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ORIGINAL PAPER

METTL3 Promotes Osteogenic Differentiation of Human Periodontal Ligament Stem Cells Under the Inflammatory Microenvironment Through the miR-141-3p/ZEB1 Axis

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Abstract

Periodontitis, a chronic inflammatory condition, often results in gum tissue damage and can lead to tooth loss. This study explores the role of methyltransferase-like 3 (METTL3) in promoting osteogenic differentiation of human periodontal ligament stem cells (hPDLSCs) within an inflammatory microenvironment. An inflammatory environment was simulated in hPDLSCs using lipopolysaccharide (LPS). Both adipogenic and osteogenic differentiation capacities of hPDLSCs were assessed. In LPS-treated hPDLSCs, METTL3 was overexpressed, and alkaline phosphatase (ALP) staining was performed alongside measurements of ALP activity, pro-inflammatory cytokines, METTL3, miR-141-3p, pri-miR-141, Zinc finger E-box binding homeobox 1 (ZEB1), runt-related transcription factor 2 (RUNX2), osteocalcin (OCN). N6-methyladenosine (m6A) and pri-miR-141 levels were quantified, and the binding of miR-141-3p to ZEB1 was analyzed. The results demonstrated that osteogenic differentiation in hPDLSCs was diminished under inflammatory conditions, coinciding with downregulated METTL3 expression. However, METTL3 overexpression enhanced osteogenic differentiation. METTL3 facilitated the conversion of pri-miR-141 into miR-141-3p via m6A modification, resulting in increased miR-141-3p levels, which in turn suppressed ZEB1 expression. Inhibition of miR-141-3p or overexpression of ZEB1 partially counteracted the positive effects of METTL3 on osteogenic differentiation. In conclusion, these findings suggest that METTL3-mediated m6A modification promotes osteogenic differentiation of hPDLSCs within an inflammatory microenvironment through the miR-141-3p/ZEB1 axis.

Keywords METTL3 · miR-141-3p · ZEB1 · hPDLSCs · Osteogenesis

Highlights

- Inflammatory microenvironment impairs osteogenic differentiation in hPDLSCs.
- METTL3 overexpression enhances osteogenic differentiation of hPDLSCs.
- METTL3 promotes miR-141-3p expression via m6A modification.
- miR-141-3p targets and suppresses ZEB1 expression.
- METTL3 enhances osteogenic differentiation of hPDLSCs through the miR-141-3p/ZEB1 axis.

Introduction

Periodontitis is a chronic, multifactorial inflammatory disease primarily triggered by the accumulation of dental plaque, leading to the irreversible destruction of the periodontal ligament and alveolar bone [1]. This severe condition affects approximately 10% of the global population, with a high prevalence across all age groups [2]. The disease is often caused by various microorganisms within the oral cavity, such as *Porphyromonas gingivalis* (*P. gingivalis*), a major pathogen in periodontitis. Lipopolysaccharide (LPS), a component of the outer membrane of *P. gingivalis*, is recognized as a key virulence factor in the progression of periodontitis [3].

In recent years, periodontal tissue engineering has emerged as a promising approach for regenerating damaged

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alveolar bone. Central to this strategy is human periodontal ligament stem cells (hPDLSCs), which play a critical role in periodontal tissue regeneration due to their mesenchymal stem cell properties, including self-renewal and multilineage differentiation potential. These properties enable hPDLSCs to differentiate into various cell types, such as osteoblasts and odontoblasts, making them indispensable for effective periodontal regeneration therapies [4]. Preserving the normal differentiation and self-renewal capacities of hPDLSCs is essential for maintaining periodontal homeostasis and preventing severe periodontitis [5]. Therefore, understanding the mechanisms that regulate the osteogenic differentiation capacity of hPDLSCs is of great importance.

N6-methyladenosine (m6A) is the most prevalent internal modification in eukaryotic RNA, and it plays a crucial role in the regulation of various biological processes. This modification is catalyzed by methyltransferases, removed by demethylases, and recognized by specific reader proteins [6]. As an m6A methyltransferase, methyltransferase like 3 (METTL3) is integral to the regulation of mRNA translation and is involved in various biological processes, such as cell differentiation and inflammatory responses [7]. Recent studies have shown that alterations in m6A modification levels significantly influence the osteogenic differentiation capacity of hPDLSCs [8]. Additionally, METTL3 has been found to promote osteogenic differentiation of hPDLSCs by regulating the stability of downstream factors mRNA through m6A methylation [9]. This study aims to further elucidate the mechanisms by which METTL3 modulates the osteogenic differentiation of hPDLSCs within an inflammatory microenvironment.

MicroRNAs (miRNAs) are a class of small, non-coding RNA molecules that play critical roles in regulating cellular processes such as growth, differentiation, development, and apoptosis [10]. One of the key mechanisms influencing miRNA gene expression is m6A modification [11]. This modification can enhance the recognition and processing of primary miRNAs (pri-miRNAs) by DiGeorge Critical Region 8 (DGCR8), thereby promoting miRNA maturation [12]. Aberrant expression of miRNAs has been linked to impaired osteogenic differentiation in periodontal ligament stem cells (PDLSCs) [13]. Among these, miR-141-3p has garnered particular interest due to its significant role in the osteogenic differentiation of hPDLSCs. miR-141-3p is highly expressed in exosome-derived hPDLSCs and has been shown to counteract cellular senescence, thereby restoring the cells' osteogenic differentiation capacity [14]. However, the precise mechanisms through which miR-141-3p regulates this process remain to be fully elucidated.

Zinc finger E-box binding homeobox 1 (ZEB1) is a transcription factor involved in a variety of cellular processes, including stemness, cell survival, longevity, cell cycle arrest, and epithelial-to-mesenchymal transition [15]. ZEB1 also plays a role in osteogenic differentiation, a process that may be regulated by miRNAs [16]. Previous research has indicated that ZEB1 is upregulated in mice with periodontitis [17]. As *P. gingivalis*, a key pathogen in periodontitis, can induce high levels of ZEB1 in gingival epithelial cells, it is believed that ZEB1 may be a critical factor in the disease's progression [18]. Importantly, emerging evidence suggests that silencing ZEB1 could promote osteogenic differentiation, offering a potential therapeutic strategy [19, 20]. Based on these findings, we hypothesize that silencing the ZEB1 gene could mitigate periodontitis by enhancing the osteogenic differentiation of hPDLSCs.

In this study, we investigated the mechanism by which METTL3-mediated m6A modification influences the osteogenic differentiation of hPDLSCs within an LPS-induced periodontitis inflammatory microenvironment characteristic of periodontitis. Our findings aim to provide new insights and potential therapeutic avenues for the treatment of periodontitis.

Materials and methods

Cell Culture

HPDLSCs (SNP-H324) were obtained from Sunncell (Wuhan, Hubei, China). The cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, 10099158, Gibco) and 100 U/mL penicillin/streptomycin (15140148, Gibco). To simulate an inflammatory microenvironment in vitro, the cells were treated with 10 µg/mL lipopolysaccharide (LPS) (I8450, Solarbio, Beijing, China) for 24 h. The control group consisted of hPDLSCs cultured under standard, non-inflammatory conditions [21].

Flow Cytometry

The hPDLSCs were digested using trypsin (40126ES60, YEASEN, Shanghai, China) and resuspended at a concentration of 1×10^7 cells in 1 mL of phosphate buffer (PBS). The cells were then incubated with antibodies targeting CD29 (ab21845, Abcam, Cambridge, MA, USA), CD90 (ab11155, Abcam), CD34 (ab18227, Abcam), and CD45 (ab27287, Abcam) at 37°C for 30 min, protected from light. Flow cytometric was subsequently performed to analyze the expression profiles of CD29, CD90, CD34, and CD45.

Adipogenic and Osteogenic Differentiation

For adipogenic differentiation, hPDLSCs were cultured in DMEM supplemented with 10% FBS, 2 µg/mL insulin (I8830, Solarbio), 500 nM 3-isobutyl-1-methylxanthine (I8450, Solarbio), and 0.5 µM dexamethasone (D8040, Solarbio) for 21 days, with the medium being refreshed every 48 h. Adipogenic differentiation was assessed using Oil Red O staining (C0157M, Beyotime, Shanghai, China).

For osteogenic differentiation, hPDLSCs were cultured in DMEM supplemented with 10% FBS, 0.01 µM dexamethasone (D8040, Solarbio), 50 µg/mL ascorbic acid (A8100, Solarbio), and 10 mM β-glycerophosphate (G8100, Solarbio) for 21 days, with medium changes every 2 days. Osteogenic differentiation was evaluated by performing Alizarin red staining (0.2%, C0138, Beyotime) to visualize calcium nodule formation. After discarding the staining solution, 10% cetylpyridinium chloride (IC5410, Solarbio) was used to dissolve the calcium nodules. The resulting solution was shaken, and 100 µL was transferred to a 96-well plate for absorbance measurement at 562 nm using a microplate reader (BioRad, Hercules, CA, USA).

Cell Transfection

Overexpression plasmids for METTL3 (oe-METTL3, NM_019852.5), ZEB1 (oe-ZEB1, NM_001128128.3), and the empty vector (oe-NC), along with miR-141-3p inhibitor, inhibitor control (inhibitor NC), miR-141-3p mimic, and mimic control (mimic NC), were obtained from GenePharma (Shanghai, China). The hPDLSCs were transfected with these RNA molecules or vectors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. After 48 h of transfection, the cells were harvested to assess transfection efficiency. Subsequently, the transfected cells were treated with LPS to mimic an inflammatory microenvironment. The sequences for the miR-141-3p inhibitor and mimic are provided in Table 1.

Cell Counting Kit-8 (CCK-8) Assay

For cell viability analysis, 5×10^3 cells were seeded into 96-well plates. After 48 h, 10 µL of CCK-8 reagent (CK04, Dojindo Laboratories, Japan) was added to each well. The cells were incubated for an additional 3 h. Absorbance at 450 nm was measured using a microplate reader. Each experiment was conducted in triplicate to ensure reproducibility.

Table 1 miRNA sequence

	Sequence
miR-141-3p inhibitor	CCAUCUUUACCAGACAGUGUUA
miR-141-3p mimic	UAACACUGUCUGGUAAAGAUGG

Enzyme-Linked Immunosorbent Assay (ELISA)

To quantify the levels of inflammatory cytokines, the following ELISA kits were used: tumor necrosis factor (TNF)-α ELISA kit (ab181421, Abcam), interleukin (IL)-1β ELISA kit (ab214025, Abcam), and IL-6 ELISA kit (ab178013, Abcam). Assays were performed according to the manufacturer's protocols, and each experiment included three independent replicates.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from hPDLSCs using TRIzol reagent (15596018CN, Invitrogen). First-strand complementary DNA (cDNA) was synthesized using the PrimeScript RT kit (RR047A, Takara, Dalian, China) for miRNA quantification, or the Mir-X miRNA first-strand synthesis kit (638315, Takara) for miRNA quantification. qRT-PCR was performed using the Premix Ex Taq kit (RR390A, Takara). Relative RNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [22], with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or U6 [23] as the internal control. Each qRT-PCR experiment was performed in triplicate. Primer sequences are listed in Table 2.

Western Blot Analysis

hPDLSCs were lysed using radioimmunoprecipitation assay buffer (Solarbio), and the protein concentration was quantified using the bicinchoninic acid method (Beyotime). Protein samples were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocking with 5% skim milk solution for 2 h, the membranes were incubated overnight at 4 °C with primary antibodies against METTL3 (1:1000, ab195352, Abcam), ZEB1 (1:500, ab203829, Abcam), and GAPDH (1:2500, ab9485, Abcam). Following three washes with Tris-buffered saline (TBST), the membranes were incubated with secondary immunoglobulin G (IgG) antibody (1:2000, ab205718, Abcam) at room temperature for 2 h. Protein bands were detected using SuperSignal West Pico PLUS chemiluminescent substrate (34580, Thermo Fisher, Waltham, MA, USA) and analyzed using Quantity One software (BioRad), with GAPDH serving as the loading control.

Table 2 qPCR primers

	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
METTL3	CGGACACGTGGAGCTCTATC	TGGAGACAATGCTGCCTCTG
miR-141-3p	GCCGAGTAACACTGTCTGGT	CTCAACTGGTGTCGTGGAGT
pri-miR-141	CCCAGCAACGCCCTTTCTAT	CAGTGGAGTCCAAGTCCCAC
ZEB1	CGGTCCCTAGCAACAAGGTT	GCAACCACCACCACATGTTC
RUNX2	TAGGCGCATTTCAGGTGCTT	GGACATACCGAGGGACATGC
OCN	ATGAGAGCCCTCACACTCCT	CTTGGACACAAAGGCTGCAC
GAPDH	GACAGTCAGCCGCATCTTCT	GCGCCCAATACGACCAAATC
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

Alkaline Phosphatase (ALP) Staining Assay and Measurement of ALP Activity

hPDLSCs were cultured in an osteogenic induction medium for 14 days. Cells were then fixed for 30 min and stained using a BCIP/NBT ALP color development kit (C3206, Beyotime) for 30 min in the dark. ALP activity was measured using an ALP activity assay kit (ab83369, Abcam) according to the manufacturer's instructions. The absorbance at 405 nm was determined using a microplate reader. A standard curve was generated based on the absorbance of the standard references, and the ALP activity of the test samples was calculated accordingly. Each experiment was conducted with three independent replicates.

Measurement of Total m6A Levels

The total m6A levels in hPDLSCs were measured using the m6A quantification kit (ab185912, Abcam). Briefly, 300 ng of RNA was immobilized on the assay plate along with m6A standard references. The plate was incubated with a capture antibody solution, followed by a secondary detection antibody solution. The m6A levels were quantified by measuring absorbance at 450 nm, and the results were calculated based on a standard curve generated from the reference standards. Each experiment was performed in triplicate.

RNA Immunoprecipitation (RIP)

Total RNA in cells was extracted from hPDLSCs, and RNA-protein complexes were immunoprecipitated using antibodies against m6A antibody (1:50, ab208577, Abcam), DiGeorge Critical Region 8 (DGCR8) antibody (1:60, ab191875, Abcam), or IgG antibody (1:50, ab172730, Abcam). The antibodies were coupled to protein A/G magnetic beads. The beads were incubated with RNA-protein complexes in immunoprecipitation (IP) buffer (140 mM NaCl, 1% NP-40, 2 mM ethylene diamine tetraacetic acid [EDTA], 20 mM Tris pH 7.5) overnight at 4 °C. The immunoprecipitated RNA was eluted, reverse-transcribed, and analyzed using qRT-PCR analysis to quantify pri-miR-141 levels. Each experiment was conducted in triplicate. Primer sequences used for qRT-PCR are listed in Table 2.

Bioinformatics Analysis

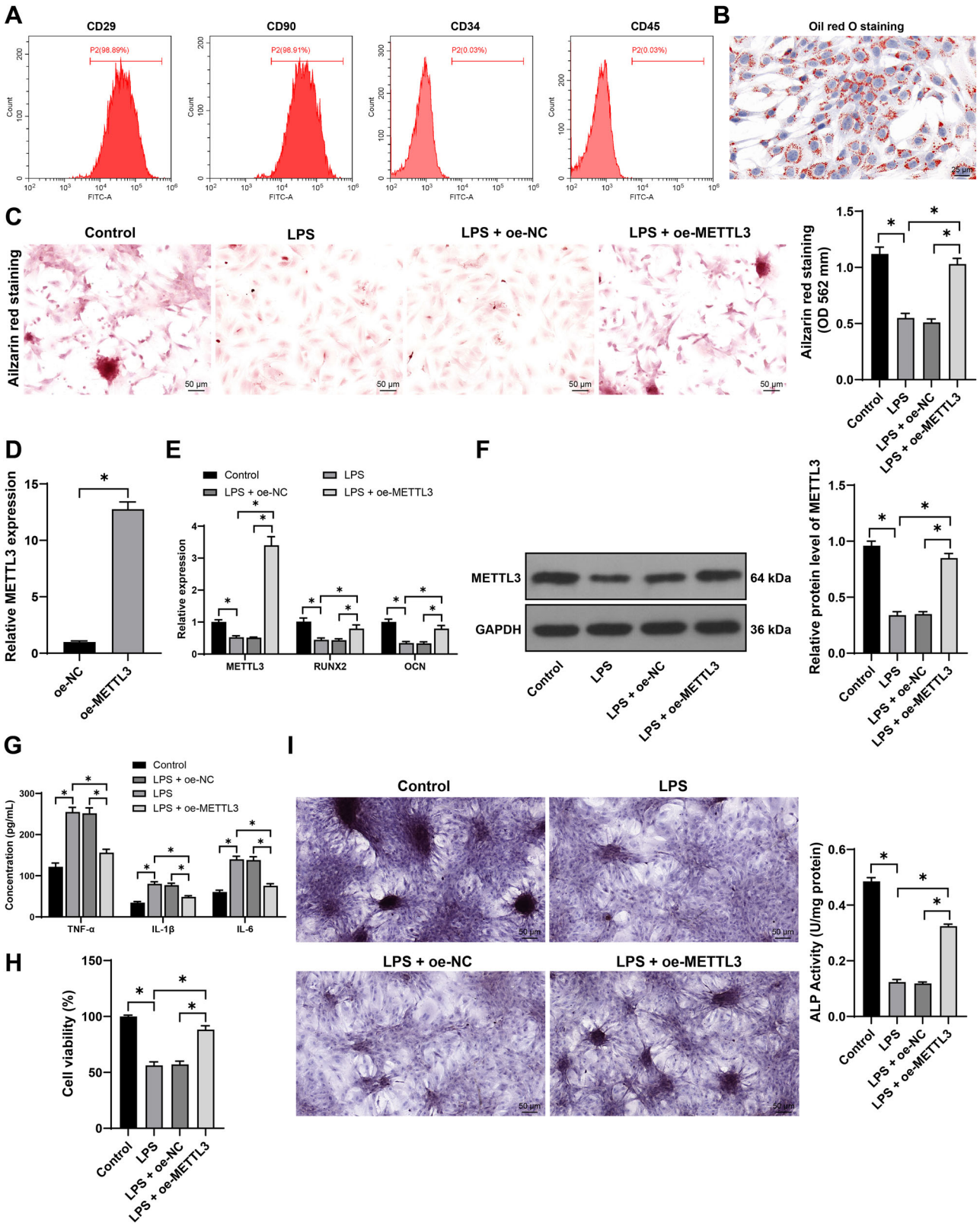
The potential downstream target genes of miR-141-3p were predicted using several bioinformatics tools, including StarBase [24] (https://rnasysu.com/encori/), TargetScan [25] (https://www.targetscan.org/vert_80/), miRDB [26] (https://mirdb.org/), and miRTarBase [27] (https://mirtarbase.cuhk.edu.cn/). These platforms provided a comprehensive analysis of the miRNA-gene interactions, identifying candidate genes that miR-141-3p may regulate.

Dual-Luciferase Reporter Gene Assay

The 3' untranslated region (UTR) of ZEB1, containing the predicted binding site for miR-141-3p (ZEB1-WT), and a mutated version of this binding site (ZEB1-MUT) were synthesized and cloned into the pMIR-reporter plasmid (AM5795, Thermo Fisher). These constructed plasmids were then transfected into hPDLSCs along with either a miR-141-3p mimic or its negative control (mimic NC). After 48 h of transfection, the cells were lysed, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (K801-200, Biovision, Mountain View, CA, USA). Each experiment was conducted in triplicate to ensure accuracy and reproducibility.

Statistical Analysis

Statistical analyses were conducted using SPSS 21.0 software (IBM, Armonk, NY, USA) and GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA). Data are presented as mean ± standard deviation. Prior to analysis, normality and homogeneity of variance were assessed to confirm that the data conformed to normal distribution and equal variance. For comparisons between two groups, an independent-sample



t-test was utilized. For comparisons involving multiple groups, one-way or two-way analysis of variance (ANOVA) was performed, followed by Tukey's multiple comparisons test for post hoc analysis. All statistical tests were two-sided, and a $p < 0.05$ was considered statistically significant.

◀ **Fig. 1** Overexpression of METTL3 enhances osteogenic differentiation of hPDLSCs under inflammatory microenvironment. **A** Flow cytometry was performed to detect the levels of surface markers in hPDLSCs (positive: CD29, CD90; negative: CD34, CD45). **B** Oil Red O staining was conducted to assess adipogenic differentiation of hPDLSCs. **C** Alizarin Red staining was performed to evaluate osteogenic differentiation of hPDLSCs. hPDLSCs were transfected with overexpression plasmids for METTL3 (oe-METTL3), and empty vector plasmids (oe-NC) were used as a control. Cells were incubated with 10 μg/mL LPS for 24 h. **D** qRT-PCR was performed to measure the transfection efficiency. **E** qRT-PCR was used to detect the expression of METTL3, RUNX2, and OCN. **F** Western blot analysis was performed to assess the expression of METTL3. **G** ELISA was conducted to measure the levels of TNF-α, IL-1β, and IL-6 in hPDLSCs. **H** Cell viability was assessed using the CCK-8 assay. **I** ALP staining assay was conducted and ALP activity was measured. These experiments were independently repeated three times, and the data are presented as mean ± standard deviation. Data in panel **D** were analyzed using *t*-test. Data in panels **C**, **F**, **H**, and **I** were analyzed by one-way ANOVA, and data in panels **E** and **G** were analyzed by two-way ANOVA, followed by Tukey's multiple comparisons test. $*p < 0.05$

Results

Overexpression of METTL3 Enhances Osteogenic Differentiation of hPDLSCs Under an Inflammatory Microenvironment

The identification of hPDLSCs through flow cytometry demonstrated high expression of CD29 and CD90, with low expression of CD34 and CD45, confirming their mesenchymal stem cell characteristics (Fig. 1A). Additionally, adipogenic and osteogenic differentiation assays confirmed the multilineage differentiation potential of hPDLSCs (Fig. 1B, C).

Previous studies have shown that METTL3 expression increases during the osteogenic differentiation of hPDLSCs [9]. To explore the specific role of METTL3 in osteogenic differentiation of hPDLSCs under an inflammatory microenvironment, we overexpressed METTL3 in hPDLSCs, which was confirmed by significant upregulation of METTL3 expression ($p < 0.05$, Fig. 1D–F).

Upon treatment with LPS to simulate an inflammatory environment, the levels of pro-inflammatory factors were significantly elevated ($p < 0.05$, Fig. 1G), accompanied by a marked reduction in cell viability ($p < 0.05$, Fig. 1H), ALP activity ($p < 0.05$, Fig. 1I), and osteogenic differentiation capacity ($p < 0.05$, Fig. 1C). Furthermore, the expression of osteogenic-related genes RUNX2 and OCN was significantly decreased ($p < 0.05$, Fig. 1E). The expression of METTL3 was also found to be downregulated in LPS-treated hPDSLSCs ($p < 0.05$, Fig. 1E,F).

Subsequent overexpression of METTL3 resulted in a significant reduction in pro-inflammatory cytokines ($p < 0.05$, Fig. 1G), alongside enhanced cell viability ($p < 0.05$, Fig. 1H), increased ALP activity ($p < 0.05$, Fig. 1I), and improved osteogenic differentiation capacity ($p < 0.05$, Fig. 1C). These findings suggest that overexpression of METTL3 effectively mitigates the negative impact of an inflammatory microenvironment on hPDLSCs and promotes osteogenic differentiation.

METTL3 Promotes the Expression of miR-141-3p via m6A Modification

Knockdown of METTL3 in hPDLSCs led to a significant decrease in total m6A levels, whereas overexpression of METTL3 resulted in a marked increase in m6A levels ($p < 0.05$, Fig. 2A). Given that miR-141-3p is downregulated in periodontitis patients [28], we hypothesized that the reduced expression of miR-141-3p could be associated with the decreased m6A modification of pri-miR-141 mediated by METTL3. Considering that pri-miRNAs are known to recruit DGCR8 for miRNA processing [10], we evaluated the levels of m6A-modified and DGCR8-bound pri-miR-141 using RIP assays.

In LPS-treated hPDLSCs, both DGCR8-bound and m6A-modified pri-miR-141 levels were significantly reduced. However, overexpression of METTL3 increased the levels of both m6A-modified and DGCR8-bound pri-miR-141 ($p < 0.05$, Fig. 2B). Pri-miR-141 serves as the precursor of miR-141-3p and undergoes processing into miR-141-3p through DGCR8-mediated modification. Post-LPS treatment, while pri-miR-141 levels were upregulated, miR-141-3p expression was downregulated in hPDLSCs. Overexpression of METTL3 reversed this trend, leading to a downregulation of pri-miR-141 and upregulation of miR-141-3p ($p < 0.05$, Fig. 2C). These findings indicate that METTL3 enhances the processing of pri-miR-141 into miR-141-3p through m6A modification, thus promoting miR-141-3p expression.

Inhibition of miR-141-3p Partially Reverses the Promotive Effect of METTL3 Overexpression on Osteogenic Differentiation of hPDLSCs under the Inflammatory Microenvironment

To assess the role of miR-141-3p in METTL3-mediated osteogenic differentiation, we inhibited miR-141-3p expression in hPDLSCs, which was confirmed by a significant downregulation of miR-141-3p levels ($p < 0.05$, Fig. 3A, B). We then conducted experiments combining miR-141-3p inhibition with METTL3 overexpression (oe-METTL3). Compared to cells transfected with oe-METTL3 alone, the combined treatment group exhibited significantly higher levels of pro-inflammatory factors ($p < 0.05$, Fig. 3C), decreased cell viability ($p < 0.05$, Fig. 3D), and a significant reduction in both ALP activity and osteogenic differentiation capacity ($p < 0.05$, Fig. 3E, F). Additionally, the expression of osteogenic-related genes RUNX2 and OCN

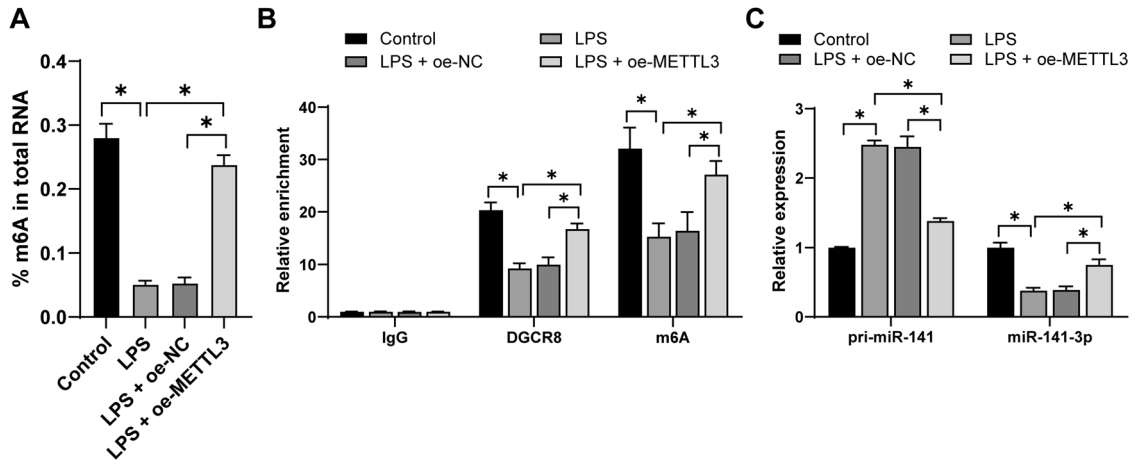


Fig. 2 METTL3 promotes the expression of miR-141-3p via m6A modification. **A** Quantification of m6A levels in total RNA of hPDLSCs. **B** RIP assay was performed to detect the levels of pri-miR-141 binding with DGCR8 and m6A modification. **C** qRT-PCR was used to measure the expression of pri-miR-141 and miR-141-3p. These experiments were independently repeated three times, and the data are presented as mean ± standard deviation. Data in panel **A** were analyzed using one-way ANOVA. Data in panels **B** and **C** were analyzed using two-way ANOVA, followed by Tukey's multiple comparisons test. *$p < 0.05$

was markedly reduced ($p < 0.05$, Fig. 3B). These findings suggest that METTL3 enhances osteogenic differentiation of hPDLSCs by upregulating miR-141-3p expression through m6A modification.

miR-141-3p Targets and Inhibits ZEB1 Expression

Using bioinformatics tools, including StarBase, TargetScan, miRDB, and miRTarBase, we predicted the downstream target genes of miR-141-3p, identifying ZEB1 as a potential target. The binding site of miR-141-3p within the ZEB1 3'UTR was confirmed using a Venn diagram (Fig. 4A-B). ZEB1 has been reported to influence osteogenic differentiation and the proliferation capacity of hPDLSCs under an inflammatory microenvironment [20]. The direct interaction between miR-141-3p and ZEB1 3'UTR was validated using a dual-luciferase reporter assay, which confirmed significant binding ($p < 0.05$, Fig. 4C). LPS treatment led to an upregulation of ZEB1 expression, while overexpression of METTL3 resulted in its downregulation. However, in the combined miR-inhi and oe-METTL3 group, ZEB1 expression was significantly increased ($p < 0.05$, Fig. 4D, E). These findings indicate that METTL3 promotes miR-141-3p expression via m6A modification, which in turn inhibits ZEB1 expression.

Overexpression of ZEB1 Partially Reverses the Promotive Effect of METTL3 Overexpression on Osteogenic Differentiation of hPDLSCs Under the Inflammatory Microenvironment

To further validate the mechanistic pathway, we conducted rescue experiments by overexpressing ZEB1 in hPDLSCs, confirmed by a significant increase in ZEB1 expression ($p < 0.05$, Fig. 5A–C). Compared to cells transfected with oe-METTL3 alone, the group with combined ZEB1 overexpression showed upregulation of pro-inflammatory cytokines ($p < 0.05$, Fig. 5D), reduced cell viability ($p < 0.05$, Fig. 5E), and decreased ALP activity ($p < 0.05$, Fig. 5F). Moreover, the osteogenic differentiation capacity and the expression of osteogenic-related genes RUNX2 and OCN were also significantly reduced in the combined group ($p < 0.05$, Fig. 5B, G). These findings suggest that METTL3 enhances the osteogenic differentiation of hPDLSCs by increasing miR-141-3p expression via m6A modification and inhibiting ZEB1 expression, and that overexpression of ZEB1 can partially reverse these effects.

Discussion

Periodontitis, primarily caused by viral and bacterial infections, is a chronic inflammatory condition that can lead to systemic diseases, significantly threatening overall health and potentially life-threatening if left untreated [29]. Current treatment strategies for periodontitis, including subgingival scaling to remove plaque and calculus, local and systemic drug therapies, and surgical interventions, although effective, have limitations [30]. This study demonstrates that METTL3 promotes the expression of miR-141-3p via m6A modification, which subsequently targets and inhibits ZEB1. The inhibition of ZEB1 facilitates the osteogenic differentiation of hPDLSCs and thereby contributes to the mitigation of periodontitis (Fig. 6).

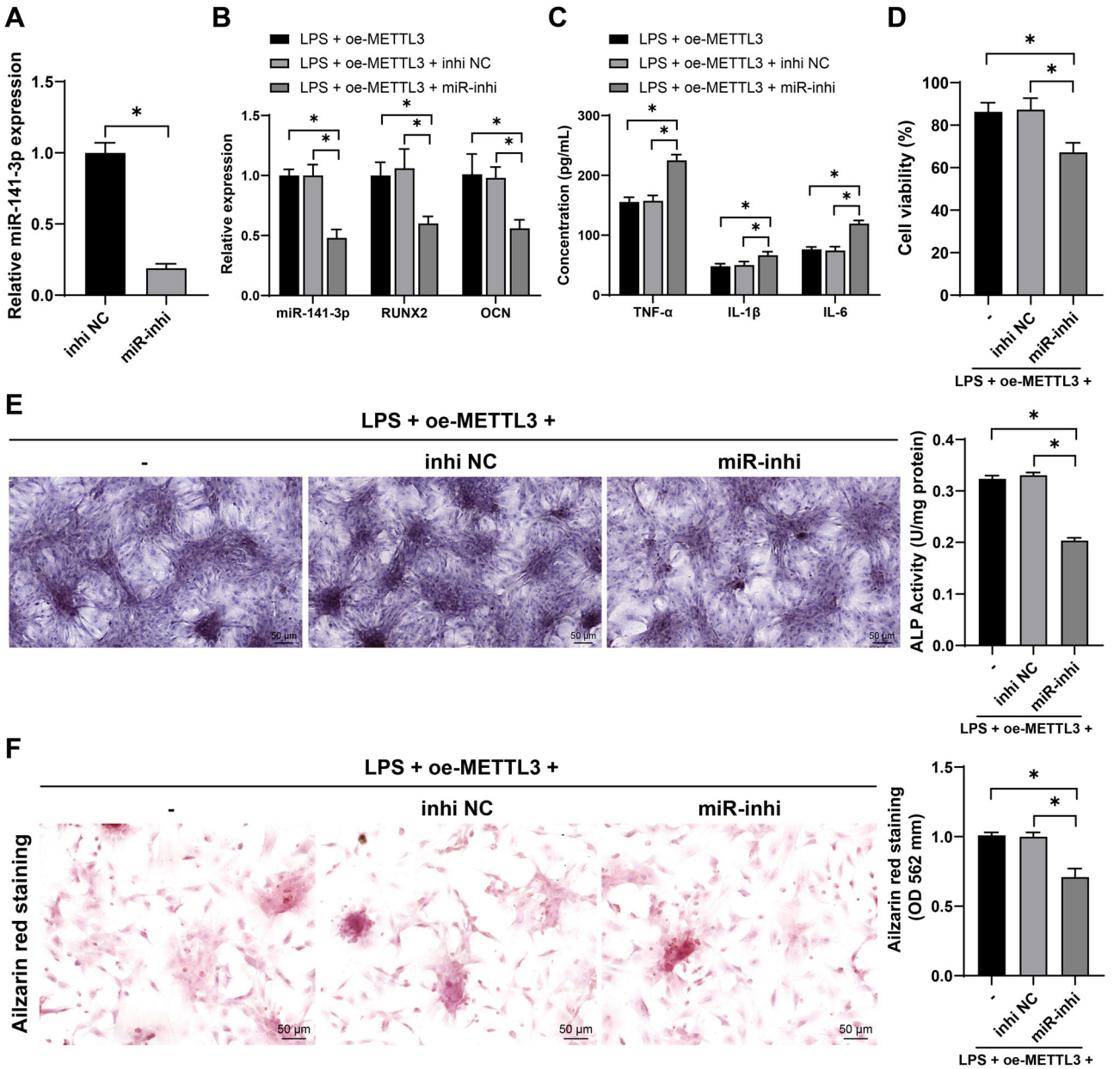


Fig. 3 Inhibition of miR-141-3p partially reverses the promotive effect of METTL3 overexpression on osteogenic differentiation of hPDLSCs under inflammatory microenvironment. hPDLSCs were transfected with miR-141-3p inhibitor (miR-inhi) and inhibitor NC (inhi NC) as a control. Combined experiments were conducted with oe-METTL3, and cells were incubated with 10 μg/mL LPS for 24 h. **A** qRT-PCR was performed to measure the transfection efficiency. **B** qRT-PCR was used to detect the expression of miR-141-3p, RUNX2, and OCN. **C** ELISA was conducted to measure the levels of TNF-α, IL-1β, and IL-6 in hPDLSCs. **D** Cell viability was assessed using the CCK-8 assay. **E** ALP staining assay was conducted and ALP activity was measured. **F** Alizarin Red staining was performed to evaluate osteogenic differentiation of hPDLSCs. These experiments were independently repeated three times, and the data are presented as mean ± standard deviation. Data in panel **A** were analyzed using *t*-test. Data in panels **D**–**F** were analyzed using one-way ANOVA, and data in panels **B** and **C** were analyzed by two-way ANOVA, followed by Tukey's multiple comparisons test. $^{*}p < 0.05$

hPDLSCs are key players in the regeneration of damaged periodontal tissues, making them promising targets for therapeutic intervention in periodontitis [31]. It is well established that both METTL3 expression and m6A modification levels are upregulated during the osteogenic differentiation process of hPDLSCs [9]. The early stages of periodontitis are characterized by significantly elevated levels of pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α [32]. In this study, we observed that LPS-treated hPDLSCs exhibited high levels of TNF-α, IL-1β, and IL-6, along with significantly reduced osteogenic differentiation and decreased expression of METTL3.

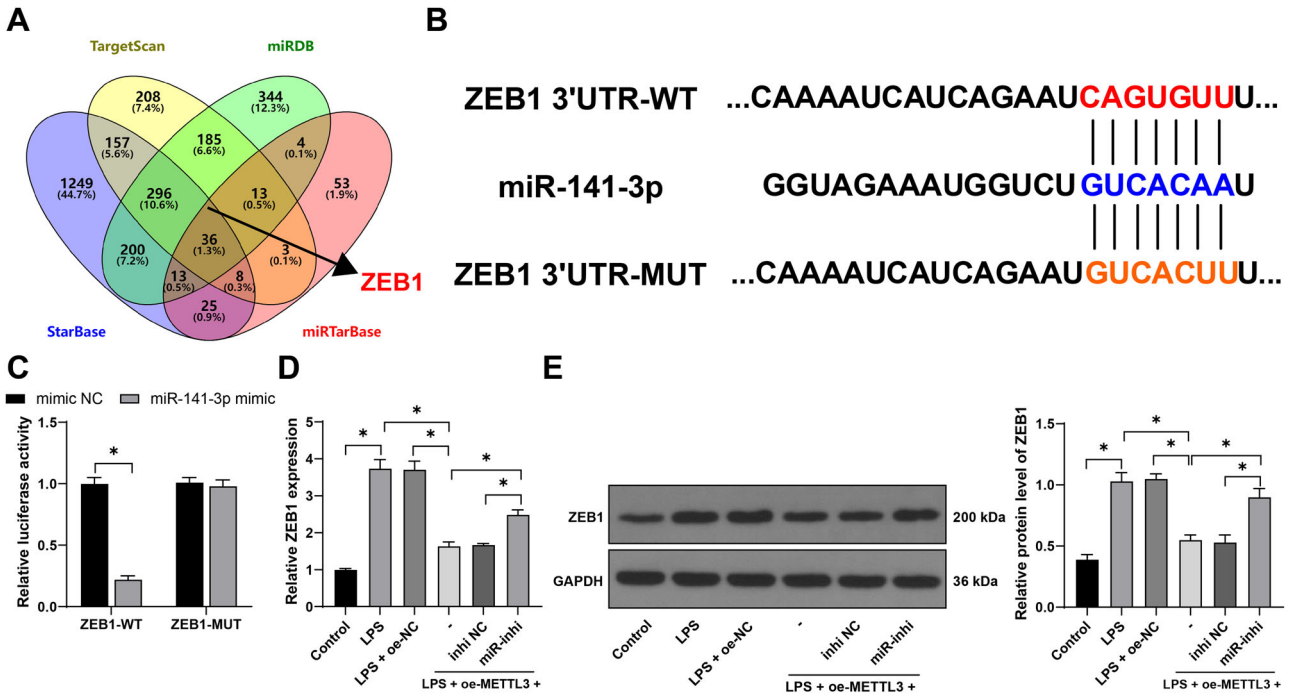


Fig. 4 miR-141-3p targets and inhibits ZEB1 expression. **A** miR-141-3p downstream target genes were predicted using STARbase, TargetScan, miRDB, and miRTarBase databases. **B** TargetScan database predicted the binding of miR-141-3p to the 3'UTR of ZEB1. **C** Dual-luciferase assay was performed to test the binding relationship between miR-141-3p and ZEB1 3'UTR. **D** qRT-PCR was used to measure the expression of ZEB1. **E** Western blot analysis was conducted to assess the expression of ZEB1. These experiments were independently repeated three times, and the data are presented as mean ± standard deviation. Data in panel **C** were analyzed using two-way ANOVA. Data in panels **D** and **E** were analyzed using one-way ANOVA, followed by Tukey's multiple comparisons test. $*p < 0.05$

Overexpression of METTL3 in hPDLSCs resulted in increased expression of osteogenic markers, such as ALP, and enhanced mineralization of the bone matrix in periodontitis-affected tissues [8]. Furthermore, hPDLSCs overexpressing METTL3 demonstrated enrichment of mature osteoblast markers and increased formation of calcium deposition nodules [33]. On the contrary, silencing METTL3 led to a decrease in both the activity and osteogenic differentiation capacity of PDLSCs [34]. Additionally, inhibiting METTL3 expression exacerbated inflammatory cell infiltration and the inflammatory response in the osteoblasts of mice with periodontitis [35]. Consistent with these findings, our study showed that METTL3 overexpression effectively reduced pro-inflammatory cytokine levels, enhanced cell viability, and upregulated the expression of osteogenic genes in hPDLSCs. Collectively, the evidence suggests that METTL3 overexpression not only alleviates inflammatory levels in hPDLSCs but also enhances their osteogenic differentiation capacity, offering a potential therapeutic approach to treat periodontitis.

Our study further elucidates the role of METTL3 in enhancing the processing of pri-miR-141 into miR-141-3p through m6A modification, thereby increasing miR-141-3p expression. A growing body of evidence highlights the critical role of miRNAs in regulating osteogenic differentiation of cells [36]. Specifically, miR-141-3p, derived from PDLSCs, has shown potential in attenuating PDLSC senescence, promoting differentiation, and enhancing proliferation [14]. Moreover, miR-141-3p overexpression has been demonstrated to reduce the release of inflammatory cytokines in various cell types, including astrocytes, intestinal epithelial cells, and synovial fibroblasts [37–39]. However, the specific anti-inflammatory effects of miR-141-3p within the inflammatory microenvironment of hPDLSCs induced by periodontitis have not been fully explored. In this study, we observed that inhibiting miR-141-3p expression in hPDLSCs led to an increase in pro-inflammatory cytokine levels, along with a decrease in ALP activity, RUNX2 and OCN expression, and overall osteogenic differentiation. These findings suggest that miR-141-3p, upregulated via METTL3-mediated m6A methylation, plays a significant role in alleviating the inflammatory environment of periodontitis and promoting osteogenic differentiation.

Subsequent investigations revealed that miR-141-3p directly binds to and suppresses ZEB1 expression. Recent studies have identified ZEB1 as being overexpressed in periodontitis, where it contributes to the upregulation of inflammatory cytokines [17]. Consistent with these findings, our study showed that ZEB1 levels were elevated in LPS-treated hPDLSCs, and overexpressing ZEB1 in these cells led

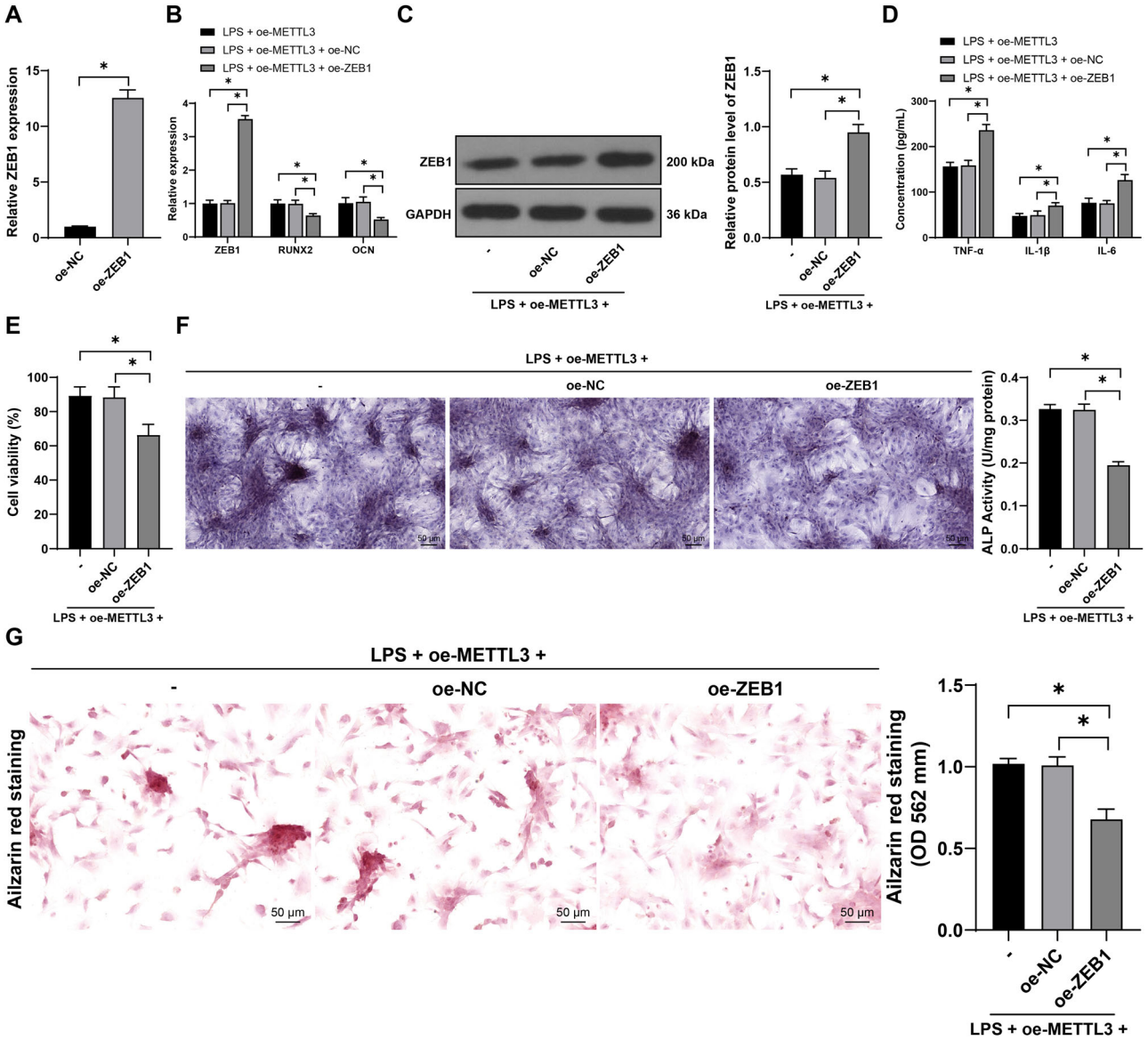


Fig. 5 Overexpression of ZEB1 partially reverses the promotive effect of METTL3 overexpression on osteogenic differentiation of hPDLSCs under inflammatory microenvironment. hPDLSCs were transfected with overexpression plasmid of ZEB1 (oe-ZEB1), with empty vector (oe-NC) as a control. Combined experiments were conducted with oe-METTL3, and cells were incubated with 10 μg/mL LPS for 24 h. **A** qRT-PCR was performed to measure the transfection efficiency. **B** qRT-PCR was used to detect the expression of ZEB1, RUNX2, and OCN. **C** Western blot analysis was conducted to assess the expression of ZEB1. **D** ELISA was conducted to measure the levels of TNF-α, IL-1β, and IL-6 in hPDLSCs. **E** Cell viability was assessed using the CCK-8 assay. **F** ALP activity was measured. **G** Alizarin Red staining was performed to evaluate osteogenic differentiation of hPDLSCs. These experiments were independently repeated three times, and the data are presented as mean ± standard deviation. Data in panel **A** were analyzed using *t*-test. Data in panels **C**, **E**–**G** were analyzed using one-way ANOVA. Data in panels **B** and **D** were analyzed using two-way ANOVA, followed by Tukey's multiple comparisons test. $^{*}p < 0.05$

to an increase in intracellular pro-inflammatory factors and a decrease in osteogenic gene activity. Silencing ZEB1 has been reported to reduce apoptosis, enhance osteogenic differentiation, and promote mineralization in hPDLSCs [20]. Furthermore, inhibiting ZEB1 in bone marrow mesenchymal stem cells has been shown to enhance osteogenic activity and mineralization while preventing bone loss [19]. Additionally, ZEB1 inhibition in inflamed gingiva has the potential to suppress IL-6 and IL-1β levels [40]. Collectively, these findings indicate that ZEB1 is upregulated following LPS induction, which contributes to increased inflammation and impaired osteogenic differentiation in hPDLSCs. Thus, targeting the METTL3/miR-141-3p/ZEB1 axis could represent a novel therapeutic approach for managing periodontitis and enhancing periodontal regeneration.

Despite the insights gained from our study, there are some limitations to acknowledge. Firstly, our research primarily focused on the role of METTL3 in promoting miR-

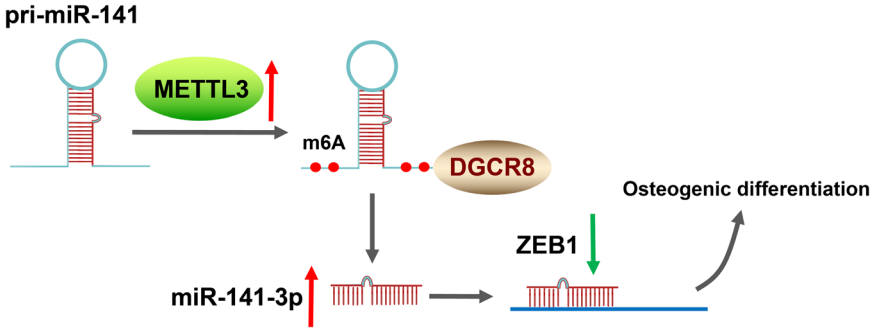


Fig. 6 Overexpression of METTL3 increases miR-141-3p expression through promoting m6A modification and enhancing the binding of DGCR8 to m6A, which in turn targets and inhibits ZEB1 expression, thus promoting the osteogenic differentiation of hPDLSCs

141-3p expression via m6A modification. The potential involvement of other miRNAs in this regulatory process remains unexplored and warrants further investigation. Secondly, while we have begun to unravel the role of METTL3 and miR-141-3p in osteogenic differentiation and inflammation, the regulatory broader mechanisms involving downstream target genes and signaling pathways of ZEB1 in the context of periodontitis require deeper exploration. Thirdly, our current findings are based solely on cellular-level studies. To validate and expand upon our conclusions, future research should investigate the METTL3-mediated regulation of additional miRNAs via m6A modification in periodontitis, delve into the downstream signaling pathways of ZEB1, and conduct in vivo experiments. These steps will be crucial in refining our understanding and providing more comprehensive insights into potential therapeutic strategies for periodontitis.

Conclusions

In summary, our study demonstrates that LPS-treated hPDLSCs exhibit decreased METTL3 expression, which contributes to increased inflammation and impaired osteogenic differentiation. By overexpressing METTL3, we were able to enhance miR-141-3p expression via m6A modification and inhibit ZEB1 expression. This, in turn, restored osteogenic differentiation, reduced inflammation, and mitigated periodontitis in LPS-treated hPDLSCs. Our findings provide a foundation for the potential application of hPDLSCs in periodontal tissue engineering and offer a theoretical basis for new clinical treatments for periodontitis.

Author contributions Weijia Li: Conceptualization, Data curation, Validation, Investigation, Visualization, Methodology, Writing – original draft, Writing – review & editing; Adili Alimujiang: Conceptualization, Formal Analysis, Supervision, Investigation, Methodology, Writing – review & editing.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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