# METTL3-Mediated lncSNHG7 m$^6$A Modification in the Osteogenic Differentiation of Human Dental Stem Cells

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**Research Article**

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# Abstract

**Background:** Human dental pulp stem cells (hDPSCs) play an important role in endodontic regeneration, with self-renewal and pluripotency capacity. N6-methyladenosine ($m^6A$) is the most common RNA modification, and noncoding RNAs have also been demonstrated to have regulatory roles in the expression of $m^6A$ regulatory proteins. This study aimed to explore the regulatory mechanism of methyltransferase 3 (METTL3)-mediated long noncoding RNA (lncRNA) $m^6A$ modification in the osteogenic differentiation of hDPSCs.

**Methods:** Single base site PCR (MazF) was used to detect the $m^6A$ modification site of lncSNHG7 before and after mineralization of hDPSCs, combined with the prediction information from the StarBase database and real-time quantitative polymerase chain reaction (qRT–PCR) to screen the target $m^6A$ modification protein, and bioinformatics analysis was used to analyse the related pathways rich in lncSNHG7. After knockdown of lncSNHG7 and METTL3, osteogenic ability was detected by alkaline phosphatase (ALP) staining, Alizarin Red S (ARS) staining, qRT–PCR and Western blotting. After METTL3 knockdown, the $m^6A$ modification level and its expression of lncSNHG7 were detected by MazF, and their binding was confirmed by RNA binding protein immunoprecipitation (RIP) analysis. Finally, Western blot analysis was used to detect the effects of lncSNHG7 and METTL3 on the Wnt/β-catenin pathway.

**Results:** MazF experiments revealed that lncSNHG7 had a $m^6A$ modification before and after mineralization of hDPSCs, and the occurrence site was 2081. The $m^6A$-modified protein METTL3 was most significantly upregulated after mineralization of hDPSCs. Knockdown of lncSNHG7 and METTL3 inhibited the osteogenic differentiation of hDPSCs. The $m^6A$ modification and expression of lncSNHG7 were both regulated by METTL3. Subsequently, lncSNHG7 and METTL3 were found to regulate the key proteins in the Wnt/β-catenin signaling pathway, β-catenin and GSK-3β.

**Conclusion:** These results revealed that METTL3 can activate the Wnt/β-catenin signaling pathway by regulating the $m^6A$ modification and expression of lncSNHG7 in hDPSCs to enhance the osteogenic differentiation of hDPSCs. Our study provides new insight into stem cell-based tissue engineering.


# Background

Bone tissue engineering is based on the concepts of stem cells, growth factors and scaffold materials [1, 2]. In the field of stomatology, bone tissue is the key supporting structure in craniofacial physiology. Many situations, such as trauma, tumour and necrosis, will lead to bone defects, which may eventually lead to extensive dysfunction [3]. Therefore, how to repair craniofacial bone defects has attracted extensive attention. Among them, stem cells have become the most commonly used cells in bone tissue engineering. Human dental pulp stem cells (hDPSCs) were initially found by Gronthos et al. [4], which were more effective in proliferation and osteogenesis, had lower immunogenicity, and had higher proliferation rates, cloning potential, and cell numbers than mesenchymal stem cells, and showed great potential in regenerative medicine for the treatment of various human diseases [5, 6]. Unfortunately, the

exact mechanism of osteogenic differentiation is still unclear, which may be necessary to achieve the best bone enhancement clinical results. Although hDPSCs are a potential candidate for bone regeneration, to better utilize the role of hDPSCs in bone regenerative medicine, the osteogenic differentiation mechanism of hDPSCs needs to be further studied.

N6 methyladenosine ($m^6A$) is the most common modification method in mRNA. It has been implicated in all aspects of posttranscriptional RNA metabolism [7]. The widespread presence of $m^6A$ in the human transcriptome has aroused great interest from researchers. Exploration of methylation patterns in cells can not only reveal the specific distribution of the $m^6A$ modification in many transcripts but also the differences in $m^6A$ status under different physiological conditions [8]. The biological function of $m^6A$ modification mainly depends on methyltransferases, demethylases and methylated reading proteins. Among them, methyltransferases such as methyltransferase 3 (METTL3) have been studied in the most detail, and their main role is to catalyse the $m^6A$ modification of adenosine on RNA [9]. It has been demonstrated that $m^6A$ modification plays an important role in cancer, metabolism, embryonic stem cell processes and tissue development [10–12]. Among them, $m^6A$ modification has also been instrumental in the osteogenic differentiation of stem cells. Studies have shown that the $m^6A$ modification mediated by METTL3 can promote the osteogenic differentiation of bone marrow mesenchymal stem cells through different pathways and help to inhibit the progression of osteoporosis [13]. In addition, the $m^6A$ modification of METTL3 can also promote osteogenic differentiation in human adipose-derived stem cells induced by NEL-like 1 protein [14]. In a study of the $m^6A$ modification of METTL3 in hDPSCs, it was shown that the $m^6A$ modification of METTL3 has a regulatory role in the cell cycle [15] and suggested that METTL3 might affect the LPS-induced inflammatory response by regulating the alternative splicing of MyD88 in hDPSCs [16]. However, research on the osteogenic differentiation of hDPSCs is still lacking. Therefore, the effect and mechanism of m 6A modification on the osteogenic differentiation of hDPSCs are still unclear, and further exploration is needed.

Many factors are involved in regulating the osteogenic differentiation of mesenchymal stem cells. Among them, long noncoding RNAs (lncRNAs), as a large class of regulatory molecules, have attracted much attention in recent years. It is a kind of noncoding RNA (ncRNA) with a length of more than 200 nucleotides and cannot be translated into protein. Studies have shown that lncRNAs are involved in a variety of biological processes and disease pathogenesis and play a significant role in the osteogenic differentiation of stem cells [17, 18]. An increasing number of studies have shown that lncRNAs can affect the osteogenic differentiation of hDPSCs by regulating the expression of downstream target genes in combination with microRNAs (miRNAs) [19–21]. However, current research on whether lncRNAs can play a regulatory role in this process in other ways is still in the preliminary stage, so the functions and mechanisms of a large number of lncRNAs are still unclear. To date, there has been no research on the regulation of the lncRNA $m^6A$ modification in the process of osteogenic differentiation of hDPSCs.

In this study, the $m^6A$ modification of lncRNAs was combined with the osteogenic differentiation pathway of hDPSCs for the first time, which confirmed the promoting effect of METTL3 in the osteogenic

differentiation of hDPSCs and the regulatory effect of METTL3 on the $m^6A$ modification of lncRNA SNHG7 and its relationship with the Wnt/β-catenin signaling pathway. The aim was to provide a new idea and method for bone tissue engineering.

# Methods

### hDPSCs Culture and characterization

hDPSCs were isolated from the teeth acquired patients who have undergone tooth extraction at the Nanfang Hospital of Southern Medical University, Guangzhou, Guangdong, China. All experimental protocols were approved by the Ethical Committee of Southern Medical University and hDPSCs were cultured as described previously [22] The hDPSC were cultured in Dulbecco's modified Eagle's medium (DMEM) added added with 10% fetal bovine serum (FBS; GIBCO, Life Technologies, Australia), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, Mo, USA) at 37°C , and the air contains 5% CO2. The medium was changed every 3 days, and hDPSCs at passages 3–5 were used for the following experiments [23,24]. We divided the samples into two groups: the undifferentiated hDPSCs group, in which cells were cultured in 10% FBS in DMEM with no supplements. And the differentiated hDPSCs group, in which cells were cultured in 50 mg/ml ascorbic acid, 100 nmol/l dexamethasone, and 10 mmol/l β-glycerophosphate (Sigma, St Louis, Mo, USA) in DMEM for 14 days. Flow cytometry was performed to identify hDPSC phenotypes by screening the surface markers against CD29, CD44, CD90, CD45, and CD34.

### Single base site PCR (MazF)

Verify the conserved motif region ($m^6A$ ACA site) of core ACA sequence on lncSNHG7. The $m^6A$ modification level in hDPSCs undifferentiated group, differentiated group and after METTL3 knockdown were detected. The RNA endonuclease MazF recognizes RNA single strand and cleaves at the 5 'end of the unmethylated ACA site, but cannot cleave the methylated $m^6A$ ACA site. The extracted total RNA samples were divided into two parts, one without MazF treatment and the other after MazF treatment. The $m^6A$ methylation level of specific ACA sites in the samples was then detected by real-time quantitative polymerase chain reaction (qRT-PCR) [25,26].

### Alkaline Phosphatase (ALP) and Alizarin Red Staining (ARS)

Samples were first washed three times with phosphate buffered saline and were fixed in 4% paraformaldehyde for 15 mins. After washing, the hDPSCs were stained with the NBT/BCIP Staining Kit and Alizarin red. The results of each group were photographed under an inverted microscope.

### Real-time polymerase chain reaction

The undifferentiated hDPSCs group and differentiated hDPSCs group were obtained by culturing as described above and total RNAs were isolated from these two groups. 1 μg of RNA per sample was

reverse transcribed into cDNA using a cDNA Reverse Transcription Kit (Takara, Tokyo, Japan). qRT-PCR was performed in a 20 μL of the reaction system. Finally, the relative expression of RNAs was calculated using the $2^{-\Delta\Delta Ct}$ method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene. Each sample was taken in triplicate, and the results were obtained from independent experiments. The primer sequences used in real-time PCR were summerized in Table 1.

### Western blot analysis

The protein of hDPSCs were lysed by radioimmunoprecipitation assay buffer (Pierce, Rockford, IL, USA). The lysate containing loading buffer (2%SDS and 1% 2-mercaptoethanol was prepared at 99°C for 5 min. The samples were separated on 10% SDS–polyacrylamide gels, and transferred to 0.22 μm polyvinylidene fluoride membranes by a semidry transfer apparatus. Afterward, the membranes were blocked with 5% skim milk powder at room temperature for 1 hour. The transferred proteins were reacted with primary antibody overnight at 4 °C and then labeled with secondary antibody for 1 h at room temperature. Primary antibodies in this study include METTL3, GAPDH, phosphorylation-GSK-3β and GSK-3β and β-catenin. Immunoreactive proteins were detected by using the ECL Kit (Beyotime Biotech, Shanghai, China), and the band densities were quantified using ImageJ software (v1.8.0).

### Gene knockdown

The undifferentiated hDPSCs group and differentiated hDPSCs group were cultured as described above and spread into six-well plates at a density of $2\times10^5$ cells per well. Transfection was performed at a cell confluence of 60%–80% according to the instruction manual. For METTL3 and lncSNHG7 knockdown, the small interfering RNAs (siRNAs) for METTL3, lncSNHG7 and control were synthesized by Genechem (Shanghai, China). The procedure of transfection was according to the manufacturer's instructions. Then the cells were collected after 48 h of incubation for subsequent experiments.

### Bioinformatic Analysis

Differentially expressed lncRNAs during the osteogenic differentiation of hDPSCs were analyzed using GEO2R in GSE138179[27] and SRP214747[28]. $m^6$Avar, WHISTLE software was used to predict the ACA sites where $m^6$A modification may occur in lncSNHG7The Starbse database was used to predict $m^6$A modifying related enzymes that may bind to lncSNHG7. Both Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) were carried out. GO (http://geneontology.org/) enrichment analysis was used to define gene attributes in organisms from three fields: biological processes (BP), cellular components (CC), and molecular functions (MF) ($P < 0.05$ was used). David software was used to test the statistical enrichment of the target gene candidates in the KEGG pathway database (KEGG; https://david.ncifcrf.gov/).

### RNA-binding protein immunoprecipitation (RIP) assay

Based on the manufacturer's instruction, the RIP assay was taken out with RNA-Binding Protein Immunoprecipitation Kit. Cells were dissolved with RIP lysis buffer. Cell lysates (100 ul) were treated with RIP buffer andcultured with Proteinase K and magnetic beads conjugated with anti-METTL3 antibody or control (anti-IgG)(Millipore). The RNA bound to the beads was purified and then reverse-transcribed into cDNA for qRT-PCR.

### Statistical analysis

All experiments were carried out three times. The data were processed by SPSS 25.0 software (SPSS, Chicago, IL). Analysis of variance and Student's t-test were used to evaluate statistical differences in different groups. All results were summarized and shown as means±standard deviation. Results were treated with statistical significance at $P<0.05$. One-way analysis of variance (ANOVA) followed by Dunnett's post hoc test was used for multiple group comparisons. Prism software (v8.2.1.441) was used to create the figures.

## Results

### Characteristics of hDPSCs

hDPSCs were extracted from the third molars of healthy people. Primary cultured hDPSCs grew around the tissue mass (Fig. 1a). Morphological observation showed that the cells had a fibroblast-like appearance (Fig. 1b). To further identify the multidirectional differentiation potential of hDPSCs, the isolated hDPSCs were induced to differentiate into osteoblasts and adipocytes. Lipid droplets were observed in the cytoplasm by oil red O staining. Matrix mineralization was increased significantly in the process of osteogenic induction compared with the undifferentiated group (Fig. 1c, d and 1e, f). Subsequently, the qRT–PCR results suggested that the expression levels of ALP, runt-related transcription factor 2 (Runx2), and osteocalcin (OCN) were upregulated (Fig. 1g). hDPSCs were identified by flow cytometry. hDPSCs exhibited high expression of CD29 (99.88%), CD44 (97.87%), and CD90 (99.37%) and were negative for CD34 (0.39%) and CD45 (0.53%) (Fig. 1h).

### lncSNHG7 m$^6$A Modification in hDPSCs

By analysing the GSE138179 and SRP214747 datasets, we found that lncSNHG7 expression was enhanced after osteogenic differentiation of hDPSCs. Through m$^6$Avar, the WHISTLE database predicted that lncSNHG7 might have 19 m$^6$A modification sites (Fig. 2a), among which there were three ACA modification sites with very high confidence. The m$^6$A single base site PCR (MazF) verified that lncSNHG7 had a m$^6$A modification on the 2081 ACA site (Fig. 2b). Then, according to the results of the StarBase database, m$^6$A-related modifying enzymes that might bind to lncSNHG7 included METTL3/14, IGF2BP1/2/3, ALKBH5, HNRNPA2B1, FTO, YTHDC1, YTHDF1, FMR1, HNRNPC and WTAP (Fig. 2c). The expression of all m$^6$A-related enzymes was detected in hDPSCs, and it was found that the expression

levels of most of them were increased in hDPSCs after osteogenic differentiation ($P<0.05$). METTL3 exhibited the highest expression (Fig. 2d).

## METTL3 Promoted Osteogenic Differentiation of hDPSCs

After osteogenic differentiation of hDPSCs, the protein level of METTL3 increased (Fig. 3a, b). Next, to better verify the role of METTL3 in the osteogenic differentiation of hDPSCs, we isolated hDPSCs and successfully knocked down METTL3 through siRNA in vitro functional experiments. qRT–PCR demonstrated not only the efficiency of knockdown (Fig. 3c) but also the decreased expression levels of the osteogenic genes ALP and Runx2 (Fig. 3d, e). In addition, the expression of osteogenic differentiation-related proteins was detected by western blotting, and the data were consistent with the qRT–PCR results. After silencing the expression of METTL3, expression of the osteogenic proteins ALP and Runx2 decreased. Similarly, as shown in Figure 3g, after METTL3 knockdown, ALP staining decreased in the siMETTL3 group compared with the control groups. ARS staining of mineralization showed reduced mineralization (Fig. 3g). These results indicated that METTL3 knockdown led to decreased osteogenic differentiation of hDPSCs.

## lncSNHG7 Promoted Osteogenic Differentiation of hDPSCs

The ability of lncSNHG7 to regulate hDPSC osteogenesis was further validated in vitro, and siRNA-SNHG7 was constructed and transduced into hDPSCs. lncSNHG7 silencing was confirmed by qRT–PCR (Fig. 4a). qRT–PCR analysis of siRNA-SNHG7 cells showed reduced expression of ALP, OCN and Runx2 after induction for 14 days (Fig. 4b). The expression of osteogenic differentiation-related proteins was detected by western blotting. After silencing the expression of lncSNHG7, expression of the osteogenic proteins ALP and Runx2 decreased (Fig. 4c, d). After lncSNHG7 knockdown, ARS staining showed reductions in mineralized nodules (Fig. 4e) and decreased ALP staining in the knockdown compared with the control group (Fig. 4e). These results indicated that lncSNHG7 was an important regulator that could promote osteogenic differentiation of hDPSCs.

To further understand the possible roles of lncSNHG7 in functional regulation, GO enrichment and KEGG pathway analyses were performed on the predicted target mRNAs of the lncRNAs based on the StarBase database. The enriched GO functions in the three GO categories (BP, MF and CC) are shown in Fig. 4f. The GO analysis results showed that the enriched GO terms for the biological process category were regulation of transcription from the RNA polymerase II promoter, signal transduction, protein phosphorylation, etc. The molecular function structured networks indicated protein binding, transcription factor activity and sequence-specific DNA binding. Through cellular component analysis, the target genes were found to be widely involved in the cytoplasm, nucleus, plasma membrane, etc. The results of the KEGG pathway analysis showed that the target mRNAs of lncSNHG7 were enriched in many pathways. These differentially expressed genes were enriched in pathways in cancer, cytokine–cytokine receptor interactions and transcriptional misregulation in cancer. Four enriched pathways were closely related to osteogenesis: MAPK, NF-kappa B, Wnt and TGF-beta (Fig. 4g). Fig. 4h shows a map of the Wnt signaling pathway.

### METTL3 Regulated the m$^6$A Modification of lncSNHG7

METTL3 has been shown to be a m$^6$A methyltransferase that is involved in regulating a variety of physiological processes. Therefore, we speculated that METTL3 could target and regulate the m$^6$A modification of lncSNHG7. First, after knocking down METTL3, it was found that the m$^6$A modification level of lncSNHG7 was reduced (Fig. 5a), and the expression level of lncSNHG7 was also reduced, indicating that METTL3 not only regulated the m$^6$A modification of lncSNHG7 but also affected its expression (Fig. 5b). In addition, the binding between METL3 and lncSNHG7 was confirmed by RIP-qPCR (Fig. 5c).

### The METTL3/lncSNHG7 axis Regulated the Wnt/β-catenin Signaling Pathway

Bioinformatics analysis predicted that the target gene of lncSNHG7 was enriched in the Wnt/β-catenin signaling pathway. We speculated that METTL3 could affect the Wnt/β-catenin signaling pathway by regulating the m$^6$A modification of lncSNHG7 and ultimately the osteogenic differentiation of hDPSCs. First, lncSNHG7 knockdown resulted in decreased phosphorylation of the key protein GSK-3β in the Wnt/β-catenin signaling pathway, and the expression of β-catenin also decreased (Fig. 6a, b), indicating that lncSNHG7 activated the Wnt/β-catenin signaling pathway. Then, after METTL3 was knocked down, western blotting showed decreased phosphorylation of GSK-3β, and the expression of β-catenin also decreased (Fig. 6c, d). These results confirmed the presence of the METTL3/lncSNHG7 axis, which could regulate the Wnt/β-catenin signaling pathway and affect the osteogenic differentiation of hDPSCs.

## Discussion

The m$^6$A modification is the most common modification in posttranscriptional RNA. It can also regulate noncoding RNAs, such as miRNAs, lncRNAs and circRNAs. The change in its level may be closely related to the metabolism and function of RNA. It has been reported that m$^6$A modification is involved in the biological processes of a variety of stem cells and plays an important role in bone metabolism. For example, the demethylase ALKBH5 can promote the expression of osteogenic genes [29]. METTL14 plays a regulatory role in osteoporosis. METTL14 can promote osteoclast activity by inhibiting miRNA expression [30]. The importance of METTL3-mediated m$^6$A methylation of XIST in OPLL provides new insights into therapeutic strategies for OPLL [31]. However, few studies have been conducted to examine m$^6$A modification of hDPSCs. Luo et al. have shown that METTL3 plays a regulatory role in the cell cycle [15]. In addition, METTL3 has been found to play an important role in the development of tooth roots. Its deletion leads to the reduction of odontogenic differentiation, shortening of molar roots and thinning of dentin by weakening the translation efficiency of nuclear factor IC (NFIC) (a key regulator of tooth roots) [32]. METTL3 can also directly interact with ATP citrate lyase (ACLY) and mitochondrial citrate transporter (SLC25A1) and then further affect the glycolysis pathway and glucose metabolism during the osteogenesis of hDPSCs [33]. However, based on the literature, the mechanism underlying the m$^6$A modification involved in bone metabolism of hDPSCs has not been fully clarified; it is still controversial

and requires further exploration. Recent studies have shown that $m^6A$ modification can also affect the stability and metabolism of lncRNAs [34–36]. However, to the best of our knowledge, there has been no research on the regulation of bone homeostasis and bone tissue engineering by $m^6A$ and lncRNA in hDPSCs. Therefore, this study is expected to provide a new theoretical basis for the study of the mechanism of hDPSC osteogenic differentiation.

In this study, we first identified the $m^6A$ modification of lncSNHG7 in hDPSCs by a MazF experiment. Its occurrence region was the 2081 site of the conserved motif region containing the core ACA sequence, but whether the $m^6A$ modification of lncSNHG7 still occurs at other sites requires further study. Then, the potential regulatory mechanism of METTL3 in the osteogenic differentiation of hDPSCs was discussed. Knockdown of METTL3 reduced expression levels of ALP and Runx2, ALP activity and the level of mineralized nodules, which indicated that deletion of METTL3 inhibited the osteogenic differentiation potential of hDPSCs and supported the positive regulatory role of METTL3 in osteogenic differentiation of hDPSCs, consistent with other studies [37, 38]. However, different studies have shown that METTL3 can inhibit osteogenesis through $m^6A$ modification [39, 40]. Altogether, these results confirm that METTL3 may be an important regulator of osteogenic differentiation, but the specific mode of action of different stem cells requires further study.

lncRNAs can regulate gene expression at the level of chromatin modification, transcription and posttranscriptional processing and are very important in almost all biological processes, including pluripotency, cell development, the immune response and differentiation. Many studies have shown that lncRNAs play an important role in the osteogenic differentiation of hDPSCs [27, 41], but to date, there has been no research on the regulation of lncRNA $m^6A$ modification in hDPSC osteogenesis. In our study, METTL3 increased $m^6A$ methylation and expression levels of lncSNHG7, leading to promotion of the osteogenic differentiation of hDPSCs. These findings revealed a new role of METTL3 in hDPSCs, showing that METTL3 could promote osteogenic differentiation of hDPSCs through the upregulation of lncSNHG7.

Osteogenic differentiation is regulated by a variety of signaling pathways, including the Wnt/β-catenin signaling pathway. The expression of β-catenin is very important for tooth formation, and β-catenin may play an important role in BMP-9-induced osteogenic and odontogenic signal transduction [42]. Recent data suggest that the treated dentin matrix directly targets GSK-3β and activates the typical Wnt/β-catenin signaling pathway to promote odontogenic differentiation of hDPSCs [43]. These reports strongly suggest that Wnt/β-catenin signaling regulates osteogenic differentiation. In the present study, through bioinformatics analysis of lncSNHG7, we found that osteogenic differentiation is enriched in the Wnt/β-catenin signaling pathway. Therefore, we speculate that METTL3 can affect the Wnt/β-catenin signaling pathway and ultimately regulate the osteogenic differentiation of hDPSCs by regulating the $m^6A$ modification of lncSNHG7. The results showed that knockdown of lncSNHG7 and METTL3 resulted in decreased expression levels of p-GSK-3β and β-catenin in the Wnt/β-catenin signaling pathway. This experiment revealed that METTL3-mediated lncSNHG7 $m^6A$ modification was involved in the Wnt/β-

catenin signaling pathway and could promote osteogenic differentiation of hDPSCs. However, due to the limited progress and lack of in-depth research on the correlation between $m^6A$ modification and the Wnt/β-catenin signaling pathway in the process of osteogenic differentiation, researchers need to invest more energy to clarify the relationship between $m^6A$ modification and the osteogenic signaling pathway in hDPSCs and to reveal the specific underlying mechanism.

# Conclusion

In summary, this study reveals that METTL3 can affect lncSNHG7, activate the Wnt/β-catenin signaling pathway, and ultimately affect the osteogenic differentiation of hDPSCs (Fig. 7). These findings can provide new insights into bone tissue engineering.

# Abbreviations

Human dental pulp stem cells (hDPSCs); N6 methyladenosine (m6A); methyltransferase 3 (METTL3); Long noncoding RNAs (lncRNAs); oncoding RNA (ncRNA); microRNA (miRNA); Dulbecco's modified Eagle's medium (DMEM); Fetal bovine serum (FBS); real-time quantitative polymerase chain reaction (qRT-PCR); Alkaline Phosphatase (ALP); Alizarin Red Staining (ARS); glyceraldehyde-3-phosphate dehydrogenase (GAPDH); small interfering RNAs (siRNAs); Gene Ontology (GO); Kyoto Encyclopedia of Genes and Genomes (KEGG); biological processes (BP), cellular components (CC), and molecular functions (MF); RNA-binding protein immunoprecipitation (RIP); runt-related transcription factor 2 (Runx2); osteocalcin (OCN)

# Declarations


## Acknowledgments

Not applicable.


## Authors' contributions

YQ. Y contributed to the conception, design,drafted and critically revised the manuscript. YQ. Y and JK. Z performed the experiments and collected data. C. J contributed to the data acquisition and analysis. JW. C critically revised the manuscript. M. C and BL. W contributed to the conception and design. All authors gave final approval and agree to be accountable for all aspects of the work.


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### Availability of data and materials

Not applicable.

### Ethics approval and consent to participate

This study was approved by the Ethics Committee of Nanfang Hospital, Southern Medical University. All subjects were informed and performed under the supervision of the Nanfang Hospital, Southern Medical University Medical Ethics Committee.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

# References


1. Zhang Z. Bone regeneration by stem cell and tissue engineering in oral and maxillofacial region. Front Med. 2011;5:401–13.
2. Euler de Souza Lucena E, Guzen FP, Lopes de Paiva Cavalcanti JR, Galvão Barboza CA, Silva do Nascimento Júnior E, Cavalcante J de S. Experimental considerations concerning the use of stem cells and tissue engineering for facial nerve regeneration: a systematic review. J Oral Maxillofac Surg. 2014;72:1001–12.
3. Ercal P, Pekozer GG, Kose GT. Dental Stem Cells in Bone Tissue Engineering: Current Overview and Challenges. In: Turksen K, editor. Cell Biology and Translational Medicine, Volume 3 [Internet]. Cham: Springer International Publishing; 2018 [cited 2022 Mar 25]. p. 113–27. Available from: http://link.springer.com/10.1007/5584_2018_171.
4. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. Proc Natl Acad Sci USA. 2000;97:13625–30.
5. Yamada Y, Nakamura-Yamada S, Kusano K, Baba S. Clinical Potential and Current Progress of Dental Pulp Stem Cells for Various Systemic Diseases in Regenerative Medicine: A Concise Review. IJMS. 2019;20:1132.
6. Ching H, Luddin N, Rahman I, Ponnuraj K. Expression of Odontogenic and Osteogenic Markers in DPSCs and SHED: A Review. CSCR. 2016;12:71–9.
7. Fazi F, Fatica A. Interplay Between N6-Methyladenosine (m6A) and Non-coding RNAs in Cell Development and Cancer. Front Cell Dev Biol. 2019;7:116.
8. Zhou C, Molinie B, Daneshvar K, Pondick JV, Wang J, Van Wittenberghe N, et al. Genome-Wide Maps of m6A circRNAs Identify Widespread and Cell-Type-Specific Methylation Patterns that Are Distinct

from mRNAs. Cell Rep. 2017;20:2262–76.

9. Wang P, Doxtader KA, Nam Y. Structural Basis for Cooperative Function of Mettl3 and Mettl14 Methyltransferases. Mol Cell. 2016;63:306–17.
10. Lin S, Zhu Y, Ji C, Yu W, Zhang C, Tan L, et al. METTL3-Induced miR-222-3p Upregulation Inhibits STK4 and Promotes the Malignant Behaviors of Thyroid Carcinoma Cells. J Clin Endocrinol Metabolism. 2022;107:474–90.
11. Xia C, Wang J, Wu Z, Miao Y, Chen C, Li R, et al. METTL3-mediated M6A methylation modification is involved in colistin-induced nephrotoxicity through apoptosis mediated by Keap1/Nrf2 signaling pathway. Toxicology. 2021;462:152961.
12. Bhattarai PY, Kim G, Poudel M, Lim S-C, Choi HS. METTL3 induces PLX4032 resistance in melanoma by promoting m6A-dependent EGFR translation. Cancer Lett. 2021;522:44–56.
13. Yan G, Yuan Y, He M, Gong R, Lei H, Zhou H, et al. m6A Methylation of Precursor-miR-320/RUNX2 Controls Osteogenic Potential of Bone Marrow-Derived Mesenchymal Stem Cells. Mol Therapy - Nucleic Acids. 2020;19:421–36.
14. Song Y, Pan Y, Wu M, Sun W, Luo L, Zhao Z, et al. METTL3-Mediated lncRNA m6A Modification in the Osteogenic Differentiation of Human Adipose-Derived Stem Cells Induced by NEL-Like 1 Protein. Stem Cell Rev and Rep. 2021;17:2276–90.
15. Luo H, Liu W, Zhang Y, Yang Y, Jiang X, Wu S, et al. METTL3-mediated m6A modification regulates cell cycle progression of dental pulp stem cells. Stem Cell Res Ther. 2021;12:159.
16. Feng Z, Li Q, Meng R, Yi B, Xu Q. METTL3 regulates alternative splicing of MyD88 upon the lipopolysaccharide-induced inflammatory response in human dental pulp cells. J Cell Mol Med. 2018;22:2558–68.
17. Liu H, Hu L, Yu G, Yang H, Cao Y, Wang S, et al. LncRNA, PLXDC2-OT promoted the osteogenesis potentials of MSCs by inhibiting the deacetylation function of RBM6/SIRT7 complex and OSX specific isoform. Stem Cells. 2021;39:1049–66.
18. Zhou Z, Hossain MS, Liu D. Involvement of the long noncoding RNA H19 in osteogenic differentiation and bone regeneration. Stem Cell Res Ther. 2021;12:74.
19. Wang J, Liu X, Wang Y, Xin B, Wang W. The role of long noncoding RNA THAP9-AS1 in the osteogenic differentiation of dental pulp stem cells via the miR-652-3p/VEGFA axis. Eur J Oral Sci. 2021;129:e12790.
20. Liao C, Zhou Y, Li M, Xia Y, Peng W. LINC00968 promotes osteogenic differentiation in vitro and bone formation in vivo via regulation of miR-3658/RUNX2. Differentiation. 2020;116:1–8.
21. Wu Y, Lian K, Sun C. LncRNA LEF1-AS1 promotes osteogenic differentiation of dental pulp stem cells via sponging miR-24-3p. Mol Cell Biochem. 2020;475:161–9.
22. Chen M, Yang Y, Zeng J, Deng Z, Wu B. circRNA Expression Profile in Dental Pulp Stem Cells during Odontogenic Differentiation. Stem Cells International. 2020;2020:1–19.

23. Jiang W, Lv H, Wang H, Wang D, Sun S, Jia Q, et al. Activation of the NLRP3/caspase-1 inflammasome in human dental pulp tissue and human dental pulp fibroblasts. Cell Tissue Res. 2015;361:541–55.
24. Wei X, Ling J, Wu L, Liu L, Xiao Y. Expression of mineralization markers in dental pulp cells. J Endod. 2007;33:703–8.
25. Garcia-Campos MA, Edelheit S, Toth U, Safra M, Shachar R, Viukov S, et al. Deciphering the “m6A Code” via Antibody-Independent Quantitative Profiling. Cell. 2019;178:731–47.e16.
26. Zhang Z, Chen L-Q, Zhao Y-L, Yang C-G, Roundtree IA, Zhang Z, et al. Single-base mapping of m6A by an antibody-independent method. Sci Adv. 2019;5:eaax0250.
27. Chen Z, Zhang K, Qiu W, Luo Y, Pan Y, Li J, et al. Genome-wide identification of long noncoding RNAs and their competing endogenous RNA networks involved in the odontogenic differentiation of human dental pulp stem cells. Stem Cell Res Ther. 2020;11:114.
28. Liu Z, Xu S, Dao J, Gan Z, Zeng X. Differential expression of lncRNA/miRNA/mRNA and their related functional networks during the osteogenic/odontogenic differentiation of dental pulp stem cells. J Cell Physiol. 2020;235:3350–61.
29. Feng L, Fan Y, Zhou J, Li S, Zhang X. The RNA demethylase ALKBH5 promotes osteoblast differentiation by modulating Runx2 mRNA stability. FEBS Lett. 2021;595:2007–14.
30. Sun Z, Wang H, Wang Y, Yuan G, Yu X, Jiang H, et al. MiR-103-3p targets the m6 A methyltransferase METTL14 to inhibit osteoblastic bone formation. Aging Cell. 2021;20:e13298.
31. Yuan X, Shi L, Guo Y, Sun J, Miao J, Shi J, et al. METTL3 Regulates Ossification of the Posterior Longitudinal Ligament via the lncRNA XIST/miR-302a-3p/USP8 Axis. Front Cell Dev Biol. 2021;9:629895.
32. Sheng R, Wang Y, Wu Y, Wang J, Zhang S, Li Q, et al. METTL3-Mediated m6 A mRNA Methylation Modulates Tooth Root Formation by Affecting NFIC Translation. J Bone Miner Res. 2021;36:412–23.
33. Cai W, Ji Y, Han L, Zhang J, Ni Y, Cheng Y, et al. METTL3-Dependent Glycolysis Regulates Dental Pulp Stem Cell Differentiation. J Dent Res. 2021;220345211051594.
34. Liu H, Xu Y, Yao B, Sui T, Lai L, Li Z. A novel N6-methyladenosine (m6A)-dependent fate decision for the lncRNA THOR. Cell Death Dis. 2020;11:613.
35. Wu Y, Yang X, Chen Z, Tian L, Jiang G, Chen F, et al. m6A-induced lncRNA RP11 triggers the dissemination of colorectal cancer cells via upregulation of Zeb1. Mol Cancer. 2019;18:87.
36. Ni W, Yao S, Zhou Y, Liu Y, Huang P, Zhou A, et al. Long noncoding RNA GAS5 inhibits progression of colorectal cancer by interacting with and triggering YAP phosphorylation and degradation and is negatively regulated by the m6A reader YTHDF3. Mol Cancer. 2019;18:143.
37. Wu Y, Xie L, Wang M, Xiong Q, Guo Y, Liang Y, et al. Mettl3-mediated m6A RNA methylation regulates the fate of bone marrow mesenchymal stem cells and osteoporosis. Nat Commun. 2018;9:4772.
38. Tian C, Huang Y, Li Q, Feng Z, Xu Q. Mettl3 Regulates Osteogenic Differentiation and Alternative Splicing of Vegfa in Bone Marrow Mesenchymal Stem Cells. Int J Mol Sci. 2019;20:E551.

39. Li D, Cai L, Meng R, Feng Z, Xu Q. METTL3 Modulates Osteoclast Differentiation and Function by Controlling RNA Stability and Nuclear Export. Int J Mol Sci. 2020;21:E1660.
40. Yu J, Shen L, Liu Y, Ming H, Zhu X, Chu M, et al. The m6A methyltransferase METTL3 cooperates with demethylase ALKBH5 to regulate osteogenic differentiation through NF-κB signaling. Mol Cell Biochem. 2020;463:203–10.
41. Zhong J, Tu X, Kong Y, Guo L, Li B, Zhong W, et al. LncRNA H19 promotes odontoblastic differentiation of human dental pulp stem cells by regulating miR-140-5p and BMP-2/FGF9. Stem Cell Res Ther. 2020;11:202.
42. Luo W, Zhang L, Huang B, Zhang H, Zhang Y, Zhang F, et al. BMP9-initiated osteogenic/odontogenic differentiation of mouse tooth germ mesenchymal cells (TGMCS) requires Wnt/β-catenin signalling activity. J Cell Mol Med. 2021;25:2666–78.
43. Lim H-M, Nam M-H, Kim Y-M, Seo Y-K. Increasing Odontoblast-like Differentiation from Dental Pulp Stem Cells through Increase of β-Catenin/p-GSK-3β Expression by Low-Frequency Electromagnetic Field. Biomedicines. 2021;9:1049.

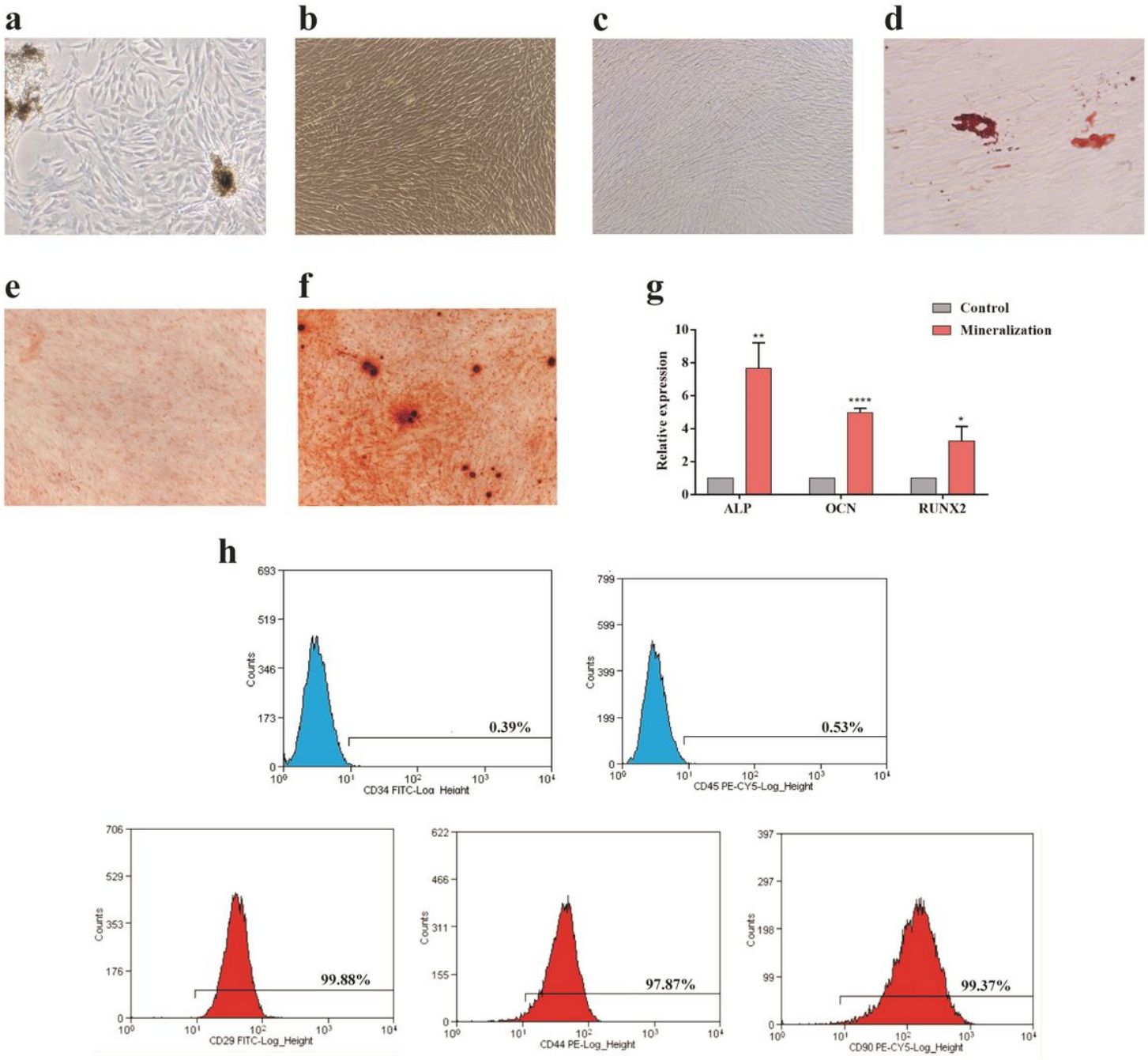
# Table

**Table 1: The sequence of primers used in PCR**

| Gene | Sequence 5’-3’ | |
|---|---|---|
| GAPDH | Forward: | TCAACAGCGACACCCACTC |
| | Reverse: | GCTGTAGCCAAATTCGTTGTC |
| ALP | Forward: | CCAAAGGCTTCTTCTTGCTG |
| | Reverse: | CCACCAAATGTGAAGACGTG |
| Runx2 | Forward: | TCGCCAGGCTTCATAGCAAA |
| | Reverse: | GGCCTTGGGTAAGGCAGATT |
| OCN | Forward: | CATGAGAGCCCTCACACTCC |
| | Reverse: | CTCCTGAAAGCCGATGTGGT |
| METTL3 | Forward: | GAGGAGTGCATGAAAGCCAG |
| | Reverse: | GGCCTCAGAATCCATGCAAG |
| METTL14 | Forward: | GACGGGGACTTCATTCATGC |
| | Reverse: | CCAGCCTGGTCGAATTGTAC |
| IGF2BP1 | Forward: | TGAAGCTGGAGACCCACATA |
| | Reverse: | GGGTCTGGTCTCTTGGTACT |
| IGF2BP2 | Forward: | AGTGGAATTGCATGGGAAAATCA |
| | Reverse: | CAACGGCGGTTTCTGTGTC |
| IGF2BP3 | Forward: | TATATCGGAAACCTCAGCGAGA |
| | Reverse: | GGACCGAGTGCTCAACTTCT |
| ALKBH5 | Forward: | ACCCCATCCACATCTTCGAG |
| | Reverse: | CTTGATGTCCTGAGGCCGTA |
| HNRNPA2B1 | Forward: | CAGTTCTCACTACAGCGCCA |
| | Reverse: | TTCCTCTCCAAAGGAACAGTTT |
| FTO | Forward: | AGACACCTGGTTTGGCGATA |
| | Reverse: | CCAAGGTTCCTGTTGAGCAC |
| YTHDC1 | Forward: | CTTCTGATGAGCAAGGGAACAA |
| | Reverse: | GGCCTCACTTCGAGTGTCATAA |
| YTHDF1 | Forward: | ACCTGTCCAGCTATTACCCG |
| | Reverse: | TGGTGAGGTATGGAATCGGAG |

| | | |
|---|---|---|
| FMR1 | Forward: | TATGCAGCATGTGATGCAACT |
| | Reverse: | TTGTGGCAGGTTTGTTGGGAT |
| HNRNPC | Forward: | GTTACCAACAAGACAGATCCTCG |
| | Reverse: | AGGCAAAGCCCTTATGAACAG |
| WTAP | Forward: | ACGCAGGGAGAACATTCTTG |
| | Reverse: | CACACTCGGCTGCTGAACT |
| lncSNHG7 | Forward: | TTGCTGGCGTCTCGGTTAAT |
| | Reverse: | GGAAGTCCATCACAGGCGAA |

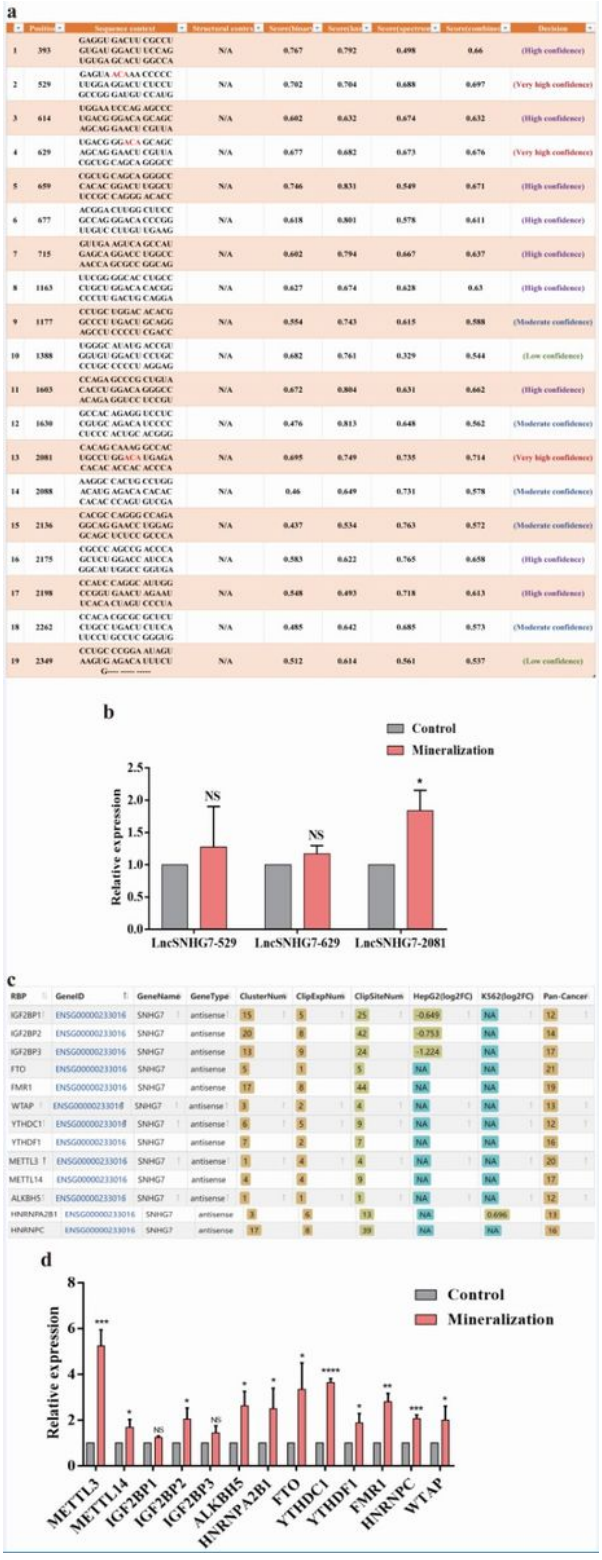
# Figures




## Figure 1

Characterizations and multiple differentiation potential of hDPSCs. **a** hDPSCs were separated from dental pulp. **b** hDPSCs were subcultured at third passage. **c** Undifferentiated hDPSCs group of osteogenic differentiation. **d** Differentiated hDPSCs group of osteogenic differentiation, mineral nodes formed in the differentiated hDPSCs group. **e** Undifferentiated hDPSCs group of adipogenic differentiation. **f** Differentiated hDPSCs group of adipogenic differentiation, lipid droplets formed in the differentiated hDPSCs group. **g** mRNA expressions of osteogenic genes-ALP, OCN, Runx2 were assayed by qRT-PCR. All samples were performed in triplicate. The data are represented as means± SD. * ***P*** < 0.05, ** ***P*** <0.01, ****

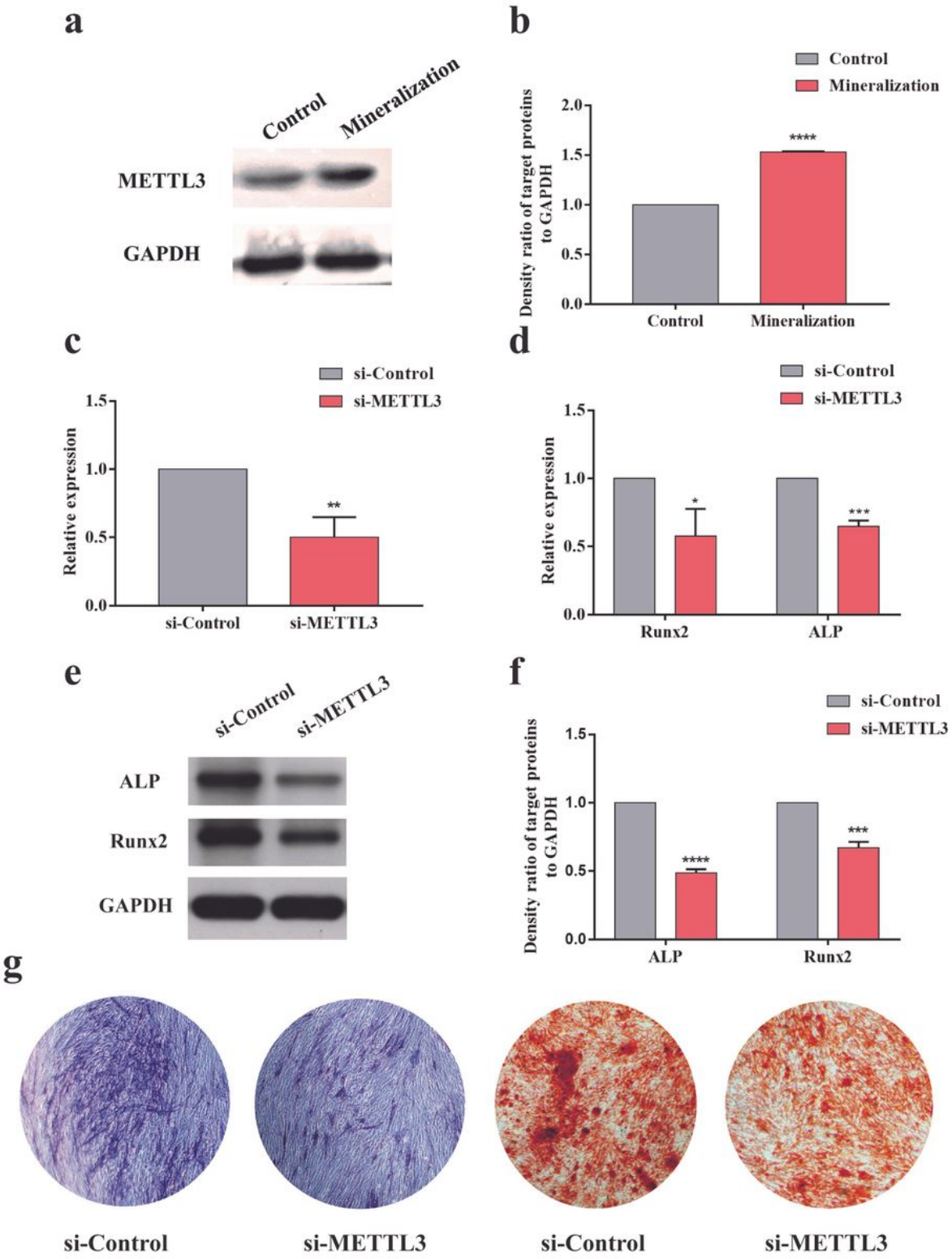
*P*0.0001. **h** Mesenchymal stem cell antigen (CD29, CD44, CD90) and hematopoietic cell antigen (CD34, CD45) expressed in hDPSCs were detected by flow cytometry.




## Figure 2

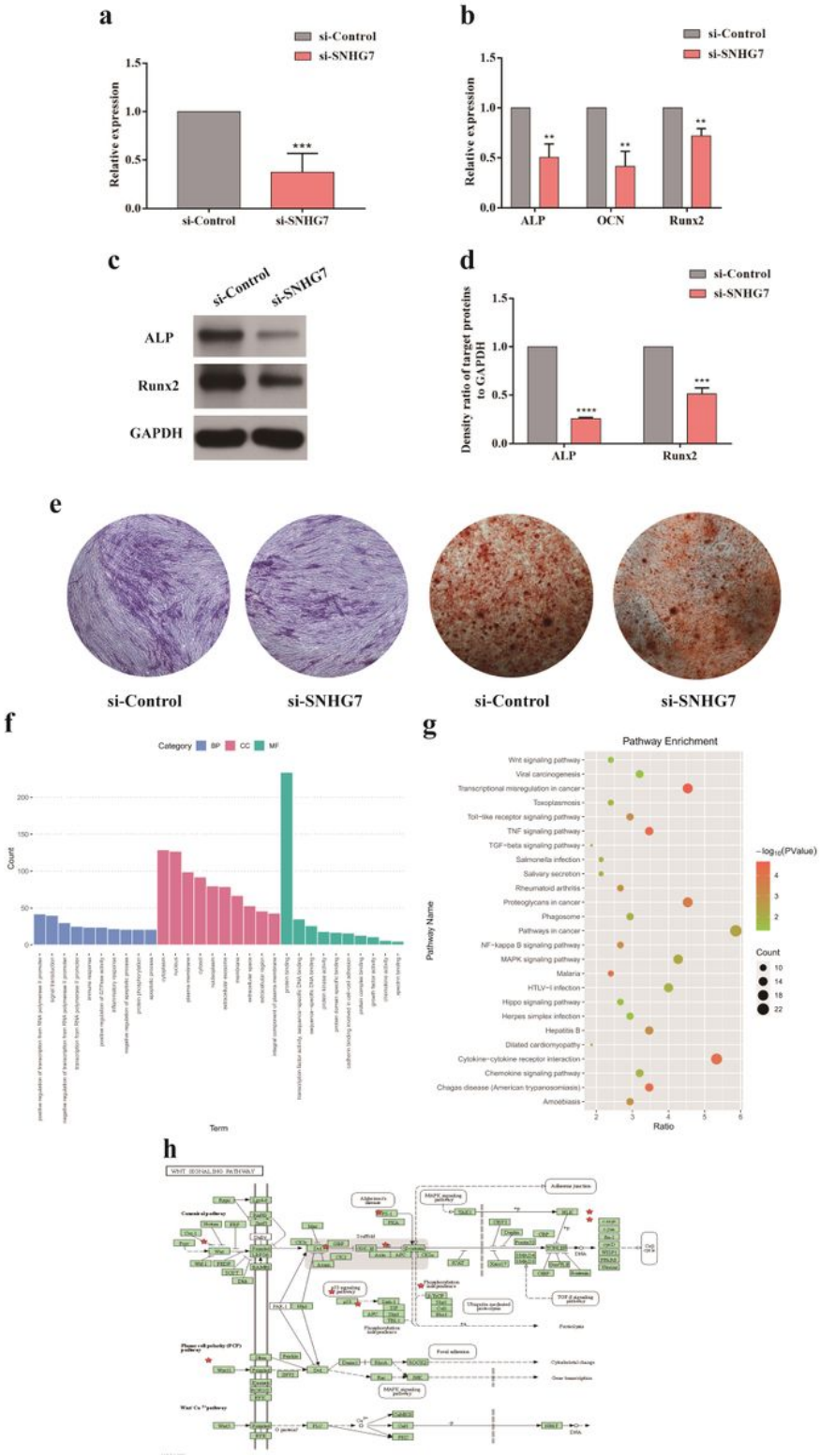
lncSNHG7 m$^6$A modification. **a** Results of m6A modification sites of lncSNHG7 predicted by m$^6$Avar and WHISTLE databases. **b** Single base site PCR (MazF) analysis of the m$^6$A modification possibility of each

point of lncSNHG7. **c** The Starbase database predicts the results of m6A modification-related enzymes that may bind to lncSNHG7. **d** Results of qRT-PCR analysis of $m^6A$ modification-related enzymes. The data were represented as means ±SD for each group:* $P$<0.05, ** $P$<0.01, *** $P$<0.001, **** $P$< 0.0001. NS: Not Statistically Significant.



## Figure 3

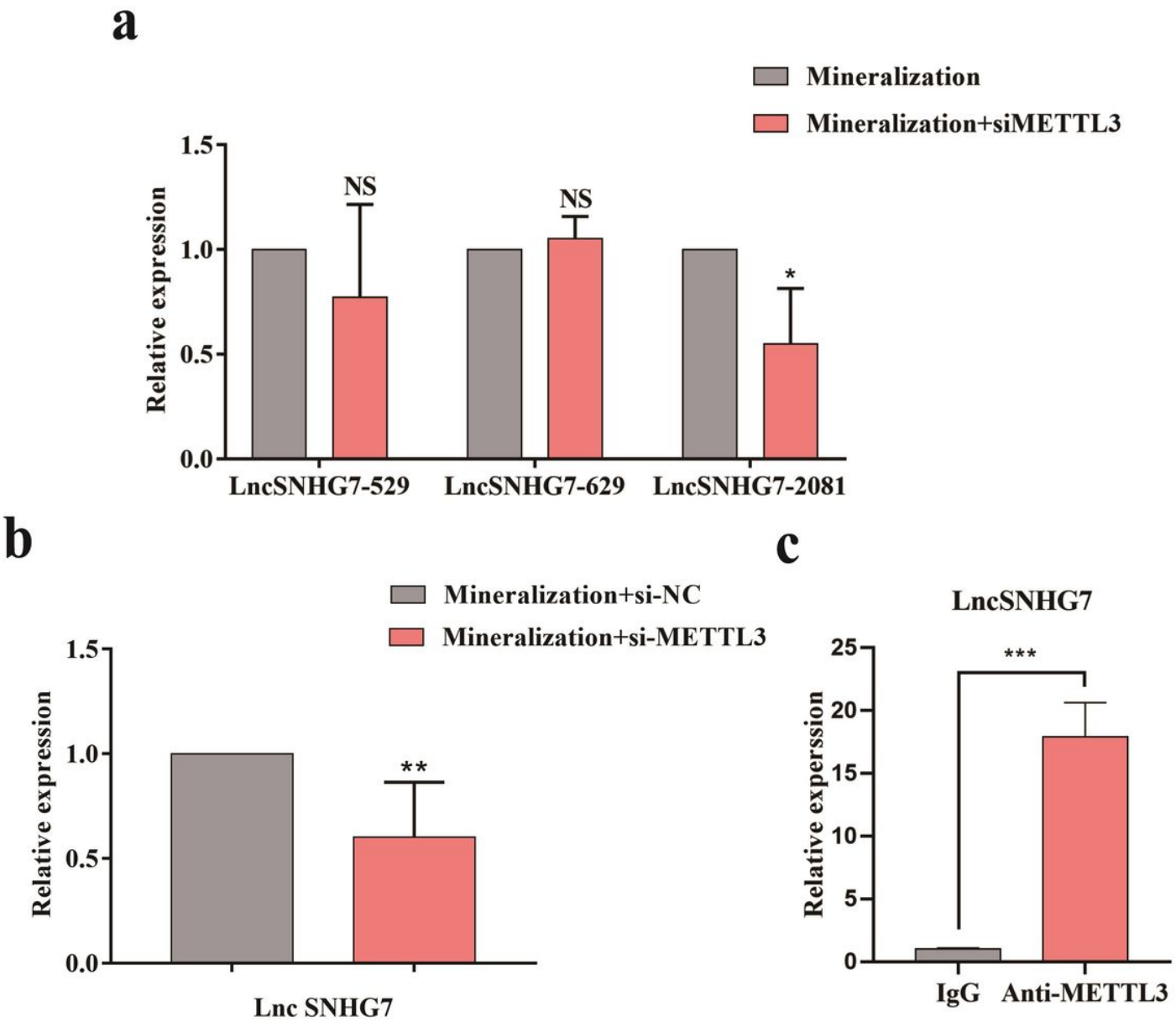
In vitro analysis of METTL3 on osteogenic differentiation of hDPSCs. **a** Western blot analysis of METTL3. **b** The density ratio of target proteins to GAPDH. **c** Transfection efficiency of METTL3 knockdown in hDPSCs. **d** qRT-PCR analysis of osteogenic genes (ALP and Runx2) in hDPSCs after METTL3 knockdown. **e** Western blot analysis shows the expression level of ALP and Runx2 decreased in the si-METTL3 group after osteogenic differentiation. **f** The density ratio of target proteins to GAPDH. **g** ARS and ALP staining after METTL3 knockdown in hDPSCs. The data were represented as means ±SD for each group:* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P< 0.0001$.




## Figure 4

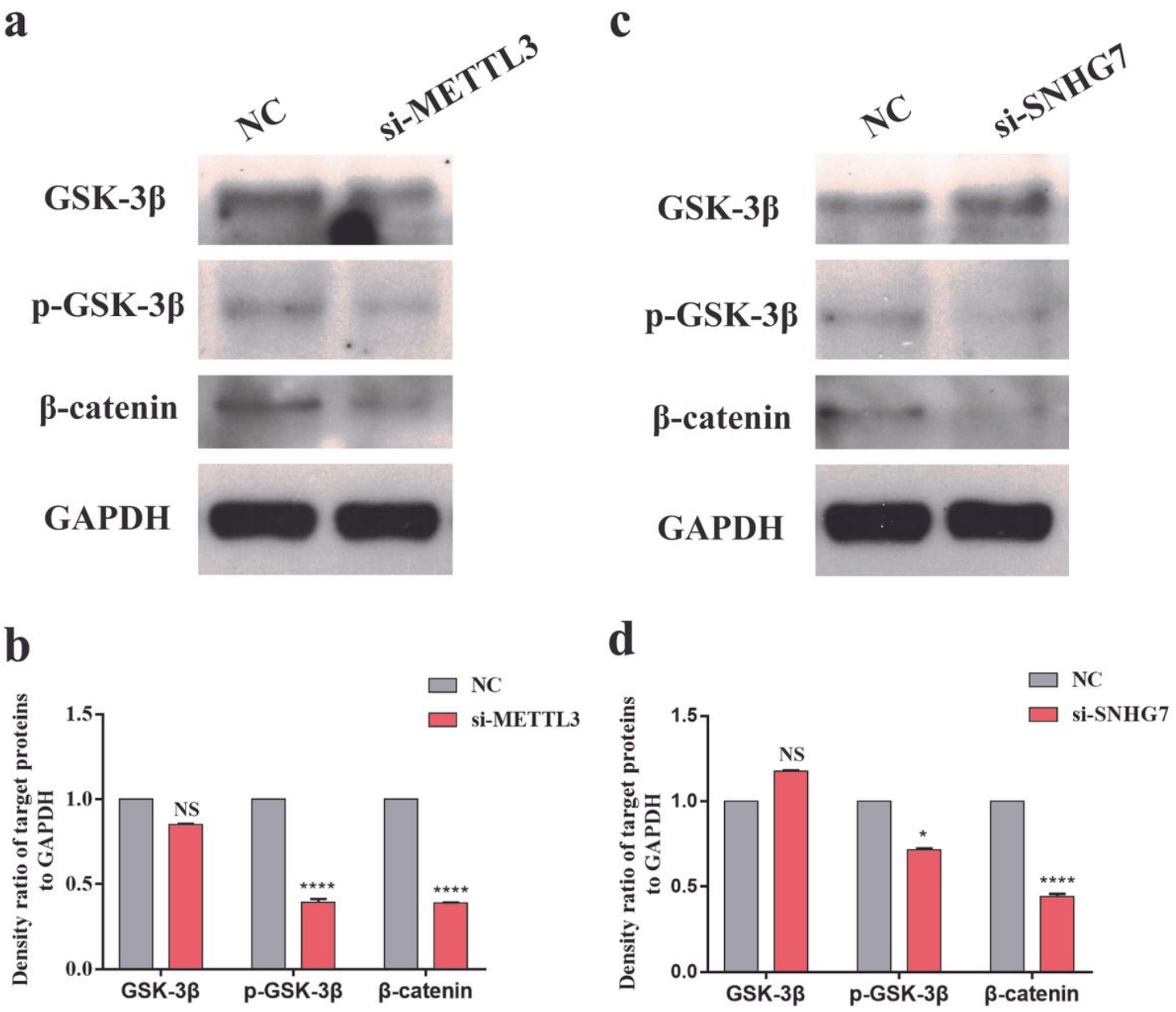
In vitro analysis of lncSNHG7 on osteogenic differentiation of hDPSCs. **a** Transfection efficiency of lncSNHG7 knockdown in hDPSCs. **b** qRT-PCR analysis of osteogenic genes (ALP and Runx2) in hDPSCs after lncSNHG7 knockdown. **c** Western blot analysis shows the expression level of ALP and Runx2 decreased in the si-SNHG7 group after osteogenic differentiation. **d** The density ratio of target proteins to GAPDH. **e** ARS and ALP staining after lncSNHG7 knockdown in hDPSCs. **f, g** GO and KEGG pathway

analysis of lncSNHG7. h Wnt signaling pathway map. The data were represented as means ±SD for each group: ** $P$<0.01, *** $P$<0.001, **** $P$< 0.0001.



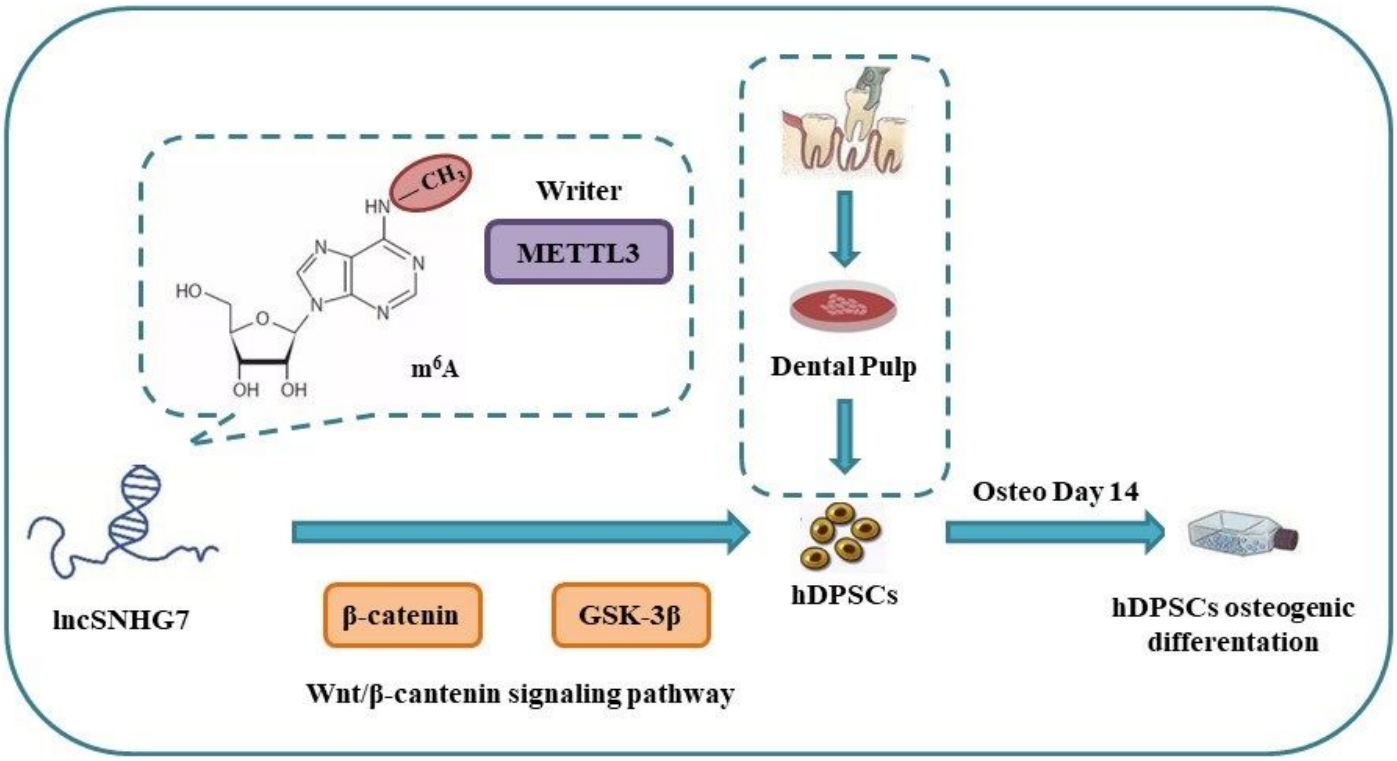

## Figure 5

Analysis of the relationship between METTL3 and lncSNHG7. **a** Single base site PCR (MazF) analysis of the m$^6$A modification possibility of each point of lncSNHG7. **b** lncSNHG7 expression levels in hDPSCs after METTL3 knockdown. **c** RIP-qPCR analysis of METTL3 and lncSNHG7 binding. The data were represented as means ±SD for each group: *$P$< 0.0005, ** $P$<0.01, *** $P$<0.001,.




## Figure 6

Demonstration of the METTL3/lncSNHG7axis and its regulatory analysis of Wnt/β-catenin signaling pathway. **a**β-catenin and GSK-3βphosphorylation levels after lncSNHG7 and METTL3 knockdown. **b, c** The density ratio of target proteins to GAPDH. The data were represented as means ±SD for each group:* $P$ <0.05, **** $P$ < 0.0001, NS: Not Statistically Significant.




## Figure 7

A schematic illustration of the molecular mechanism of lncSNHG7 promoting osteogenic differentiation of hDPSCs