

CircRNA circFADS2 is Downregulated in Osteoporosis and Suppresses LPS-induced Apoptosis of Osteoblast by Downregulating miR-16-5p

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Research

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Abstract

Background: CircRNA circFADS2 plays protective roles in LPS-induced cell injury, which promotes osteoporosis (OS), suggesting the involvement of circFADS2 in OS. This study aimed to explore the role of circFADS2 in OS.

Methods: RT-qPCRs were performed to analyze the expression of circFADS2 and miR-16-5p in plasma samples from OS patients (n=64) and healthy controls (n=64). Correlations between circFADS2 and miR-16-5p were explored by Pearson's correlation coefficient. In primary osteoblasts, circFADS2 and miR-16-5p were overexpressed to study the interactions between them. Cell apoptosis assay was performed to study the functions of circFADS2 and miR-16-5p in the apoptosis of osteoblasts induced by LPS.

Results: CircFADS2 was downregulated in OA and inversely correlated with miR-16-5p. After treatment, circFADS2 was upregulated and miR-16-5p was downregulated. In osteoblasts, LPS treatment decreased the expression of circFADS2 but increased the expression of miR-16-5p. Overexpression of circFADS2 decreased the expression of miR-16-5p. Cell apoptosis analysis showed that circFADS2 overexpression reduced the apoptosis of osteoblasts under LPS-treatment, while miR-16-5p overexpression increased cell apoptosis. Moreover, overexpression of miR-16-5p reduced the effects of circFADS2 overexpression on the apoptosis of osteoblasts.

Conclusion: Therefore, CircFADS2 is downregulated in OS and promotes LPS-induced apoptosis of osteoblast by downregulating miR-16-5p.

Background

Osteoporosis (OS) is a type of progressive bone disease caused by bone loss and is associated with high risk of fractures [1]. OS is closely correlated with menopause and aging [2]. It is estimated that more than 10% of women older than 60 years are suffering from OS, which is two times higher than that in men [3]. At present, OS patients are mostly treated with bisphosphonates (such as alendronate, risedronate, and zoledronic acid) or hormones (such as estrogen) [4, 5]. However, these treatments only strengthen or protect bones, or just slow the breakdown of bones [6]. Only in rare cases new bone will growth [7]. At present, there is no cure for OS.

Treatment of OS requires the development of novel therapeutic approaches. Previous studies on the pathogenesis of OS have identified the involvement of multiple molecular pathways, such as p53 and LPS signaling pathways [7–11]. In effect, some of these molecular pathways have been proven to be potential molecular targets for the development of targeted therapy, which suppresses disease progression by regulating gene expression, to treat OS [10, 11]. Circular RNAs (circRNAs) participate in human diseases including OS mainly by regulating gene expression rather than protein-coding [12]. So, circRNAs are potential targets for molecular targeted therapy. CircRNA circFADS2 plays protective roles in LPS-induced cell apoptosis [13], which contributes to OS [14]. We performed preliminary microarray analysis and observed the inverse correlation between circFADS2 and miR-16-5p, which can suppress

bone fracture healing [15]. We therefore analyzed the interaction between circFADS2 and miR-16-5p in OS.

Methods

Research subjects

This study was approved by The First Hospital of Fuyang Ethics Committee. From June 2018 to July 2019, this study enrolled both OS patients (n=64, 40 males and 24 females) and healthy controls (n=64, 40 males and 24 females) at this hospital. Age range of both groups was 42 to 68 years, and the mean age was 55.3 ± 5.8 years. It is known that initiated therapy and other clinical disorders can also affect gene expression, the 64 OS patients were excluded the ones with initiated therapy and the ones diagnosed with multiple clinical disorders. All the 64 OS patients were newly diagnosed OS cases. Range of T score was -2.83 to -4.99 in OS group. All healthy controls showed normal physiological functions in systemic physiological exam. Healthy controls with a history of OS were excluded. Range of T score was 3.19 to -0.47 in OS group. All participants signed informed consent.

Treatment, blood extraction and plasma preparation

Patients were treated with bisphosphonates (alendronate, risedronate, and zoledronic acid) and/or hormones (estrogen). The dosage was determined based on their conditions.

Prior to therapy, blood (3ml) was extracted under fasting from both OS patients and healthy controls. At 1 year after the initiation of therapy, fasting blood (3 ml) was also extracted from each patient. To prepare plasma samples, blood samples were mixed with citric acids to the ratio of 10:1, followed by centrifuging the mixture for 15 min at 1200g to collect the supernatant.

Osteoblasts

This study included primary osteoblasts from Sigma-Aldrich (USA) to perform in vitro cell experiments. Cells were cultivated in osteoblast growth medium (Promocell) at 37°C, 95% humidity and 5% CO₂. Cells were cultivated to reach about 85% confluence prior to the subsequent assays. To study the effects of LPS treatment on gene expression, osteoblasts were cultivated in medium supplemented with 0, 2, 4, 6 and 8 µg/ml LPS for 48h prior to the subsequent assays.

Vector, miRNA and cell transfections

To overexpress circFADS2 and miR-16-5p, osteoblasts were transfected with either circFADS2 expression vector (1µg) or miRNA (40 nM) using Lipofectamine 2000 (Invitrogen). Expression vector of circFADS2 was constructed with pcDNA3.1 backbone vector (Invitrogen). Mimic of miR-16-5p and negative control

(NC) miRNA were from Sigma-Aldrich. Empty vector- or NC miRNA-transfected cells (NC cells) and untransfected cells (Control cells, C) were included in each experiment. In fresh medium, cells were cultivated for further 48h after transfections prior to the subsequent assays.

RNA preparations

Total RNA extractions from plasma samples and osteoblasts were performed using Trizol reagent (Invitrogen). Genomic DNA removal was performed by incubating the RNA samples with DNase I (Invitrogen) at 37°C for 85 min. RNA integrity was analyzed using 5% urea-PAGE gel. OD 260/280 ratios were determined to analyze RNA purity.

QRT-PCR

SS-III-RT system (Invitrogen) was used to prepare cDNA samples through reverse transcriptions (RTs) with RNA samples with good integrity and an OD value close to 2.0 (pure RNA) as templates. To determine the expression of circFADS2, SYBR® Green Realtime PCR Master Mix (Toyobo) was used to perform qPCRs with 18S rRNA as an internal control.

To determine the expression of miR-16-5p, poly (A) addition was performed, followed by using poly (T) as reverse primer to carry out RTs and qPCRs with U6 as an internal control. All steps were completed using All-in-One™ miRNA qRT-PCR Detection Kit (Genecopoeia).

Ct values were normalized to corresponding internal controls using $2^{-\Delta\Delta Ct}$ method.

Cell apoptosis assay

Osteoblasts with transfections were subjected to cell apoptosis assay using FITC/PI apoptosis kit (Life Technologies, USA). Briefly, osteoblasts were cultivated in medium containing 8 µg/ml LPS for further 48h, followed by washing with pre-cold PBS. After that, osteoblasts were resuspended in binding buffer, followed by staining with PI and Annexin-V for 20 min in dark. Finally, flow cytometry was performed to analyze cell apoptosis

Statistical analysis

Unpaired t test was used to compare OS and control groups. Paired t test was used to compare two time points. ANOVA Tukey's test was used to compare multiple independent groups. Pearson's correlation coefficient was used to analyze correlations. $p < 0.05$ was statistically significant.

Results

CircFADS2 and miR-16-5p were inversely correlated in OS

Plasma samples (collected prior to therapy) from both OS patients (n = 64) and controls (n = 64) were subjected to RNA isolation and RT-qPCRs to analyze the expression of circFADS2 and miR-16-5p. Compared to control group, OS group exhibited significantly downregulated circFADS2 (Fig. 1A, $p < 0.001$) and significantly overexpressed miR-16-5p (Fig. 1B, $p < 0.001$). Pearson's correlation coefficient analysis showed that circFADS2 and miR-16-5p were inversely correlated across OS samples (Fig. 1C), but not control samples (Fig. 1D). Therefore, circFADS2 and miR-16-5p may have crosstalk in OS.

Treatment altered the expression of circFADS2 and miR-16-5p in OS patients

Expression of circFADS2 and miR-16-5p in plasma from OS patients was also determined at 1 year after the initiation of therapy. Heatmaps were plotted using Heml 1.0 software to reflect the changes in gene expression during treatment. It was observed that circFADS2 was upregulated (Fig. 2A) and miR-16-5p was downregulated (Fig. 2B) at 1 year after the initiation of therapy compared to pre-treatment levels.

Overexpression of circFADS2 decreased the expression of miR-16-5p in osteoblasts

To study the crosstalk between circFADS2 and miR-16-5p, osteoblasts were transfected with circFADS2 expression vector or miR-16-5p mimic, followed by the confirmation of circFADS2 and miR-16-5p every 24 h until 96 h. It was observed that circFADS2 and miR-16-5p were significantly overexpressed from 24 h through 96 h (Fig. 3A, $p < 0.05$). Overexpression of circFADS2 decreased the expression of miR-16-5p from 48 h to 96 h (Fig. 3B, $p < 0.05$). In contrast, miR-16-5p overexpression failed to significantly alter the expression of circFADS2 (Fig. 3C). Therefore, circFADS2 may downregulate miR-16-5p in osteoblasts.

CircFADS2 suppressed the apoptosis of osteoblasts induced by LPS through miR-16-5p

To study the effects of LPS treatment on the expression of circFADS2 and miR-16-5p, osteoblasts were cultivated in medium supplemented with 0, 2, 4, 6 and 8 $\mu\text{g/ml}$ LPS for 48 h, followed by RT-qPCRs. LPS treatment decreased the expression of circFADS2 (Fig. 4A, $p < 0.05$), but increased the expression of miR-16-5p (Fig. 4B, $p < 0.05$). Cell apoptosis analysis showed that circFADS2 overexpression reduced the apoptosis of osteoblasts under LPS-treatment, while miR-16-5p overexpression increased cell apoptosis. Moreover, overexpression of miR-16-5p reversed the effects of circFADS2 overexpression on the apoptosis of osteoblasts (Fig. 4C, $p < 0.05$).

Discussion

This study mainly explored the involvement of circFADS2 and miR-16-5p in OS. We found that circFADS2 was downregulated in OS and miR-16-5p was upregulated in OS. In addition, circFADS2 could downregulate miR-16-5p to suppress the apoptosis of osteoblasts induced by LPS.

LPS-mediated inflammation plays a critical role in the occurrence and progression of osteoporosis [11]. In a recent study, Li et al. reported that circFADS2 could interact with the axis of miR-498/mTOR to suppress the apoptosis of chondrocytes induced by LPS treatment [13], suggesting the potential involvement of circFADS2 in OS. In this study we first reported the downregulation of circFADS2 in OS. In addition, LPS treatment mediated the downregulation of circFADS2 in osteoblasts. It has been well established that LPS can induce the apoptosis and suppress the differentiation of osteoblasts [14]. Osteoblasts are responsible for the formation of new bones and OS is characterized by the increased apoptosis of osteoblasts [16]. In this study, overexpression of circFADS2 suppressed the apoptosis of osteoblasts under LPS treatment. Therefore, LPS may promote OS by increasing the apoptosis of osteoblasts through the downregulation of circFADS2, and overexpression of circFADS2 may serve as a potential target for OS.

It has been reported that miR-16-5p can increase the apoptosis of osteoblasts and suppress their proliferation to slow bone fracture healing [15]. However, its involvement in OS is unknown. We found that miR-16-5p was overexpressed in OS and promoted the apoptosis of osteoblasts induced by LPS. Interestingly, circFADS2 overexpression decreased the expression of miR-16-5p in osteoblasts. However, the mechanism is unclear. We observed an inverse correlation between circFADS2 and miR-16-5p across OS samples, but not control samples. Therefore, the interaction between them may be mediated by certain OS-related factors.

Conclusion

In conclusion, circFADS2 is under-expressed in OS and miR-16-5p is overexpressed in OS. In addition, circFADS2 overexpression may downregulate miR-16-5p to suppress the apoptosis of osteoblasts induced by LPS.

Declarations

Ethics approval and consent to participate

This study was approved by The First Hospital of Fuyang Ethics Committee and all the patients or parents/ guardians of patients provided written informed consent.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contributions

Zhiwei Zhang designed the study. Hailong Zhou carried out experiments and wrote the manuscript, Zhiwei Zhang revised the paper, Jianmin Jiang and Xiaohua Chen collected patient specimens and related information. Hailong Zhou, Jianmin Jiang and Xiaohua Chen contributed to analysing the data. All authors reviewed the results and approved the final version of the manuscript.

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Figures

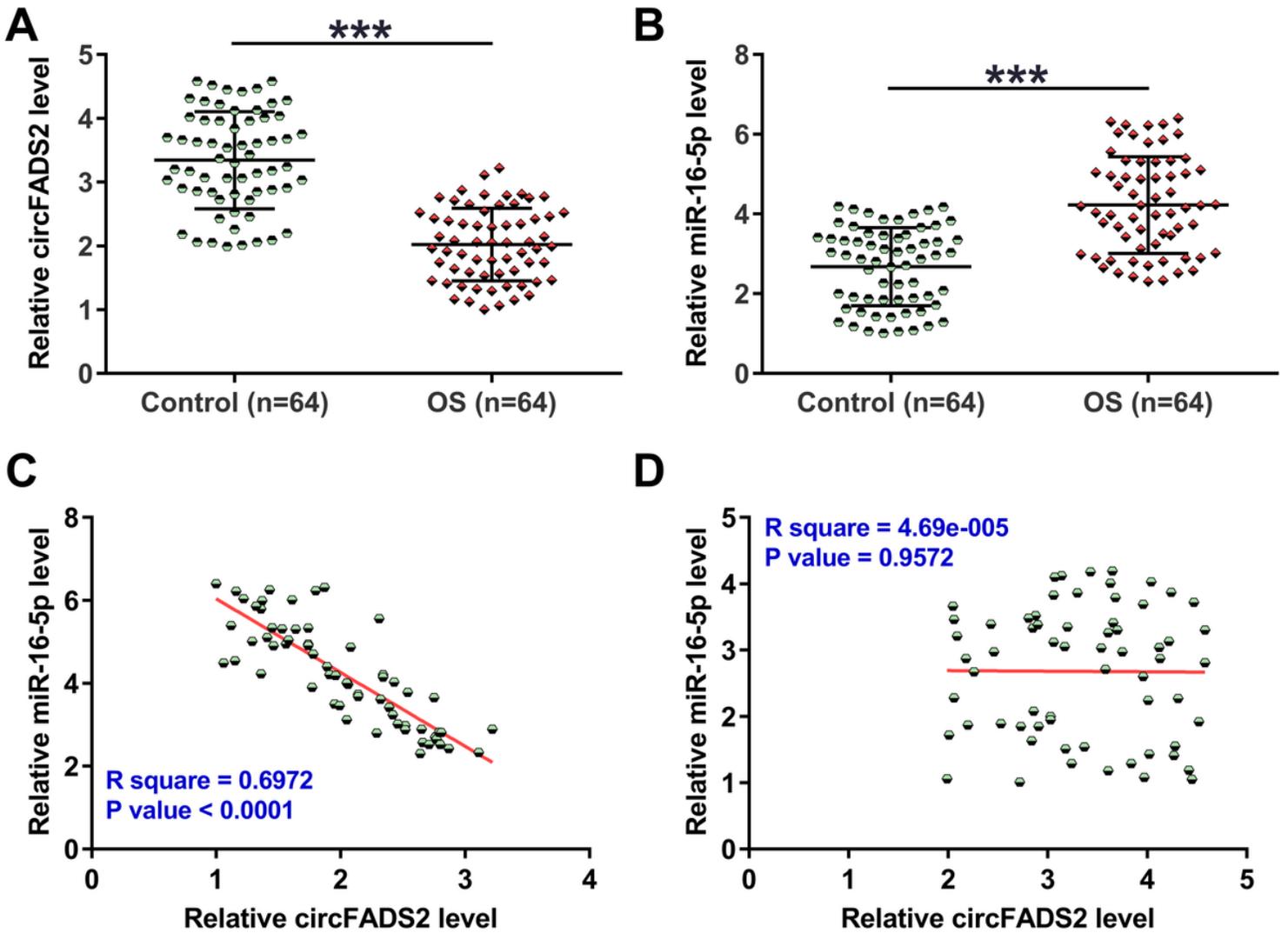


Figure 1

CircFADS2 and miR-16-5p were inversely correlated in OS Plasma samples from both OS patients (n=64) and controls (n=64) were subjected to RNA isolation and RT-qPCRs to analyze the expression of circFADS2 (A) and miR-16-5p (B).***,p<0.0001. Pearson's correlation coefficient analysis was performed to analyze the correlations between circFADS2 and miR-16-5p across OS samples (C) and control samples (D).

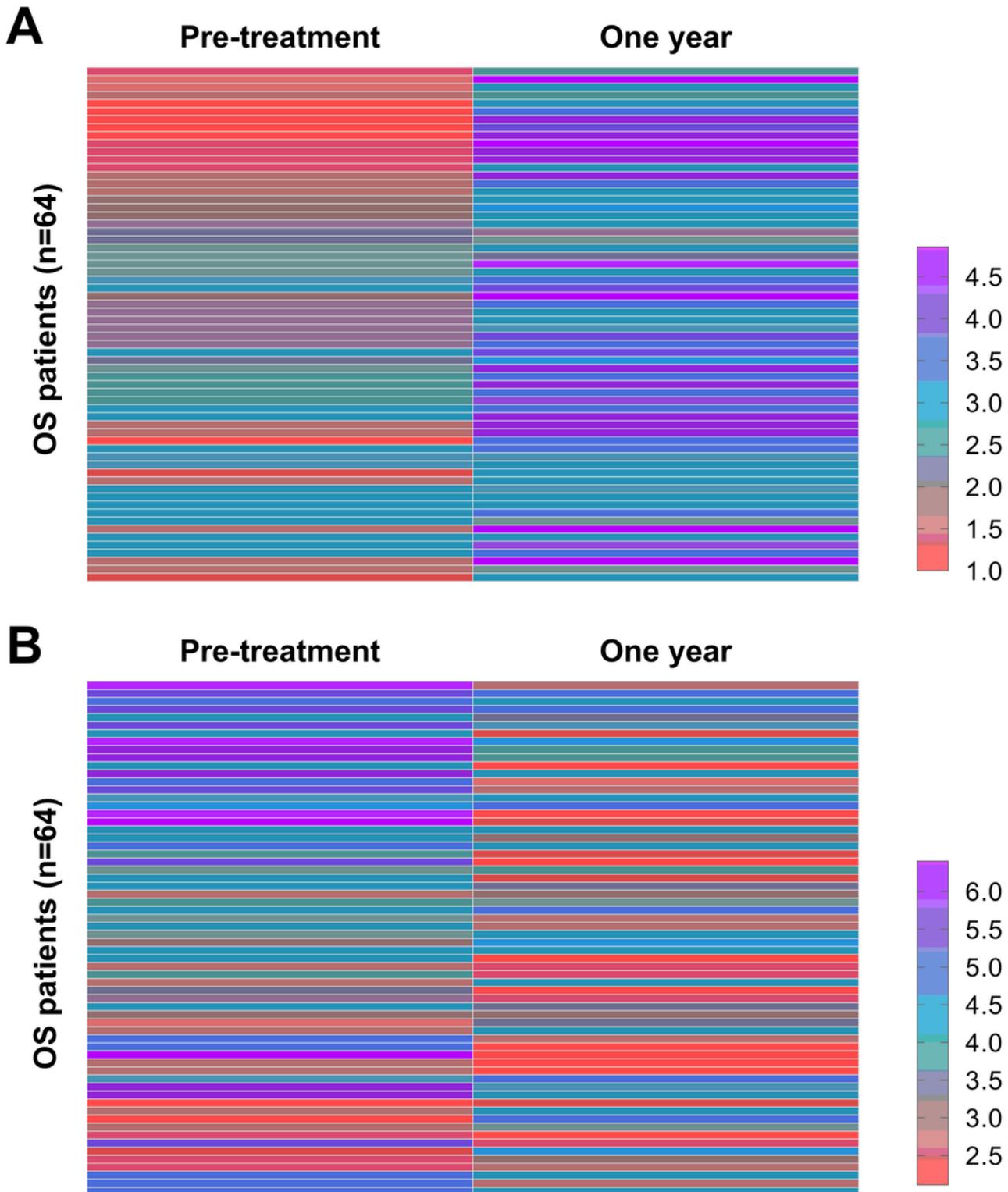


Figure 2

Treatment altered the expression of circFADS2 and miR-16-5p in OS patients. Expression of circFADS2 (A) and miR-16-5p (B) in plasma from OS patients was also determined at 1 year after the initiation of therapy. Heatmaps were plotted using Heml 1.0 software to reflect the changes in gene expression during treatment.

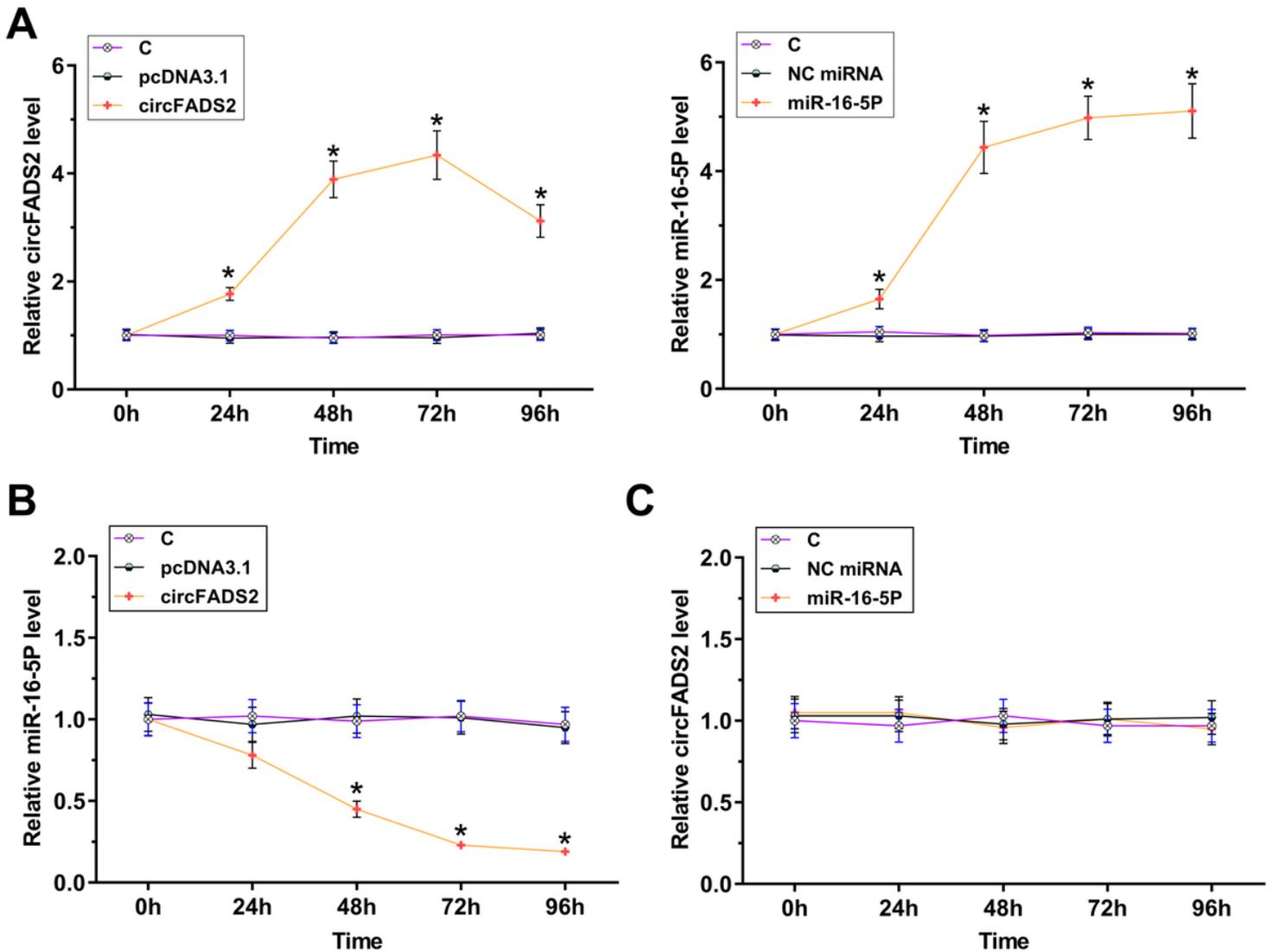


Figure 3

Overexpression of circFADS2 decreased the expression of miR-16-5p in osteoblasts To study the crosstalk between circFADS2 and miR-16-5p, osteoblasts were transfected with circFADS2 expression vector or miR-16-5p mimic, followed by the confirmation of circFADS2 and miR-16-5p every 24h until 96h (A). The effects of circFADS2 overexpression on the expression of miR-16-5p (B) and the effects of miR-16-5p overexpression on the expression of circFADS2 (C) were also analyzed by RT-qPCR. *, $p < 0.05$.

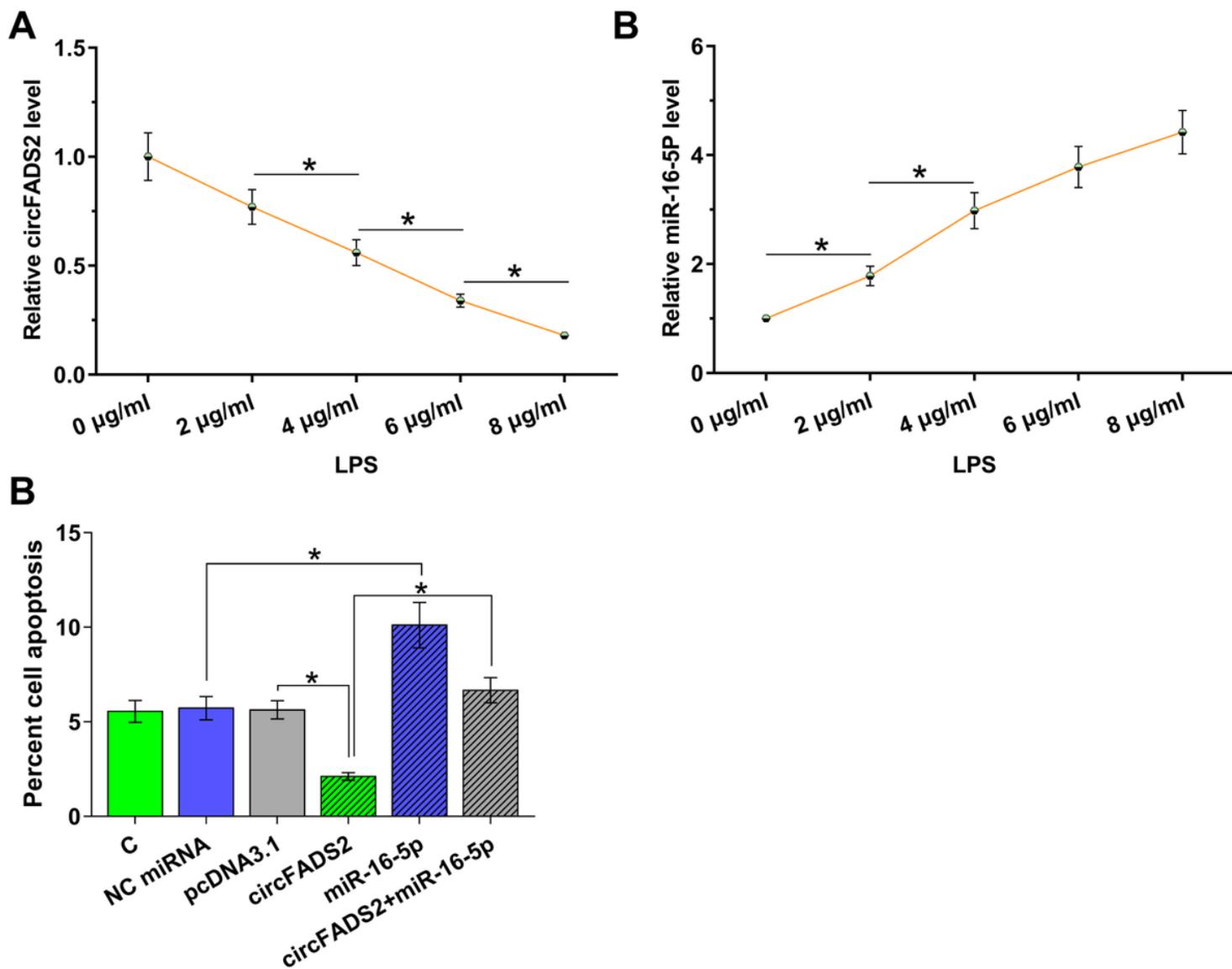


Figure 4

CircFADS2 suppressed the apoptosis of osteoblasts induced by LPS through miR-16-5p To study the effects of LPS treatment on the expression of circFADS2 (A) and miR-16-5p (B), osteoblasts were cultivated in medium supplemented with 0, 2, 4, 6 and 8 μg/ml LPS for 48h, followed by RT-qPCRs. Cell apoptosis assay was performed to study the functions of circFADS2 and miR-16-5p in the apoptosis of osteoblasts induced by LPS (C).*,p<0.05.