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Increased serum miR-300 level serves as a potential biomarker of lipopolysaccharide-induced lung injury by targeting IκBα

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Received July 26, 2016, accepted August 26, 2016

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Pharmazie 72: 5–9 (2017) *doi: 10.1691/ph.2017.6776*

MicroRNAs (miRs) are reported to play key roles in various disease models. In this study, the functional role of miR-300 in the regulation of lung injury was explored to assess the feasibility of serum miR-300 as a potential biomarker for lung injury. Firstly, the expression of miR-300 was studied in the serum of 50 lung injury patients and 50 healthy controls. And the expression of miR-300 was also explored in the serum and lung tissues of mouse models. To further explore the possible mechanism in which miR-300 may contribute to lung injury, the target genes of miR-300 were predicted by TargetScan and validated using dual luciferase reporter assay. Moreover, the expression of inflammation factors was studied after transfection of miR-300 mimics and inhibitors into A549 cells. Here, we first identified that the level of miR-300 was significantly upregulated in the blood samples of acute lung injury patients compared with healthy control. Meanwhile, miR-300 was also found to be enhanced in the blood samples and lung tissues of LPS-induced mouse models. Further study showed that miR-300 significantly suppressed the expression of IκBα and luciferase reporter assay showed that IκBα was a target gene of miR-300. More importantly, the levels of inflammatory factors, such as TNFα, COX-2, iNOS, IL-6 and IL8, were significantly upregulated accompanied by overexpression of miR-300 in A549 cells. In summary, enhanced miR-300 expression in the peripheral blood contributed to the lung injury mainly by inhibiting the expression of IκBα.

1. Introduction

Toll like receptor (TLR) and nuclear factor kappa B factor (NF-κB) signaling are the major contributors of inflammatory responses (Marcu et al. 2010; Houard et al. 2013). Abnormal expression of NF-κB activation has been identified in various disease models, especially considering the production of procatabolic mediators, such as the proinflammatory cytokins (Rigoglou and Papavassiliou 2013). It was reported that the activation of NF-κB signaling can execrate the inflammatory responses through forming a positive feedback circuit (Saito et al. 2010; Quan et al. 2013; Rigoglou and Papavassiliou 2013). At normal status, the inhibitory κB (IκB) proteins retains the NF-κB dimmers in the cytoplasm (Hoffmann et al. 2006). However, after lung injury, the NF-κB signaling was activated and then acts as a sensor of inflammatory insults, which initiates the onset of inflammatory responses (Zhang et al. 2008).

MicroRNAs (miRNAs, miRs) are small noncoding RNAs that are widely involved in gene expression at the post-transcriptional level (Chen and Liu 2015). Through the incomplete base pairing mechanism, miRNA induces mRNA degradation. Nearly 30% of human genes are reported to be regulated by miRNAs (Li et al. 2011). Studies have indicated that miRNAs play a key role in the progression of autoimmunity (Xie et al. 2012; Wu et al. 2013). For instance, reduced miR-152 and miR-30a-3p expression levels as well as increased miR-133b and miR-7 expression levels were found in the older-age patients with bronchopulmonary dysplasia compared to the younger-age set (Wu et al. 2013). In addition, miR-127 was suggested to suppress lung inflammation mainly by binding the 3'untranslated region of IgG Fcγ receptor I (Xie et al. 2012).

MiR-300 a microRNA that was widely studied in tumors, including glioblastoma, human oral squamous cell carcinoma and breast cancer (Xu et al. 2015; Lin et al. 2016; Zhou et al. 2016). However, the functional role of miR-300 in acute lung injury (ALI) has never been explored. Here, we first report that the level of miR-300 was significantly enhanced in the peripheral blood of acute lung injury patients. And through targeting IκBα, enhanced miR-300 expression activates NF-κB signaling which then execrates the progression of lung injury.

2. Investigations, results and discussion

2.1. *The level of miR-300 was increased in the peripheral blood of ALI patients*

Serum level of c-reactive protein (CRP) was significantly enhanced in ALI patients compared with healthy control (Fig. 1A), indicating an inflammatory response in ALI patients. Meanwhile, we also explored the level of miR-300 and found it was significantly increased in the peripheral blood samples of ALI patients compared with healthy control (Fig. 1B).

2.2. *Enhanced miR-300 expression in the peripheral blood and lung tissues of ALI mice models*

To further validate the functional role of miR-300 in ALI, we established ALI mice models with LPS treatment. Firstly, we collected the peripheral blood of ALI mice and explored the expression of CRP as well as miR-300. As shown in Fig. 2A, the level of CRP was significantly increased in the peripheral blood of ALI mice compared with vehicle control. Meanwhile, the expression of miR-300 was increased in the peripheral blood of ALI mice

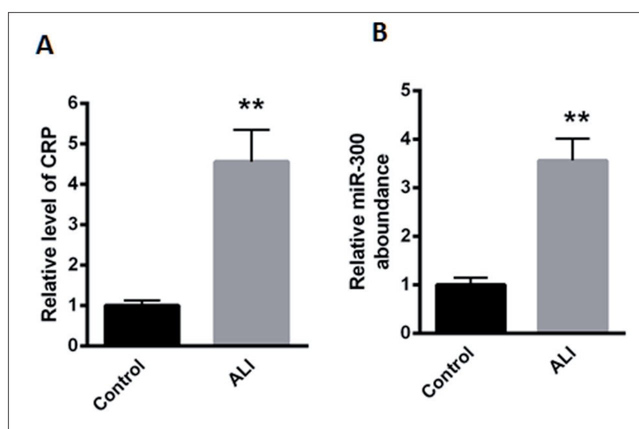

Fig. 1: The level of miR-300 was increased in the peripheral blood of ALI patients. (A) Serum level of c-reactive protein (CRP) was significantly enhanced in ALI patients (n=50) compared with healthy control (n=50). (B) The level of miR-300 was significantly increased in the peripheral blood samples of ALI patients (n=50) compared with healthy control (n=50). Data were presented as mean ± SE from 3 independent experiments. **P<0.01.

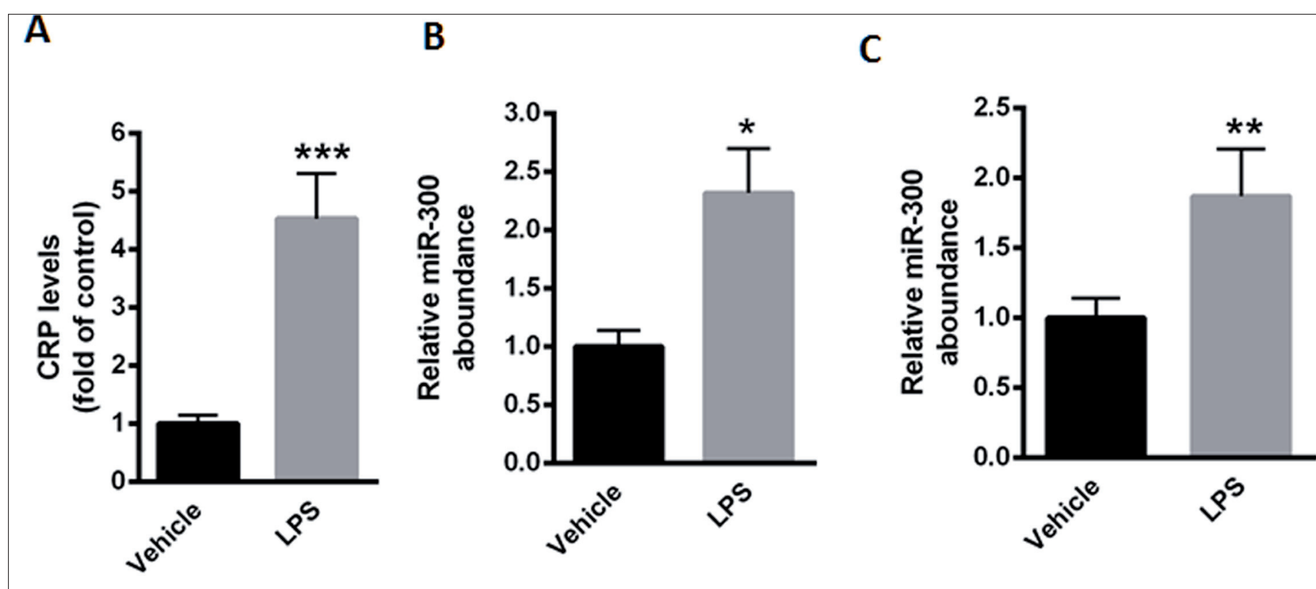

Fig. 2: The expression of miR-300 was increased in both the peripheral blood and lung tissues of ALI mice models. (A) The level of CRP was determined in the peripheral blood of ALI mice models. (B) The expression of miR-300 was explored in the peripheral blood of ALI mice models. (C) The expression of miR-300 was explored in the lung tissues of ALI mice models (n=6). Data were presented as mean ± SE from 3 independent experiments. *P<0.05; **P<0.01; ***P<0.001.

(Fig. 2B). Moreover, we also collected the lung tissues of ALI mice and control mice. And the level of miR-300 was also demonstrated to be markedly increased in the lung tissues of ALI mice and control mice (Fig. 2C). Together, these data showed that increased miR-300 level was related to the inflammatory response of ALI.

2.3. IκBα is the target gene of miR-300

Then, we try to serve the potential target gene of miR-300 that is correlated with inflammatory response. Interestingly, we found that there was a conserved binding site of miR-300 on the 3'UTR of IκBα (Fig. 3A). Then, the 3'UTR of IκBα was cloned into the pmirGLO plasmid. Dual luciferase reporter assay indicated that the luciferase activity of pmirGLO-IκBα-3'UTR was significantly suppressed by miR-300 (Fig. 3B). Moreover, overexpression of miR-300 significantly repressed the expression of IκBα, while inhibition of miR-300 increased the protein level of IκBα in A549 cells (Figs. 3C and 3D). These data indicated that IκBα was a target gene of miR-300.

2.4. NF-κB activation is enhanced by miR-300 through IκBα

Furthermore, we also determined the downstream inflammatory factors of NF-κB signaling. As shown in Fig. 4A, the expression of TNFα, COX-2, iNOS, IL-6 and IL8 was markedly enhanced when miR-300 was overexpressed. Furthermore, the phosphorylation level of NF-κB was increased when miR-300 mimics was transfected into A549 cells (Fig. 4B). More importantly, a specific siRNA targeting IκBα was applied to identify whether miR-300 exerts its role through IκBα. As shown in Fig. 4C, silence of IκBα significantly activated the NF-κB signaling, even in cells transfected with miR-300 inhibitors (Fig. 4C).

Acute lung injury (ALI) is well recognized as typical characteristics of severe lung edema and an increase in the inflammatory reaction (Wang et al. 2015). Considerable evidence has indicated that microRNAs (miRNAs or miRs) are involved in various human diseases (Liu et al. 2015; Yang et al. 2015). NF-κB signaling plays a key role in the inflammatory and oxidative stress induced injury, which suggests a potential therapeutic target for lung injury patients (Liu et al. 2015, Zhao et al. 2015). Abnormal activation of NF-κB signaling significantly enhanced the expression of proinflammatory cytokines, including TNFα and IL-1β (Tong et al. 2015).

MiRNAs are small noncoding RNAs that widely regulate gene expression by an incomplete binding mechanism (Rao et al. 2015). Previous studies have indicated the correlation between miRNAs and lung injury (Guo et al. 2014; Rao et al. 2014, Ying et al. 2015). For instance, overexpression of miR-127 in macrophages led to obvious enhanced production of proinflammatory cytokines, whereas deletion of miR-127 reduced M1 gene expression and resulted in a M2-biased response, which may cause potential lung injury (Ying et al. 2015). Besides, enforced expression of miR-125b was reported to effectively improve the LPS-induced ALI, indicating a potential application for miR-125b-based therapy to treat ALI among patients (Guo et al. 2014). And miR-155 was found to enhance Staphylococcal enterotoxin B (SEB)-induced inflammation in the lungs mainly by targeting Socs1 (Rao et al. 2014).

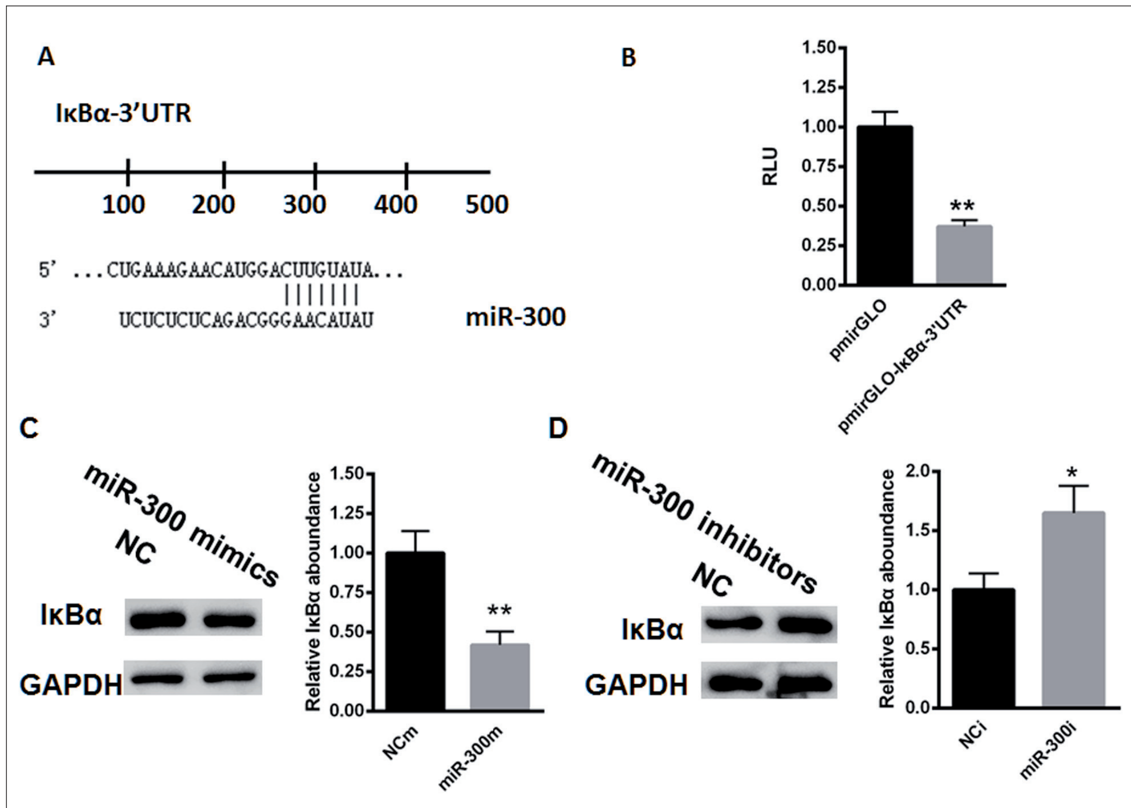

Fig. 3: IκBα is the target gene of miR-300. (A) Schematic analysis of miR-300 on the 3'UTR of IκBα. (B) Dual luciferase reporter assay indicated that the luciferase activity of pmirGLO-IκBα-3'UTR was significantly suppressed by miR-300. (C) Overexpression of miR-300 significantly repressed the expression of IκBα. (D) inhibition of miR-300 increased the protein level of IκBα. Data were presented as mean ± SE from 3 independent experiments. *P<0.05, **P<0.01.

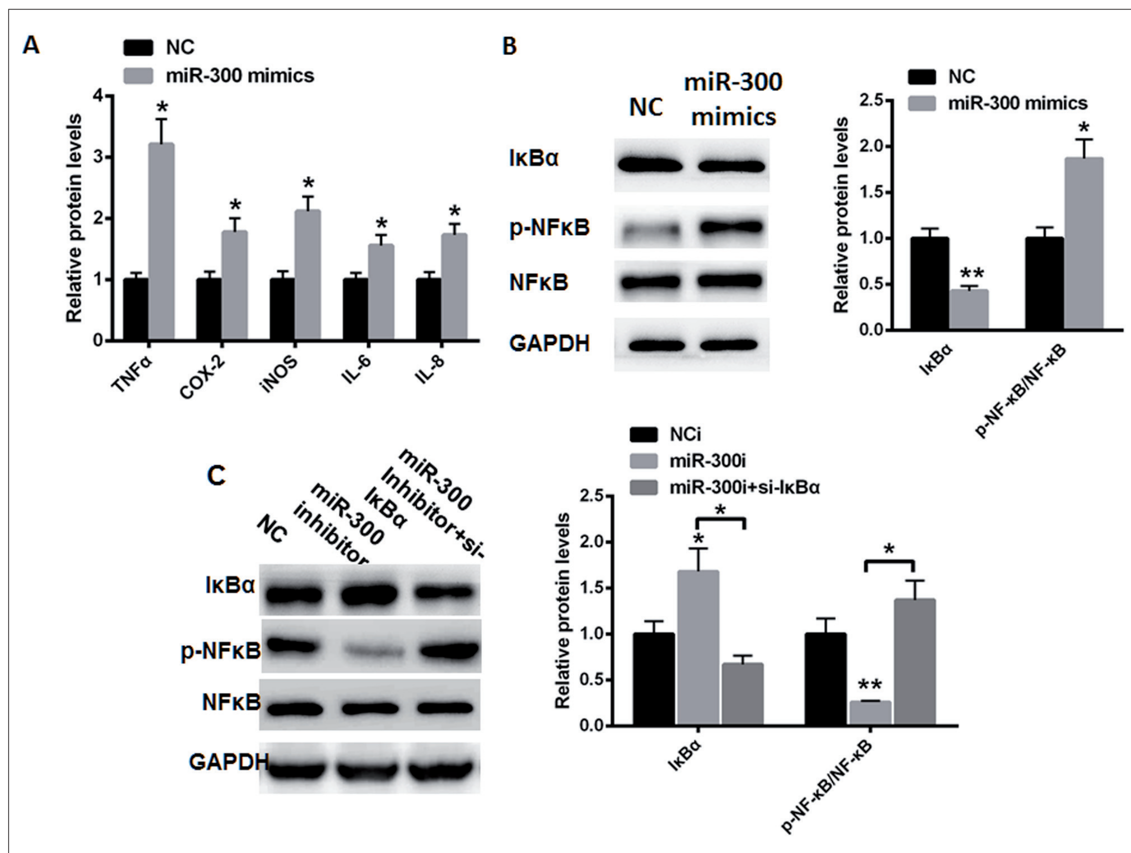

Fig. 4: NF-κB activation is enhanced by miR-300 through IκBα. (A) The expression of TNFα, COX-2, iNOS, IL-6 and IL8 was markedly enhanced when miR-300 was overexpressed. (B) The phosphorylation level of NF-κB was increased when miR-300 mimics was transfected into A549 cells. (C) Silence of IκBα significantly activated the NF-κB signaling, even in cells transfected with miR-300 inhibitors. Data were presented as mean ± SE from 3 independent experiments. *P<0.05, **P<0.01.

In this study, we mainly focus on miR-300, which has been extensively explored in tumors. Here, we first demonstrated that the level of miR-300 was significantly enhanced in the peripheral blood of ALI patients compared with healthy control. Meanwhile, in the blood samples of LPS-induced ALI mice models, the level of miR-300 was also found to be increased. We also determined the inflammation status in the patients as well as the mice models with the prognostic biomarker of CRP. Interestingly, the enhanced level of miR-300 showed a positive correlation of CRP levels in the peripheral blood samples of ALI. Therefore, we hypothesized that enhanced miR-300 in peripheral blood may be a possible marker of ALI.

To further identify the correlation between miR-300 and ALI, the possible target gene of miR-300 was studied. And we first validated that IκBα, a well-known inhibitor of NF-κB signaling, is a target gene of miR-300. Canonical NF-κB signaling directly enhances the transcription of downstream regulators, which links inflammation and oxidative stress responses (Zhang et al. 2015). Thus, we explored the expression of downstream inflammation factors of NF-κB signaling, including TNFα, COX-2, iNOS, IL-6 and IL8. We found that these inflammatory factors were all increased when miR-300 was upregulated in A549 cells.

To conclude, enhanced miR-300 level in peripheral blood of ALI patients may serve as a prognostic biomarker to screen the inflammation response.

3. Experimental

3.1. Patients and blood samples

Patients were eligible for enrollment if they fulfilled the criteria of sepsis and ALI (National Heartung Blood Institute et al. 2014). ALI was defined as acute onset of the illness and all of the three criteria: 1) a ratio of the partial pressure of arterial oxygen (Pao2) to the fraction of inspired oxygen (Fio2) of 300 or less; 2) bilateral infiltrates on chest radiography that were consistent with pulmonary edema, without evidence of left atrial hypertension; and 3) receiving positive-pressure mechanical ventilation through an endotracheal tube (Bernard et al. 1994). All peripheral whole blood samples of lung injury patients (50, aged at 54±8.9, male/female (25/25)), and healthy individuals (50, aged at 52±9.4, male/female (25/25)) were obtained from the Second Xiangya Hospital between June 2015 and January 2016. The application of patient-derived materials was approved by the Research Ethics Committee of the Second Xiangya Hospital, and written consent was obtained from all patients.

3.2. Establishment of a mouse model for lung injury

Briefly, eight-week-old mice were treated with intrapulmonary instillation (1 $mg.kg^{-1}$; V = 100 μl) of lipopolysaccharide (LPS) from Escherichia coli (Santa Cruz Biotechnology, Inc., CA, USA) using a MicroSprayer aerosolizer (Penn-Century Inc., PA, USA). At 48 h post LPS challenge, the lung tissue was collected. All experiments were performed in accordance with the national guidelines for the care of laboratory animals and the study was approved by the Ethical Committee of Hunan Normal University.

3.3. Cell culture

A549 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

3.4. miRNA Transfection

A549 cells were seeded at the concentration of 2×10^5 cells/well. Twenty four hours later, miRNA Control (NC) or miR-300 mimics or inhibitors was transfected into cells at a final concentration of 50 nM using Hiperfect transfection reagent according to the manufacturers' instructions (Qiagen, Hilden, Germany).

3.5. Western blot

Cells were first washed with PBS for three times and then followed by lysis in RIPA buffer (1% TritonX-100, 150 mmol/L NaCl, 5 mmol/L EDTA, and 10 mmol/L Tris-HCl (pH 7.0) (Solarbio, Beijing, China). The cell lysates were collected after centrifugation at 12,000 rpm for 15 min. Protein concentration was determined using a BCA protein assay kit (Merck Millipore, Darmstadt, Germany). The cell lysates were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a PVDF membrane. The membranes were blocked with 5% non-fat dried milk in TBST for 1 h at room temperature. And then, the specific primary antibodies were added for incubation overnight at 4 °C. Subsequently, the membranes were washed with TBST for three times and incubated with the appropriate HRP-conjugated anti-rabbit IgG secondary antibodies (Zhongshanjinqiao, Beijing, China) at room temperature for 1 h. The blots were detected using the ECL plus detection system (Millipore, Darmstadt, Germany) and digitized data were quantified by Image J software.

3.6. Reverse transcription-PCR

RNA was extracted with RNAvizol reagent (Vigorous, Beijing, China) and reverse transcribed with the EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix (AE311-03, Transgene, Beijing, China). The PCR program was described as follows: 48 °C for 45 min; 96 °C for 2 min; 25 repeats of 94 °C for 30 s, 55 °C for 45 s, and 68 °C for 80 s; followed by a final extension at 65 °C for 6 min. In addition, Taqman miRNA assays (Applied Biosystems) were applied to detect the expression level of mature miRNAs using the stem-loop method. All PCRs were run in triplicate and gene expression, relative to U6 small nuclear RNA (RNU6), was calculated by the comparative ΔCt method.

3.7. Luciferase reporter assay

To verify whether miR-300 targets the 3'untranslated region (3'UTR) of IκBα, the sequence containing the predicted binding sites was included into the pmirGLO plasmids (Promega, Madison, WI, USA). For luciferase reporter assay, A549 cells were seeded into 96-well plates at 10000 cells per well the day before transfection. Then, a mixture of 100 ng pmirGLO-IκBα–3'UTR and 50 nM miRNA mimic or negative control were transfected into A549 cells using Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). After 48 h, firefly and Renilla luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The firefly luciferase activities served as an internal control for transfection efficiency.

3.8. Statistical analysis

All results are expressed as mean values±S.E.M. (standard error of mean). The evaluation of real-time PCR data was done by one-way ANOVA with a post-hoc Tukey's test using $2^{-\Delta\Delta ct}$ values of each sample. A value of $P<0.05$ was considered significant.

Conflicts of interest: None declared.

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