

MiR-4262 regulates differentiation and osteogenesis of human periodontal stem cells by targeting suppressor of cytokine signaling 4

ZIMING WEI^{1,2}; YARONG WANG³; MINGYUAN DU^{1,4}; MINJIE LI²; HONG HE^{1,4,*}

¹ The State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) & Key Laboratory of Oral Biomedicine Ministry of Education, School & Hospital of Stomatology, Wuhan University, Wuhan, 430079, China

² Department of Orthodontics, Stomatological Hospital, Southern Medical University, Guangzhou, 510280, China

³ Department of Periodontology and Implantology, Stomatological Hospital, Southern Medical University, Guangzhou, 510280, China

⁴ Department of Orthodontics, School and Hospital of Stomatology, Wuhan University, Wuhan, 430079, China

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Abstract: Periodontitis, as a chronic inflammatory disease, remains unsolved, and the pathogenesis of this disease has not been fully elucidated. In this study, the effect of miR-4262 was investigated in tumor necrosis factor- α (TNF- α) induced human periodontal stem cells (hPDLSCs) for the first time. The gene expression involved in this study was determined using polymerase chain reaction (PCR), the expressions of relevant proteins were determined by western blot analysis, and the levels of IL-1 β , IL-6, and MCP-1 were estimated by enzyme-linked immunosorbent assay (ELISA) assay. The luciferase reporter assay was performed for verification of the target gene, alkaline phosphatase (ALP) activity detection was used for differentiation capacity, and alizarin red staining assay was used for mineralization capacity. The inhibition of miRNA-4262, which resulted in the upregulation of suppressor of cytokine signaling 4 (SOCS4), showed protective effects, including anti-inflammation and promotional effects on osteogenesis as well as differentiation in TNF- α induced hPDLSCs. These results provided insights into the roles of miRNAs in regulating the inflammatory response, differentiation, and osteogenesis in hPDLSCs, which may promote the understanding of the mechanisms of periodontitis and find out a better therapeutic application.

Introduction

Periodontitis, the main cause of tooth loss in adults, has gained widespread attention. Damage of tooth-supporting tissues is one of the main features of periodontitis (Gaudilliere *et al.*, 2019). As a chronic infectious disease induced by bacteria, periodontitis is difficult to cure, and the therapeutic efficacy is not satisfactory. Periodontitis often results in different inflammatory responses that depend on the individual (Bartold, 2018). Therefore, there is a pressing need to elucidate the disease mechanisms and discovering new treatments with better therapeutic efficacy for periodontitis.

hPDLSCs connect the tooth to the alveolar bone socket, aiding in the regeneration of the injured tissue and homeostasis (Bojic *et al.*, 2014; Nagata *et al.*, 2017; Sedgley

and Botero, 2012). The regeneration of destroyed tissues plays an important role in the treatment of periodontitis, so it is of high value to explicitly identify the regeneration mechanism of hPDLSCs to repair the damaged tissues (Feng *et al.*, 2012). However, the regeneration mechanism of the cells is still not fully clear. Previous studies have reported many vital endogenous substances, including key regulators, molecular markers, and related signaling pathways, that are involved in the regeneration mechanism. For example, the Runt-related transcription factor 2 (RUNX2) induced by fibrinogen has been found to play a pivotal role in osteogenic differentiation (Kidwai *et al.*, 2016). Moreover, osteoprotegerin (OPG) has been found to mediate the inhibitory effect of TSH on osteoclast differentiation (Ma *et al.*, 2011). ALP activity has been found to serve as the marker of early osteogenic stem differentiation (Wang *et al.*, 2017; Wu *et al.*, 2018).

Similarly, collagen I, as the vital natural matrix component, has also been used as the biomarker for osteogenesis evaluation. All the relevant endogenous

*Address correspondence to: Hong He, drhehong@whu.edu.cn

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substances described above were evaluated herein to illuminate the relevant mechanisms.

Inflammation is the focus of periodontitis in many studies. TNF- α , a pro-inflammatory cytokine, has been the primary focus of the research (Fredriksson *et al.*, 2002; Noh *et al.*, 2013). TNF- α is considered to have an inhibitory effect on osteogenic differentiation and is involved in the development of periodontitis (Ding *et al.*, 2014; Lacey *et al.*, 2009; Li *et al.*, 2010). Furthermore, the vital biomarker miRNAs have been widely studied in diabetes, inflammatory diseases, cardiovascular diseases, and various cancers (Ambs *et al.*, 2008; Feng *et al.*, 2014; Feng *et al.*, 2012; Yuan *et al.*, 2011). They mainly regulate gene expression at the post-translational level, which illuminates the regulatory mechanism of many fields at the gene level. MiRNAs not only serve as the regulators in many physiological processes but also act as predictive markers and therapeutic targets. For instance, miRNA-128 has been found to regulate the secretion of inflammatory cytokines, which could be applied to periodontitis therapy (Na *et al.*, 2016). Similarly, miRNA-203 has been reported to play an important role in the pathogenesis of *Porphyromonas gingivalis*-challenged gingival epithelial cells (Moffatt and Lamont, 2011). MiR-4262 has been found to be related to osteoarthritis, gastric cancer, and colon cancer cells (Sun *et al.*, 2018; Weng *et al.*, 2018; Zhang *et al.*, 2019). It regulates the apoptosis and autophagy of chondrocytes; however, the role of miR-4262 in periodontitis remained largely unknown. In this study, we aimed to investigate the effect of miR-4262 in TNF- α -induced hPDLSCs and explore the underlying mechanism.

Materials and Methods

Cell culture and treatment

hPDLSCs were purchased from iCell Bioscience Inc (Shanghai, China) and cultured in α -MEM medium containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/mL of penicillin, 100 mg/mL of streptomycin, and 2 mmol of glutamine. Cells were maintained in an atmosphere of 5% CO₂ at 37°C in saturated humidity. The medium was refreshed every three days. hPDLSCs were treated with TNF- α (10 ng/mL) for 0 d, 3 d, 7 d, and 14 d, respectively.

Cell transfection

MiR-4262 mimic (chemical synthesis of mature miRNA double chain), miR-4262 inhibitor (chemical modification of mature miRNA complementary single chain), and corresponding negative control (mimic-NC or inhibitor-NC) were purchased from RiboBio (Guangzhou, China). Cells were seeded into a six-well plate and transfected with mimic or inhibitor at 70–80% confluence according to the manufacturers' instruction for 48 h. shRNA-SOCS4-1, shRNA-SOCS4-2, and shRNA-NC (GenePharma, Shanghai, China) were generated to interfere with the expression of SOCS4. The shRNA was transfected into cells by Lipofectamine 3000 (Thermo Fisher Scientific) and incubated for 48 h.

ELISA assay

The cells in different groups lysed by pancreatin were subjected to centrifugation (500 \times g, 8 min). The levels of IL-1 β , IL-6, and MCP-1 in the supernatants for each group

were detected using the ELISA kits (Beyotime, Jiangsu, China). The absorbance values at 450 nm were obtained to determine the levels of IL-1 β , IL-6, and MCP-1 using a spectrophotometer (Bio-Rad, CA, USA).

Quantitative real-time PCR

The gene expressions involved in this study were detected using quantitative real-time PCR (qRT-PCR). QRT-PCR was performed as follows: 95°C for 15 min, 40 cycles of 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s. The primers of miRNA 4262 and U6 (internal control) were purchased from Applied Biosystems (Foster City, CA, USA). The remaining primers for DNA synthesis were as follows: RUNX2: Forward 5'-TCAACGATCTGAGATTTGTGGG-3', Reverse 5'-TCAA CGATCTGAGATTTGTGGG-3'; OPG: Forward 5'-TGCTGT TCCTACAAAGTTTAC-3', Reverse 5'-CTTTGAGTGCT TTAGTGCGTG-3'; Col1a1 Forward 5'-ACGTCCTGGTGA AGTTGGTC-3', Reverse: 5'-CAGGGAAGCCTCTTT CTCCT-3'; GAPDH (internal control): Forward: 5'-GAAGG TGAAGGTCGGAGTC-3', Reverse: 5'-GAAGATGGTG ATGGGATTTC-3'; 2- $\Delta\Delta$ Ct methods was used to calculate the relative gene expression level.

Western blot analysis

The total proteins in each group were collected. Then, the samples were transferred to SDS-PAGE for separation and transferred onto polyvinylidene difluoride membranes. Furthermore, 5% fat-free milk in the Tris-buffered saline-Tween-20 solution (TBST) was used to block the membranes. Next, the primary antibodies, including the anti-RUNX2 antibody, anti-OPG antibody, collagen I antibody, anti-SOCS4 antibody, and anti-GAPDH antibody, were incubated with polyvinylidene difluoride membranes. The GAPDH level was detected as an internal reference. All the antibodies described above were purchased from the Abcam Company. After the membrane was washed with TBST, it was incubated with the secondary antibody for 1 h. Image Quant_LAS500 (GE, Japan) was used to obtain the images.

Luciferase reporter assay

SOCS4 was considered to be a target gene of miR-4262 through searching the MicroRNA Target Prediction Database. The 3'-UTR of SOCS4 was found to be the binding site of miR-4262. The gene sequence of the binding site at 3'-UTR of SOCS4 was mutated using the site-directed mutagenesis kit (TransGen, Beijing, China). The mutated and wild type of SOCS4 were individually cloned into the pGL3-Basic Luciferase reporter vector (Promega, Madison, WI, USA). Then, SOCS4 (mutated or wild) were co-transfected with either the miR-4262 mimic or negative control, individually. The Luciferase Reporter Gene Assay Kit (Abnova, Taibei, China) was used to determine the luciferase activity.

ALP activity detection

ALP activity was evaluated using an ALP activity assay kit (Sigma-Aldrich, St. Louis, Missouri US). The cell lysate was collected and incubated with detection buffer for 5 min at 37°C, the absorbance was measured at 405 nm according to

the manufacturer's protocol. For the ALP staining assay, cells were seeded in a six-well plated at a density of 3×10^4 under the same conditions described above. After a week, the medium was removed, and the cells were washed three times, with 1 mL of PBS. Then, 10% neutral formalin was used to fix

the cells. Next, 50 μ L of the chromogenic substrate (Boppar, Guangzhou, China) was added into each well of the cells, and the cells were incubated at 37°C for 30 min. After that, the cells were washed with deionized water. The images of the cells were obtained using a light microscope (Nikon Corporation).

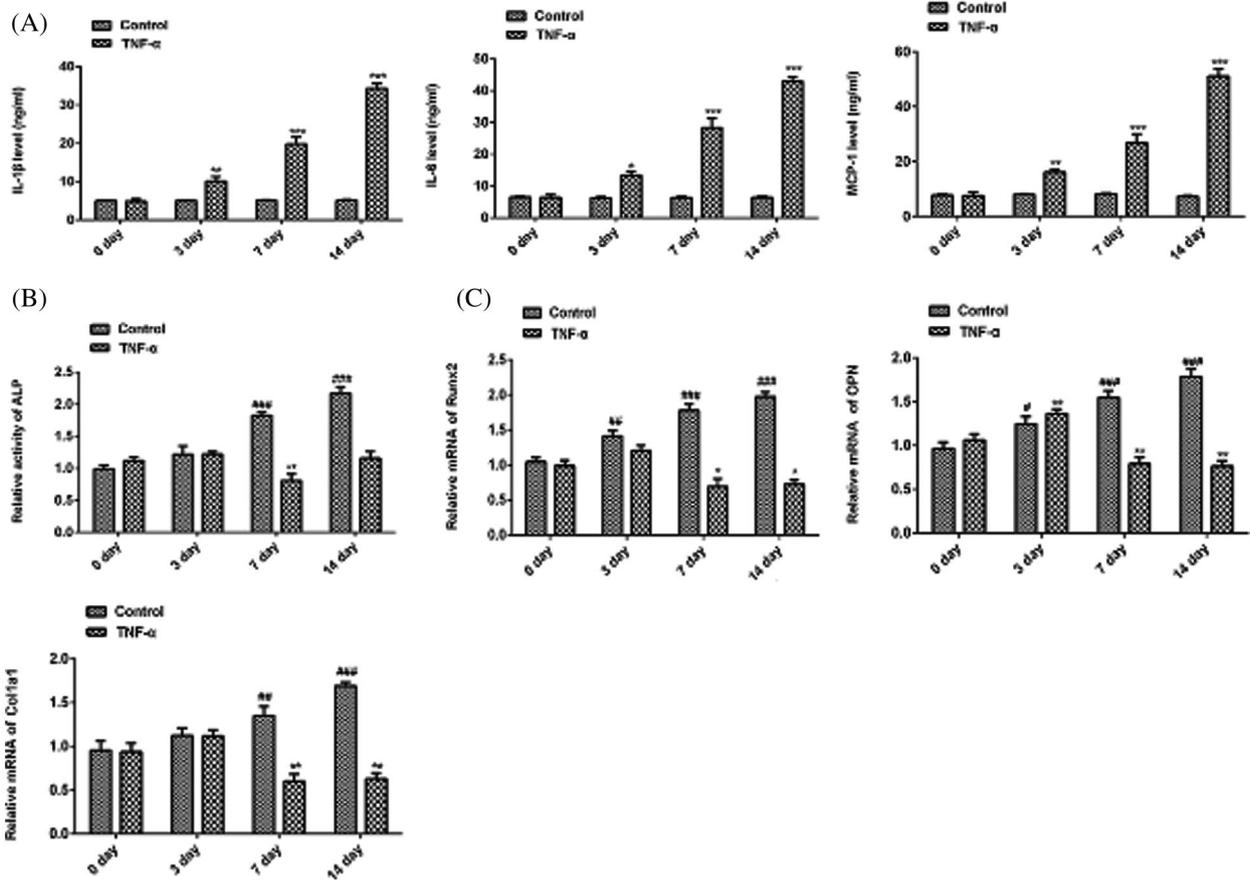


FIGURE 1. A. The levels of IL-1 β , IL-6, and MCP-1 in hPDLSCs were estimated on 0, 3, 7, 17 d by ELISA kits. B. The relative activity of ALP was determined by an ALP activity assay kit. C. The mRNA levels of RUNX2, OPN and Col1a1 were evaluated by qRT-PCR. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. control group (0 day). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. TNF- α group (0 day).

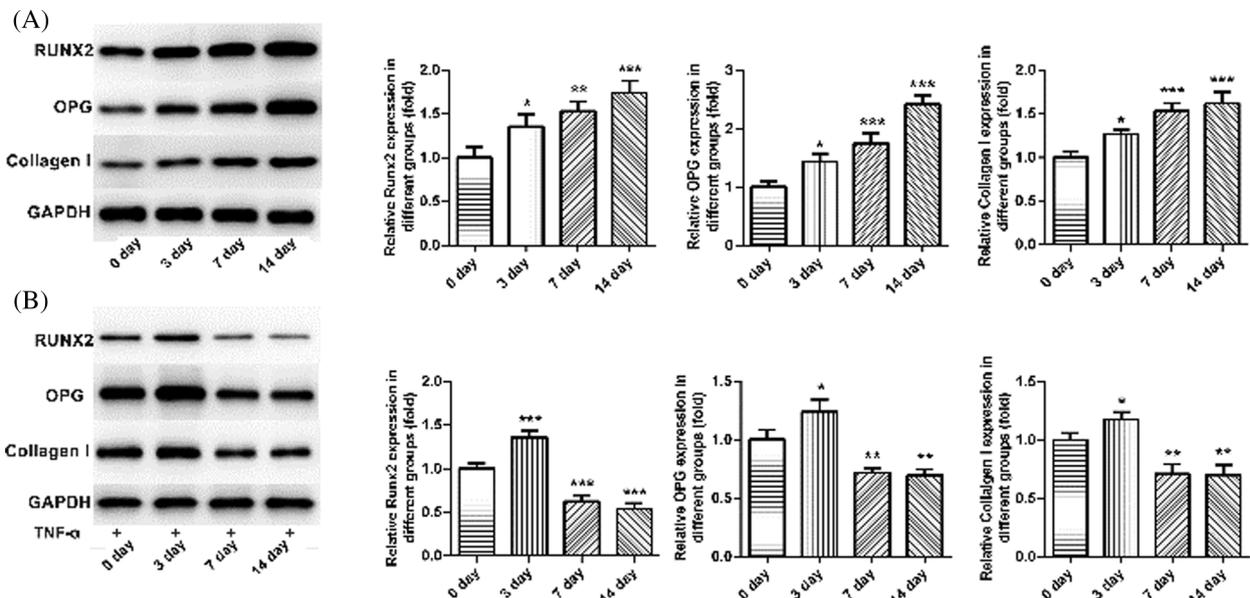


FIGURE 2. A. The protein levels of RUNX2, OPN, and collagen I in hPDLSCs were determined by western blot analysis on 0, 3, 7, 17 d. B. The protein levels of RUNX2, OPN, and collagen I in TNF- α -induced hPDLSCs were determined by western blot analysis on 0, 3, 7, 17 d. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. 0 days.

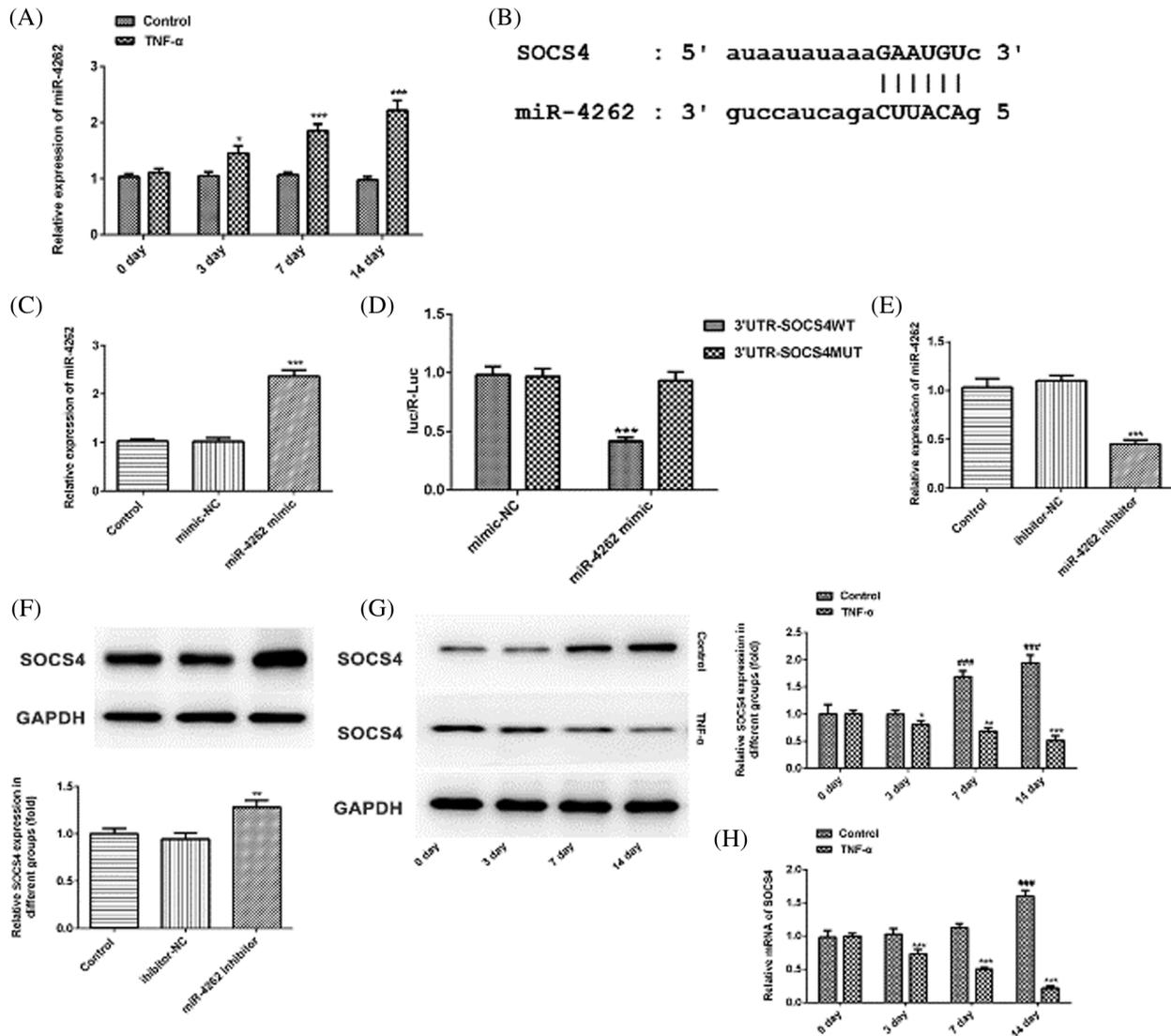


FIGURE 3. A. The level of miR-4262 was evaluated by qRT-PCR. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. TNF- α group (0 day). B. The binding sites were predicted via Starbase V2.0. C. The transfection efficiency of miR-4262 mimic was evaluated by qRT-PCR. $***p < 0.001$ vs. mimic-NC group. D. Luciferase reporter gene assay was conducted to validate the association between miR-4262 and SOCS4. $***p < 0.001$ vs. SOCS4 MUT group. E. The transfection efficiency of miR-4262 inhibitor was evaluated by qRT-PCR. $***p < 0.001$ vs. inhibitor-NC group. F. The protein levels of SOCS4 in hPDLSs transfected with miR-4262 inhibitor or inhibitor-NC were determined by western blot analysis. $**p < 0.01$ vs. inhibitor-NC group. G-H, The protein and mRNA levels of SOCS4 in hPDLSs were determined on 0, 3, 7, 17 d. $***p < 0.001$ vs. control group (0 day). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. TNF- α group (0 day).

Alizarin red staining assay

The mineralization capacity for the cells in each group after transfection with inhibitor-NC, miR-4262 inhibitor, miR-4262 inhibitor + shRNA-NC, and the miR-4262 inhibitor + shRNA-SOCS4-1 was evaluated by using an Alizarin red staining assay. The cells without transfection were shown as the control group. The cells in the groups described above were fixed with 10% formalin and then incubated with 1% Alizarin red S solution (Sigma-Aldrich) for 10 min. After washed with distilled water, the cells were examined under a microscope (Olympus, Shanghai, China).

Statistical analysis

The data were processed using the GraphPad Prism 6.0. All the data are presented as mean \pm SD. One-way or two-way variance analyses were used to determine the significant

differences between groups, and p -value < 0.05 was considered to be statistically significant.

Results

Effects of TNF- α on inflammation, ALP activity, and relevant protein expressions of bone formation in hPDLSs

TNF- α was used to stimulate hPDLSs for 0 d, 3 d, 7 d, and 14 d, respectively. The inflammatory cytokines were determined by ELISA kits. As shown in Fig. 1A, the levels of IL-1 β , IL-6, and MCP-1 in hPDLSs without TNF- α treatment remained unchanged during 0–14 d, and there was no significance between groups. However, the levels of IL-1 β , IL-6, and MCP-1 were increased over time following treatment of TNF- α . The ALP activity increased with the time in the control group without TNF- α treatment,

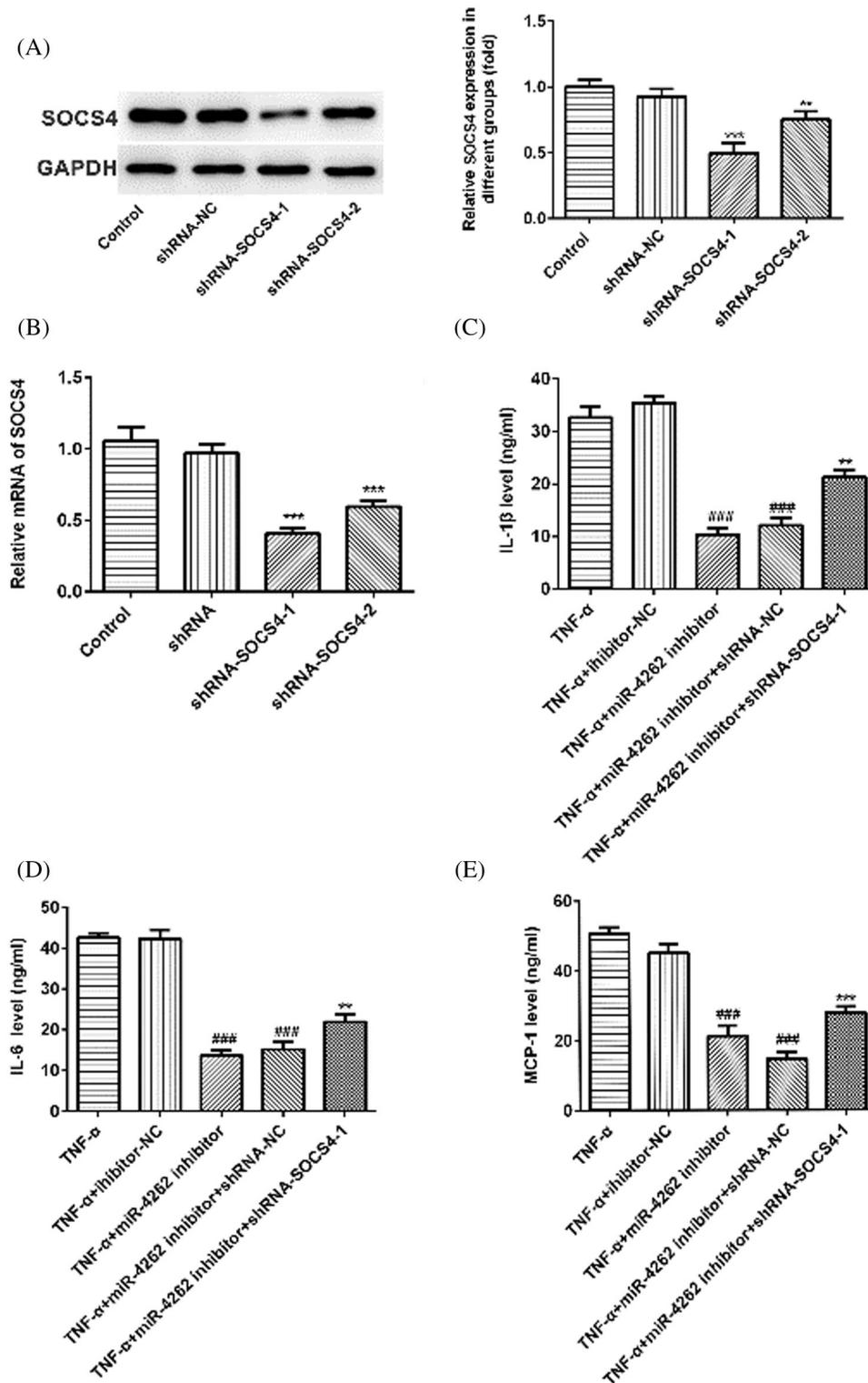


FIGURE 4. A-B. The transfection efficiency of shRNA-SOCS4-1 or shRNA-SOCS4-2 was evaluated by western blot analysis and qRT-PCR. $**p < 0.01$, $***p < 0.001$ vs. shRNA-NC group. C-E. The levels of IL-1 β , IL-6, and MCP-1 in different groups were estimated by ELISA kits. $###p < 0.001$ vs. TNF- α + inhibitor-NC group, $**p < 0.01$, $***p < 0.001$ vs. TNF- α +miR-4262 inhibitor + shRNA-NC group.

whereas hPDLSCs treated with TNF- α exhibited a decrease of ALP activity at 7 d (Fig. 1B). Moreover, the mRNA levels of RUNX2, OPG, and Col1a1 in hPDLSCs of the control group increased in a time-dependent manner. Compared with the original level (0 d), the expressions of RUNX2, OPG, and Col1a1 in the TNF- α -induced hPDLSCs were increased on the third day and then decreased until 14 d (Fig. 1C). The protein levels of them, estimated via western blot analysis, exhibited a similar trend (Figs. 2A and 2B), confirming that the activity of

ALP and the expressions of bone formation-associated proteins were inhibited by TNF- α after 3 d.

SOCS4 was confirmed as the target gene of MiR-4262

In order to clarify the effect of miR-4262 in hPDLSCs, the miR-4262 expression in cells was estimated. Results indicated that miR-4262 in hPDLSCs without TNF- α treatment remained nearly unchanged during the 14 d, and there was no significance between groups of cells without TNF- α treatment. In contrast, the level of miR-4262 was

increased over time in the TNF- α group (Fig. 3A). Subsequently, the target gene of miR-4262 was predicted via Starbase V2.0 (Fig. 3B). Luciferase reporter gene assay was performed to further validate the association between them. The expression of miR-4262 in hPDLSCs transfected with miR-4262 mimic was elevated, indicating the transfection was successful (Fig. 3C). Luciferase activity in the SOCS4 WT + miR-4262 mimic group was significantly decreased, confirming that SOCS4 was the target gene of miR-4262 (Fig. 3D). The transfection efficiency of the miR-4262 inhibitor was verified by PCR (Fig. 3E). hPDLSCs transfected with miR-4262 inhibitor presented higher protein expression of SOCS4 (Fig. 3F). Furthermore, the level of SOCS4 in hPDLSCs was monitored during 0–14 d, as shown in Figs. 3G and 3H, the SOCS4 expression was downregulated in TNF- α -induced hPDLSCs in comparison with the cells without TNF- α treatment. Taken together, these results suggested that SOCS4 may serve as a target gene of miR-4262.

Inhibition of miR-4262 expression protected against the inflammation effect via targeting SOCS4 in TNF- α -induced hPDLSCs

To specify the role of SOCS4 in TNF- α -induced hPDLSCs, the SOCS4 expression was interfered with by shRNA. Results

revealed that the protein and mRNA levels of SOCS4 were downregulated by shRNA-SOCS4-1 more significantly than by shRNA-SOCS4-2 (Figs. 4A and 4B). Therefore, shRNA-SOCS4-1 was chosen for the following experiment. It was found that inflammatory response was relieved by miRNA-4262 inhibitor, indicating that the downregulation of miR-4262 can suppress the inflammation induced by TNF- α in hPDLSCs (Figs. 4C–4E). In addition, this anti-inflammatory effect of miRNA-4262 inhibitor was weakened following the downregulation of SOCS4 by shRNA-SOCS4-1, indicating that the anti-inflammatory effect of miR-4262 inhibitor was realized via targeting SOCS4.

Inhibition of miR-4262 expression enhanced ALP activity and mineralization capacity via targeting SOCS4 in TNF- α -induced hPDLSCs

The activity of ALP in TNF- α -treated cells was increased obviously following the transfection of the miR-4262 inhibitor. However, co-transfected with shRNA-SOCS4-1 weakened the effect of miR-4262 inhibitor, indicating that the inhibition of miR-4262 expression enhances ALP activity via upregulating the expression of SOCS4 (Fig. 5A), which was consistent with the results of ALP staining assay (Fig. 5B). Moreover, compared with the TNF- α group, the mineralization capacity in the miR-4262 inhibitor group was

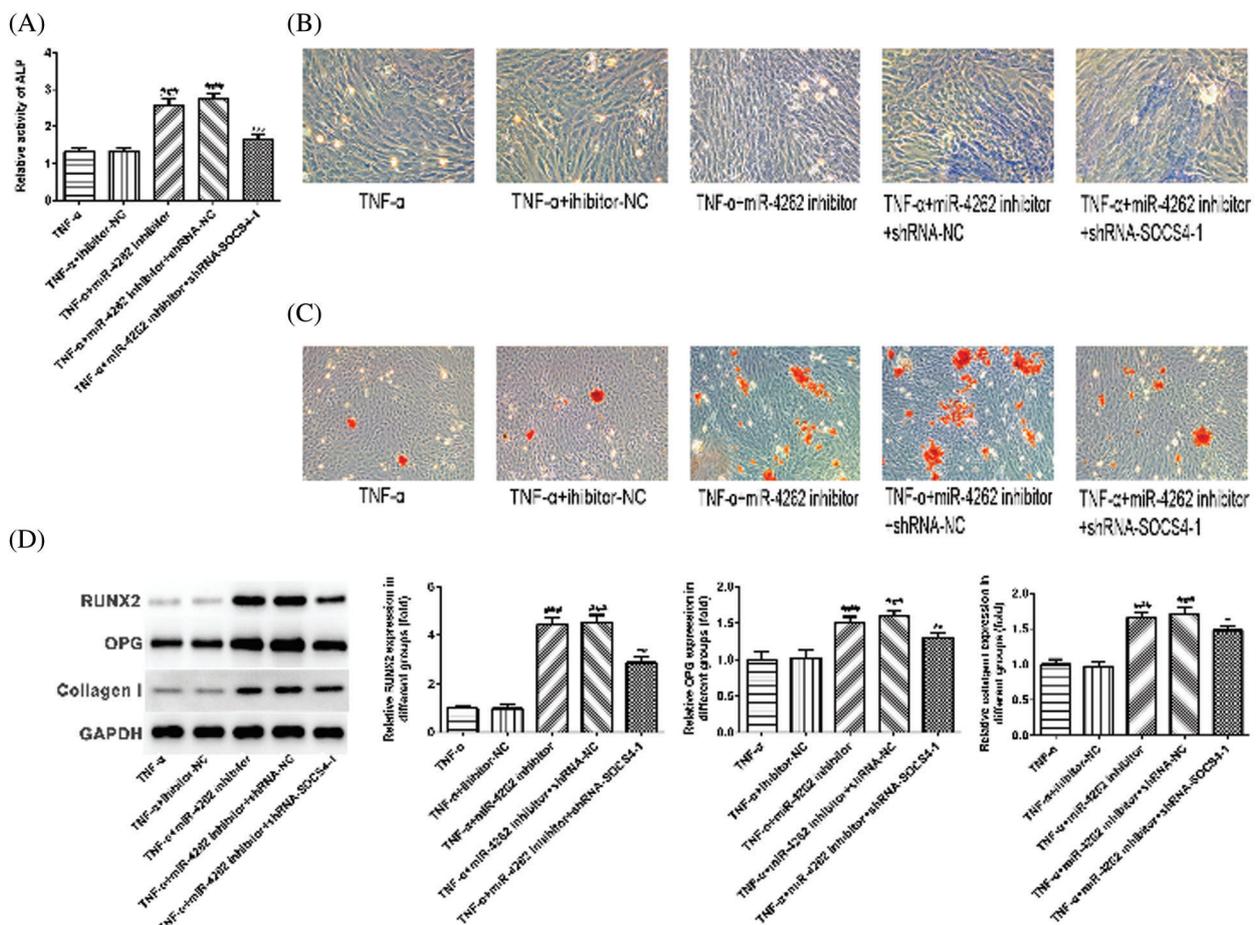


FIGURE 5. A. The relative activity of ALP was determined by an ALP activity assay kit. B. The expression of ALP was stained by an ALP staining kit. C. Alizarin red staining assay was performed to estimate the mineralization capacity. D. The protein levels of RUNX2, OPN, and collagen I were determined by western blot analysis. ### $p < 0.001$ vs. TNF- α + inhibitor-NC group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. TNF- α + miR-4262 inhibitor + shRNA-NC group.

increased obviously, as shown by the red-stained area (Fig. 5C), whereas this effect was impeded by co-transfected with shRNA-SOCS4-1. As shown in Fig. 5D, the protein levels of RUNX2, OPG, and collagen I were upregulated significantly following the transfection of the miR-4262 inhibitor. Whereas, this effect was partially reversed by shRNA-SOCS4-1, suggesting that miR-4262 modulated the bone mineralization and bone formation via targeting SOCS4.

Discussion

Periodontitis is an inflammatory disease that is the main cause of alveolar bone loss (Hienz *et al.*, 2015). Much research has been conducted on the inflammation regulatory proteins and regeneration of destroyed tissues, but because of poor clinical prediction, the outcomes of current methods for periodontal disease are limited (Hu *et al.*, 2018). Thus, there is a pressing need to illuminate the mechanism of inflammation in periodontitis and identify new avenues for prediction and treatment in periodontitis. hPDLSCs induced by TNF- α , which is the main inflammatory mediator of periodontitis, served as the model of periodontitis for a series of experiments in this study.

MiRNAs, promising functional biomolecules, have been the focus in many fields for the prediction and treatment of various diseases. In this study, we found that miR-4262 was highly expressed in TNF- α -induced hPDLSCs. An in-depth study was conducted on the effects of miR-4262 in hPDLSCs. The level of inflammatory factors, including IL-1 β , IL-6, and MCP-1, increased with the treatment time of TNF- α . Meanwhile, the ALP activity and bone formation proteins were inhibited by TNF- α . The pro-inflammatory effect and the suppression of bone formation induced by TNF- α were consistent with previous studies, confirming that the model of periodontitis was successfully constructed (Kim *et al.*, 2017).

The expression of miR-4262 was enhanced in TNF- α -induced hPDLSCs. SOCS4, as a target gene of miR-4262, was investigated in this study. SOCS4 was found to have an anti-inflammatory effect on the influenza infection (Kedzierski *et al.*, 2015; Kedzierski *et al.*, 2014). In this study, the downregulation of miR-4262 enhanced the ALP activity and mineralization in TNF- α -induced hPDLSCs, indicating important regulatory effects of miR-4262 on the differentiation and bone formation in hPDLSCs. However, the effects of the miR-4262 inhibitor were weakened by shRNA-SOCS4.

Conclusion

In this study, it was found that miR-4262 was upregulated in TNF- α -treated hPDLSCs, while the downregulation of miRNA-4262 could promote differentiation and mineralization. Moreover, the facilitation of miRNA-4262 inhibitor on osteogenesis was abrogated partially following the SOCS4 knockdown. Taken together, miRNA-4262 knockdown promoted differentiation and mineralization of hPDLSCs via elevating the expression of SOCS4. However, further *in vivo* study is required to illustrate whether

miRNA-4262 or SOCS4 could act as a potential target for periodontitis.

Availability of Data and Materials: The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: All authors have no conflicts of interest to declare.

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