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CREB1 alleviates the apoptosis and potentiates the osteogenic differentiation of zoledronic acid-treated human periodontal ligament stem cells via up-regulating VEGF

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ABSTRACT

Periodontitis represents a severe inflammatory illness in tooth supporting tissue. It has been supported that cAMP response element binding protein 1 (CREB1), a common transcription factor, extensively participates in osteogenic differentiation. Here, the current study was to look into the impacts of CREB1 on the process of periodontitis and its possible action mechanism. After human periodontal ligament stem cells (PDLSCs) were challenged with zoledronic acid (ZA), CREB1 expression was examined with RT-qPCR and western blotting. CCK-8 assay appraised cell activity. Following CREB1 elevation or/and vascular endothelial growth factor (VEGF) silencing in ZA-treated PDLSCs, CCK-8 and TUNEL assays separately estimated cell viability and apoptosis. Western blotting tested the expression of apoptosis- and osteogenic differentiation-associated proteins. ALP staining measured PDLSCs osteogenic ability and ARS staining estimated mineralized nodule formation. JASPAR predicted the potential binding of CREB1 with VEGF promoter, which was then testified by ChIP and luciferase reporter assays. RT-qPCR and western blotting tested VEGF expression. CREB1 expression was declined in ZA-exposed PDLSCs and CREB1 elevation exacerbated the viability and osteogenic differentiation while obstructed the apoptosis of PDLSCs. Additionally, CREB1 bond to VEGF promoter and transcriptionally activated VEGF expression. Further, VEGF absence partially stimulated the apoptosis while suppressed the osteogenic differentiation of CREB1-overexpressing PDLSCs treated by ZA. To be concluded, CREB1 might activate VEGF transcription to obstruct the apoptosis while contribute to the osteogenic differentiation of ZA-treated PDLSCs.

1. Introduction

Periodontitis represents a highly prevalent chronic dental infection featured by progressive periodontal support loss (Kwon et al., 2021). As reported, more than 50% of the global population suffers from periodontitis, leading to short- or long-term loss of health (2017). Risk factors including smoking, drinking, and osteoporosis have been deemed as pivotal contributors to the initiation and progression of periodontitis (Baumeister et al., 2021; Yu and Wang, 2022). Periodontitis has been identified as a major cause of tooth loss among adults through resulting in continuous and irreversible destruction of periodontal tissue including alveolar bone, periodontal ligament, and root cementum (Kwon et al., 2021; Ramseier et al., 2017). Accordingly, ascertaining the potential molecular targets may contribute to the therapy for periodontitis due to the currently limited effective treatments (Trindade et al., 2014).

Bisphosphonates are widely used compounds for the management of oncology and osteoporosis patients. Although bisphosphonates are highly effective in reducing bone loss, pain, and other skeletal clinical manifestations, they can also cause some adverse effects, such as inflammation and bisphosphonate-related osteonecrosis of the jaw (BRONJ) (Yoshiga et al., 2014). Zoledronic acid (ZA), a nitrogen-containing bisphosphonate, possesses the most potent anti-bone-resorptive effect, but it also lead to a significant 30-fold

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increased risk of injury to jawbones (Roelofs et al., 2006; Wessel et al., 2008; Woo et al., 2006). Most often, BRONJ occurs in extraction patients with active periodontal or periapical disease. Importantly, this clinical complication may also be due to the lack of appropriate postoperative treatment (Marx et al., 2007; Marx et al., 2005). Several investigators have investigated the role of periodontitis and the role of ZA in the development of BRONJ through different animal models (Aghaloo et al., 2011; Aguirre et al., 2012). In humans, several case reports suggest that periodontitis may predispose patients to BRONJ (Kos, 2014; Thumbigere-Math et al., 2014). In particular, the exposure to ZA at concentrations $\geq$ 1 μM may impair the osteogenic potential of PDLSCs under osteogenic conditions (Di Vito et al., 2020). Thesefore, this study aimed to explore the possible mechanism underlying periodontitis after ZA exposure by using a ZA-treated human periodontal ligament stem cell model.

CAMP-response element-binding protein 1 (CREB1) is a master nuclear transcription factor belonging to the leucine zipper transcription factor family (Shaywitz and Greenberg, 1999). Through regulating the expression of target genes, CREB1 extensively participates in a variety of physiological processes, including metabolism and DNA repair (Moore et al., 2016; Wang et al., 2016). Accumulating researches have elaborated that CREB1 suppresses apoptosis of cells in many animal and cellular disease models. For instance, knockdown of CREB1 promotes apoptosis in mouse granulosa cells (Zhang et al., 2018). Lactoferrin restrains IL-1β-induced chondrocyte apoptosis through AKT1-induced CREB1 upregulation (Xue et al., 2015). Besides, CREB1 overexpression suppresses the apoptosis in renal tissue of kidney stone disease rats through regulating apoptosis-associated proteins (Yu et al., 2021). Additionally, CREB is involved in the biomineralization process of adult human molar odontoblasts and cementoblasts (Klinz et al., 2013). Notably, emerging evidence has proposed that CREB1 is involved in bone metabolism. Previous literatures have highlighted the significant role of CREB1 in the osteogenic differentiation of mouse pre-osteoblastic MC3T3-E1 cells and bone marrow stromal cells (Fu et al., 2021; Zhang et al., 2022). Through the activation of CREB1, tauroursodeoxycholic acid enhances osteogenic differentiation in mesenchymal stem cells (Kang et al., 2023). Importantly, CREB1 has been identified as a core protein participating in tension force-induced bone formation in PDLSCs (Chang et al., 2015). Nevertheless, the impacts of CREB1 on periodontitis remain elusive. Vascular endothelial growth factor (VEGF), a potent angiogenic factor, is a glycosylated, multifunctional cytokine that has been well documented to be involved in extracellular matrix remodeling, angiogenesis and bone formation (Hu and Olsen, 2016; Melincovici et al., 2018). More importantly, Ren et al. (2021) have mentioned that increased VEGF expression is closely implicated in the development of periodontitis. Further, VEGF secreted by periodontal ligament stem cells (PDLSCs) regulates the osteogenic potential of PDLSCs (Abd Rahman et al., 2016; Huang et al., 2020).

Accordingly, this study is conducted with the aim of shedding light on the role of CREB1 in periodontitis after ZA exposure by using a ZA-treated human periodontal ligament stem cell model, and determining the possible action mechanism of CREB1 and VEGF in periodontitis.

2. Materials and methods

2.1. Bioinformatics tools

JASPAR database (https://jaspar.genereg.net/) predicted the binding between CREB1 and VEGF promoter.

2.2. Cell culture

DMEM medium (VivaCell, Shanghai, China) containing 10% fetal bovine serum (FBS; Rongye Biotechnology Co., Ltd., Lanzhou, China) was used to culture human PDLSCs (Procell, Wuhan, China) at 37 °C in an incubator with 5% CO_2. Increasing concentrations (0.1, 1, 1.5, 2 and 3 μM) of zoledronic acid (ZA; Aladdin, Shanghai, China) were employed to treat PDLSCs for 72 h (Di Vito et al., 2020). For osteogenic induction, PDLSCs were maintained in α-MEM (VivaCell, Shanghai, China) composed of 10 mM beta-glycerophosphate, 10% FBS, 50 mM ascorbic acid and 10 nM dexamethasone for 14 days in a 37 °C incubator with 5% CO_2. The Ethics Committee of Peking University School and Hospital of Stomatology (Beijing, China) waived the requirement for ethics approval for using the purchased PDLSCs.

2.3. Transfection protocol

The transfection of short hairpin RNAs (shRNAs) for VEGF (sh-VEGF#1/2) or control shRNA (sh-NC) synthesized by Biotend Biotechnology (Shanghai, China) and CREB1 overexpression vector (Oe-CREB1) or empty overexpression vector (Oe-NC) designed by EK-Bioscience (Shanghai, China) was performed in the presence of Lipofectamine™ 3000 Transfection Reagent (Beijing Novo Technology Co., Ltd., Beijing, China) in line with the manufacturer's protocol. Cells were harvested for follow-up experiments at 48 h post-transfection. The experiment was repeated three times.

2.4. Reverse transcription-quantitative PCR (RT-qPCR)

With the aid of cDNA first strand synthesis kit (Simgen, Hangzhou, China), total RNA was prepared from human PDLSCs, from which cDNA was produced adopting Trizol reagent (Simgen, Hangzhou, China). Following the implementation of PCR analysis with the employment of 2 ×SYBR Green PCR Mix (Simgen, Hangzhou, China), the alternations in mRNA levels were reflected in compliance with the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008), viewing GAPDH as a housekeeping gene. The primer sequences used are as follows: CREB1, forward, 5′-ATTCACAGGAGTCAGTGGATAGT-3′ and reverse, 5′- CACCGTTAC AGTGGTGATGG-3′; VEGF, forward, 5′- ACTGCCATCCAATCGAGACC-3′ and reverse, 5′- GCTCCAGGGCATTAGACAGC-3′; GAPDH, forward, 5′-AATGGGCAGCCGTTAGGAAA-3′ and reverse, 5′- GCGCCCAATACG ACCAAATC-3′. The experiment was repeated three times.

2.5. Western blotting

PVDF membranes immersed in 5% BSA were to transfer the acquired total protein extracts from cells adopting RIPA buffer (EpiZyme, Shanghai, China) resolved on 10% SDS-PAGE. Afterwards, the membranes were successively immunoblotted with primary antibodies overnight at 4 °C and goat anti-rabbit HRP antibody (cat. no. ab205718; 1/2000; Abcam) for 1 h. Protein band visualization was carried out with the Omni-ECL™Femto Light Chemiluminescence Kit (EpiZyme, Shanghai, China) and the gray analysis was implemented with ImageLab4.0 software. CREB1 (cat. no. ab32515; 1/1000), VEGF (cat. no. ab46154; 1/1000), B cell lymphoma-2 (Bcl-2; cat. no. ab182858; 1/2000), BCL-2 associated X (Bax; cat. no. ab32503; 1/1000), osteocalcin (OCN; cat. no. ab133612; 1/1000), runt-related transcription factor 2 (RUNX2; cat. no. ab236639; 1/1000), bone morphogenic protein 2 (BMP2; cat. no. ab284387; 1/1000) and GAPDH (cat. no. ab9485; 1/2500) primary antibodies were all provided by Abcam. The experiment was repeated three times.

2.6. Cell counting kit-8 (CCK-8)

In short words, untransfected or transfected human PDLSCs seeded in a 96-well plate at 37 ℃ received treatment with ZA (0.1, 1, 1.5, 2 and 3 μM) or 1.5 μM ZA. Prior to the determination of OD 450 nm value with a microplate reader (Thermo, Varioskan Flash), the addition of 10 μl CCK-8 solution (Tiandz, Beijing, China) lasted for extra 2 h in conformity to the product manual. The experiment was repeated three times.

2.7. Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay

Following indicated treatment and transfection, human PDLSCs received immobilization and permeabilization respectively adopting 4% paraformaldehyde and 0.1% Triton X-100. TUNEL assay kit (Qihaifutai, Shanghai, China) was supplemented for 60 min of cultivation in the light of the manufacturer's instructions prior to cultivation in the mounting medium containing 10 mg/ml DAPI for 10 min. Finally, a fluorescence microscope (Leica, Germany) was employed to capture images. The experiment was repeated three times.

2.8. Alkaline phosphatase (ALP) and Alizarin red S (ARS) staining

At the 14th day after osteoblastic induction, treated and transfected human PDLSCs were submerged in 4% paraformaldehyde at room temperature for 20 min and rinsed in distilled water prior to the respective cultivation with BCIP/NBT ALP staining buffer (CoWin Biotech, Beijing, China) or 2% ARS solution (Saiye, China) for 20 min. The images were recorded by an inverted microscope (Leica, Germany). The experiment was repeated three times.

2.9. Chromatin immunoprecipitation (ChIP)

ChIP assay was executed employing the SimpleChIP kit (Cell Signaling Technology) in the light of the manufacturer's guidelines. The obtained cell lysates were sonicated following the immobilization of human PDLSCs in 1% formaldehyde. Afterwards, the acquired chromatin fragments were cultivated with CREB1 antibody (cat. no. #9197; Cell Signaling Technology) or IgG antibody (cat. no. ab205718; Abcam) overnight. The purified DNA fragments were subjected to PCR analysis. The experiment was repeated three times.

2.10. Luciferase reporter assay

The pGL3 vectors containing the wild-type (WT) VEGF promoter sequence (5′-CTTTGATGTCTGC-3′) or the corresponding mutant (MUT) VEGF promoter sequence were established by Promega Corporation (WI, USA). These vectors were co-transfected with Oe-NC and Oe-CREB1 into human PDLSCs by using Lipofectamine® 3000 Transfection Reagent (Thermo Fisher Scientific, Inc). After 48 h, the luciferase activity was evaluated using the Dual Luciferase Reporter Assay Kit (KeyGen, Nanjing, China). The experiment was repeated three times.

2.11. Statistical analyses

Data from three independent replicates were presented as mean ± standard deviation (SD). GraphPad Prism 8.0 software (GraphPad software, Inc.) was used for all statistical analyses. Statistical test was conducted using the one-way analysis of variance followed by Tukey's test among multiple groups and the student's t-test among two groups. Two-way analysis of variance was used to analyze the significant difference in luciferase reporter assay. $P < 0.05$ was considered statistically significant.

3. Results

3.1. CREB1 exhibits declined expression in ZA-treated PDLSCs

To elucidate the role of CREB1 in periodontitis, CREB1 expression was examined when human PDLSCs were exposed to ascending concentrations of ZA (0.1, 1, 1.5, 2 and 3 μM) for 72 h (Di Vito et al., 2020). Through RT-qPCR and western blotting, it was noted that ZA exposure led to a downward trend on CREB1 expression in PDLSCs (Fig. 1A-B). Moreover, CREB1 expression was the lowest in PDLSCs when treated by 1.5 μM of ZA. Additionally, the influence of ZA after 72 h exposure on the viability of PDLSCs was also detected and the experimental data from CCK-8 assay manifested that 1.5, 2 and 3 μM of ZA prominently

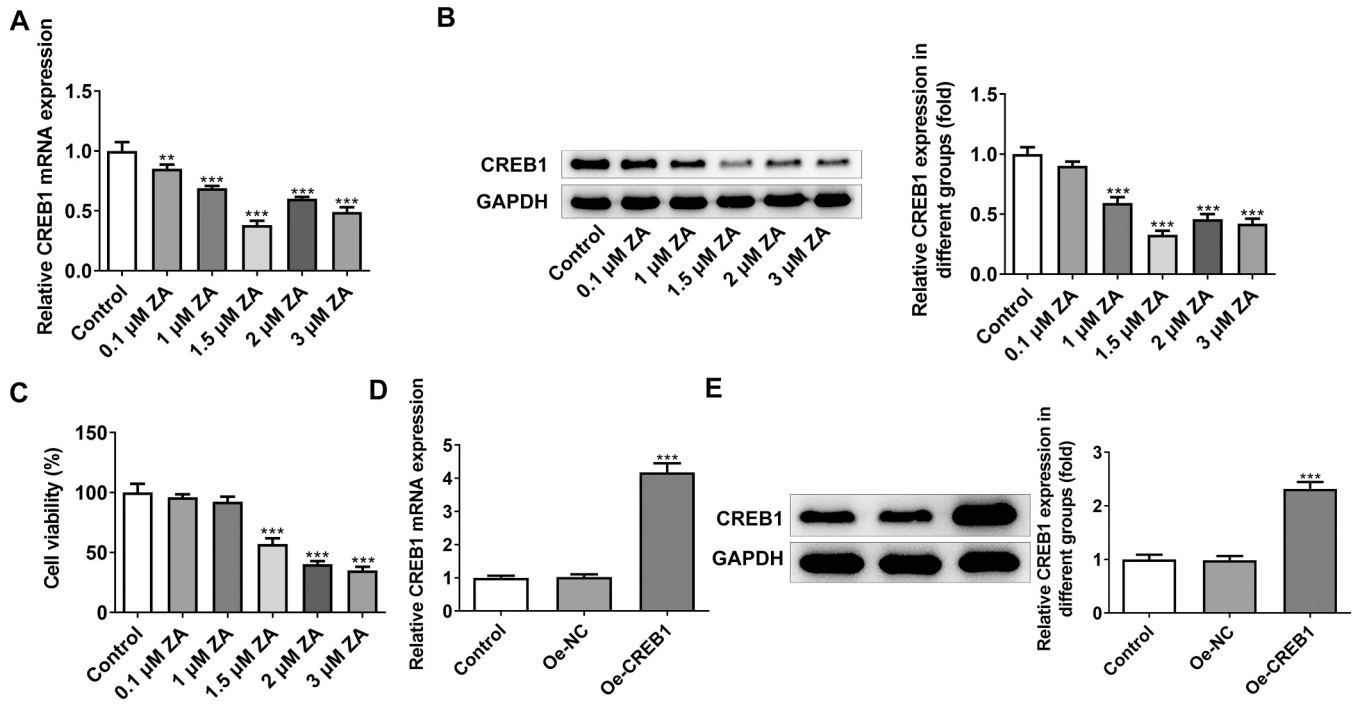

Fig. 1. CREB1 exhibits declined expression in ZA-treated PDLSCs. (A-B) RT-qPCR and western blotting tested CREB1 expression in human PDLSCs exposed to varying concentrations of ZA. (C) CCK-8 assay appraised PDLSCs viability upon exposure to varying concentrations of ZA. Data from three independent replicates were presented as mean ± standard deviation (SD). * **P < 0.01, * **P < 0.001 vs. Control. (D-E) RT-qPCR and western blotting tested the transfection efficacy of CREB1 overexpression plasmids. Data from three independent replicates were presented as mean ± standard deviation (SD). * **P < 0.001 vs. Oe-NC.

diminished PDLSCs viability while no apparent alternations were observed in the viability of PDLSCs when exposed to 0.1 and 1 μM of ZA (Fig. 1C). Hence, 1.5 μM of ZA was adopted to treat PDLSCs in the follow-up experiments. To evaluate the specific impacts of CREB1 on the biological behaviors of ZA-challenged PDLSCs, CREB1 overexpression plasmids were transfected into PDLSCs. As depicted in Fig. 1D-E, following transfection of Oe-CREB1, CREB1 expression was markedly raised. Above all, CREB1 expression was depleted in human PDLSCs upon exposure to ZA.

3.2. CREB1 elevation potentiates the viability while hampers the apoptosis of ZA-challenged PDLSCs

The decrease in viability and apoptosis of PDLSCs play a significant role in the pathogenic progression of periodontitis (Taskan et al., 2019). To determine the effects of CREB1 on the viability and apoptosis of ZA-treated PDLSCs, CCK-8 and TUNEL assays were conducted. As illustrated in Fig. 2A, the declined viability of PDLSCs exposed to ZA was enhanced after CREB1 was overexpressed. Conversely, the results of TUNEL assay illuminated that ZA treatment significantly aggravated PDLSCs apoptosis, which was then suppressed by CREB1 elevation (Fig. 2B). Also, western blotting analysis hinted that anti-apoptotic Bcl-2 expression was lessened and pro-apoptotic Bax expression was augmented in ZA-exposed PDLSCs while CREB1 up-regulation raised Bcl-2 expression and cut down Bax expression in ZA-treated PDLSCs (Fig. 2C). To be concluded, CREB1 up-regulation elevated the viability and protected against the apoptosis of PDLSCs exposed to ZA.

3.3. CREB1 elevation strengthens the differentiative capacity of PDLSCs into osteoblasts

At the same time, it was observed from ALP staining that the lowered ALP activity in ZA-challenged PDLSCs was remarkably augmented when CREB1 was up-regulated (Fig. 3A). Besides, ARS staining demonstrated that ZA treatment resulted in the declined number of mineralized nodules in PDLSCs, which was then elevated following CREB1 elevation (Fig. 3B). As expected, Western blot analysis manifested that ZA exposure prominently down-regulated osteogenic markers including OCN, RUNX2 and BMP2 in PDLSCs, which were all fortified after CREB1 was overexpressed (Fig. 3C). In summary, CREB1 up-regulation facilitated PDLSCs osteogenic differentiation upon exposure to ZA.

3.4. CREB1 activates the transcription of VEGF

By using JASPAR database, we obtained the sequence logo of CREB1 transcription factor (Fig. 4A). And CREB1 was predicted to bind to VEGF promoter and the potential binding regions (5′-CTTTGATGTCTGC-3′) was displayed in Fig. 4B. Importantly, it turned out that VEGF expression at mRNA level and protein level was also descending in ZA-treated PDLSCs (Fig. 4C-D). Luciferase reporter assay corroborated that CREB1 overexpression noticeably improved the luciferase activity of WT VEGF promoter instead of MUT VEGF promoter (Fig. 4E). ChIP assay also proved that VEGF promoter was abundant in CREB1 antibody (Fig. 4F). Further, RT-qPCR and western blotting implied that VEGF expression was increased in CREB1-overexpressing PDLSCs (Fig. 4G-H). Overall,

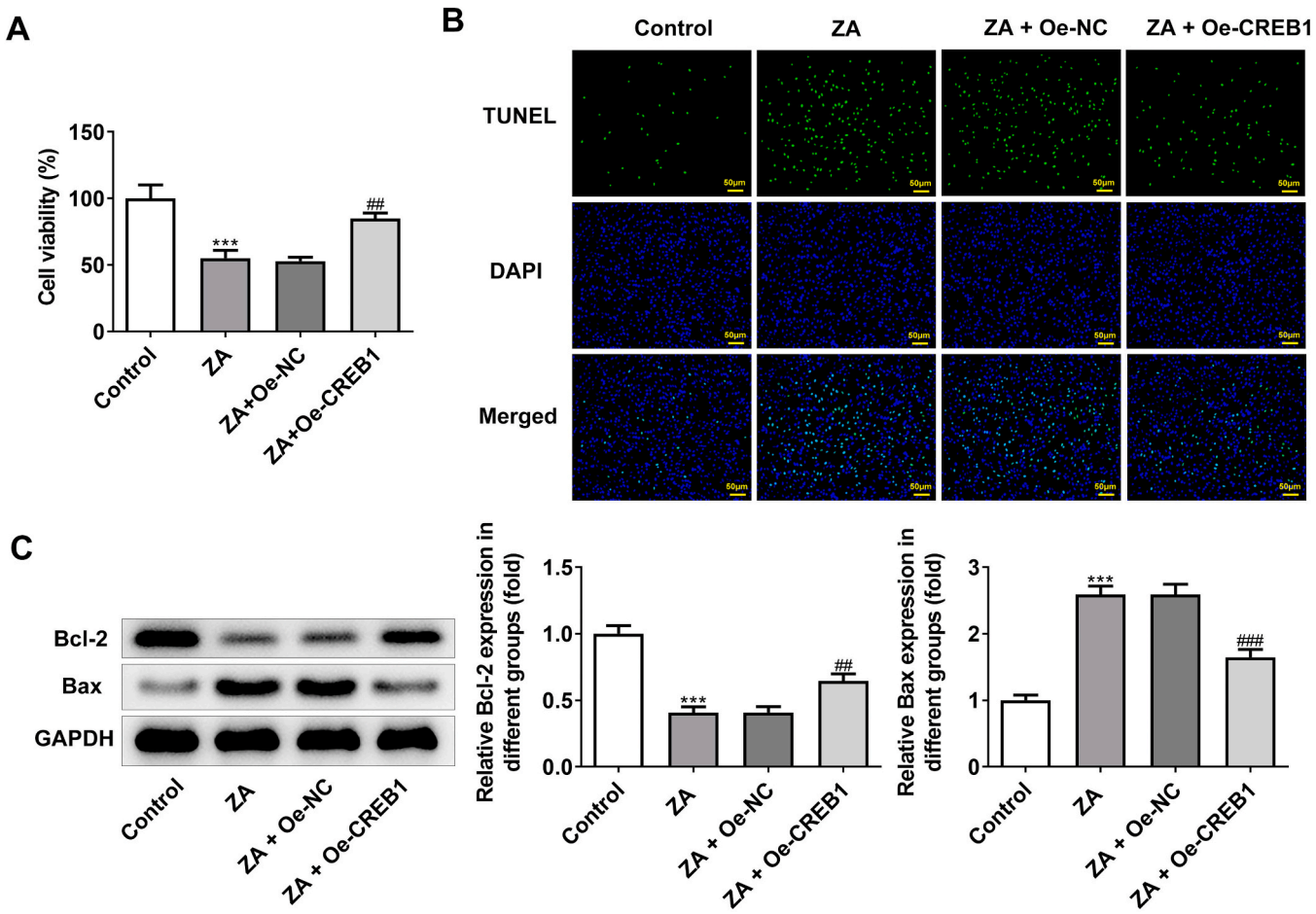

Fig. 2. CREB1 elevation potentiates the viability while hampers the apoptosis of ZA-challenged PDLSCs. (A) CCK-8 assay appraised PDLSCs viability. (B) TUNEL assay appraised PDLSCs apoptosis. (C) Western blotting examined the expression of proteins implicated in apoptosis. Data from three independent replicates were presented as mean ± standard deviation (SD). * **P < 0.001 vs. Control. ##P < 0.01, ###P < 0.001 vs. ZA + Oe-NC.

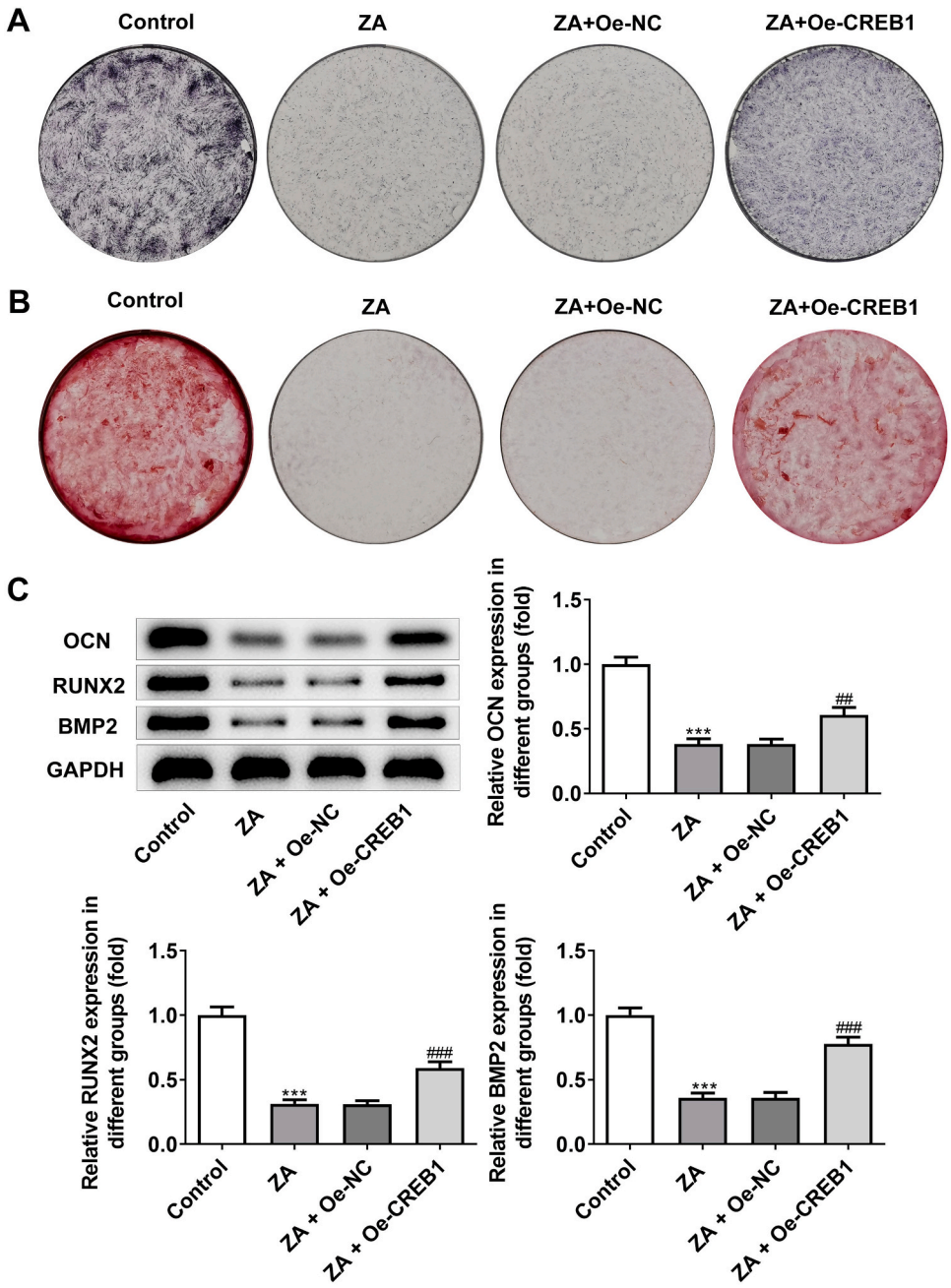

Fig. 3. CREB1 elevation strengthens the differentiative capacity of PDLSCs into osteoblasts. (A) ALP level was appraised by ALP staining. (B) ARS staining measured mineralized nodules. (C) Western blotting tested the expression of osteogenic markers. Data from three independent replicates were presented as mean ± standard deviation (SD). * **P < 0.001 vs. Control. ##P < 0.01, ###P < 0.001 vs. ZA + Oe-NC.

CREB1 might bind to VEGF promoter and serve as a transcriptional activator of VEGF.

3.5. CREB1 up-regulates VEGF to obstruct the apoptosis and accelerate the osteogenic differentiation of PDLSCs exposed to ZA

To substantiate that CREB1 participated in the apoptosis and osteogenic differentiation of ZA-exposed PDLSCs through activating the transcription of VEGF, VEGF was silenced following transfection of sh-VEGF#1/2 plasmids. Moreover, sh-VEGF#2 was selected for the ensuing experiments for its prominent interference efficacy (Fig. 5A-B). As denoted by TUNEL assay, the weakened apoptotic rate of PDLSCs treated by ZA imposed by CREB1 was elevated after VEGF was depleted (Fig. 5C). This finding was also evidenced by the results that absence of VEGF diminished Bcl-2 expression while fortified Bax expression in ZA-challenged PDLSCs transfected with CREB1 overexpression plasmids (Fig. 5D-E). In the same way, ALP staining and ARS staining assays testified that VEGF knockdown partially reduced the increased ALP activity and mineralized nodules in the CREB1-overexpressing PDLSCs treated by ZA (Fig. 6A-B). In addition, CREB1 raised OCN, RUNX2 and BMP2 expression in ZA-challenged PDLSCs, which were then partially abolished by VEGF deficiency (Fig. 6C-D). Taken together, VEGF absence counteracted the effects of CREB1 overexpression on the apoptosis and osteogenic differentiation of ZA-treated PDLSCs.

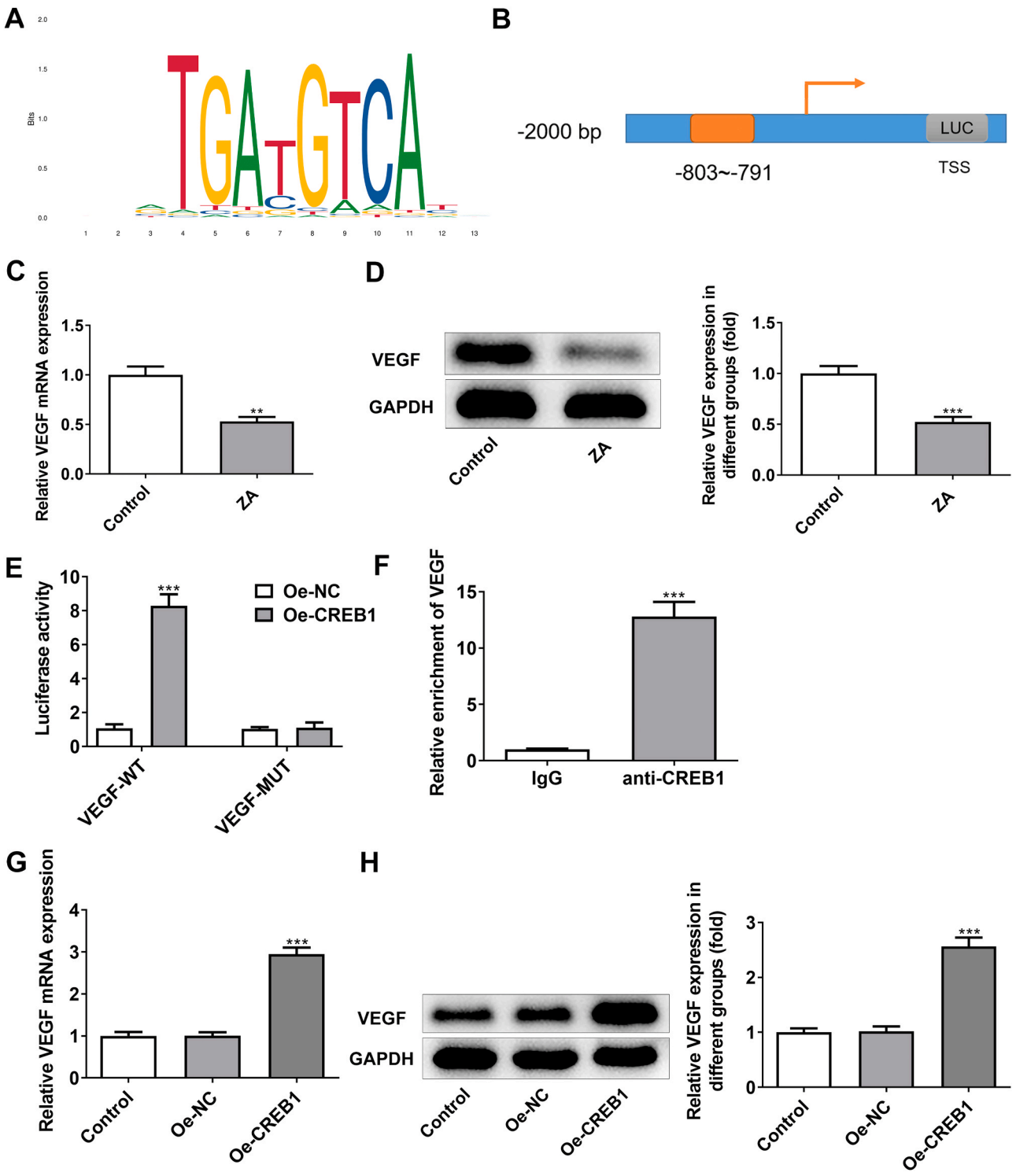

Fig. 4. CREB1 activates the transcription of VEGF. (A) The sequence logo of CREB1 transcription factor was obtained from JASPAR database. (B) JASPAR database predicted the binding regions of CREB1 with VEGF promoter. (C-D) RT-qPCR and western blotting tested VEGF expression in human PDLSCs exposed to ZA. Data from three independent replicates were presented as mean ± standard deviation (SD). * **P < 0.001 vs. Control. (E) Luciferase reporter assay assessed the luciferase activity of VEGF promoter. Data from three independent replicates were presented as mean ± standard deviation (SD). * **P < 0.001 vs. Oe-NC. (F) ChIP assay evaluated the abundance of VEGF promoter in CREB1 antibody. Data from three independent replicates were presented as mean ± standard deviation (SD). * **P < 0.001 vs. IgG. (G-H) RT-qPCR and western blotting tested VEGF expression after CREB1 was overexpressed. Data from three independent replicates were presented as mean ± standard deviation (SD). * **P < 0.001 vs. Oe-NC.

4. Discussion

Periodontitis is commonly acknowledged as a frequently encountered infectious disease featured by pathologic destruction of periodontium composed of gingiva, alveolar bone, periodontal ligament and cementum (Kinane et al., 2017). Periodontal ligament is a soft tissue that supports the teeth, and drives tooth nutrition, alveolar bone remodeling, and orthodontic tooth movement (Jiang et al., 2016). As

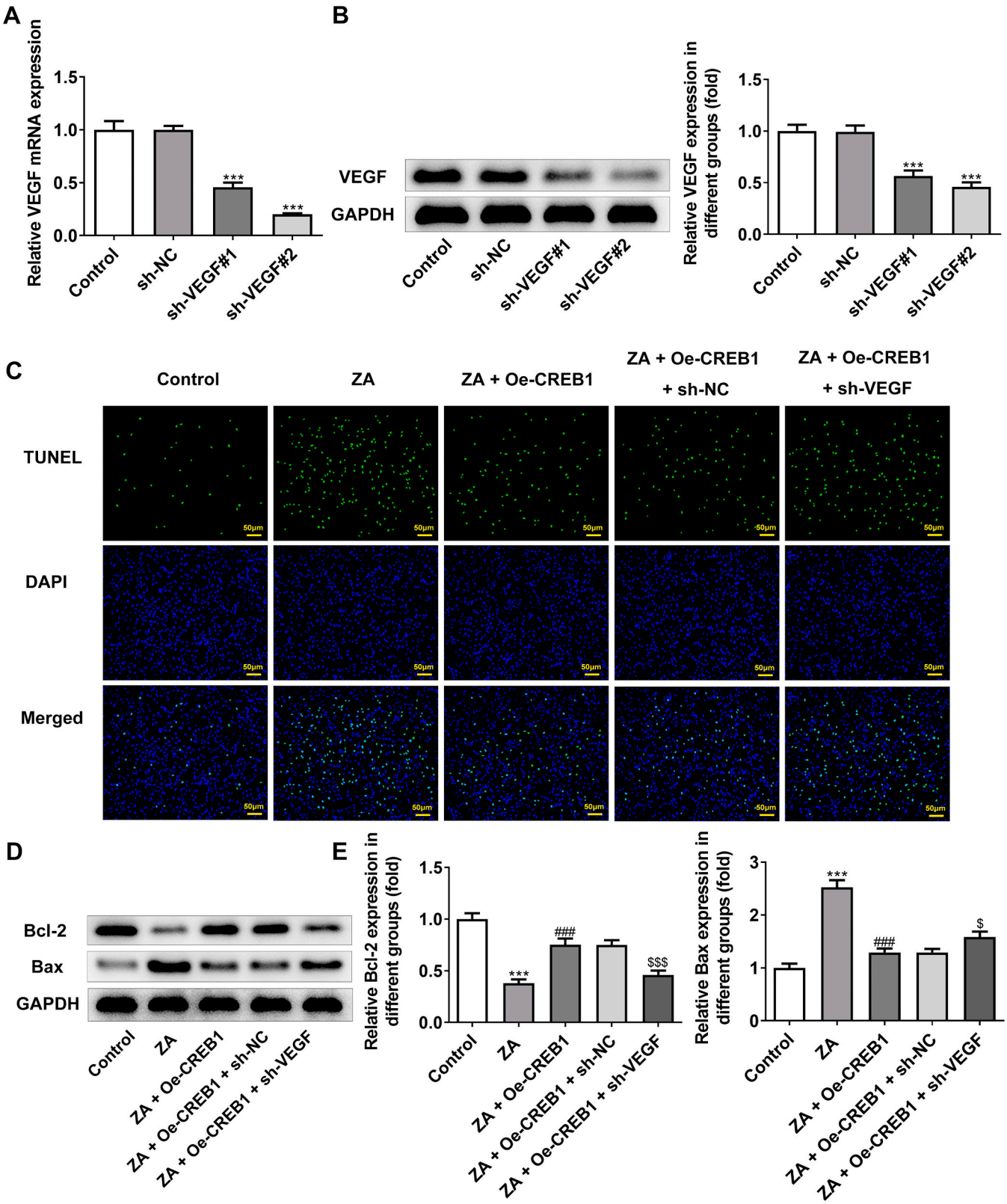

Fig. 5. CREB1 up-regulates VEGF to obstruct ZA-stimulated PDLSCs apoptosis. (A-B) RT-qPCR and western blotting tested the transfection efficacy of VEGF interference plasmids. Data from three independent replicates were presented as mean ± standard deviation (SD). * **P < 0.001 vs. sh-NC. (C) TUNEL assay appraised PDLSCs apoptosis. (D-E) Western blotting examined the expression of proteins implicated in apoptosis. Data from three independent replicates were presented as mean ± standard deviation (SD). * **P < 0.001 vs. Control. ###P < 0.001 vs. ZA. $ P < 0.05, $$$ P < 0.001 vs. ZA + Oe-CREB1 + sh-NC.

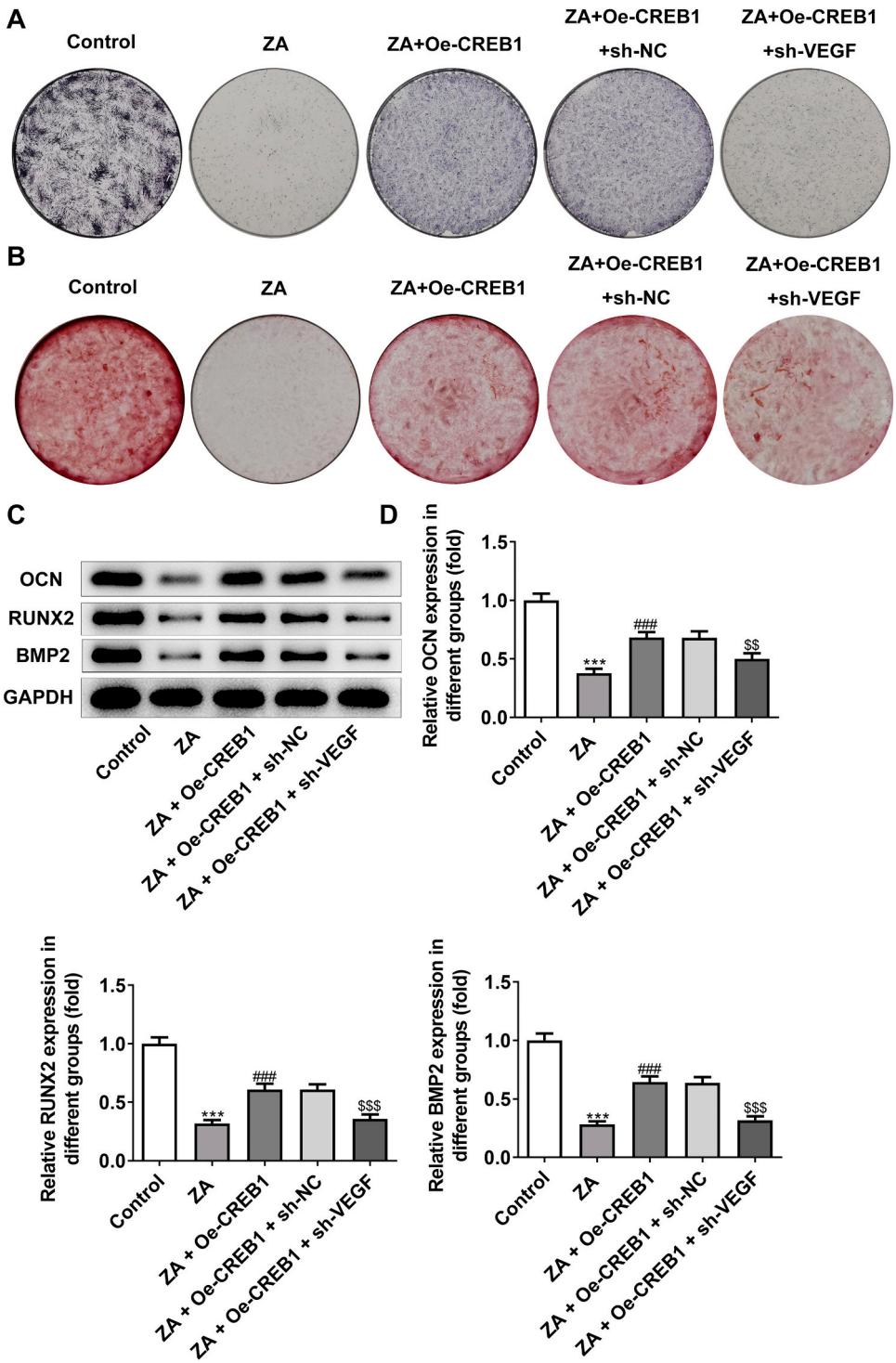

Fig. 6. CREB1 up-regulates VEGF to accelerate the osteogenic differentiation of PDLSCs exposed to ZA. (A) ALP level was appraised by ALP staining. (B) ARS staining measured mineralized nodules. (C-D) Western blotting tested the expression of osteogenic markers. Data from three independent replicates were presented as mean ± standard deviation (SD). * **P < 0.001 vs. Control. ###P < 0.001 vs. ZA. $$ P < 0.01, $$$ P < 0.001 vs. ZA + Oe-CREB1 + sh-NC.

heterogeneous mesenchymal stem cells (MSCs) located in the periodontal ligament, PDLSCs are mainly responsible for maintaining the dynamic balance of periodontal tissue and repair defects by means of their self-renewal and multipotential differentiation abilities (Tomokiyo et al., 2019). Previous researches have uncovered that PDLSCs are viewed as good candidates for periodontal and alveolar bone regeneration since PDLSCs can differentiate into multiple types of cells including osteoblasts and cementoblasts under certain circumstances dependent on their osteogenic capacity (Bright et al., 2015; Chamila Prageeth Pandula et al., 2014). Thus, figuring out the molecular mechanisms underlying the osteogenic differentiation of PDLSCs may contribute to the development of regenerative therapies for periodontal and bone diseases (Chamila Prageeth Pandula et al., 2014). In recent years, ZA has been widely applied to stomatology as a highly effective bone resorption inhibitor via accelerating bone formation and suppressing bone resorption (Mardas et al., 2017; Qi et al., 2012). As a

third-generation nitrogen-containing bisphosphonate, ZA treatment may lead to interference with tooth movement, difficulty in closing dental spaces after tooth extractions for orthodontic purposes as well as the occurrence of osteonecrosis in some rats with experimental periodontitis (de Sousa et al., 2021; Leite de Marcelos et al., 2021). In particular, the exposure to ZA at concentrations $\geq$ 1 μM may impair the osteogenic potential of PDLSCs under osteogenic conditions (Di Vito et al., 2020). Moreover, PDLSCs show reduced mesenchymal stem cell-like characteristics in response to ZA (Rodríguez-Lozano et al., 2015). All these findings imply that ZA may result in damage to periodontal tissues. Based on these findings, varying concentrations of ZA were to treat human PDLSCs here and it was observed that ZA at concentrations of 1.5, 2 and 3 μM apparently diminished PDLSCs viability while ZA at concentrations of 0.1 and 1 μM exerted no obvious influence on PDLSCs viability. Thereafter, PDLSCs were induced by 1.5 μM of ZA in the ensuing experiments.

Previous research has mentioned that CREB is activated in unstimulated peripheral blood neutrophils in patients with localized aggressive periodontitis (Buchmann et al., 2009). Emerging evidence has proposed that CREB1 is involved in bone metabolism. For instance, activation of CREB1 signaling blocks osteoclastic bone resorption (Liu et al., 2021) and facilitates osteoclastogenesis (Park et al., 2017). Notably, CREB1 has been identified as a core protein participating in tension force-induced bone formation in PDLSCs (Chang et al., 2015). In the present study, CREB1 expression was discovered to be depleted in ZA-treated PDLSCs, suggesting that CREB1 might be involved in periodontitis. Periodontitis can lead to the progressive destruction of periodontal tissue, accompanied by PDLSCs dysfunction or loss. Through detecting PDLSCs viability and apoptosis upon exposure to ZA, whether CREB1 participates in the ZA-induced periodontal damage was confirmed. It was observed that CREB1 up-regulation elevated the diminished viability of PDLSCs challenged with ZA. On the contrary, ZA treatment markedly boosted the apoptotic ability, lowered anti-apoptotic Bcl-2 expression and raised pro-apoptotic Bax expression in PDLSCs. In ZA-exposed PDLSCs, further CREB1 overexpression notably impeded PDLSCs apoptosis, accompanied with the fortified Bcl-2 expression and reduced Bax expression.

PDLSCs are known as a promising method for regenerative treatment of periodontitis due to their highly effective osteogenic differentiation capacity (Cao et al., 2022). Numerous literatures have underlined that CREB1 regulates the osteogenic differentiation of mesenchymal stem cells (Lin et al., 2023; Zhang et al., 2015). ALP that can enhance calcium and phosphorus concentration in cells is viewed as a marker of the early stage of osteogenic differentiation (Brauer et al., 2016). The deposition of calcium salt is a frequent event during mid-late stages of cell differentiation (Brauer et al., 2016). Through investigation, the present paper disclosed that when CREB1 was up-regulated, the declined ALP activity and mineralized nodules in ZA-exposed PDLSCs were both remarkably elevated. Gao et al. (2017) have supported that the expression of osteogenic markers OCN and RUNX2 were descending in the conditioned medium from preosteoclasts treated by ZA. As expected, ZA exposure prominently down-regulated OCN, RUNX2 and BMP2 in PDLSCs, which were all fortified after CREB1 was overexpressed. Therefore, CREB1 might act as a regulatory molecule in the development of ZA-induced periodontal damage. This study is the first to explore the role of CREB1 in periodontitis disease.

CREB1 is a leucine zipper-type transcription factor that recognizes CRE elements in the regulatory region of target gene to activate gene transcription (Moore et al., 2016). For instance, CREB1 has been determined as a transcription factor that can promote BMP2 expression through binding to the BMP2 promoter in osteoblasts (Zhang et al., 2011). Zhang et al. (2017) have introduced that in gastric cancer, VEGF signaling pathway is targeted by KIF26B, in which CREB1 is involved. In our study, the specific relationship between CREB1 and VEGF was revealed. As predicted by JASPAR database, CREB1 might bind to VEGF promoter, which was also verified by mechanism assays. Moreover, CREB1 elevation might result in the increase on VEGF expression in PDLSCs. Considering that, it was speculated that CREB1 might function in the process of periodontitis via mediating VEGF expression. Previous research has also expounded that ZA treatment down-regulated VEGF expression in the conditioned medium from preosteoclasts (Gao et al., 2017). Similarly, our study implied that VEGF expression was also descending in ZA-treated PDLSCs. Mounting evidence has also proved that VEGF may act as a potential molecular target in periodontitis and contribute to PDLSCs angiogenesis and osteogenic differentiation (Huang et al., 2020; Iwasaki et al., 2021; Ren et al., 2021). In addition to the potential role of VEGF in PDLSCs osteogenic differentiation, our experimental data also elaborated that VEGF deletion reversed the impacts of CREB1 on PDLSCs in response to ZA, which was manifested by the finding that VEGF absence aggravated the apoptosis while suppressed the osteogenic differentiation of CREB1-overexpressing PDLSCs exposed to ZA.

5. Conclusion

Taken together, CREB1 inhibited the apoptosis and promoted the osteogenic differentiation of ZA-induced PDLSCs through transcriptional activation of VEGF (graphical abstract). This study hinted the potential protective role of CREB1 in ZA-induced osteogenic differentiation impairment of PDLSCs and suggested that targeted modulation of CREB1 expression might be a potential therapeutic strategy for postoperative treatment of periodontitis-related BRONJ.

Ethics approval and consent to participate

The Ethics Committee of Peking University School and Hospital of Stomatology (Beijing, China) waived the requirement for ethics approval for using the purchased human periodontal ligament stem cells.

Author statement

We would like to submit the enclosed manuscript entitled "**CREB1 alleviates the apoptosis and potentiates the osteogenic differentiation of zoledronic acid-treated human periodontal ligament stem cells via up-regulating VEGF**" to be considered for publication in **Tissue and cell**. The authors declare that they have no competing interests. Your kind considerations will be greatly appreciated.

Competing interests

The authors declare no competing financial interests.

Data availability

Data will be made available on request.

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Patient consent for publication

Not applicable.

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