

# The Modulation of Autophagy in Bone Marrow Mesenchymal Stem Cells Via Hoxa11-as Activation of the PI3K-AKT-mTOR Pathway

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**Abstract:** Objective: To investigate the regulatory mechanisms by which long non-coding RNA (lncRNA) Hoxa11-as modulates autophagy in bone marrow mesenchymal stem cells (BMSCs) via the PI3K-AKT-mTOR signaling pathway, and to elucidate its specific roles and potential implications in BMSC autophagy. Methods: BMSCs were divided into three groups: a blank control group, an Hoxa11-as overexpression group, and an si-Hoxa11-as knockdown group. Cells were seeded in six-well plates at a density of  $3 \times 10^5$  cells/mL and cultured in complete medium. The blank control group received no treatment and had the complete culture medium replaced every 48 hours. For the Hoxa11-as overexpression group, BMSCs were transfected with the Hoxa11-as plasmid. After transfection, cells were cultured in complete medium for an additional 48 hours, after which the BMSCs carrying the Hoxa11-as plasmid were harvested. For the si-Hoxa11-as knockdown group, BMSCs were transfected with the si-Hoxa11-as plasmid. After transfection, cells were cultured in complete medium for an additional 48 hours, after which the BMSCs carrying the si-Hoxa11-as plasmid were harvested. Real-time quantitative PCR (qPCR) was performed on the blank control group, the Hoxa11-as overexpression group, and the si-Hoxa11-as knockdown group to quantify the mRNA expression levels of Hoxa11-as, PI3K, AKT, mTOR, Foxo1, LC3, and Beclin1. Immunofluorescence staining was performed on the blank control group, the Hoxa11-as overexpression group, and the si-Hoxa11-as knockdown group to quantify the average fluorescence intensity of Hoxa11-as, PI3K, AKT, mTOR, Foxo1, LC3, and Beclin1 proteins. Results: The qPCR results showed that in the Hoxa11-as overexpression group, the mRNA levels of Hoxa11-as, PI3K, AKT, mTOR, and Foxo1 were significantly increased compared to the blank control group ( $P > 0.05$ ), while those of LC3 and Beclin1 were significantly decreased ( $P < 0.05$ ). In the si-Hoxa11-as knockdown group, the mRNA levels of Hoxa11-as, PI3K, AKT, mTOR, and Foxo1 were significantly reduced ( $P < 0.05$ ), with a more pronounced decrease observed for Hoxa11-as ( $P < 0.05$ ). The mRNA expression levels of LC3 and Beclin1 in the experimental group were significantly increased compared with the blank control group ( $P < 0.05$ ). Additionally, there was a significant positive correlation between the mRNA expression levels of LC3/Beclin1 and mTOR ( $P < 0.05$ ). Immunofluorescence results demonstrated that in the Hoxa11-as group, PI3K, AKT, mTOR, and Foxo1 were significantly upregulated ( $P < 0.05$ ), while LC3 was significantly downregulated ( $P < 0.05$ ). Beclin1 expression did not differ significantly ( $P > 0.05$ ). In contrast, in the si-Hoxa11-as group, PI3K, AKT, mTOR, and Foxo1 were significantly downregulated ( $P < 0.05$ ), whereas LC3 was significantly upregulated ( $P < 0.05$ ). Conclusion: Long non-coding RNA Hoxa11-as significantly activates the PI3K-AKT-mTOR signaling pathway, thereby inhibiting the expression of autophagy-related proteins in BMSCs.

**Keywords:** Hoxa11-as; BMSCs; Autophagy; PI3K-AKT-mTOR Signaling Pathway.

## 1. Introduction

Bone marrow mesenchymal stem cells (BMSCs) exhibit robust differentiation potential, enabling them to differentiate into osteoblasts, adipocytes, or chondrocytes in response to environmental cues[1]. The differentiation process of BMSCs is regulated by multiple signaling pathways, with the PI3K-AKT-mTOR pathway being a key regulator involved in osteoblast formation. Osteoblast differentiation is also regulated by lncRNAs, which play a crucial role in controlling mRNA transcription and translation during this process[2]. Moreover, lncRNAs can generate smaller non-coding molecules, such as miRNAs, which promote osteoblast differentiation by activating key transcription factors RUNX2 and SP7[3].

The PI3K-AKT-mTOR signaling pathway is a critical intracellular signaling cascade that regulates key cellular processes, including metabolism, proliferation, cell survival, growth, and cell cycle progression[4]. The PI3K-AKT-mTOR signaling pathway plays a significant role in various diseases,

including cancer, endocrine disorders, and osteoporosis[5]. The downstream target of the PI3K-AKT-mTOR signaling pathway is the mammalian target of rapamycin (mTOR). Through its regulation of mTORC1, this pathway can either inhibit or promote autophagy, thereby controlling cell growth and metabolism[6]. Autophagy is a cellular self-degradation process that primarily functions to clear damaged organelles, such as mitochondria, endoplasmic reticulum, and peroxisomes, and to eliminate intracellular pathogens. When cellular nutrients or energy are insufficient, cells degrade non-essential proteins and excess organelles via lysosomes to provide essential substances and energy for survival[7].

Studies have shown that inhibiting mTOR phosphorylation can activate the autophagy system. Autophagy selectively regulates osteoblast apoptosis and modulates osteoblast differentiation, thereby maintaining adequate bone mass and enhancing bone strength[8]. Therefore, autophagy plays a crucial role in osteoblast apoptosis. Activating autophagy in osteoblasts can mitigate apoptosis and thereby contribute to the preservation of bone mass.

In recent years, long non-coding RNAs (lncRNAs) have garnered significant attention for their roles in bone metabolism and cancer diagnosis[9]. Among these, lncRNA Hoxa11-as participates in scar formation via the Wnt signaling pathway and serves as an indispensable initiating and promoting factor in the proliferation and metastasis of malignant tumors. According to relevant studies, lncRNA Hoxa11-as promotes the activation of the PI3K-AKT pathway in cells. The subsequent activation of the PI3K-AKT-mTOR signaling cascade increases the expression of downstream mTOR, thereby reducing autophagy in osteoblasts[10]. This suggests that regulating cellular autophagy could be a promising approach for treating osteoporosis, offering a novel therapeutic option.

This study primarily investigates the regulatory effects of lncRNA Hoxa11-as-transfected BMSCs on the PI3K-AKT-mTOR signaling pathway. Leveraging the ability of long non-coding RNA Hoxa11-as to activate the PI3K-AKT-mTOR signaling pathway, this study investigates its promoting effects on the differentiation of BMSCs and the underlying mechanisms, as well as its impact on autophagy and the associated mechanisms.

## 2. Materials

### 2.1. Bone Marrow Mesenchymal Stem Cells

Rat bone marrow mesenchymal stem cells (CP-R131), supplied at a density of  $5 \times 10^5$  cells per T25 culture flask, were purchased from Wuhan Pricella Biotechnology Co., Ltd. The cells were cultured in the cell facility of the Life Science Research Center at the Affiliated to Youjiang Medical College for Nationalities for Nationalities using a constant-temperature incubator set to 5% CO<sub>2</sub> and 37°C.

### 2.2. Medicine

HOXA11-AS lncRNA and si-HOXA11-AS lncRNA were obtained from Wuhan Miaoling Biotechnology Co., Ltd. Lipofectamine 3000 (Cat. No. L3000015) transfection reagent was obtained from Thermo Fisher Scientific. The primers for PI3K, AKT, mTOR, Foxo, GAPDH, and Hoxa11-as were designed by Wuhan SAIWELL Biotechnology Co., Ltd.

## 2.3. Instrument

Low-speed refrigerated centrifuge (Zhongke Zhongjia Scientific Instrument Co., Ltd.), High-speed refrigerated benchtop centrifuge (Sigma Laboratory Centrifuge Co., Ltd.), Multifunctional microplate reader (PerkinElmer Inc.), Real-time fluorescent quantitative PCR instrument (Roche Diagnostics Products Co., Ltd.), Inverted fluorescence microscope (Zeiss, Germany).

## 3. Method

### 3.1. Grouping and Model Establishment

BMSCs were divided into three groups: a blank control group, a Hoxa11-as overexpression group, and a si-Hoxa11-as knockdown group. Cells were seeded in six-well plates at a density of  $3 \times 10^5$  cells/mL and cultured in complete medium. In the blank control group, no treatment was performed, and the complete culture medium was replaced every 48 hours. In the Hoxa11-as overexpression group, BMSCs were transfected with the Hoxa11-as plasmid. After transfection, cells were cultured in complete medium for an additional 48 hours before harvesting the BMSCs carrying the Hoxa11-as plasmid. In the si-Hoxa11-as knockdown group, BMSCs were transfected with the si-Hoxa11-as plasmid. After transfection, cells were cultured in complete medium for an additional 48 hours before harvesting the BMSCs that had incorporated the si-Hoxa11-as plasmid.

### 3.2. Detection Indicators and Methods

#### 3.2.1. Quantitative real-time PCR (qPCR) was Employed to Quantify the mRNA Expression Levels of Hoxa11-as, PI3K, AKT, mTOR, Foxo1, LC3, and Beclin1

Cells were lysed using TRIzol reagent, and total RNA was extracted. The concentration and purity of the RNA were assessed using a spectrophotometer. Following reverse transcription, cDNA was amplified by quantitative real-time PCR (qPCR). The relative mRNA expression levels of each target gene were calculated using the  $2^{-\Delta\Delta Ct}$  method, with GAPDH serving as the internal control. Data were subsequently analyzed statistically. The primer sequences for each target gene are listed in Table 1.

**Table 1.** Primer Sequences

| Primer Information | Primer Names  | Primer sequence (5'-3')   |
|--------------------|---------------|---------------------------|
| GAPDH              | R-GAPDH-S     | CTGGAGAAACCTGCCAAGTATG    |
|                    | R-GAPDH-A     | GGTGAAGAATGGGAGTTGCT      |
| Hoxa11-as          | R-Hoxa11-as-S | CGAACCTTGGGCTGCTTTA       |
|                    | R-Hoxa11-as-A | AGGACTGGAAGCTCGGACTTGG    |
| Pi3k               | R-pi3k-S      | AGAGCTTGGAGGACGATGACG     |
|                    | R-pi3k-A      | TGGACTGGGCTATCTCACTTCG    |
| AKT                | R-AKT1-S      | CGACGTAGCCATTGTGAAGGAG    |
|                    | R-AKT1-A      | ATTGTGCCACTGAGAAGTTGTTG   |
| mTOR               | R-mTOR-S      | CTCTGACTACAGCACCAGTGAAATG |
|                    | R-mTOR-A      | CAGGGACTTGAAGATGAAGGTGAT  |
| FOXO1              | R-FOXO1-S     | CTTCAAGGATAAGGGCGACAG     |
|                    | R-FOXO1-A     | GCCATTAGAAAAGTGAAGACCA    |
| LC3                | R-LC3-S       | ACAGCATGGTGAGTGTGTCC      |
|                    | R-LC3-A       | AAGGTTTCCTGGGAGGCGTA      |

#### 3.2.2. The Average Fluorescence Intensity of Hoxa11-as, PI3K, AKT, mTOR, Foxo1, LC3, and Beclin1 was Measured Using Immunohistochemistry

Cells were fixed with 4% paraformaldehyde for 15 minutes

at room temperature. Following fixation, 0.5% Triton X-100 solution was added to each well of the 6-well plate for permeabilization. After permeabilization, cells were blocked with 10% goat serum blocking solution for 1 hour at room temperature. Subsequently, the primary antibody was

incubated overnight at 4°C. Following the overnight incubation with the primary antibody, the secondary antibody was incubated for 1 hour at room temperature in the dark. After the incubation is completed, add an appropriate amount of anti-fade mounting medium containing DAPI. The samples can then be observed under a fluorescence microscope.

### 3.2.3. Statistical Methods

Statistical analysis was conducted using GraphPad Prism 10. Continuous data were presented as mean  $\pm$  standard deviation (SD). For normally distributed data with homogeneity of variance, one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test was used for multiple comparisons between groups. If the data did not meet the assumptions of normality or homogeneity of variance, non-parametric tests for multiple independent samples (e.g., Kruskal-Wallis test) were employed. Categorical data were expressed as percentages and analyzed using the chi-square test. A P value  $< 0.05$  was considered statistically significant.

## 4. Result

### 4.1. The mRNA Expression Levels of Hoxa11-as, PI3K, AKT, mTOR, Foxo1, LC3, and Beclin1 Were Analyzed

Following the described experimental protocol, qPCR

analysis was performed on cells transfected with Hoxa11-as and si-Hoxa11-as plasmids, yielding the results presented below. In Figure A, there was no significant difference in mRNA expression between the blank group and the si-Hoxa11-as group ( $P > 0.05$ ). In contrast, the Hoxa11-as group exhibited significantly higher mRNA expression compared to the si-Hoxa11-as group ( $P < 0.05$ ). These results indicate that the Hoxa11-as plasmid was successfully transfected into the cells and effectively expressed. As shown in Figure B, in the Hoxa11-as group, the mRNA expressions of Hoxa11-as, PI3K, AKT, mTOR, and Foxo1 were significantly increased compared to the blank group ( $P > 0.05$ ), whereas the mRNA expressions of LC3 and Beclin1 were significantly decreased ( $P < 0.05$ ). As shown in Figure C, in the si-Hoxa11-as group, the mRNA expressions of Hoxa11-as, PI3K, AKT, mTOR, and Foxo1 were all significantly decreased ( $P < 0.05$ ). Notably, the reduction in the mRNA expression of Hoxa11-as was particularly pronounced ( $P < 0.05$ ). The mRNA expressions of LC3 and Beclin1 were significantly increased compared with the blank group ( $P < 0.05$ ). Additionally, there was a significant positive correlation between the mRNA expression levels of LC3/Beclin1 and mTOR ( $P < 0.05$ ). For detailed information, please see Figure 1.

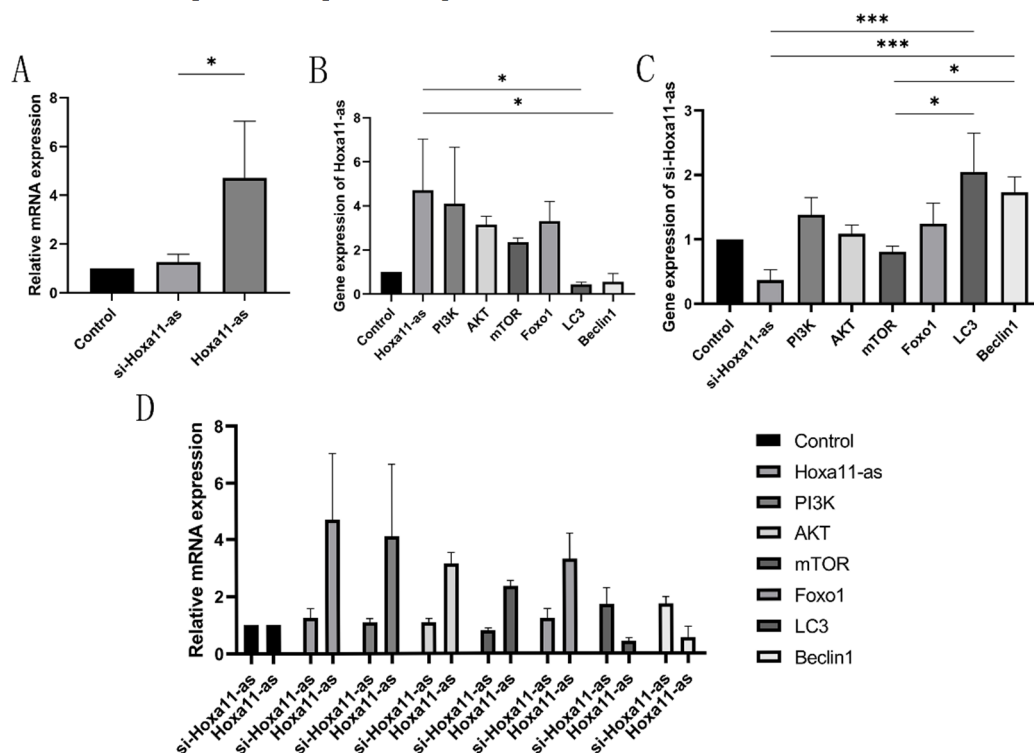


Figure 1. mRNA expression results for Hoxa11-as, PI3K, AKT, mTOR, Foxo1, LC3, and Beclin1

### 4.2. The Average Fluorescence Intensity Results for Hoxa11-as, PI3K, AKT, mTOR, Foxo1, LC3, and Beclin1

As shown in Figure 2, the protein expression levels of Hoxa11-as, PI3K, AKT, mTOR, Foxo1, LC3, and Beclin1 were analyzed in the blank group, Hoxa11-as group, and si-Hoxa11-as group. The average fluorescence intensity of the cells was quantified using ImageJ, normalized, and plotted using GraphPad Prism 10. In Figure A, the average

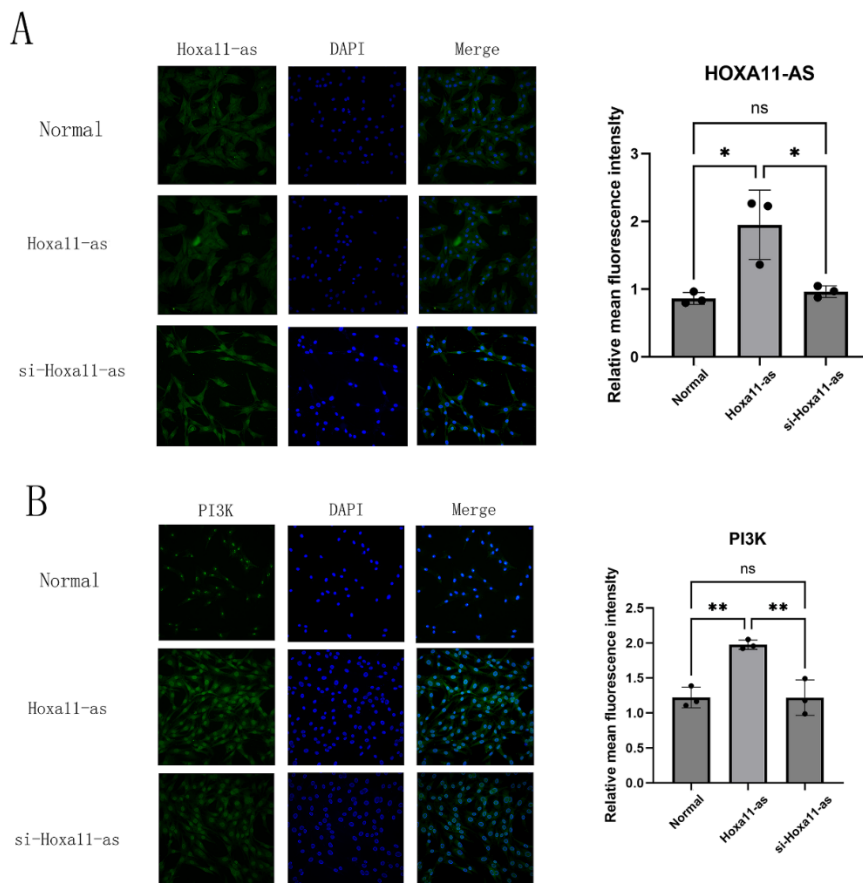
fluorescence intensity of Hoxa11-as protein in the Hoxa11-as group was significantly higher than that in both the blank group and the si-Hoxa11-as group. Statistical analysis revealed significant differences between the Hoxa11-as group and the other two groups ( $P < 0.05$ ). There was no significant difference in fluorescence intensity between the blank group and the si-Hoxa11-as group ( $P > 0.05$ ). In Figure B, the average fluorescence intensity of PI3K protein in the Hoxa11-as group was significantly higher than that in both the blank group and the si-Hoxa11-as group, with statistically

significant differences observed between the Hoxa11-as group and the other two groups ( $P < 0.05$ ). There was no statistically significant difference in fluorescence intensity between the blank group and the si-Hoxa11-as group ( $P > 0.05$ ). In Figure C, the average fluorescence intensity of AKT protein in the Hoxa11-as group was significantly higher than that in both the blank group and the si-Hoxa11-as group. Statistical analysis revealed significant differences between the Hoxa11-as group and the other two groups ( $P < 0.05$ ). In contrast, there was no statistically significant difference in fluorescence intensity between the blank group and the si-Hoxa11-as group ( $P > 0.05$ ). In Figure D, the average fluorescence intensity of mTOR protein in the Hoxa11-as group was significantly higher than that in both the blank group and the si-Hoxa11-as group. Statistical analysis revealed significant differences between the Hoxa11-as group and the other two groups ( $P < 0.05$ ). In contrast, there was no statistically significant difference in fluorescence intensity between the blank group and the si-Hoxa11-as group ( $P > 0.05$ ). In Figure E, the average fluorescence intensity of Foxo1 protein in the Hoxa11-as group was significantly higher than that in both the blank group and the si-Hoxa11-as group. Statistical analysis revealed significant differences between the Hoxa11-as group and the other two groups ( $P < 0.05$ ). In contrast, there was no statistically significant difference in fluorescence intensity between the blank group and the si-Hoxa11-as group ( $P > 0.05$ ). In Figure F, there was no statistically significant difference in the fluorescence intensity of LC3 protein between the Hoxa11-as group and the blank group ( $P > 0.05$ ). In contrast, the fluorescence intensity of LC3 protein in the si-Hoxa11-as group was significantly different from that in the blank group ( $P < 0.05$ ). In Figure G, there was no statistically significant difference in

the fluorescence intensity of Beclin1 protein among the blank group, the Hoxa11-as group, and the si-Hoxa11-as group ( $P > 0.05$ ). As shown in Figure 2.

## 5. Discussion

In this study, we cultured mesenchymal stem cells (BMSCs) derived from rat bone marrow and investigated the regulatory effects of the Hoxa11-as plasmid on the PI3K-AKT-mTOR signaling pathway. Our results show that in the Hoxa11-as group, transfection with the Hoxa11-as plasmid led to increased mRNA expression levels of PI3K and AKT, two key upstream regulators of the PI3K-AKT-mTOR pathway. This suggests that Hoxa11-as promotes activation of the PI3K-AKT-mTOR signaling pathway. The activation of the PI3K-AKT signaling pathway not only promotes the activation of the downstream mTOR factor but also leads to an increase in its mRNA expression level. According to relevant studies, the mTOR factor plays a crucial role in cellular anabolic processes, including the synthesis of proteins, lipids, and cholesterol. Additionally, it promotes extracellular nutrient uptake and inhibits autophagy. The mTOR complex mTORC1 regulates autophagy at the lysosomal surface by interacting with key lysosome-related proteins, including ULK1, ATG13, and TFEB. This interaction restricts autophagic processes mediated by lysosomes[11][12]. Beclin1 is a key regulator of autophagy. Its activation promotes the initiation of autophagic processes. The interaction between Beclin1 and BCL-2 plays a critical role in modulating the level of autophagy[13]. LC3 is a homolog of Atg8, and its lipidated form has been confirmed as a marker of autophagosomes. It is widely utilized in autophagy research, particularly in studies of neuromuscular diseases and tumors[14].



(a)

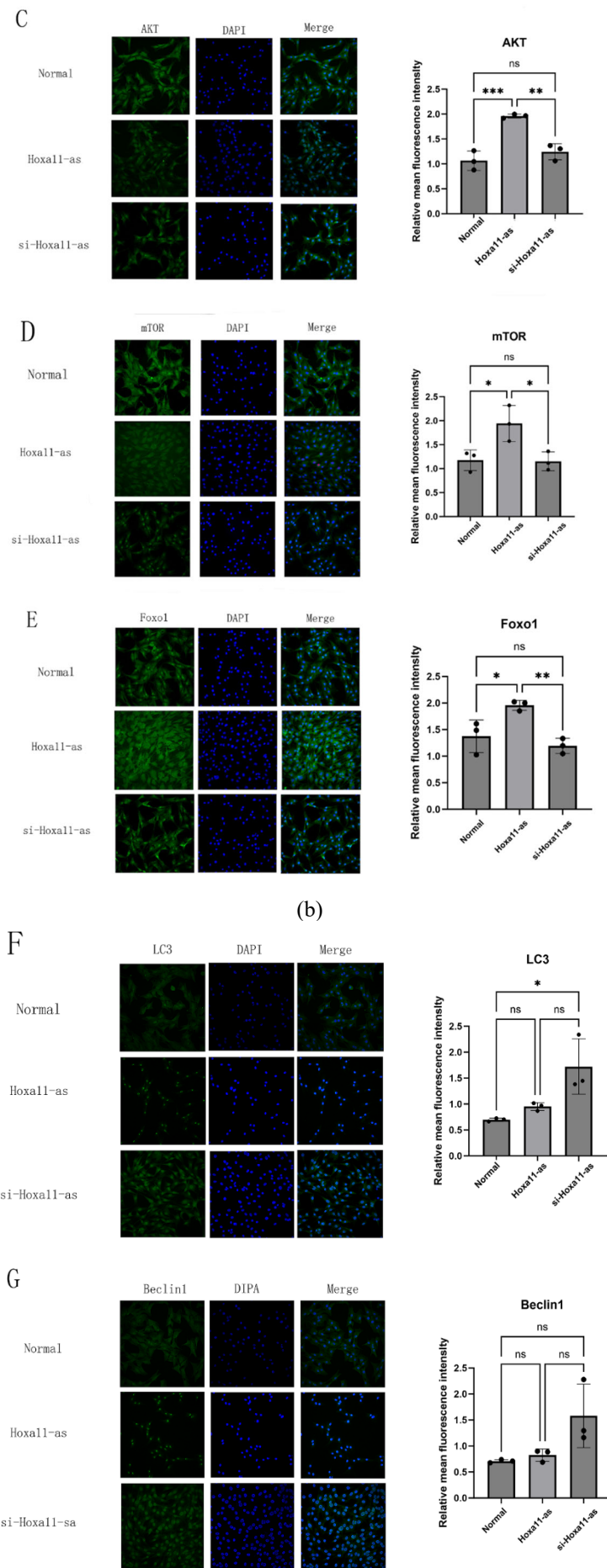


**Figure 2.** Average Fluorescence Intensity of Hoxa11-as, PI3K, AKT, mTOR, Foxo1, LC3, and Beclin1

In the qPCR experiment, the mRNA expression levels of PI3K, AKT, mTOR, and Foxo1 were significantly elevated in the Hoxa11-as group compared to the blank group, while the mRNA expression of LC3 and Beclin1 showed a decreasing trend. In contrast, in the si-Hoxa11-as group, the mRNA expression of PI3K, AKT, mTOR, and Foxo1 did not differ significantly from that of the blank group, whereas the mRNA expression of LC3 and Beclin1 exhibited an increasing trend. In the Hoxa11-as group, the Hoxa11-as plasmid promotes the PI3K-AKT-mTOR signaling pathway, leading to mTOR activation and subsequent inhibition of autophagy. In the si-Hoxa11-as group, the absence of Hoxa11-as plasmid intervention resulted in reduced activation of the PI3K-AKT-mTOR signaling pathway and lower mTOR activation levels, thereby promoting autophagy in BMSCs. In the immunofluorescence experiment, the fluorescence intensities of Hoxa11-as, PI3K, AKT, mTOR, and Foxo1 were significantly higher in the Hoxa11-as group compared to the si-Hoxa11-as group. These results confirmed the promoting effect of the Hoxa11-as plasmid on the PI3K-AKT-mTOR signaling pathway. However, in the protein immunofluorescence analysis of LC3 and Beclin1, no significant differences in autophagy-related fluorescence intensity were observed in the si-Hoxa11-as group compared to the control.

In this experiment, the results from Real-time qPCR and immunofluorescence clearly demonstrate that the long non-coding RNA Hoxa11-as significantly promotes the PI3K-AKT-mTOR signaling pathway. However, the regulatory mechanism by which Hoxa11-as influences autophagy in BMSCs remains to be elucidated. However, the influence and regulatory role of long non-coding RNA (Hoxa11-as) on autophagy have not yet been validated through relevant animal experiments. Future studies will include additional animal experiments to further investigate the impact of Hoxa11-as on autophagy.

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