, siCON, siATG5 group 4 weeks after osteoinduction culture and semi-quantitative analysis. *$p < 0.05$ *vs.* siCON group.

there were no statistical differences among ATG5 group, GFP group, and CON group. However, the gene and protein expressions of Runx2, OCN, and COL-I in siATG5 group were decreased compared with those in the CON group, suggesting that ATG5 plays an essential role in osteoblast differentiation (Figure 4).

### Impacts of ATG5 on Osteoblast Apoptosis

In order to clarify the effects of ATG5 on osteoblast apoptosis, the impacts of ATG5 on osteoblast apoptosis under the conditions of oxidative and non-oxidative stress were detected. Flow sorting of apoptosis was conducted after Annexin V-PE/7-AAD double staining. Apoptotic cells in the early, middle and late stages were combined as total apoptotic cells in this experiment for counting. The apoptosis rates of cells in ATG5 group, GFP group, and CON group were 4.30%, 4.52%, and 4.74%, respectively under non-oxidative condition, and they were 23.36%, 24.08%, and 17.56%, respectively under oxidative stress condition. In addition, the apoptosis rate of cells in siATG5 group was 10.56%, which was significantly higher than those in siCON group (4.55%) and CON group (4.26%). After 12-h treatment with 0.5 mM hydrogen peroxide, the apoptosis rate of cells in siATG5 group was 36.81%, which was remarkably higher than the apoptosis rate in siCON group and that in CON group. The results indicate that ATG5 knockout can lead to increased osteoblasts apoptosis, while the overexpression of ATG5 can partially inhibit osteoblast apoptosis. From the above results, it is initially believed that ATG5 can inhibit the injury of oxidative stress on osteoblasts (Figure 5).

## Discussion

In this experiment, it was found that the overexpression of ATG5 had no significant effect on the proliferation and cycle of the cells. However, the results of measurement with cell growth curve showed that siATG5 could inhibit cell proliferation. Moreover, it was found in flow cell sorting that the cell cycle of ATG5 was stagnated in S-phase, which on the other hand verified the important roles of ATG5 in cell proliferation and

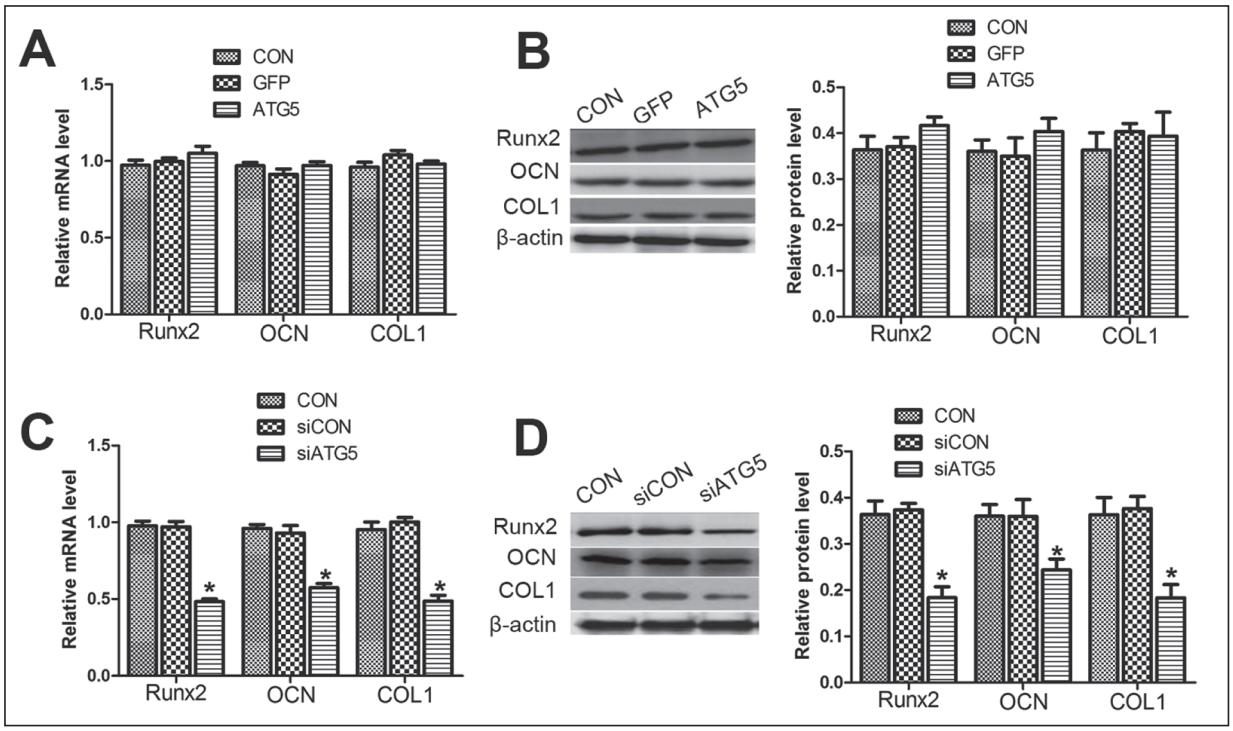


**Figure 4.** mRNA and protein levels of genes related to osteogenic differentiation. ***A,*** Relative mRNA level of Runx2, OCN, COL1 in CON, GFP, ATG5 group. ***B,*** Relative protein level of Runx2, OCN, COL1 in CON, GFP, ATG5 group. ***C,*** Relative mRNA level of Runx2, OCN, COL1 in CON, siCON, siATG5 group. ***D,*** Relative protein level of Runx2, OCN, COL1 in CON, siCON, siATG5 group. *$p < 0.05$ *vs.* siCON group.

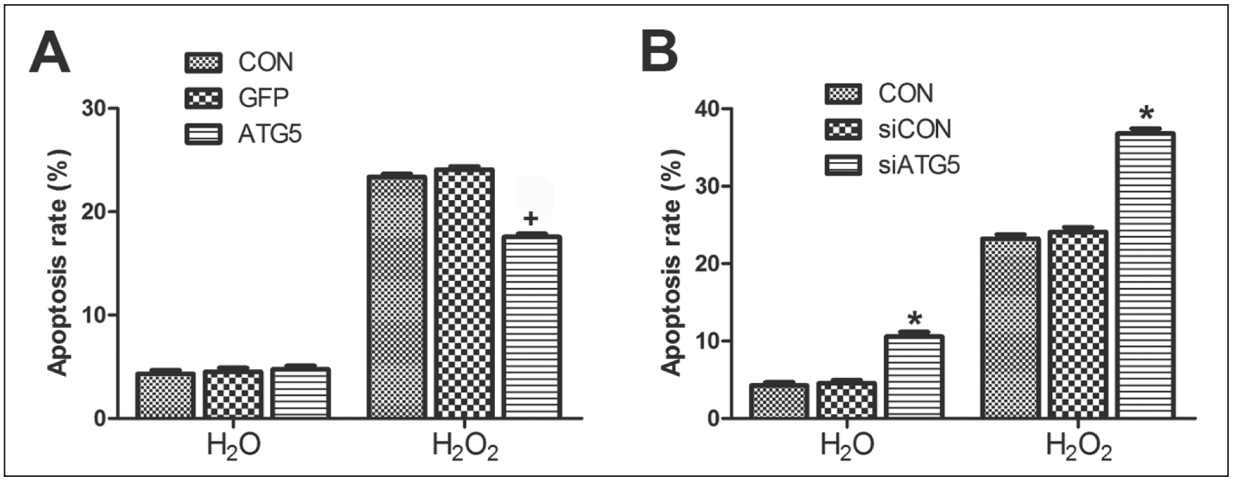


**Figure 5.** Impacts of ATG5 on Mc3T3-E1 osteoblast apoptosis were detected by flow cytometry. ***A,*** Analysis of the apoptosis rate of osteocytes in CON, GFP, ATG5 group at different conditions. ***B,*** Analysis of the apoptosis rate of osteocytes in CON, siCON, siATG5 group at different conditions. $+p < 0.05$ *vs.* GFP group. $*p < 0.05$ *vs.* siCON group.

cycle regulation. A key step in osteoblast differentiation is the arrest of cell cycle in G1 phase[16]. When the cells develop from G1 phase to G0 phase, they stop dividing, and start to differentiate. However, we found that siATG5 could arrest a large amount of cells in S-phase, indicating that siATG5 can prevent cell replication, rather than promoting cell differentiation. Runx2 is a specific transcriptional regulation factor for the differentiation of bone marrow mesenchymal stem cells into osteoblasts[17]. OCN plays an important role in promoting mineral deposit in bone tissues[18]. COL-I is the primary extracellular matrix of osteoblast synthesis[19]. Therefore, Runx2, COL-I, and OCN are the three key genes that reflect the activity of osteoblasts. To further clarify the effects of ATG5 on the activity of osteoblasts, changes in osteoblast differentiation-related indicators were detected. The detection of osteoblasts with qRT-PCR and Western blotting revealed that siATG5 could reduce the levels of gene and protein expressions of Runx2, OCN, and COL-I, while the overexpression of ATG5 had no obvious changes in the expressions of osteogenesis-related genes and proteins. This data is identical with that recently obtained by Nollet et al[20]. They found that siATG5 can result in a rise of oxygen free radicals of osteoblasts and disorders of osteoblast differentiation and mineralization.

Since previous investigations have shown that autophagy also plays an important role in oxidative stress injury, the role of ATG5 in oxidative stress injury of osteogenesis was observed. The overexpression of ATG5 did not affect the apoptosis rate of osteoblasts under non-oxidative stress condition, but the apoptosis rate of osteoblasts was reduced when ATG5 was overexpressed compared with the condition in the control group after treatment with 0.5 mM hydrogen peroxide. It indicates that ATG5 protein does not change the apoptosis rate of osteoblasts under the non-stressed condition, but ATG5 can inhibit apoptosis induced by the oxidative stress of osteoblasts. More importantly, after ATG5 was silenced, the apoptosis rate was significantly increased compared with the condition in the control group, and silenced ATG5 resulted in a further increase in oxidative stress-induced apoptosis rate. The result illustrates to a certain extent that the expression of ATG5 protein plays a key role in maintaining normal functions of osteoblasts. Su et al[21] confirmed that the oxidative stress of ATG5-deficient cells is exacerbated and the apoptosis is excessive, suggesting that this protein may be a key regulatory protein that inhibits apoptosis. The change in bone microenvironment and the imbalance between osteogenesis and osteoclasis under pathological conditions will eventually lead to bone loss. This loss of bone mass differs a lot in the same post-menopausal women and the elderly, suggesting that aging and estrogen withdrawal are not the entire causes of bone loss. In recent years, the relationship between autophagy and bone metabolism has attracted wide attention based on the experimental reports[22,23]. In addition, some researchers found that cell autophagy is of great importance in stress factors such as glucocorticoid, urate crystals and high

glucose[24-26]. Autophagy inhibits cell apoptosis induced by stress conditions. However, the role of autophagy in bone metabolism still lies in the preliminary stage. Autophagy is involved in the pathogenesis of osteoporosis. To further clarify the role of autophagy in osteoblast proliferation, differentiation, and apoptosis, we conducted this experiment. The result suggested that autophagy exerts great effects on osteoblast proliferation, differentiation, and apoptosis. Although our *in vitro* studies revealed that ATG5 knockout could lead to disorders of osteoblast proliferation and differentiation, the overexpression of ATG5 could not promote proliferation and differentiation of osteoblasts, but inhibits oxidative stress-induced apoptosis of osteoblasts. It suggested that ATG5 may participate in regulating the expressions of antioxidant genes of osteoblasts.

## Conclusions

ATG5 silence can lead to inhibition of osteoblast proliferation and differentiation. Moreover, it makes the cells easier to be damaged by oxidative stress, and causes an increase in apoptosis. However, the overexpression of ATG5 strengthens the anti-oxidative capacity of osteoblasts, and reduces apoptosis. ATG5 may be an effective target of anti-oxidative therapy for osteoporosis, which brings a new direction for the treatment of osteoporosis.

### Acknowledgements

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### Conflict of Interest

The Authors declare that they have no conflict of interests.

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