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Role of METTL3-mediated m6A modification in osteogenic differentiation of periodontal ligament stem cells extracted from adult periodontal ligaments *ex-vivo*

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ABSTRACT

Periodontal ligament stem cells (PDLSCs) are identified as candidate cells for the regeneration of periodontal and alveolar bone tissues. This research was to analyze the effect of methyltransferase-like 3 (METTL3)-mediated m6A modification on the osteogenic differentiation of PDLSCs extracted from adult periodontal ligaments (PDLs) ex-vivo. From June 2022 to October 2022, 27 patients undergoing orthodontic treatment in our hospital were selected as the research population, with 31 teeth extracted in total. PDLSCs were isolated from PDLs by tissue block culture, and the results were analyzed. Then PDLSCs were induced to differentiate into osteoblasts, and changes in METTL3 and m6A levels during differentiation were observed. Additionally, abnormal METTL3 expression vectors were constructed and transfected into PDLSCs to observe the influence of METTL3 on the biological behavior and osteogenic differentiation of PDLSCs. PDLSCs isolated from ex-vivo PDLs were predominantly spindle-shaped, with high CD73, CD90 and CD105 levels and low CD11b, CD34 and CD45 levels, showing the characteristics of stem cells. Spearman correlation coefficients identified a positive connection between Runx2, Sp7, Alp, Bglap, METTL3 and m6A levels and osteogenic differentiation incubation time ($P<0.05$). As METTL3 expression was increased, the proliferation capacity and osteogenic differentiation ability of PDLSCs were enhanced ($P<0.05$), and the content of m6A was increased ($P<0.05$). However, the activity and osteogenic differentiation ability of PDLSCs was decreased after silencing METTL3 ($P<0.05$). In conclusion, METTL3-mediated m6A modification promoted the osteogenic differentiation of PDLSCs extracted from adult PDLs ex vivo. This study offered a novel understanding of the mechanisms underlying osteogenic differentiation, and implied a possible method for accelerating bone formation.

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Introduction

Stem cells (SCs), a type of cell with an infinite or immortal capacity to self-renew, have a multidirectional differentiation potential and can produce at least one type of highly differentiated progeny cells (1). Embryonic SCs in blastocysts, for example, are considered high-grade SCs that are able to differentiate into any kind of tissues and organs in the body (2). However, SCs in various tissues and organs of mature individuals have a low differentiation grade and are also known as adult stem cells (ASCs) (3). Among them, bone marrow mesenchymal stem cells (BMSCs) are the representatives of ASCs, which can differentiate into various tissue cells such as osteoblasts, chondrocytes, adipocytes, and vascular endothelial cells under characteristic conditions (4). It is precise because of the multiple differentiation characteristics of SCs that SC transplantation therapy is also hailed as a breakthrough in the treatment of a wide spectrum of diseases in the future (5). At present, the related research on SCs focuses on BMSCs isolated from bone marrow, which is one of the most important hematopoietic and immune organs of the human body. It is not only difficult to extract but also harmful to the donors after extraction (6). Therefore, it is of great significance for the follow-up clinical research to find a kind of SCs that is convenient for separation and safe for the donor. Periodontal ligament stem cells (PDLSCs) are a kind of SCs isolated from periodontal tissues. Compared with bone marrow samples, PDLSCs can be extracted from periodontal specimens obtained from orthodontic treatment, children's tooth replacement, wisdom teeth extraction and other ways, with higher diversity of access and convenience (7). But at present, PDLSCs have not been extensively studied.

In addition, N6-methyladenosine (m6A), which is dynamic methylation at the N6 site of adenosine, is known to be the most common post-transcriptional internal mRNA modification. In mammals, m6A is regulated by its methy-

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lating enzyme methyltransferase-like 3 (METTL3) to exert various biological effects (8). At the same time, it is reversed by demethylases, such as FTO and ALKBH5. In addition to 'writers' and 'erasers', this modification is recognized and bound by m6A 'readers', mainly YTHDF1-3 and YTHDC1, 2, thus participating in a variety of biological processes, including tumorigenesis, and osteogenic differentiation (9, 10). Accumulating evidence has pointed the importance of m6A methylation in cell function, and abnormal changes in methyltransferase and demethylase can lead to dysfunction or disease. A recent study has shown that the inhibited m6A modification in BMSCs interrupts parathyroid hormone-stimulated osteogenesis, leading to severe bone loss (11). Besides, a number of existing studies have shown that METTL3-mediated m6A is involved in SC differentiation (12, 13), but its role in PDLSCs remains to be characterized.

Consequently, PDLSCs were extracted from human periodontal ligaments (PDLs) ex-vivo in this study to analyze the impact of METTL3-mediated m6A on PDLSC differentiation, providing a new reference basis for future clinical SC research.

Materials and Methods

Study population

The study population consisted of 27 patients who received orthodontic treatment in our hospital between June 2022 and October 2022. All patients needed tooth extraction, with 31 teeth extracted in total. With the consent of the patients, PDLSCs were extracted from the extracted teeth for analysis. The study received ethical approval was obtained from the hospital ethics committee, as well as written informed consent from all research participants.

Eligibility criteria

Patients (age range: 18-50) who underwent tooth extraction due to orthodontic treatment and agreed to cooperate with this research were enrolled. In contrast, those with defects of the extracted teeth or chronic periodontal diseases (caries, periodontal disease, etc.) were excluded.

Cell culture

After rinsing the extracted teeth using phosphate buffer saline (PBS), the PDL of 1/3 of the root was scraped and cut into 1 mm × 1 mm tissue blocks, which were spread evenly on the bottom of the cell culture flask. A tissue block culture was used until the cells swam out from the periphery of the tissue block, and the primary cells were digested with 0.25% trypsin after confluence. After cell counting using a cell counting chamber, the cell concentration was adjusted to 1-2 cells/well for incubation in the wells of a 96-well plate. Cells were then routinely cultivated for 7-14 days until cell clones appeared, followed by cell digestion and transfer to a 24-well plate for expanded culture before routine passage or cryopreservation. The remaining tissue blocks were digested with 0.2% type I collagenase and 0.2% neutral protease for 1 h, and then filtered (pore size 100 μm) to obtain a single cell suspension, which was inoculated into a 96-well plate at the concentration of 1-2 cells/well and routinely cultured until the presence of cell clones. The expanded culture was then carried out.

PDLSCs identification

After being digested with 0.02% ethylene diamine tetraacetic acid (EDTA), the 3rd generation PDLSCs were removed from the culture medium and immersed in trypsin and EDTA, each at a concentration of 0.2% and volume of 2.5 mL, followed by microscopy observations of cell morphology. A digestion termination solution was added when most cells become round, followed by centrifugation to remove the supernatant. Flow cytometry was then performed to quantify the expression of the SC marker antibodies CD11b, CD34, CD45, CD90, CD73 and CD105, which all obtained from Cell Signal Technology (USA).

Osteogenic differentiation

When PDLSCs became 80% confluent, they were digested with 0.04% EDTA, inoculated in a 6-well plate at the adjusted density of 2×10^4 cells/mL, and added with 2 mL PDLSC complete osteogenic differentiation medium with the medium changed once every 3 days. The PDLSCS were stained with alizarin red S (ARS, Sigma-Aldrich) 2 weeks later.

Quantitative real time polymerase chain reaction (qRT-PCR)

As suggested in the kit manual, purity verification was performed for total RNA of PDLSCs extracted from Trizol (Invitrogen, USA) before reverse transcription. Reverse transcription was performed using HiScript II (Vazyme, China) at 50℃ for 30 min, and inactivated at 94℃ for 2 min. Subsequently, qPCR was performed with SYBR Green reagent (Bio-Rad Laboratories, Inc.) on 20 μg cDNA under the following conditions: 94℃/45 s, 56℃/45 s, and 72℃/60 s, for 35 cycles, extension at 72℃ for 7min. Sequences of primers can be found in Tab 1, and the $2^{-\Delta\Delta CT}$ method was employed for the calculation of

Table 1. Primer names and sequences.

Name	F (5'-3')	R (5'-3')
Runx2	GGTACTTCGTCAGCATCCTATCAG	GCTTCCGTCAGCGTCAACAC
Sp7	ATGGCGTCCTCTCTGCTTG	TGAAAGGTCAGCGTATGGCTT
Alp	AACCCAGACACAAGCATTCC	GCCTTTGAGGTTTTTGGTCA
Bglap	TTGGTGCACACCTAGCAGAC	ACCTTATTGCCCTCCTGCTT
GAPDH (i)	ACTGAGGACCAGGTTGTC	TGCTGTAGCCGTATTCATTG
METTL3	TTGTCTCCAACCTTCCGTAGT	CCAGATCAGAGAGGTGGTGTAG
GAPDH (ii)	AGCCTCAAGATCATCAGC	GAGTCCTTCCACGATACC

Note: GAPDH (i) is an internal reference gene for Runx2, Sp7, Alp and Bglap, and GAPDH (ii) is an internal reference gene for METTL3.

METTL3, runt-related transcription factor 2 (Runx2), Sp7 transcription factor (Sp7), alkaline phosphatase (Alp) and bone gamma-carboxyglutamate protein (Bglap) expression levels.

Western blotting

PDLSCs were lysed on ice and centrifuged to obtain supernatant. Next, it was added with 5×sodium dodecyl sulfate (SDS) loading buffer, electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore), sealed with 5% skim milk for 2 h, and washed with METTL3 (Abcam, ab195352, 1:1000) and GAPDH antibodies (Abcam, ab9485, 1:2500) for overnight incubation at 4°C. The next day, anti-II (Abcam, ab6721, 1:2000) was added to the rinsed membrane, and electrochemiluminescence (ECL, Bio-Rad Laboratories, Inc.) was developed after 2 h of incubation. The target bands' gray values were analyzed by Image J software.

Colorimetric determination of m6A content

200 g of the Trizol-extracted total RNA from PDLSCs was inoculated into microwells and incubated at 37°C for 90 min. The PDLSCs were placed at room temperature for 30 min after the addition of a specific antibody against m6A, followed by washing and the addition of 100 μL chromogenic agent for 5 min of light-tight incubation. Finally, a microplate reader was utilized to measure the optical density (OD) at 450 nm wavelength. m6A content (%) = [(sample OD- negative control OD)/ loading amount of the sample to be tested]/[(positive control OD- negative control OD)/ positive control loading amount]×100%.

Cell transfection

Guangzhou Yunzhou Biotechnology Co., Ltd. was entrusted to design and construct METTL3 over-expression vector (ov-METTL3), over-expressed control vector (ov-control), METTL3 silencing vector (si-METTL3) and silenced control vector (si-control). This was followed by the transfection of the above abnormal METTL3 expression vectors into PDLSCs as per the instructions of the LipofectamiTM2000 kit, with the transfection success rate verified by qRT-PCR detection of METTL3 expression.

MTT assay

200 μL cells were inoculated into the wells of a 96-well plate after adjusting the cell density to 1×10^5 cells/mL, and 20 μL MTT solution (Abcam) was added at 0, 24, 48 and 72 h, respectively. 150 μL dimethyl sulfoxide (DMSO) was placed to stop the reaction after 4 hours of incubation. Finally, OD values were detected at 490 nm wavelength with a microplate reader and cell growth curves were drawn.

Statistical processing

The results obtained after three repeated measurements for each experiment were denoted by ($\chi\pm s$), and the statistical method for inter-group comparisons was the independent samples t-test. To identify statistical significance among multiple groups, variance analysis and least significant difference (LSD) post-hoc tests were used. Correlation analysis was performed using Spearman correlation coefficients. Statistical significance was indicated by $P<0.05$ in this paper.

Results

PDLSCs isolation results

The results of the flow cytometry analysis showed thatthe PE-labeled positive surface antigens CD73, CD90 and CD105 showed high expression levels in PDLSCs, while the FITC-labeled negative surface antigens CD11b, CD34 and CD45 showed low expression in PDLSCs (Fig 1).

Relationship between METTL3, m6A and osteogenic differentiation of PDLSCs

After 14 days of cultivation for osteogenic differentiation, there was obvious red calcium salt deposition in PDLSCs, indicating the stable osteogenic capacity of PDLSCs (Fig 2A). Then, according to Spearman correlation coefficient analysis, the osteogenic differentiation marker proteins Runx2, Sp7, Alp and Bglap levels in PDLSCs, as well as METTL3 expression and m6A content in total RNA were positively correlated with the osteogenic differentiation cultivation time ($P<0.05$) (Fig 2B-2G).

Influence of METTL3 on PDLSCs

After transfection, METTL3 expression was found to be higher in an ov-METTL3 group than in the ov-control group, while that in the si-METTL3 group was lower as compared to the si-control group ($P<0.05$), confirming

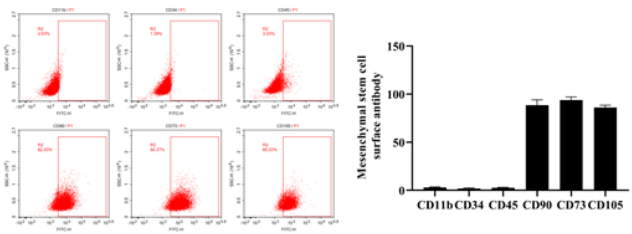

Figure 1. Stem cell surface antigen test results.

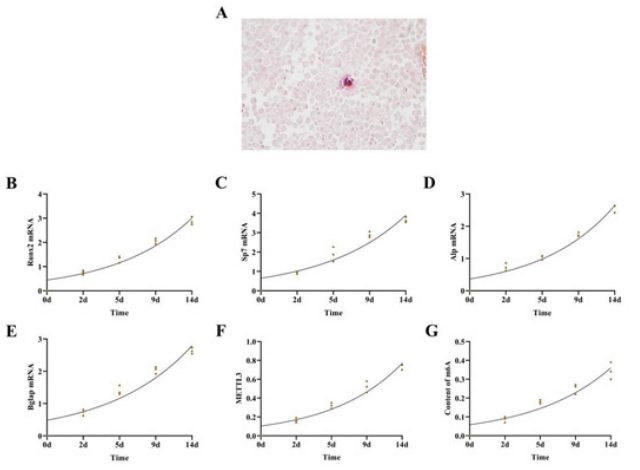

Figure 2. Relationship between METTL3, m6A and osteogenic differentiation of PDLSCs. (A) Results of alizarin red staining of PDLSCs (40×). (B) Relationship between Runx2 and osteogenic differentiation of PDLSCs. (C) Relationship between Sp7 and osteogenic differentiation of PDLSCs. (D) Relationship between Alp and osteogenic differentiation of PDLSCs. (E) Relationship between Bglap and osteogenic differentiation of PDLSCs. (F) Relationship between METTL3 and osteogenic differentiation of PDLSCs. (G) Relationship between m6A and osteogenic differentiation of PDLSCs. *P<0.05.

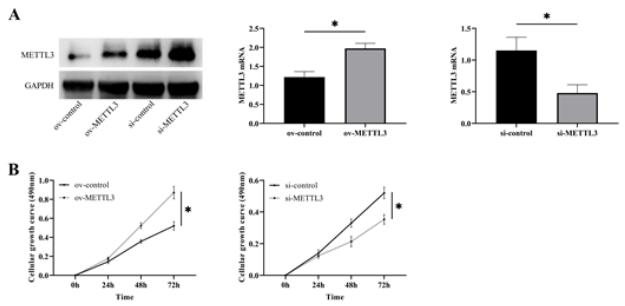

Figure 3. Influence of METTL3 on PDLSCs. (A) METTL3 expression was detected by qRT-PCR to evaluate the transfection success rate. (B) Effects of METTL3 on proliferation capacity of PDLSCs. *P<0.05.

successful transfection (Fig 3A). MTT assay showed that the ov-METTL3 group had statistically higher cell proliferation capacity than the ov-control group, while the cell growth was lower in the si-METTL3 group versus the si-control group (P<0.05) (Fig 3B).

Influence of METTL3 on osteogenic differentiation of PDLSCs

Then, the results of ARS staining showed a significantly elevated red calcium salt deposition in the ov-METTL3 group compared to the ov-control group, and statistically lower deposition in the si-METTL3 group compared to the si-control group (Fig 4A). Furthermore, the ov-METTL3 group had higher Runx2, Sp7, Alp and Bglap levels than the ov-control group, while the si-METTL3 group showed lower levels of these genes than the si-control group (P<0.05) (Fig 4B-4E).

Influence of METTL3 on m6A modification level in PDLSCs

Finally, as indicated by the colorimetric assay, the m6A content of total RNA in the ov-METTL3 group was (0.44±0.02), significantly higher than that in the ov-control group (P<0.05) (Fig 5A), while the content of m6A in the si-METTL3 group was (0.23±0.03), which was notably reduced as compared to the si-control group (P<0.05) (Fig 5B).

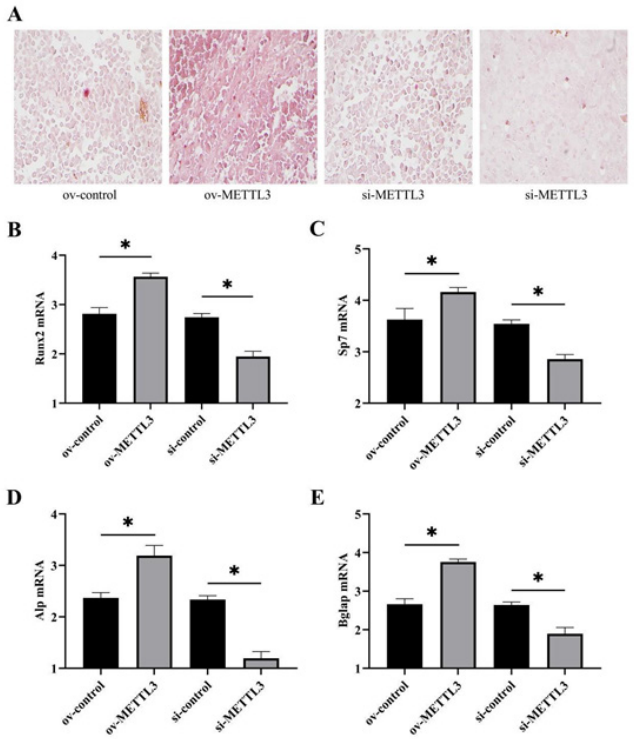

Figure 4. Influence of TTL 3 on osteogenic differentiation of PDLSCs. (A) Results of alizarin red staining (40×). (B) Effects of METTL3 on Runx2 mRNA expression in PDLSCs. (C) Effects of METTL3 on Sp7 mRNA expression in PDLSCs. (D) Effects of METTL3 on Alp mRNA expression in PDLSCs. (E) Effects of METTL3 on Bglap mRNA expression in PDLSCs. *P<0.05.

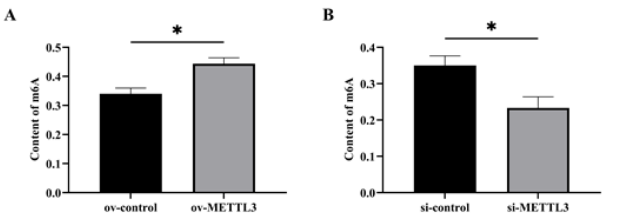

Figure 5. Influence of METTL3 on m6A modification level in PDLSCs. (A) Effect of increasing METTL3 expression on m6A content. (B) Effect of silencing METTL3 expression on m6A content. *P<0.05.

Discussion

SC research has been the focus of clinical research in recent years. At present, ASCs have been found in various organisms and mature tissues and organs (14). However, in the field of stomatology, although researchers have found that PDLSCs have the potential to differentiate into fibroblasts and cementoblasts that form their own fibrous connective tissue, further studies and analyses have not been carried out (15). In 2004, Seo BM et al. put forward the research concept of PDLSCs and isolated PDLSCs from human-impacted teeth for the first time (16). However, follow-up-related research is still rare. Therefore, by extracting PDLSCs from human PDLs and analyzing the influence of METTL3-mediated m6A modification on their osteogenic differentiation ability, this study had important reference significance for PDLSCs and SC research in stomatology in the future.

In this study, we isolated PDLSCs from ex vivo PDLs, and ARS staining showed that PDLSCs had obvious red calcium salt deposition, suggesting that PDLSCs also had excellent osteogenic differentiation potential. This was also consistent with the results of previous studies (17), According to the correlation analysis, which supports the accuracy of our findings. According to the correlation analysis, Runx2, Sp7, Alp, and Bglap in PDLSCs were positively correlated with the cultivation time of osteogenic differentiation. Runx2, Sp7, Alp and Bglap are marker proteins of SC osteogenic differentiation (18, 19), so this result could be expected. Second, at present, the important role of m6A modification in different physiological and pathological processes such as self-renewal and differentiation of stem cells, immune response, as well as tumorigenesis has become a research focus in epitaxial transcriptomics (20). METTL3, as the most famous methyltransferase of m6A, is known to be involved in various cell life activities (21, 22). Although no studies have confirmed the effect of METTL3 on PDLSCs, its role in BMSCs, chondrocytes and osteoblasts has been confirmed (23-25), with impor-

tant potential significance for the osteogenic differentiation of mesenchymal SCs (26, 27). Therefore, we also detected METTL3 and m6A levels in PDLSCs. The results showed that METTL3 and m6A levels were also strongly correlated with the osteogenic differentiation of PDLSCs.

Then, in order to further confirm the effects of METTL3 and m6A on PDLSCs, we constructed abnormal expression vectors of METTL3 and transfected them into PDLSCs to detect changes in their biological behaviors. We found that increased expression of METTL3 could enhance the differentiation potential of PDLSCs. In previous studies, we also found that the loss of METTL3 reduced the osteogenic differentiation ability of BMSCs (28), which is consistent with our experimental results. Finally, the detection of m6A content in the total RNA of PDLSCs transfected with METTL3 also showed that increasing METTL3 expression could enhance the osteogenic differentiation capacity of PDLSCs modified by m6A, which was consistent with the results of previous studies on m6A and mesenchymal SCs (9, 29, 30).

However, due to limited experimental conditions, it is not clear whether METTL3 affects the osteogenic differentiation of PDLSCs through m6A methylation, and further studies and validation of METTL3 downstream genes are required. Meanwhile, we need to carry out animal experiments as soon as possible to confirm the actual osteogenesis of PDLSCs isolated from PDLs and the effect of METTL3.

METTL3-mediated m6A modification can promote the osteogenic differentiation of PDLSCs extracted from adult PDLs ex vivo, laying the foundation for future applications of mesenchymal SCs in periodontal tissue engineering and providing a new idea for cartilage tissue engineering.

Declaration

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Zhenjiang First people's Hospital (Approve number:2022-128).

Data availability

The data in this article can be obtained from the corresponding author under reasonable circumstances.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Authors contributions

Y.J, designed the study and provided theoretical guidance, C.Y, and HY X, wrote and revised the paper, S.L, and P.S, collected data, YH.M, and M.H, analyzed data and Supervised the research, C.Y, and HY.X, have the same contribution in this work.All authors read and approved the final submitted manuscript.

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