RUVBL1 and CRAF promote periodontal ligament stem cell osteogenic differentiation via the MEK/ERK signaling cascade

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Abstract. MAPK signaling is key for osteogenic differentiation of periodontal ligament stem cells (PDLSCs), a process important in treating periodontal injury. RuvB-like AAA ATPase-1 (RUVBL1) binds C-Raf proto-oncogene serine/threonine-protein kinase (CRAF) to activate the MAPK signaling pathway, but the specific roles and mechanisms of RUVBL1 and CRAF in PDLSCs remain unclear. In the present study, PDLSCs were subjected to lentiviral transfection for RUVBL1 and CRAF expression manipulation. Flow cytometry was performed to characterize mesenchymal SC (MSC) features. A Cell Counting Kit-8 assay was used to assess PDLSC proliferative viability. Lipogenic differentiation potential was evaluated using Oil Red O staining following lipogenic induction. Alkaline phosphatase and alizarin red staining were utilized to assess osteogenic differentiation potential and mineralization, respectively, following osteogenic induction. Reverse transcription-quantitative PCR and western blotting were conducted to determine the effects of RUVBL1 and CRAF on the MAPK signaling pathway in PCLSCs. PDLSCs were determined to be SCs exhibiting self-renewal and MSC characteristics, including lipogenic and osteogenic differentiation potential. RUVBL1 overexpression enhanced PDLSC osteogenic differentiation while inhibiting proliferative activity and lipogenic differentiation. Conversely, CRAF overexpression promoted PDLSC proliferative activity, as well as osteogenic and lipogenic differentiation. Knockdown of RUVBL1 reduced osteogenic differentiation while enhancing proliferative activity and lipogenic differentiation, whereas knockdown of CRAF suppressed proliferative activity, osteogenic differentiation and lipogenic differentiation. RUVBL1 did not regulate CRAF in PDLSCs. However, overexpression of RUVBL1 or CRAF promoted MEK and ERK phosphorylation, activating the MEK/ERK signaling pathway. Overall, RUVBL1 and CRAF activated the MEK/ERK signaling pathway to promote osteogenic differentiation in PDLSCs, although RUVBL1 did not regulate CRAF.

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Introduction

Periodontitis is a chronic inflammatory disease of the oral cavity that arises from untreated gingivitis, progressively destroying the tooth-supporting structures and leading to tooth loss. The condition is typically associated with immune dysregulation, bacterial infection and systemic disease such as diabetes (1). Although the prevalence of severe periodontitis increases with age, it can affect individuals across all age groups. In 2011-2020, the estimated rate of dentate periodontitis in adults was ~62% and the rate of severe periodontitis was 23.6% (2). Diagnosis typically involves clinical and radiographic evaluation of periodontal tissue, with hallmark features including alveolar bone resorption and deepened periodontal pockets. Treatment for mild periodontitis typically involves mechanical debridement and antibiotic therapy. However, severe periodontitis results in irreversible destruction of periodontal tissue and therapeutic efforts typically focus on promoting alveolar bone regeneration (3-5). The periodontal ligament, a specialized connective tissue layer, anchors the tooth to the alveolar bone, supplies nutrients and contributes to tissue repair through the regulation of cell proliferation, growth factors and calcification homeostasis (6-8). Periodontal ligament stem cells (PDLSCs) possess multipotent differentiation capacity and can give rise to osteoblasts, adipocytes and periodontal neuronal cells (9). Owing to their regenerative potential and compatibility with biomaterials, PDLSCs are regarded as good seed cells for periodontal tissue regeneration (10,11).

The MAPK signaling pathway is a key intracellular cascade regulating cell proliferation, differentiation, apoptosis and stress responses (12,13). This pathway is markedly involved in the pathogenesis of chronic periodontitis, influencing inflammatory responses and alveolar bone remodeling (14).

Furthermore, the osteogenic differentiation potential of PDLSCs is associated with MAPK signaling (15), underscoring its dual importance in both disease progression and tissue regeneration. The MAPK pathway operates through a phosphorylation cascade in which MAP kinase kinase kinases (MAPKKKs), such as RAF family members, activate MAP kinase kinases, which phosphorylate MAPKs that translocate to the nucleus to directly phosphorylate transcription factors or activate downstream kinases to modulate gene expression (16). C-Raf proto-oncogene serine/threonine-protein kinase (CRAF), a representative member of the RAF family, contains three conserved regions (CR1, CR2 and CR3) and participates in cellular processes through downstream MAPK signaling by dimerizing and phosphorylating MEK to activate the MEK-ERK pathway (17). RuvB-like AAA ATPase-1 (RUVBL1), a multifunctional ATPase, is implicated in SC maintenance, differentiation and migration by functioning as an essential component of chromatin remodeling complexes thereby regulating transcription, DNA repair and protein assembly (18). RUVBL1 is a CRAF-binding protein that activates the RAF/MEK/ERK pathway by preventing phosphorylation at serine 259 within the CR2 domain of CRAF (19). RUVBL1 may interact with CRAF to promote or inhibit MAPK pathway activation in PDLSCs, potentially impacting periodontal regeneration. However, this hypothesis remains to be experimentally validated. In addition, oligodeoxynucleotide (ODN)-MT01, an inhibitory ODN designed based on human mitochondrial DNA, attenuates alveolar bone resorption and enhances osteogenic differentiation of bone marrow mesenchymal SCs (MSCs) in periodontitis models via ERK- and p38-mediated MAPK signaling (20,21). However, whether ODN MT01 enhances PDLSC-mediated periodontal regeneration remains unclear.

The present study aimed to elucidate the roles of RUVBL1 and CRAF in PDLSCs and their involvement in the MAPK signaling pathway in the treatment of periodontitis. The present findings may provide mechanistic insight into the osteogenic potential of PDLSCs and their application in periodontal tissue regeneration.

Materials and methods

Cell culture. Primary human PDLSCs (cat. no. CP-H234; passage three) were obtained from Procell Life Science & Technology Co., Ltd. and cultured in DMEM/F12 basal medium (88%; cat. no. 12400-024; Gibco; Thermo Fisher Scientific, Inc.) containing FBS (10%; cat. no. 10099-141; Gibco; Thermo Fisher Scientific, Inc.), penicillin-streptomycin (P/S, 1%; cat. no. 1902417; Gibco; Thermo Fisher Scientific, Inc.) and glutamine (1%; cat. no. 1894153; Gibco; Thermo Fisher Scientific, Inc.). The cells were incubated at 37˚C with 5% CO_2 and 95% humidity.

Flow cytometry. Cell suspension was placed in a flow tube at the $1x10^6$ cells/ml and 5 μl anti-human CD90 FITC (cat. no. 555595; dilution 1:20; BD Biosciences), CD105 APC (cat. no. 562408; dilution 1:20; BD Biosciences) and CD45 PE-Cy7 (cat. no. 557748; dilution 1:20; BD Biosciences) fluorescent-labeled antibodies was added. Cells were incubated at 4˚C in the dark for 30 min. Following incubation, the cells were washed with PBS containing 2% FBS and centrifuged at 4˚C at 300 x g for 5 min. The cell pellet was resuspended in 500 μl PBS and analyzed immediately. Flow cytometry was performed using a BD FACSCanto II flow cytometer (BD Biosciences) equipped with 488 and 633 nm lasers. Data acquisition and analysis were performed using BD FACSDiva software (version 8.0; BD Biosciences).

Cell induction. PDLSCs were cultured for 14 days at 37˚C with 5% CO_2 in a DMEM/F12 medium (cat. no. 12400-024; Gibco; Thermo Fisher Scientific, Inc.) containing osteogenic or lipogenic inducers: Osteogenic inducers were as follows: 10^{-8} mol/l dexamethasone (cat. no. D4902; MilliporeSigma), 50 μg/ml vitamin C (cat. no. A8960; MilliporeSigma) and 10 mmol/l sodium β-glycerophosphate (cat. no. G9422; MilliporeSigma). Lipogenic inducers were as follows: 200 μmol/ml indomethacin (cat. no. I7378; MilliporeSigma), 10 μg/ml insulin (cat. no. I3536; MilliporeSigma), 0.5 mmol/ml IBMX (cat. no. I5879; MilliporeSigma) and 1 μmol/ml dexamethasone (cat. no. D4902; MilliporeSigma). To explore the potential of ODN MT01, a synthetic ODN known to promote osteoblast maturation (20), in PDLSC osteogenesis, cells were treated with ODN MT01 (0.5-4.0 μg/ml) following osteogenic induction.

Construction of overexpression and interference plasmids. Overexpression or knockdown plasmids and negative controls (NCs) were constructed by Genomeditech (Shanghai) Co., Ltd. The CRAF overexpression vector was PGMLV-CMV-MCS-3xFlag-PGK-Puro, the RUVBL1 overexpression vector was PGMLV-CMV-MCS1-3xFIag-PGK-PuroxFIag-PGK-Puro and the interference vector was pGMLV-SC5 RNA inteference. For the overexpression NC, insert-free PGMLV-CMV-MCS-3xFlag-PGK-Puro (for CRAF) and PGMLV-CMV-MCS1-3xFIag-PGK-Puro xFIag-PGK-Puro (for RUVBL1) were used. NC and short hairpin (sh)RNA targets were as follows: sh-NC, 5'-TTC TCCGAACGTGTCACGT-3'; sh-CRAF-1, 5'-GGAGTA ACATCAGACAACTCT-3'; sh-CRAF-2, 5'-GGATTTCGA TGTCAGACTTGT-3'; sh-CRAF-3, 5'-GAAGACGTTCCT GAAGCTTGC-3'; sh-RUVBL1-1, 5'-GGGAGTGAAGTT TACTCAACT-3'; sh-RUVBL1-2, 5'-GCCACAGAATTC GACCTTGAA-3'; and sh-RUVBL1-3, 5'-GTCCATGAT GGGCCAGCTAAT-3'. Lentiviral particles were produced using a third-generation packaging system in 293T cells (ATCC CRL-3216). For packaging, 20 μg lentiviral plasmid (pGMLV-CRAF, pGMLV-RUVBL1 or pGMLV-shRNA) was co-transfected with 10 μg psPAX2 (cat. no. 12260; Addgene) and 5 μg pMD2.G envelope plasmid (cat. no. 12259; Addgene) at a 4:2:1 mass ratio using Lipofectamine® 3000 (cat. no. L3000015; Thermo Fisher Scientific, Inc.). The transfection complex was incubated with 293T cells for 6-8 h at 37˚C, and viral supernatants were collected at 48 h and 72 h, filtered (0.45 μm; cat. no. SLHV033RS; MilliporeSigma) and concentrated using Lenti-X Concentrator (cat. no. 631232; Takara Bio, Inc.). PDLSCs were infected at an MOI of 10-20 with 8 μg/ml polybrene (cat. no. H9268; MilliporeSigma) for 24 h at 37˚C. Puromycin (cat. no. P8833; MilliporeSigma; 2 μg/ml for selection and 1 μg/ml for maintenance) was added 48 h post-infection. Reverse transcription-quantitative (RT-q)

Table I. Primer information.

Gene	Forward (5'-3')	Reverse (5'-3')
GAPDH	AAAGGGTCATCATCTCTG	GCTGTTGTCATACTTCTC
CRAF	ACTCCTATGGCATCGTATT	TCTGATCTCGGTTGTTGA
RUVBL1	GATACCTATGCCACAGAAT	ATTAGCCACATCCAAGTC
ARAF	TTCTGTGACTTCTGCCTTA	ATGCTGGTGGAACTTGTA
BRAF	CTTGTATCACCATCTCCATA	GGCGTGTAAGTAATCCAT
MEK1	CAGTGGAGTGTTCAGTCT	ACTTCCTCAGCATCAGAT
MEK2	CTGGACTATATTGTGAAC	TTGATGAGGCATTTATTG
ERK1	GATGGAGACTGACCTGTA	CTGGTAGAGGAAGTAGCA
ERK2	TGGTGTGCTCTGCTTATG	AGTAGGTCTGGTGCTCAA

RUVBL1, RuvB-like AAA ATPase-1; CRAF, C-Raf proto-oncogene serine/threonine-protein kinase; ARAF, A-Raf proto-oncogene serine/threonine-protein kinase; BRAF, B-Raf proto-oncogene serine/threonine-protein kinase.

PCR and western blotting were used to perform expression validation and shRNA lentiviral screening.

Cell Counting Kit-8 (CCK-8) assay. Cells were inoculated into 96-well plates at a density of $2.5x10^3$ cells/well. The medium was replaced with complete medium [DMEM/F12 (cat. no. 11330032; Gibco; Thermo Fisher Scientific, Inc.) without P/S when the cell fusion reached 40-50%. CCK-8 assay was performed according to the manufacturer's instructions (cat,. no. CK-04; Dojindo Laboratories). Briefly, 10 μl CCK-8 reagent was added to each well and incubated for 2 h at 37˚C with 5% CO_2. Absorbance was measured at 450 nm.

RT-qPCR. RNA was extracted using using Trizol® solution (Thermo Fisher Scientific, Inc.) in an ice bath. cDNA was obtained according to the instructions of the FastKing cDNA First Strand Synthesis kit (cat. no. KR116; Tiangen Biotech Co., Ltd.) and amplified. The color developer was the Taq Pro Universal SYBR qPCR Master Mix (cat. no. Q712-03; Vazyme Biotech, Co., Ltd.). Thermocycling conditions were as follows: 95˚C for 30 sec; followed by 40 cycles of 95˚C for 10 sec and 60˚C for 30 sec. GAPDH was used as an internal reference gene. Experimental results were quantified using the $2^{-\Delta\Delta Cq}$ method (22). Primer information is listed in Table I.

Western blotting. Protein extraction from cells was performed with RIPA (Beyotime Biotechnology) containing protease inhibitors [Roche Diagnostics (Shanghai) Co., Ltd]. Protein concentration was determined using the BCA Protein Assay Kit (cat. no. P0012; Beyotime Biotechnology). Sample proteins (60 μg per lane) were subjected to SDS-PAGE (10% gel) electrophoresis, PVDF membrane transfer and incubated with skimmed milk (5%) for 2 h at room temperature. Primary antibodies (5 ml) were added overnight at 4˚C in a refrigerator. Enzyme-labeled secondary antibody was added and incubated for 2 h at room temperature. Color development was performed using an ECL kit (cat. no. P0018; Beyotime Biotechnology). Chemiluminescent signals were detected using ChemiDoc XRS+ Imaging System (Bio-Rad Laboratories) and analyzed using Image Lab software (version 6.1; Bio-Rad Laboratories). The antibodies were as follows: GAPDH (1:1,000; cat. no. P30008M; Abmart Pharmaceutical Technology Co., Ltd.), A-Raf proto-oncogene serine/threonine-protein kinase (ARAF) (cat. no. bs-2251R), CRAF (cat. no. bs-23170R), MEK1/2 (cat. no. bs-1041R), phosphorylated (p)-MEK1/2 (cat. no. bs-3270R), ERK1/2 (cat. no. bsm-33232M), p-ERK1/2 (cat. no. bs-3016R; all BIOSS), RUVBL1 (cat. no. 74775; Cell Signaling Technology, Inc.), ERK1/2 (all 1:500; cat. no. bsm-33232M; BIOSS), B-Raf proto-oncogene serine/threonine-protein kinase (BRAF) (1:2,000; cat. no. ab33899; Abcam) and HRP-conjugated universal secondary antibody [cat. nos. 7074 (anti-rabbit) and 7076 (anti-mouse); Cell Signaling Technology, Inc.].

Oil Red O staining. Cell slides were fixed in paraformaldehyde (4%) for 10 min at room temperature. The slides were washed in 60% isopropanol for 20 sec and stained for 10 min with Oil Red O at room temperature. Slides were differentiated with 60% isopropanol and the excess dye was removed. Finally, the slides were restained in hematoxylin (0.5%) for 2 min at room temperature and rinsed in distilled water before being dried and sealed for microscopic observation. The stained sections (5-μm thick) were observed under a light microscope (Eclipse E100; Nikon Corporation).

Alkaline phosphatase (ALP) staining. ALP staining was performed using ALP Staining Solution (cat. no. G1480; Solarbio Science & Technology Co., Ltd.) according to the manufacturer's instructions. Cells were fixed with ALP fixative (4% paraformaldehyde; 0.5 ml per well) for 3 min at room temperature and incubated with ALP incubation solution (BCIP/NBT working solution; 0.5 ml per well) for 30 min at room temperature, with PBS washing after each step. Finally, microscopic examination was performed using a light microscope (Eclipse E100; Nikon Corporation).

Alizarin red staining. Cells were fixed using a paraformaldehyde solution (4%) for 10 min at room temperature. Staining was performed with 0.1% alizarin red-Tris-HCl (pH 8.3) solution for 30 min at room temperature. Rinsing was performed

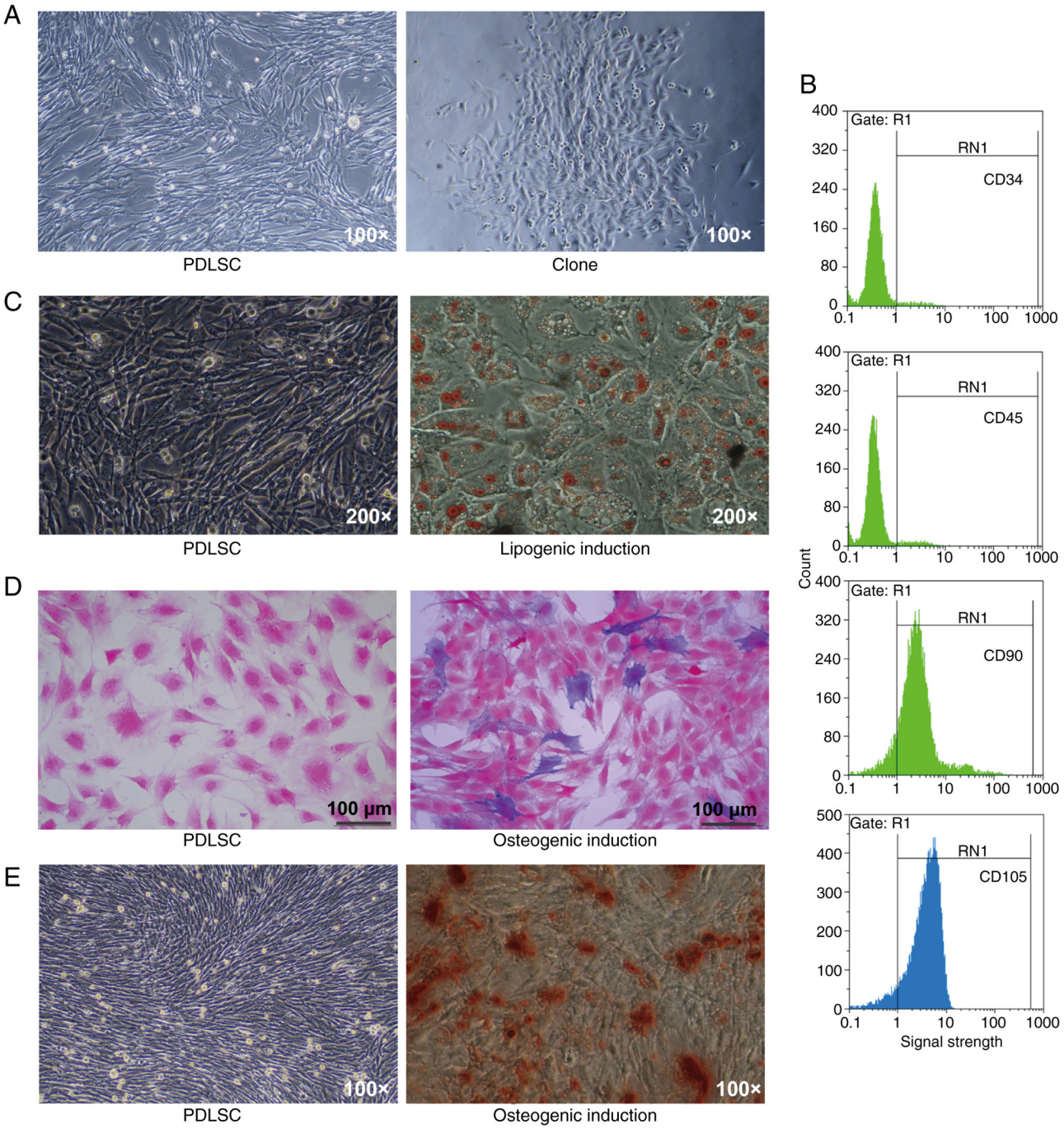

Figure 1. PDLSCs exhibit SC characteristics. (A) Microscopy was used to assess the self-cloning potential. (B) Flow cytometry was used to assess mesechmyal SC properties. (C) Oil Red O staining was performed to assess the lipogenic differentiation potential. (D) Alkaline phosphatase staining was performed to assess the osteogenic differentiation potential. (E) Alizarin red staining was performed to assess the degree of mineralization. PDLSC, periodontal ligament stem cell; RN1, region negative 1.

with distilled water and drying were performed for sealing. The degree of cell mineralization was observed under the light microscope (Eclipse E100; Nikon Corporation).

Statistical analysis. Data are presented as the mean ± SD from three independent experiments (n=3). One-way ANOVA followed by Tukey's HSD post hoc test was performed using SPSS (version 23.0; IBM Corp.) under a two-tailed significance level. Visualization was conducted using Origin® 2021 software (OriginLab Corporation). $P<0.05$ was considered to indicate a statistically significant difference.

Results

PDLSCs exhibit clonogenic capacity and multilineage differentiation potential. Following successful cloning (Fig. 1A), the expression of MSC surface markers in PDLSCs was analyzed by flow cytometry. The cells were positive for CD90 and CD105 but negative for CD34 and CD45 (Fig. 1B), demonstrating their MSC phenotype. PDLSCs possessed both adipogenic and osteogenic differentiation potential (Fig. 1C and D). Alizarin red staining revealed a marked increase in mineralized nodule formation following osteogenic induction (Fig. 1E).

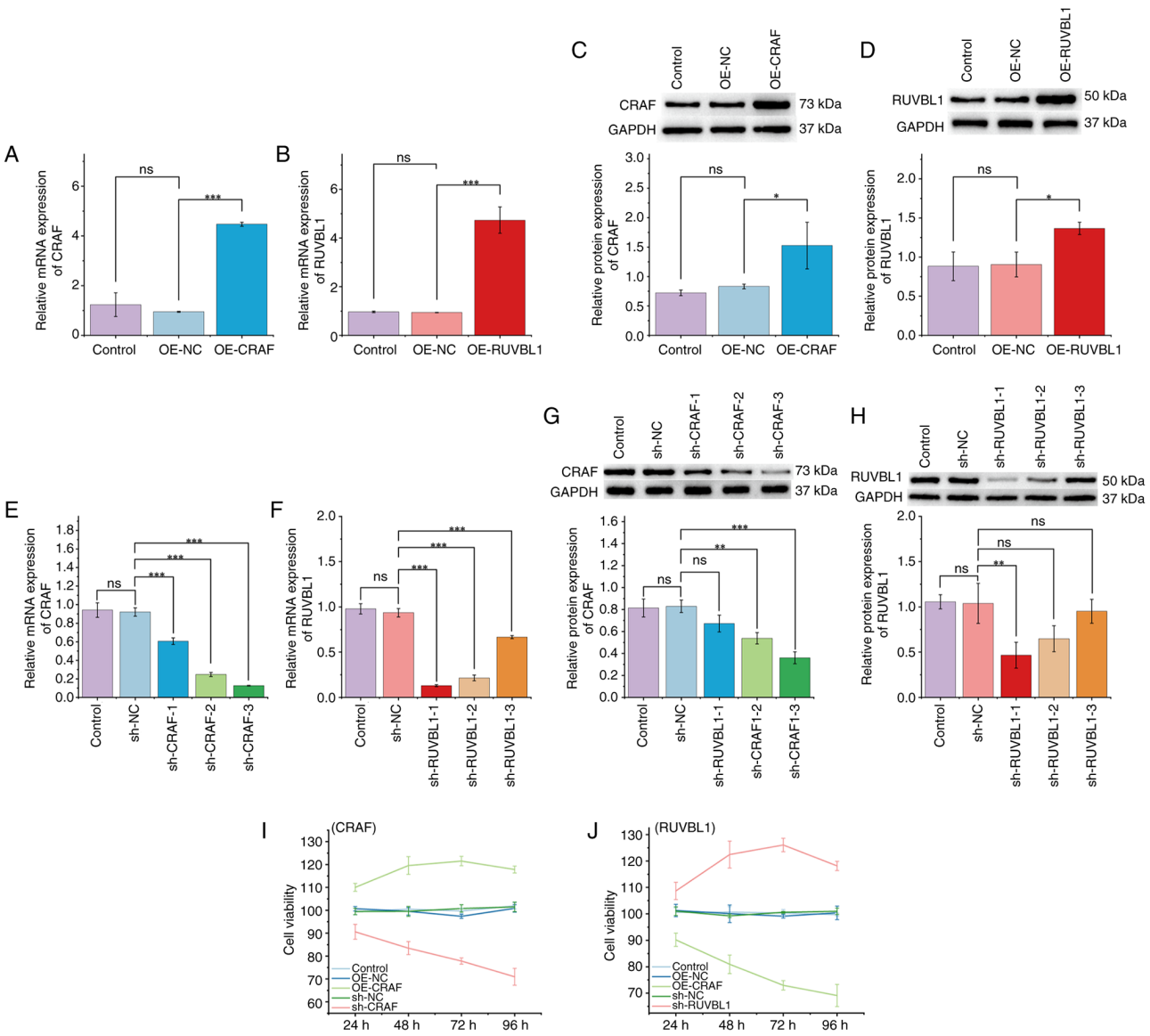

Figure 2. CRAF promotes the proliferative activity of PDLSCs, whereas RUVBL1 inhibits it. RT-qPCR was used to analyze the mRNA expression levels following OE of (A) CRAF and (B) RUVBL1. Western blotting was used to analyze the protein expression after OE of (C) CRAF and (D) RUVBL1. RT-qPCR was performed to analyze mRNA expression following knockdown of (E) CRAF and RUVBL1 (F) Western blotting was used to analyze protein expression following knockdown of (G) CRAF and (H) RUVBL1. Cell Counting Kit-8 assay was performed to analyze the effects of (I) CRAF and (J) RUVBL1 on the viability of PDLSCs. *P<0.05, **P<0.01 and ***P<0.001. PDLSC, periodontal ligament stem cell; RT-q, reverse transcription-quantitative; ns, not significant; OE, overexpression; NC, negative control; sh, short hairpin; RUVBL1, RuvB-like AAA ATPase-1.

Collectively, these results indicated that the isolated PDLSCs exhibited robust self-renewal and multipotent differentiation capabilities.

PDLSC viability is positively associated with CRAF and negatively associated with RUVBL1. To investigate the roles of CRAF and RUVBL1 in PDLSC viability, lentiviral transfection was performed. qPCR and western blotting analyses determined the successful overexpression of CRAF (Fig. 2A and C) and RUVBL1 (Fig. 2B, D), as well as effective knockdown using sh-CRAF-3 and sh-RUVBL1-1 (Fig. 2E-H). CCK-8 assay demonstrated that CRAF overexpression markedly enhanced PDLSC viability, whereas CRAF knockdown suppressed it (Fig. 2I). Conversely, RUVBL1 overexpression decreased PDLSC viability, whereas RUVBL1 knockdown restored it (Fig. 2J). These findings indicated that PDLSC viability was positively regulated by CRAF and negatively regulated by RUVBL1.

RUVBL1 and CRAF enhance osteogenic differentiation, whereas CRAF promotes while RUVBL1 inhibits lipid accumulation. To examine the effects of RUVBL1 and CRAF on PDLSC differentiation, Oil Red O, ALP and alizarin red staining were performed. CRAF overexpression enhanced adipogenic differentiation, whereas CRAF knockdown suppressed it (Fig. 3A). By contrast, RUVBL1 overexpression inhibited adipogenic differentiation, while its knockdown enhanced lipid accumulation (Fig. 3B). Both CRAF and RUVBL1 overexpression increased ALP activity and mineralized nodule formation, indicating enhanced osteogenic differentiation (Fig. 3C-F). Knockdown of either gene reversed these effects, leading to decreased ALP activity

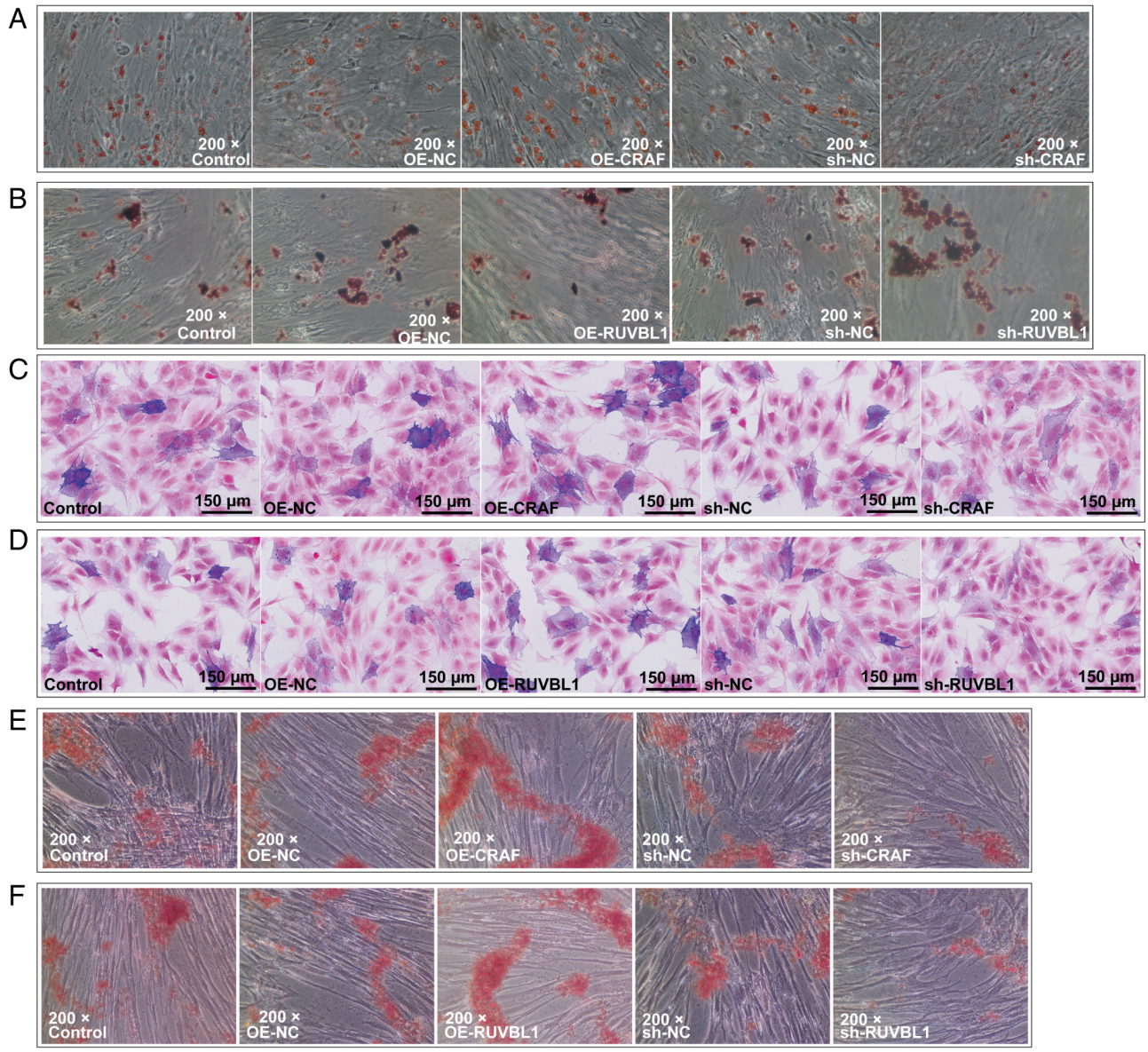

Figure 3. Effects of CRAF and RUVBL1 on the lipogenic and osteogenic differentiation potential of PDLSCs. Oil Red O staining was performed to assess the effect of (A) CRAF and (B) RUVBL1 on the lipogenic differentiation. Alkaline phosphatase staining was used to assess the effect of (C) CRAF and (D) RUVBL1 on osteogenic differentiation. Alizarin red staining was used to assess the effect of (E) CRAF and (F) RUVBL1 on the degree of mineralization. RUVBL1, RuvB-like AAA ATPase-1; PDLSC, periodontal ligament stem cell; OE, overexpression; NC, negative control; sh, short hairpin.

and mineralization. Collectively, these data demonstrated that both RUVBL1 and CRAF promoted osteogenic differentiation of PDLSCs, while exhibiting divergent effects on adipogenesis.

RUVBL1 does not regulate CRAF, but both activate the MEK/ERK signaling pathway. As the MAPK pathway serves a key role in PDLSC differentiation (12), the association between RUVBL1, CRAF and this pathway was assessed. Overexpression of CRAF did not significantly affect ARAF or BRAF expression at either the mRNA or protein level (Fig. 4A, B, H and I). Similarly, RUVBL1 overexpression did not significantly alter the expression of ARAF, BRAF or CRAF (Fig. 5A-C and H-J), suggesting that RUVBL1 did not regulate CRAF. However, CRAF overexpression significantly increased MEK1/2 and ERK1/2 expression and phosphorylation compared with NCs, while CRAF knockdown resulted in the opposite effect (Fig. 4C-G and J-P). Similarly, RUVBL1 overexpression significantly elevated MEK1/2 and ERK1/2 mRNA and protein levels, as well as their phosphorylation, whereas RUVBL1 knockdown significantly suppressed them compared with NCs (Fig. 5D-G and K-P). These results indicate that although RUVBL1 did not directly regulate CRAF, both proteins may have independently activated the MEK/ERK signaling pathway in PDLSCs.

ODN MT01 enhances RUVBL1- and CRAF-mediated osteogenic differentiation. ALP activity increased in a dose-dependent manner and 4.0 μg/ml was used for subsequent experiments (Fig. 6A and B). ODN MT01 markedly enhanced osteogenic differentiation of PDLSCs

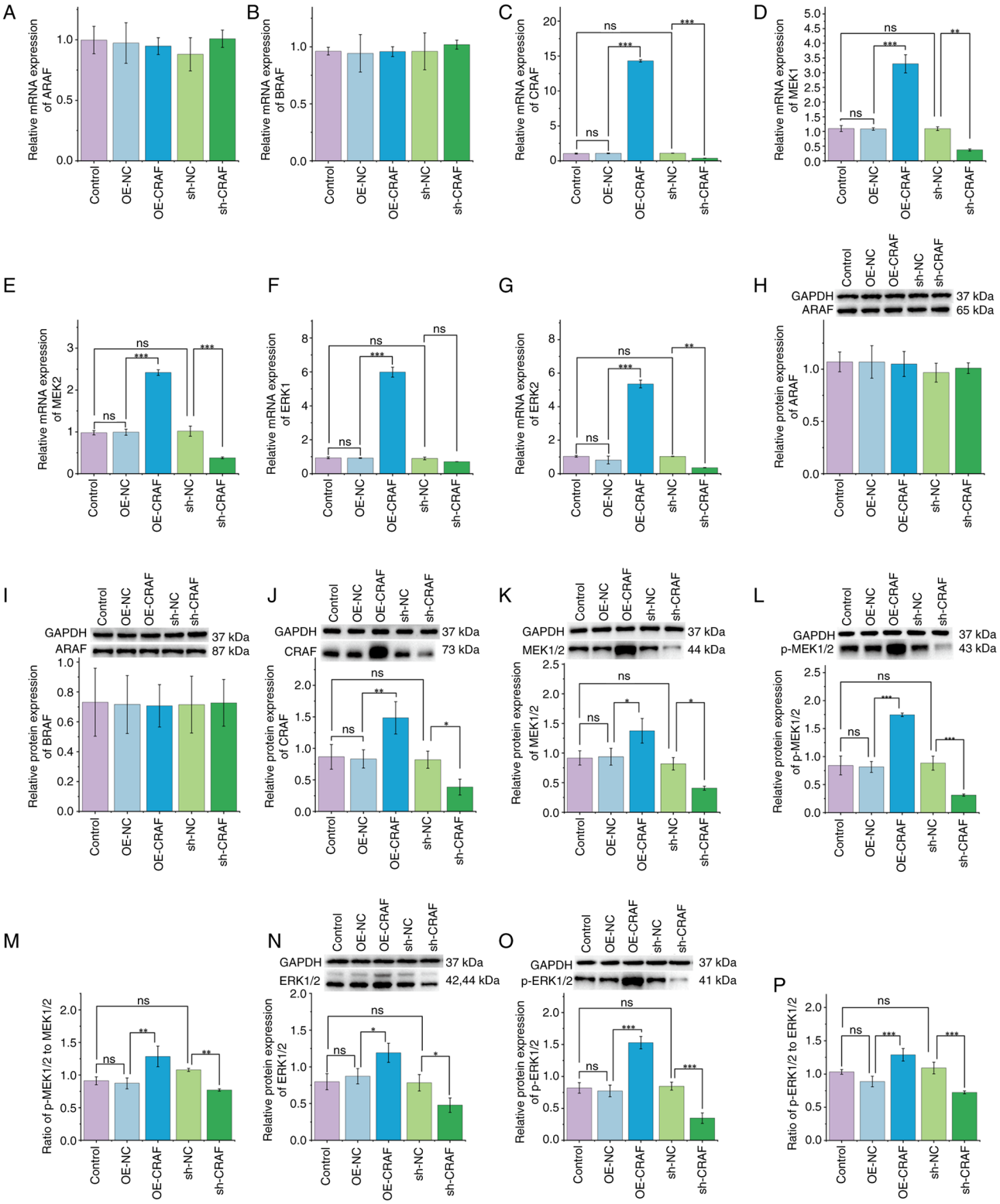

Figure 4. Overexpression of CRAF activates the RAF/MEK/ERK signaling pathway. Reverse transcription-quantitative PCR was used to analyze the mRNA expression of (A) ARAF, (B) BRAF, (C) CRAF, (D) MEK1, (E) MEK2, (F) ERK1and (G) ERK2. Western blotting was used to analyze the protein levels of (H) ARAF, (I) BRAF, (J) CRAF, (K) MEK1/2, (L) p-MEK1/2, (M) p-MEK1/2 to MEK1/2, (N) ERK1/2, (O) p-ERK1/2 and (P) p-ERK1/2 to ERK1/2. *P<0.05, **P<0.01 and ***P<0.001. p-, phosphorylated; OE, overexpression; NC, negative control; sh, short hairpin; ns, not significant; ARAF, A-Raf proto-oncogene serine/threonine-protein kinase; BRAF, B-Raf proto-oncogene serine/threonine-protein kinase; CRAF, C-Raf proto-oncogene serine/threonine-protein kinase.

(Fig. 6C and D). In addition, combined treatment with ODN MT01 and overexpression of CRAF or RUVBL1 further augmented osteogenic differentiation (Fig. 6E and F). Conversely, knockdown of CRAF or RUVBL1 decreased osteogenesis, however this inhibitory effect was partially rescued by ODN MT01 treatment (Fig. 6G and H). These

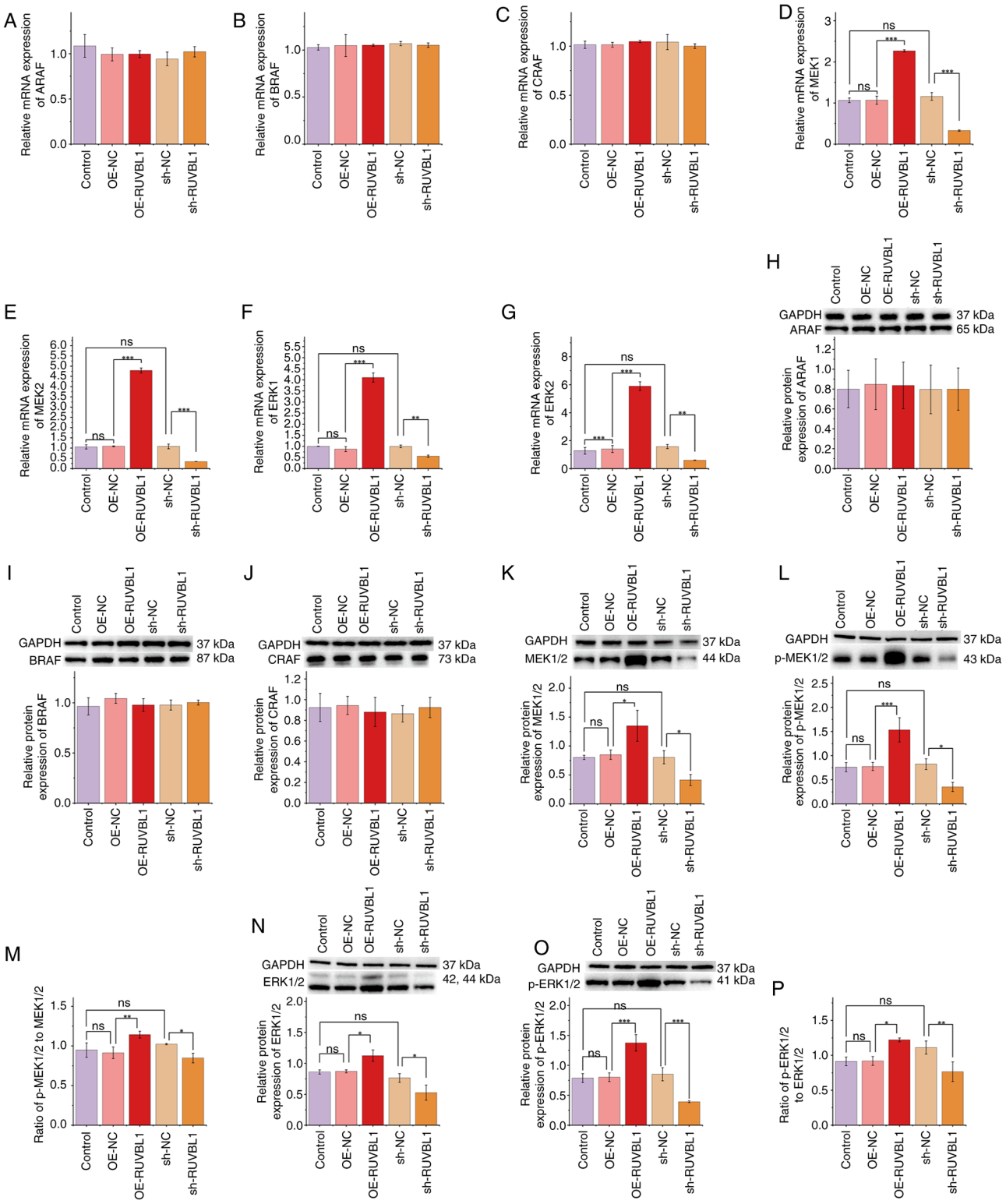

Figure 5. Overexpression of RUVBL1 activates the MEK/ERK signaling pathway. Reverse transcription-quantitative PCR was used to analyze the mRNA expression of (A) ARAF, (B) BRAF, (C) CRAF, (D) MEK1, (E) MEK2, (F) ERK1 and (G) ERK2. Western blotting was used to analyze protein levels of (H) ARAF, (I) BRAF, (J) CRAF, (K) MEK1/2, (L) p-MEK1/2, (M) p-MEK1/2 to MEK1/2, (N) ERK1/2, (O) p-ERK1/2 and (P) p-ERK1/2 to ERK1/2. *P<0.05, **P<0.01 and ***P<0.001. RUVBL1, RuvB-like AAA ATPase-1; p-, phosphorylated; OE, overexpression; NC, negative control; sh, short hairpin; ns, not significant; ARAF, A-Raf proto-oncogene serine/threonine-protein kinase; BRAF, B-Raf proto-oncogene serine/threonine-protein kinase; CRAF, C-Raf proto-oncogene serine/threonine-protein kinase.

findings suggested that ODN MT01 promoted PDLSC osteogenic differentiation and acted additively with with RUVBL1 and CRAF activation to potentially enhance bone-forming potential.

Discussion

As pluripotent SCs with multilineage differentiation potential, PDLSCs serve a key role in the regeneration of periodontal

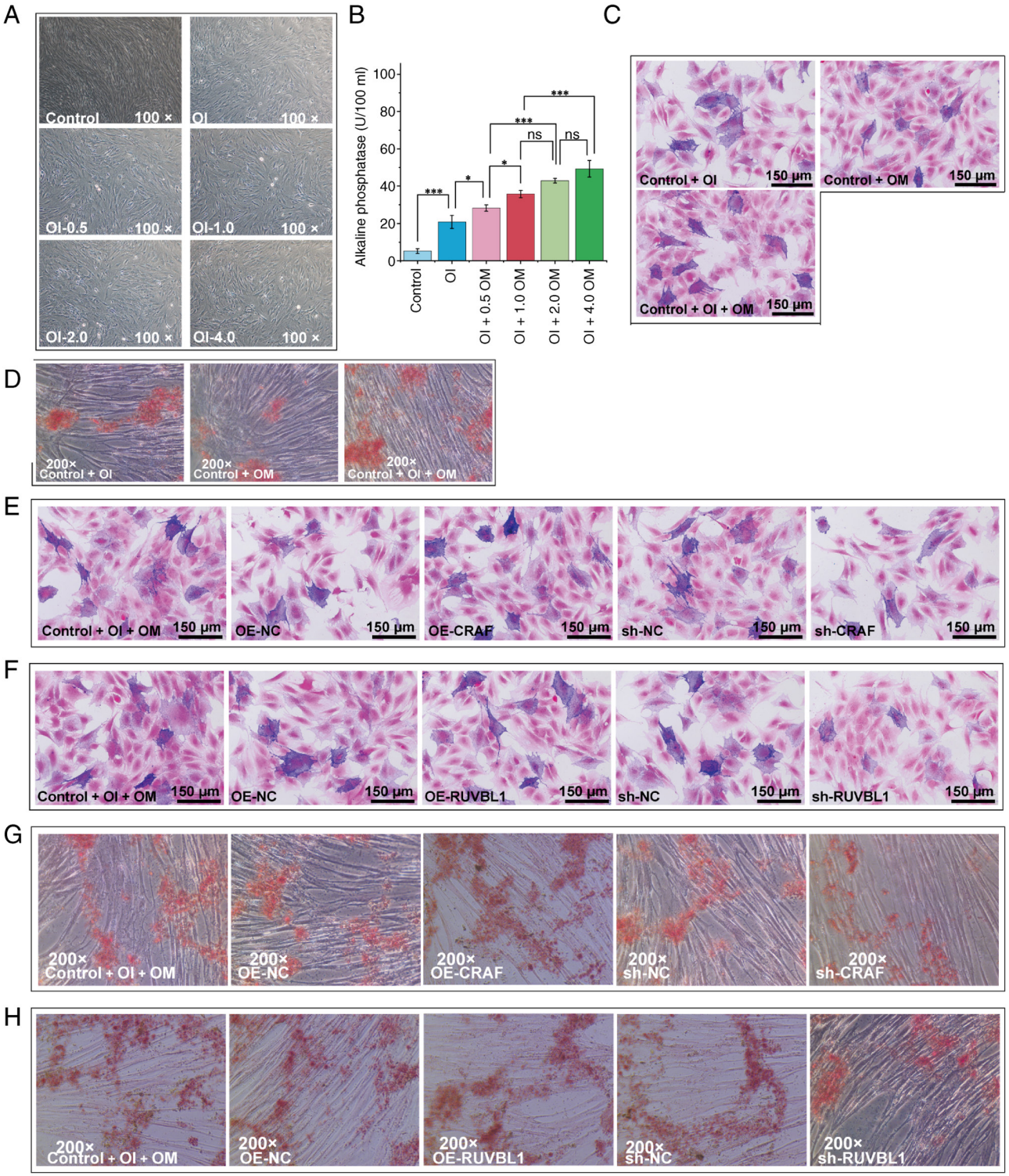

Figure 6. ODN MT01 promotes osteogenic differentiation of PDLSCs. (A) PDLSC proliferation and (B) ALP staining following treatment with varying concentrations of ODN MT01. (C) Osteogenic differentiation and (D) mineralization after the addition of ODN MT01 and osteogenic inducers. ALP staining was used to assess the effect of (E) CRAF and (F) RUVBL1 on osteogenic differentiation of PDLSCs following the addition of ODN MT01 and osteogenic inducers. Alizarin Red staining was used to assess the effect of (G) CRAF and (H) RUVBL1 on the degree of mineralization of PDLSCs following the addition of ODN MT01 and osteogenic inducers. *P<0.05 and ***P<0.001. ODN, oligodeoxynucleotide; PDLSC, periodontal ligament stem cell; ALP, alkaline phosphatase, RUVBL1, RuvB-like AAA ATPase-1; NC, negative control; OE, overexpression; sh, short hairpin; ns, not significant; CRAF, C-Raf proto-oncogene serine/threonine-protein kinase; OI, osteogenic induction; OM, ODN MT01.

tissue. Their osteogenic and adipogenic differentiation capacities are important for periodontitis therapy. RUVBL1, an ATP-binding protein belonging to the AAA^{+} ATPase family, participates in cellular processes, including DNA damage repair, transcriptional regulation and chromatin remodeling (23). A previous study has suggested that RUVBL1 serves as a negative regulator of cell differentiation (24). However, its specific role in PDLSC differentiation has not

been fully elucidated. The present study demonstrated that RUVBL1 overexpression enhanced the osteogenic potential of PDLSCs while suppressing adipogenic differentiation. Notably, although RUVBL1 positively regulates cell proliferation (25), the present results indicated RUVBL1 overexpression reduced PDLSC viability. Excessive RUVBL1 expression may induce replication stress, leading to cell cycle arrest and a shift from proliferation toward osteogenic differentiation (26). CRAF, a member of the RAF kinase family, regulates SC function through both MAPK-dependent and -independent mechanisms (27), though to the best of our knowledge, its role in PDLSCs has not been previously reported. In the present study, CRAF overexpression enhanced PDLSC viability and increased both osteogenic and adipogenic differentiation. By contrast, RUVBL1 promoted osteogenesis but suppressed viability and adipogenesis. RUVBL1 promotes a shift from mTOR-driven lipogenesis towards AMPK-induced fatty acid catabolism in a hepatocellular carcinoma model (28). Although this differed from the present research model and may reflect cell-specificity, it offers a potential explanation of the present findings, suggesting that the divergent effects of RUVBL1 and CRAF on lipid synthesis may stem from distinct signaling pathways. Collectively, these data suggested that both RUVBL1 and CRAF enhance osteogenic differentiation through distinct regulatory mechanisms.

The MAPK signaling pathway is implicated in PDLSC osteogenic differentiation (12,15). Extracellular stimuli are important in activation of the MAPK pathway (29). ERK, a traditional MAPK, primarily regulates cell proliferation and differentiation (30). As a MAPKKK, CRAF activates MEK1/2, which phosphorylates ERK1/2 to regulate downstream transcriptional responses associated with proliferation, differentiation and apoptosis (31). Consistent with these canonical functions, the present study demonstrated that CRAF overexpression increased MEK and ERK phosphorylation, thereby activating the MEK/ERK signaling pathway to enhance PDLSC proliferation and differentiation. RUVBL1 is a CRAF-binding protein capable of activating the RAF/MEK/ERK pathway by preventing phosphorylation at serine 259 within the CR2 domain of CRAF (19). This suggests RUVBL1 may affect PDLSC function by binding to CRAF and promoting its kinase activity through dephosphorylation of the inhibitory S259 site, thereby facilitating CRAF-mediated MEK/ERK activation.. However, the present results did not reveal a regulatory association between RUVBL1 and CRAF in PDLSCs. Despite this, RUVBL1 overexpression independently enhanced MEK/ERK phosphorylation, indicating that it may activate this pathway via a CRAF-independent mechanism. Specifically, RUVBL1 has been demonstrated to exert its effects through numerous pathways, including regulating chromatin remodeling via its AAA+ ATPase activity to alter the transcriptional activity of oncogenes such as CTNNB1 (25), inducing transcription-dependent replication stress and DNA damage when expression levels are deregulated (26), promoting cell cycle progression through direct interaction with AHNAK2 (32). These findings indicate that RUVBL function does not necessarily require RAF activation. Collectively, these results indicate that RUVBL1 and CRAF function independently yet converge on the MEK/ERK pathway to promote osteogenic differentiation in PDLSCs. In addition, ODN MT01, a synthetic ODN derived from human mitochondrial DNA, upregulates osteoblast differentiation markers such as RUNX family transcription factor 2 and osteocalcin (21) and exerts anti-inflammatory effects (33). In the present study, ODN MT01 enhanced PDLSC osteogenesis and partially rescued the inhibitory effects of RUVBL1 or CRAF knockdown. These results suggested that ODN MT01 may have potentiated RUVBL1- and CRAF-mediated activation of the MEK/ERK pathway, thereby amplifying PDLSC osteogenic differentiation.

The present study demonstrated that the overexpression of RUVBL1 or CRAF in PDLSCs activated the MEK/ERK signaling pathway and promoted osteogenic differentiation, an effect augmented by ODN MT01 (Fig. S1). This has marked implications for the therapeutic management of periodontal tissue. The present results warrant further investigation, including additional replication studies and *in vivo* research to assess their clinical translational potential. Furthermore, the delivery potential of RUVBL1/CRAF modulators based on biomaterials requires development. In addition, studies investigating the upstream pathways regulating RUVBL1 and CRAF as well as whether the osteogenic differentiation-promoting effect of ODN MT01 occurs through direct interaction with PDLSCs or other cytokine-mediated pathways are required.

In PDLSCs, RUVBL1 and CRAF independently activated the MEK/ERK signaling pathway to promote osteogenic differentiation, despite the absence of a direct regulatory association between them. The present study identified RUVBL1 and CRAF as potential molecular targets for enhancing periodontal tissue regeneration. ODN MT01 further augmented this osteogenic effect, suggesting its potential as an adjunctive therapeutic agent for periodontitis. Collectively, the present study provided novel mechanistic insight and a basis for future translational research into PDLSC-based periodontal regeneration.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

XYZ conceived and designed the study, performed the experiments, analyzed the data and wrote the manuscript.

PH performed the experiments and analyzed the data. MYQ performed the experiments, analyzed the data and wrote the manuscript. HG conceived and designed the experiments, performed the experiments, analyzed the data and edited the manuscript. HG and XYZ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Caton JG, Armitage G, Berglundh T, Chapple ILC, Jepsen S, Kornman KS, Mealey BL, Papapanou PN, Sanz M and Tonetti MS: A new classification scheme for periodontal and peri-implant diseases and conditions - Introduction and key changes from the 1999 classification. J Periodontol 89 (Suppl 1): S1-S8, 2018.
2. Trindade D, Carvalho R, Machado V, Chambrone L, Mendes JJ and Botelho J: Prevalence of periodontitis in dentate people between 2011 and 2020: A systematic review and meta-analysis of epidemiological studies. J Clin Periodontol 50: 604-626, 2023.
3. Ramanauskaite A, Becker K, Cafferata EA and Schwarz F: Clinical efficacy of guided bone regeneration in peri-implantitis defects. A network meta-analysis. Periodontol 2000 93: 236-253, 2023.
4. Wang C, Zhao Q, Chen C, Li J, Zhang J, Qu S, Tang H, Zeng H and Zhang Y: CD301b(+) macrophage: the new booster for activating bone regeneration in periodontitis treatment. Int J Oral Sci 15: 19, 2023.
5. Wei J, Wang K, Li Y, Huang J, Deng P, Xia X, Yang C, Xu L and Xu J: Metformin carbon dots-based osteogenic and protein delivery system to promote bone regeneration in periodontitis. Bioact Mater 53: 459-479, 2025.
6. Zhang JJ, Li X, Tian Y, Zou JK, Gan D, Deng DK, Jiao C, Yin Y, Tian BM, Wu RX, *et al*: Harnessing mechanical stress with viscoelastic biomaterials for periodontal ligament regeneration. Adv Sci (Weinh) 11: e2309562, 2024.
7. Zhang S, Liu J, Feng F, Jia Y, Xu F, Wei Z and Zhang M: Rational design of viscoelastic hydrogels for periodontal ligament remodeling and repair. Acta Biomater 174: 69-90, 2024.
8. Shimono M, Ishikawa T, Ishikawa H, Matsuzaki H, Hashimoto S, Muramatsu T, Shima K, Matsuzaka K and Inoue T: Regulatory mechanisms of periodontal regeneration. Microsc Res Tech 60: 491-502, 2003.
9. Trubiani O, Pizzicannella J, Caputi S, Marchisio M, Mazzon E, Paganelli R, Paganelli A and Diomede F: Periodontal ligament stem cells: current knowledge and future perspectives. Stem Cells Dev 28: 995-1003, 2019.
10. Wang Y, Papagerakis S, Faulk D, Badylak SF, Zhao Y, Ge L, Qin M and Papagerakis P: Extracellular matrix membrane induces cementoblastic/osteogenic properties of human periodontal ligament stem cells. Front Physiol 9: 942, 2018.
11. Zhu Y, Wang W, Chen Q, Ren T, Yang J, Li G, Qi Y, Yuan C and Wang P: Bioprinted PDLSCs with high-concentration GelMA hydrogels exhibit enhanced osteogenic differentiation in vitro and promote bone regeneration in vivo. Clin Oral Investig 27: 5153-5170, 2023.
12. Liu X, Zhao W, Peng Y, Liu N and Liu Q: The relationship between MAPK signaling pathways and osteogenic differentiation of periodontal ligament stem cells: A literature review. PeerJ 13: e19193, 2025.
13. Kim HJ and Bar-Sagi D: Modulation of signalling by Sprouty: A developing story. Nat Rev Mol Cell Biol 5: 441-450, 2004.
14. Zhao N, Fang H, Tang YP and Liu Q: Advancement on Correction of MAPK Signal Pathway and Chronic Periodontitis. J Oral Sci Res 33: 1012-1015, 2017 (In Chinese).
15. Wu Y, Yang Y, Yang P, Gu Y, Zhao Z, Tan L, Zhao L, Tang T and Li Y: The osteogenic differentiation of PDLSCs is mediated through MEK/ERK and p38 MAPK signalling under hypoxia. Arch Oral Biol 58: 1357-1368, 2013.
16. Yang SH, Sharrocks AD and Whitmarsh AJ: MAP kinase signalling cascades and transcriptional regulation. Gene 513: 1-13, 2013.
17. Riaud M, Maxwell J, Soria-Bretones I, Dankner M, Li M and Rose AAN: The role of CRAF in cancer progression: from molecular mechanisms to precision therapies. Nat Rev Cancer 24: 105-122, 2024.
18. Jha S and Dutta A: RVB1/RVB2: Running rings around molecular biology. Mol Cell 34: 521-533, 2009.
19. Guo H, Zhang XY, Peng J, Huang Y, Yang Y, Liu Y, Guo XX, Hao Q, An S and Xu TR: RUVBL1, a novel C-RAF-binding protein, activates the RAF/MEK/ERK pathway to promote lung cancer tumorigenesis. Biochem Biophys Res Commun 498: 932-939, 2018.
20. Zhang Q, Qu X, Liang C, Li H, Du S, Wang C, Xie Y, Zheng Y and Wang L: Effect of oligonucleotide MT01 delivered by N-isopropylacrylamide modified polyethyleneimine for bone regeneration. Front Bioeng Biotechnol 11: 1204571, 2023.
21. Hou X, Shen Y, Zhang C, Zhang L, Qin Y, Yu Y, Wang L and Sun X: A specific oligodeoxynucleotide promotes the differentiation of osteoblasts via ERK and p38 MAPK pathways. Int J Mol Sci 13: 7902-7914, 2012.
22. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
23. Yenerall P, Das AK, Wang S, Kollipara RK, Li LS, Villalobos P, Flaming J, Lin YF, Huffman K, Timmons BC, *et al*: RUVBL1/RUVBL2 ATPase activity drives PAQosome maturation, DNA replication and radioresistance in lung cancer. Cell Chem Biol 27: 105-121.e14, 2020.
24. Lin D, Lin B, Bhanot H, Riou R, Abt NB, Rajagopal J and Saladi SV: RUVBL1 is an amplified epigenetic factor promoting proliferation and inhibiting differentiation program in head and neck squamous cancers. Oral Oncol 111: 104930, 2020.
25. Zhang C and Wu S: RUVBL1-modulated chromatin remodeling alters the transcriptional activity of oncogenic CTNNB1 in uveal melanoma. Cell Death Discov 9: 132, 2023.
26. Hristova RH, Stoynov SS, Tsaneva IR and Gospodinov AG: Deregulated levels of RUVBL1 induce transcription-dependent replication stress. Int J Biochem Cell Biol 128: 105839, 2020.
27. Khacho M, Clark A, Svoboda DS, Azzi J, MacLaurin JG, Meghaizel C, Sesaki H, Lagace DC, Germain M, Harper ME, *et al*: Mitochondrial dynamics impacts stem cell identity and fate decisions by regulating a nuclear transcriptional program. Cell Stem Cell 19: 232-247, 2016.
28. Guida A, Simeone I, Papini D, Polvani S, Dragoni G, Ceni E, Picariello L, Galli A and Mello T: Targeting RuvBL1 reduces mTOR-driven NASH-HCC progression in conditional PTEN-KO mice. Dig Liver Dis 56: S96, 2024.
29. Xie B, He X, Guo Y, Shen J, Yang B, Cai R, Chen J and He Y: Cyclic tensile stress promotes osteogenic differentiation via upregulation of Piezo1 in human dental follicle stem cells. Hum Cell 37: 1649-1662, 2024.
30. Lavoie H, Gagnon J and Therrien M: ERK signalling: A master regulator of cell behaviour, life and fate. Nat Rev Mol Cell Biol 21: 607-632, 2020.
31. Bahar ME, Kim HJ and Kim DR: Targeting the RAS/RAF/MAPK pathway for cancer therapy: From mechanism to clinical studies. Signal Transduct Target Ther 8: 455, 2023.
32. Li X, Li H, Shao MM, Miao J, Fu Y and Hu B: Downregulation of AHNAK2 inhibits cell cycle of lung adenocarcinoma cells by interacting with RUVBL1. Thorac Cancer 14: 2093-2104, 2023.
33. Yu H, Shen Y, Liu Y, Gao H, Zhou Y, Hu T and Lin C: Effect of specific sequence oligodeoxynucleotide MT01 on the proliferation, apoptosis, and cell cycle of osteoblasts invaded by *Porphyromonas gingivalis*. West China Journal of Stomatology 33: 617-621, 2015 (In Chinese).