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Original Article

METTL3 promotes the osteogenic differentiation of periosteum-derived MSCs via regulation of the HOXD8/ITGA5 axis in congenital pseudarthrosis

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ABSTRACT

Background: Congenital pseudarthrosis of the tibia (CPT) is a dominant health challenge in pediatric orthopedics. The essential process in the development of CPT is the limited capacity of mesenchymal stem cells (MSCs) derived from CPT to undergo osteogenic differentiation. Our research aimed to elucidate the role and mechanism of methyltransferase-like 3 (METTL3) in the osteogenic differentiation process of CPT MSCs.

Methods: The osteogenic differentiation medium was used to culture MSCs, and the detection of osteogenic differentiation was performed using Alizarin Red S and alkaline phosphatase (ALP) assays. Gene or protein expression was assessed by quantitative real-time polymerase chain reaction (qRT-PCR), Western blot, or immunofluorescence (IF) staining. The m^6A modification of Homeobox D8 (HOXD8) was verified by methylated RNA immunoprecipitation (MeRIP) assay. Interactions between METTL3 and HOXD8 or HOXD8 and integrin alpha 5 (ITGA5) promoter were validated by the luciferase reporter gene, RIP, and chromatin immunoprecipitation (ChIP) assays.

Results: METTL3 overexpression enhanced CPT MSCs' osteogenic differentiation. METTL3 stabilized the HOXD8 in an m^6A-dependent manner. Moreover, the overexpressed ITGA5 up-regulated the CPT MSCs' osteogenic differentiation. Further, HOXD8 could transcriptionally activate ITGA5. METTL3 increased the transcription of ITGA5 via HOXD8 to enhance the osteogenic differentiation of CPT MSCs.

Conclusion: METTL3 promoted osteogenic differentiation via modulating the HOXD8/ITGA5 axis in CPT MSCs.

1. Introduction

Congenital pseudarthrosis of the tibia (CPT) may be recognized as one of the most difficult diseases needing clinical treatment for children [1]. It belongs to bone non-union disease and presents as pseudarthrosis or pathological fracture of the bone, in childhood [2]. And CPT's clinical symptoms include the angulation of the anterolateral tibial and complete non-union with bone defects [3]. Currently, its major treatment is surgery, such as intramedullary nailing (bone graft), vascularized fibular transferring, and the Ilizarov technique [4]. While that is not fully capable of attaining the purpose of preventing fracture recurrence and ideal union in the pseudarthrosis area [5]. Periosteum is the major contributor to bone formation, regeneration, and fracture healing processes [6,7]. Moreover, the increasing evidence has indicated that the pathologically changed periosteum in pseudarthrosis may essentially account for the CPT progression [8,9]. While its precise function or mechanism in the healing process of CPT has not been clearly illustrated. That needs further exploration to enrich the clinical treatment of CPT.

Mesenchymal stem cells (MSCs) exert a vital role in the formation and regeneration of bone. And its osteogenic differentiation ability is vital for bone integrity maintenance and fracture healing [10]. For instance, abnormal osteogenic differentiation of MSCs is linked to diverse bone disorders including osteoporosis, ankylosing spondylitis, and even bone cancer [11,12]. As described, the restrained osteogenic differentiation of MSCs has been found in CPT patients

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and the osteogenic differentiation enhancement may facilitate the CPT therapy improvement [13]. Specifically, our previous study has demonstrated that there was a significant discrepancy in the osteogenic differentiation capacity between CPT periosteum-derived MSCs (extremely low, CPT MSCs) and MSCs sourced from the normal iliac periosteum [14]. The detailed mechanism underlying the cells' decreased capability to undergo osteogenic differentiation remains unclear. Further, the abnormally low-expressed osteogenic markers including runt-related transcription factor 2 (RUNX2), integrin-binding sialoprotein (IBSP), osteocalcin (OCN), and osteopontin (OPN) have been identified in the CPT pathogenesis [14,15]. Consistently, the upstream regulatory mechanisms among them in the CPT progression have not been clearly illustrated.

Homeobox D8 (HOXD8) is a member of HOX family, a transcription factor family. The silenced HOXD8 has also inhibited the bone marrow-derived MSCs' osteogenic differentiation process [16]. Similarly, our previous study identified that HOXD8 overexpression upregulated the osteogenic differentiation of CPT [14]. Furthermore, N^6-methyladenosine (m^6A) modification is engaged with the osteogenic differentiation regulation in MSCs [11]. And Methyltransferase-like 3 (METTL3) is an extensively explored catalytic subunit of methyltransferases complex in the m^6A modification process [17]. The inhibition of METTL3 has restrained the RUNX2 expression and down-regulated the bone marrow stem cell's osteogenic differentiation capacity [18–21]. While its specific role in CPT MSCs is still unknown.

Integrin alpha5 (ITGA5) exerts a key function in cell surface adhesion and signaling reaction, as one subtype of integrin. Moreover, ITGA5 is a well-documented positive correlator of the osteogenesis process. It can induce osteoprogenitor cell proliferation and promote osteogenic differentiation to induce bone formation [22]. ITGA5 has exerted a potential role in facilitating the repair of non-healing bone defects [23]. At the same time, the precise role of ITGA5 in the CPT progression and osteogenic differentiation of CPT MSCs has been not discussed yet.

So, the purpose of this research was to examine how METTL3 influences the regulation of osteogenic differentiation in CPT periosteum-derived MSCs. The findings of this study indicate that METTL3 enhances the process of osteogenic differentiation by promoting ITGA5 transcription via HOXD8 in CPT MSCs. It indicated that METTL3/HOXD8/ITGA5 axis could be utilized as the therapeutic cellular target in the clinical treatment to increase the healing rate and ameliorate the high refracture of CPT patients.

2. Material and methods

2.1. Cell culture

All procedures in our work were kept in line with the approved principles from the Medical Ethics Committee of The Children's Hospital of Hunan Province. And the primary isolation and culture of periosteum-derived mesenchymal stem cells (MSCs) from CPT patients or healthy iliac periosteum (6 CPT patients or normal people, respectively) were in line with our previous protocol [14]. HEK-293T cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), and were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Indianapolis, IN, USA) containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (P/S). The cells were incubated in a humidified incubator at a temperature of 37 °C and a CO_2 concentration of 5%.

2.2. MSCs' osteogenic differentiation

Osteogenic differentiation induction was performed utilizing MSCs osteogenic differentiation medium (HUXUC-90021, Cyagen Biotech, Suzhou, China). And early osteogenic differentiation was identified by the Alkaline Phosphatase Assay Kit (ALP, P0321S, Beyotime Biotech, Shanghai, China), after the 7 days' induction. After a period of 21 days of induction, the distinction in osteogenesis was stained employing the Alizarin Red S Staining Kit for Osteogenesis (ARS, C0148S, Beyotime Biotech).

2.3. ALP activity determination

The ALP activity was measured by the above-mentioned ALP Assay Kit (P0321S, Beyotime Biotech). Briefly, MSCs were lysed in the lysis buffer (P0013J, Beyotime Biotech) after the wash of PBS. And then, the detection buffer (30 μL), chromogenic substrate (50 μL), and cell lysate (20 μL) were supplemented to the 96-well plate for incubation (10 min, 37 °C). The corresponding absorbance of 405 nm was read on the Multiskan SkyHigh microplate (Thermo Fisher, Waltham, USA). Furthermore, The ALP Detection Kit (SCR004, Merck, Beijing, China) was used for the ALP staining of paraformaldehyde-fixed MSCs (4%, 25 °C, 30 min), in line with the kits' appendant instructions. And the stained MSCs were counted and captured with the microscope (Thermo Fisher).

2.4. ARS staining

To assess the MSCs' matrix mineralization condition, the cells were fixed with paraformaldehyde (4%, 25 °C, 30 min), washed with PBS, and dyed with ARS (C0148S, Beyotime Biotech) for 30 min at 37 °C, according to the kits' guidelines. And the matrix mineralization condition was observed under a microscope (Thermo Fisher).

2.5. Cell transfection

For silencing ITGA5, the small interfering RNAs (siRNAs) targeting it and associative negative control (NC) were purchased from Genechem (Shanghai, China). For the overexpression of HOXD8, the overexpression vector (HOXD8-pcDNA3.1) and control vector (pcDNA3.1) were also obtained from Genechem. And then, MSCs received the transfection of the above siRNAs with the Lipofectamine 3000 Transfection Kits (#L3000015, Invitrogen, Carlsbad, CA, USA) for 48 h, as instructed by the appendant guidelines.

2.6. Lentivirus vector infection

To overexpress METTL3, ITGA5, and HOXD8 in MSCs or 293T cells, their coding sequences of them were cloned and inserted into the lenti-vector plasmid pLVX (Hanbio Biotech, Shanghai, China). The pLVX plasmids with the fusion sequence were transduced into 293T cells using the lentiviral packaging plasmid mix (Hanbio Biotech) for 24 h. And the culture medium was collected and centrifuged at 25,000 rpm for 1 h to obtain the precipitate. After that, the preparation was supplemented in the medium of MSCs, containing 3 μg/mL polybrene. The infection efficacy was validated by protein and mRNA expression detection.

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

MSCs' total RNA was isolated applying the MolPure TRIeasy™ Plus Total RNA Kit (19211ES60, Yeasen Biotech, Shanghai, China). And the mRNA levels' determination was performed using the Hifair III One Step RT-qPCR SYBR Green Kit (11143ES70, Yeasen Biotech) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA, USA), following the provider's protocol. GAPDH was selected to be the control gene for normalization purposes. The comparative change in the target genes was analyzed

using the $2^{-\Delta\Delta Ct}$ method. The primer sequences employed in this study provided below:

ITGA5-F: 5′-CATGATGAGTTTGGCCGATTTG-3′,
ITGA5-R: 5′-CCCCCAGGAAATACAAACACTA-3’;
HOXD8-F: 5′-GTTTTGAACCGCCCTTGTAA-3′,
HOXD8-R: 5′-GTGAGGCTATCGCTTTCCTG-3’;
β-actin-F: 5′-CTGGCACCACACCTTCTACAATG-3′,
β-actin-R: 5′-GGCGTACAGGGATAGCACAGC-3’.

2.8. Western blot analysis

The total protein of MSCs was isolated and extracted with the application of Total Protein Extraction Kits (EX1102, Solarbio Biotech, Beijing, China). And the protein concentration determination was assessed using the BCA Protein Assay Kit (PC0020, Solarbio Biotech). An equal amount of protein samples (20 μg) were separated and then electrotransferred. Thereafter, the blocking of membranes was performed for 1 h at room temperature using a 5% solution of NON-Fat Powdered Milk (D8340, Solarbio Biotech). Then the membranes were incubated with the primary antibodies of the targeted proteins for another 12 h at 4 °C. The membranes received incubation for one more hour at room temperature with the corresponding horseradish peroxidase (HRP) that were conjugated secondary antibodies. And the targeted proteins' chemiluminescent signals were visualized with the Low background luminescence ECL detection kit (SNM425, Biolab Biosciences, Beijing, China) on the ImageQuant LAS 4000 mini system (GE Healthcare, Sunnyvale, CA, USA). Primary antibodies including METTL3 (1:1000, ab195352, Abcam, Cambridge, UK), HOXD8 (1:1000, ab228450, Abcam), ITGA5 (1:1000, ab150361, Abcam), RUNX2 (1:1000, ab236639, Abcam), Osteopontin (OPN, 1:1000, ab214050, Abcam), osteocalcin (OCN, 1:1000, ab93876, Abcam), and GAPDH (1:1000, ab9485, Abcam) were purchased from Abcam. IBSP antibody (1:1000, #5468, CST, Danvers, MA) was purchased from CST. GAPDH was selected as the loading control for the quantification of protein expression data.

2.9. Immunofluorescence (IF) staining

MSCs were fixed with 4% paraformaldehyde for 15 min, washed with PBST solution three times, and blocked in the normal goat-blocking buffer for 1 h. Thereafter, the cells were incubated with the METTL3 (1:1000, ab195352, Abcam) primary antibody at 4 °C overnight. Then, the cells were further incubated with the secondary anti-rabbit antibody for 1 h at 37 °C and incubated with the DAPI Reagent for 5 min in darkness. After washing with PBST solution 4 times and the supplement of the anti-fluorescence quenching agent, the fluorescence images of MSCs were observed using the microscope (Thermo Fisher).

2.10. Luciferase reporter gene assay

The promoter of ITGA5 (ITGA5-wt) and the mutated sites (ITGA5-mut) were constructed and subcloned into the firefly luciferase gene in the psiCheck-2 vector (Promega, Madison, WI, USA). Then, HEK-293T cells were co-transfected with the ITGA5-wt/ITGA5-mut reporter gene vector and HOXD8 overexpression or empty vectors (HOXD8-pcDNA3.1 or pcDNA3.1 vector) using Lipofectamine 3000 Transfection Kits (Invitrogen). The Dual-Luciferase Reporter Assay System (Promega) was utilized to assess luciferase activities.

2.11. Chromatin immunoprecipitation (ChIP) assay

The interaction between HOXD8 and ITGA5 promoter region was validated using the ChIP Assay Kit (P2078, Beyotime Biotech), based on the kits' instructions. The lenti-HOXD8-HA infected MSCs were fixed with paraformaldehyde (37%, 270 μL, 37 °C) for 10 min, and the culture was supplemented with the glycine solution (10×, 1.1 mL) for 5 min at room temperature. And then, MSCs were administrated with centrifugation, lysing, and ultrasonic treatment, as instructed by the kit's protocol. Furthermore, the cell solution was supplemented with ChIP Dilution Buffer (1.8 mL) and Protein A + G Agarose/Salmon Sperm DNA (70 μL) for 30 min at 4 °C. Next, the centrifugation obtained samples were incubated with Anti-HA tag antibody-ChIP Grade (1:500, ab9110, Abcam) or immunoglobulin G (1:500, IgG, ab37355, Abcam) antibodies overnight at 4 °C. And following the elution and purification, the centrifugation was determined by qPCR analysis.

2.12. RNA immunoprecipitation (RIP)/Methylated RIP-qPCR (MeRIP-qPCR) assay

To analyze the interaction between HOXD8 mRNA and METTL3, RIP assays were employed. MeRIP-qPCR was applied for the determination of m^6A-modified levels of HOXD8, with the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (17-700, Merck Chemicals, Shanghai, China). In short, MSCs were lysed and the got cellular extraction was incubated with 5 μg of antibodies of anti-normal mouse IgG or METTL3 (1:500, ab195352, Abcam) and anti-m^6A (1:500, ab208577, Abcam) for 1 h, at 4 °C. And the kit's Protein A/G Magnetic Beads in immunoprecipitation buffer were supplemented to the cell extraction and received further incubation for 12 h at 4 °C, in line with the manufacturer's working manual. After the digestion and isolation of the RNAs in the immunoprecipitated complex, qRT-PCR was employed to examine the relative expression change. The m^6A enrichment was calculated by normalizing to the input (%Input = % (IP/Input)).

2.13. RNA stability assay

For determining the RNA stability of HOXD8, the MSCs were treated with Actinomycin D (5 μg/mL, HY-17559, MedChemExpress) for 0, 2, and 4 h. Then, the total RNA was extracted and the gene expression of HOXD8 was detected by qRT-PCR.

2.14. Statistical analysis

The SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA) was utilized for conducting statistical analysis. To compare two groups, the Student's t-test was employed, whereas for comparing multiple groups, One-Way ANOVA followed by Tukey's test was performed. The data was represented as means ± standard deviation (SD). Each experiment was repetitively conducted three times in an independent manner. Significant experiment outcomes were identified when the *p*-value was less than 0.05.

3. Results

3.1. Overexpression of METTL3 promoted the osteogenic differentiation of CPT MSCs

The expression of METTL3 was significantly decreased in CPT MSCs, compared to MSCs derived from the normal iliac periosteum, and its expression was concentrated on the cell nucleus, from the IF analysis (Fig. 1A&B). Then, the CPT MSCs received the lentiviral infection for the overexpression of METTL3 (lenti-vector or lenti-

METTL3), and they were administrated with the osteogenic differentiation induction. METTL3 protein expression was markedly enhanced in the lenti-METTL3 infection group than the control or lenti-vector groups, suggesting an ideal overexpression (Fig. 1C). Moreover, the ALP activity was significantly enhanced in the METTL3 overexpression group (Fig. 1D&E). And the osteogenic differentiation ability was also increased by METTL3 overexpression, with the higher extracellular matrix mineralization from ARS staining (Fig. 1F). Congruously, the protein abundance of osteogenic marker proteins including RUNX2, OPN, IBSP, and OCN were significantly promoted by the overexpression of METTL3 in CPT-derived MSCs (Fig. 1G). Overall, METTL3 expression was found to be lower in CPT MSCs compared to that derived from normal iliac periosteum, and its overexpression could enhance the osteogenic differentiation ability.

3.2. METTL3 stabilized HOXD8 mRNA in an m^{6}A-dependent manner

As Fig. 2A described, the HOXD8's m^{6}A levels for CPT MSCs were significantly lower than that of the normal iliac periosteum-derived MSCs, proving that m^{6}A modification of HOXD8 was decreased in CPT MSCs. Furthermore, the direct interactions between HOXD8 and METTL3 were validated by RIP analysis, as described that the relative expressions of HOXD8 of METTL3 immunoprecipitation groups were higher than in the IgG groups (Fig. 2B). Further, the CPT MSCs had received the METTL3 overexpression, and we identified that METTL3 overexpression dramatically increased the m^{6}A levels of HOXD8 (Fig. 2C). The overexpressed METTL3 markedly enhanced the mRNA or protein expressions of HOXD8 (Fig. 2D&E). Moreover, the mRNA stability of HOXD8 was also increased in the METTL3 overexpression group (Fig. 2F). In summary, these data demonstrated that the HOXD8 m^{6}A levels declined in CPT MSCs, and the overexpressed METTL3 could promote HOXD8 expression and stabilize its mRNA in an m^{6}A-dependent manner.

3.3. Overexpressed ITGA5 enhanced the osteogenic differentiation of CPT MSCs

ITGA5 protein abundance was significantly lower in CPT MSCs than that derived from the normal iliac periosteum (Fig. 3A). ITGA5 protein abundance was markedly enhanced in the lenti-ITGA5 infection group, compared with the control groups (Fig. 3B).

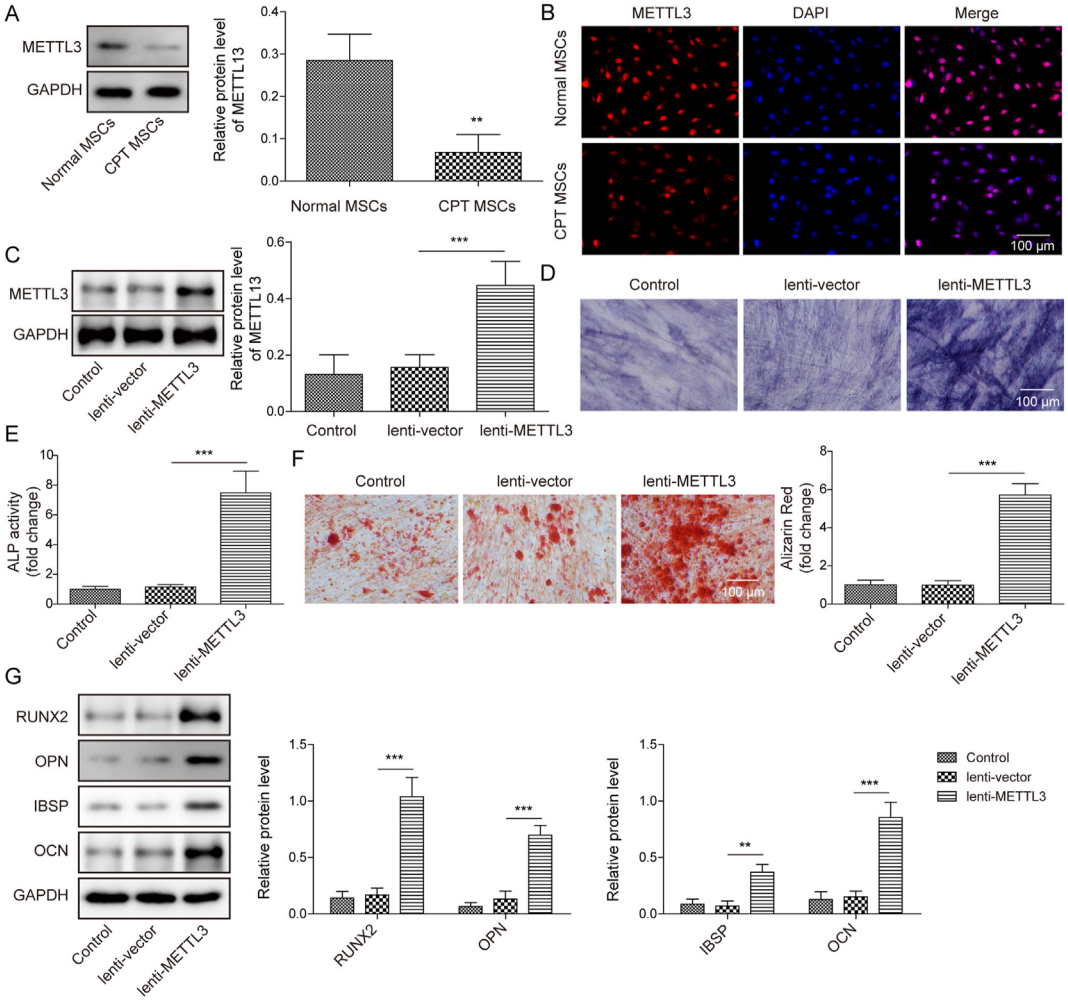

Fig. 1. Overexpression of METTL3 promoted the osteogenic differentiation of CPT MSCs. (A) Western blot detected the protein abundance of METTL3 in normal iliac periosteum-derived MSCs or CPT MSCs. (B) IF staining detected the protein expression of METTL3 in normal iliac periosteum-derived MSCs or CPT MSCs (scale bar = 100 μm). CPT MSCs were infected with lenti-vector or lenti-METTL3 and then received the osteogenic differentiation induction. (C) Western blot detected the protein abundance of METTL3. (D) ALP staining was conducted by ALP Detection Kit (scale bar = 100 μm). (E) ALP activity was detected by ALP Assay Kit. (F) Osteogenic differentiation was assessed by ARS staining (scale bar = 100 μm). (G) Western blot detected RUNX2, OPN, IBSP, and OCN protein abundance. Each experiment was repeated in triplicates. Experimental data are indicated as means ± SD, n = 3. $^{**}P < 0.01$; $^{***}P < 0.001$.

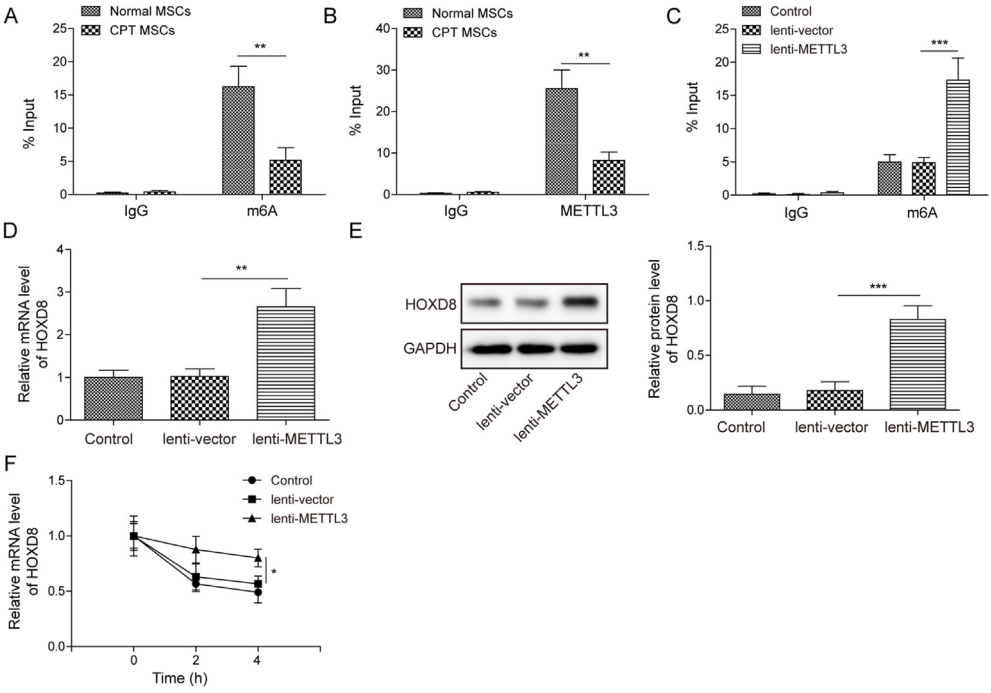

Fig. 2. METTL3 stabilized HOXD8 mRNA in an m^{6}A-dependent manner. (A) MeRIP assay analyzed the m^{6}A modification levels. (B) RIP verified the binding between METTL3 and HOXD8, anti-IgG and anti-METTL3 antibodies were utilized for incubation, and the immunoprecipitated HOXD8 mRNA level of Normal MSCs and CPT MSCs groups were determined using qRT-PCR. Then, CPT MSCs were infected with lenti-vector or lenti-METTL3 and then received the osteogenic differentiation induction. (C) HOXD8's m^{6}A modification levels were analyzed by MeRIP assays, anti-IgG and anti-m^{6}A antibodies were utilized for incubation, and the immunoprecipitated HOXD8 mRNA level of control, lenti-vector infected CPT MSCs, and lenti-METTL3 CPT MSCs groups were determined using qRT-PCR. (D) qRT-PCR investigated the expression level of HOXD8. (E) Western blot detected the protein abundance of HOXD8. (F) qRT-PCR investigated the RNA stability of HOXD8, after treatment with Actinomycin D (5 μg/mL, 0, 2, 4 h). Each experiment was repeated in triplicates. Experimental data are indicated as means ± SD, n = 3. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Subsequently, ITGA5 overexpression increased the ALP activity (Fig. 3C&D). Consistently, the overexpressed ITGA5 promoted the extracellular matrix mineralization conditions, suggesting the elevation of osteogenic differentiation (Fig. 3E). Protein abundance of osteogenic marker proteins (RUNX2, OPN, IBSP, and OCN) were up-regulated by ITGA5 overexpression (Fig. 3F). Collectively, these data identified that ITGA5 expression was repressed in CPT MSCs, and overexpression of it promoted osteogenic differentiation.

HOXD8 transcriptionally activated ITGA5 expression.

As displayed in Fig. 4A, the potential binding sites of HOXD8 on the promoter region of ITGA5 existed, from the JASPAR online database (http://www.jaspar.genereg.net). Overexpression of HOXD8 markedly up-regulated the luciferase activities in ITGA5 promoter-wt groups, while there was no obvious change in ITGA5 promoter-mut groups (Fig. 4B). Furthermore, CPT MSCs were infected with the HOXD8-HA lentivirus to overexpress HA-HOXD8. And in accordance with that, the binding of HOXD8 in the promoter of ITGA5 was further validated by ChIP analysis (Fig. 4C). Consistently, the mRNAs or protein expression of ITGA5 were significantly enhanced by HOXD8 overexpression (Fig. 4D&E). Overall, HOXD8 could transcriptionally activate ITGA5 expression.

3.4. METTL3 augmented ITGA5 expression via HOXD8 to enhance the osteogenic differentiation of CPT MSCs

CPT MSCs were overexpressed METTL3 or cotreated with METTL3 overexpression and ITGA5 silencing. Compared with the empty lentivirus infection, the overexpression of METTL3 markedly enhanced the expression of HOXD8 and ITGA5 at mRNA and protein levels, whereas the cotreatment of ITGA5 silencing only reversed the up-regulatory effects of METTL3 overexpression on ITGA5 expression (Fig. 5A&B). Similarly, the overexpressed METTL3 increased the ALP activities of CPT MSCs, whereas the ITGA5 silencing reversed the promotive effects of METTL3 on ALP activities (Fig. 5C&D). METTL3 overexpression markedly enhanced the formation of mineralized nodules, while ITGA5 silencing overturned the regulatory trend of METTL3 (Fig. 5E). In addition, the protein expressions of osteogenic markers were up-regulated by METTL3 overexpression, the cotreatment of ITGA5 silencing similarly reversed these effects of METTL3 (Fig. 5F). In summary, METTL3 could enhance osteogenic differentiation by promoting ITGA5 transcription via HOXD8 in CPT MSCs.

4. Discussion

CPT is accompanied by the tibia's advanced varus, ante-curation malformation, bone fracture, and pseudarthrosis in neonates [24]. Moreover, the osteogenesis of periosteum-derived mesenchymal stem cells (MSCs) is the primary process in bone regeneration and fracture healing of CPT [10,14]. This study found that METTL3 could promote CPT MSCs osteogenic differentiation by enhancing ITGA5 transcription via HOXD8. Our study initially offered experimental evidence that the METTL3/HOXD8/ITGA5 axis could be employed as the potential therapeutical target to enhance healing and reduce the refracture appearance of CPT patients.

The m^{6}A modification is the key epitranscriptomic regulator in bone disorders pathogenesis. METTL3, a principal subunit of the m^{6}A methyltransferase complex, catalyzes the m^{6}A mRNA formation [25]. Knockdown of METTL3 has restrained the osteogenic differentiation and osteogenic marker expression such as RUNX2 in bone marrow stem cells [18,26]. Our study identified that METTL3 expression was down-regulated in the CPT MSCs. And we first revealed that overexpression of METTL3 could enhance the osteogenic differentiation markers expression and promote CPT MSCs'

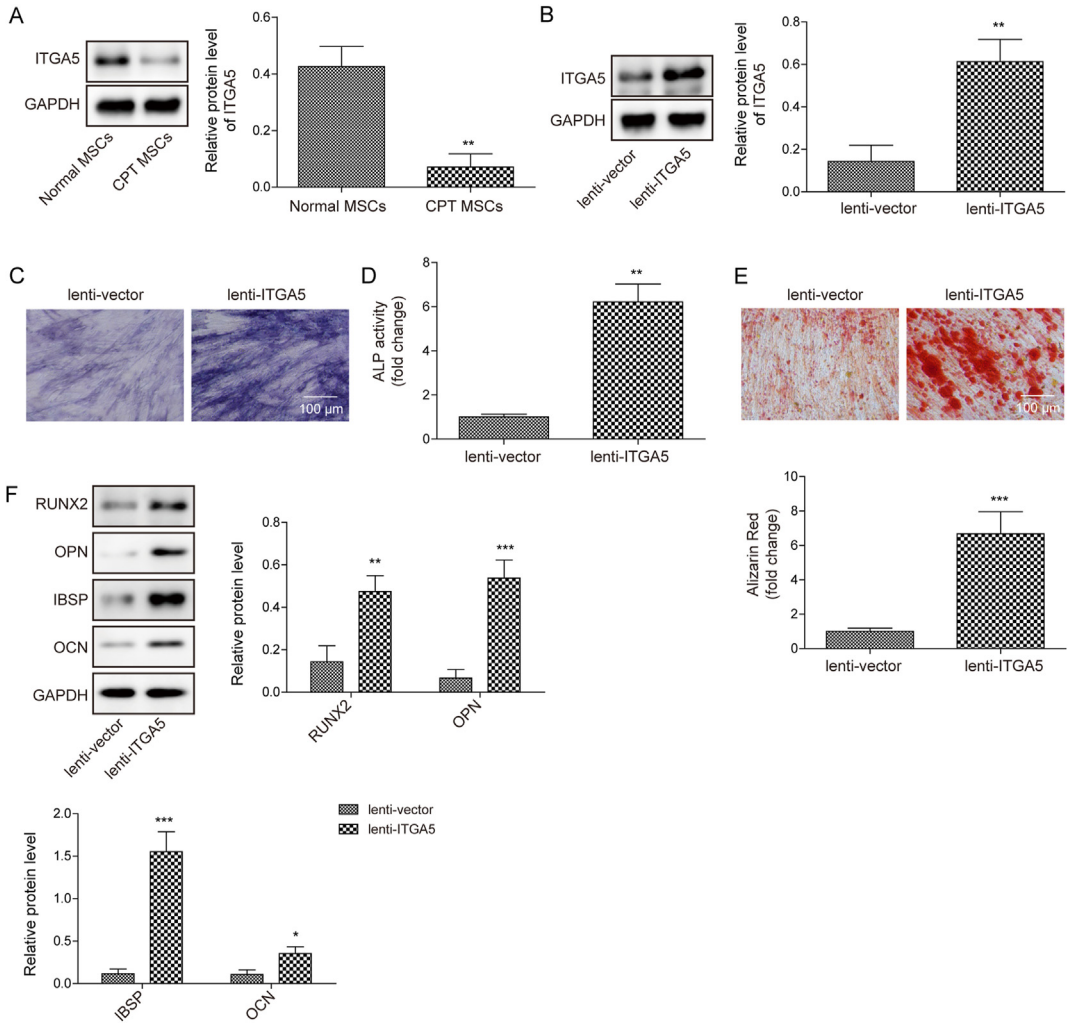

Fig. 3. Overexpressed ITGA5 enhanced the osteogenic differentiation of CPT MSCs. (A) Western blot detected the protein abundance of ITGA5. (B) Western blot detected the protein abundance of ITGA5. (C) ALP staining was conducted by ALP Detection Kit (scale bar = 100 μm). (D) ALP activity was detected by ALP Assay Kit. (E) Osteogenic differentiation was assessed by ARS staining (scale bar = 100 μm). (F) Western blot detected RUNX2, OPN, IBSP, and OCN protein abundance. Each experiment was repeated in triplicates. Experimental data are indicated as means ± SD, n = 3. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

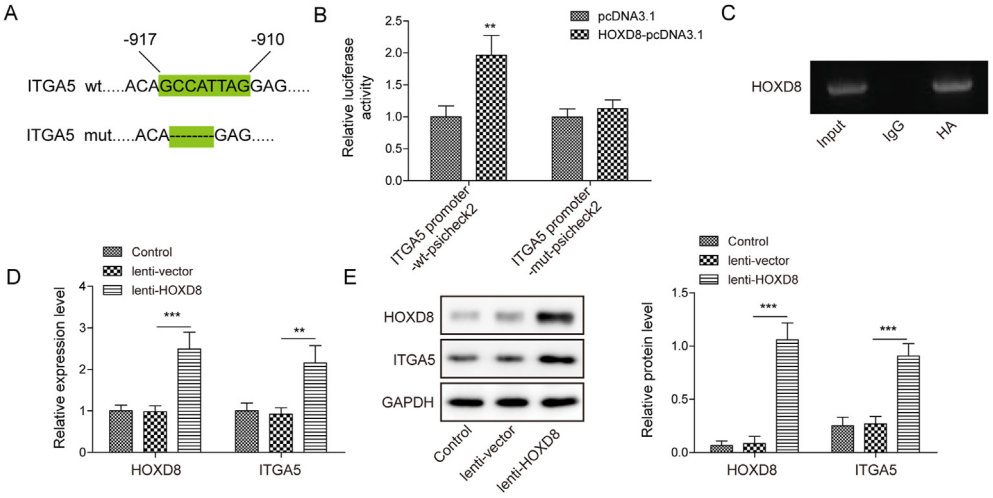

Fig. 4. HOXD8 transcriptionally activated ITGA5 expression. (A) JASPAR predicted the binding sites of HOXD8 in the promoter region of ITGA5. (B) The dual luciferase reporter gene assessed the activation of ITGA5 by HOXD8. CPT MSCs received lenti-PLVX-HA or HOXD8-HA infection. (C) ChIP-PCR validated the interaction between HOXD8 and the promoter region of ITGA5. (D) qRT-PCR investigated the expression levels of HOXD8 and ITGA5. (E) Western blot detected the protein abundance of HOXD8 and ITGA5. Each experiment was repeated in triplicates. Experimental data are indicated as means ± SD, n = 3. $^{**}P < 0.01$; $^{***}P < 0.001$.

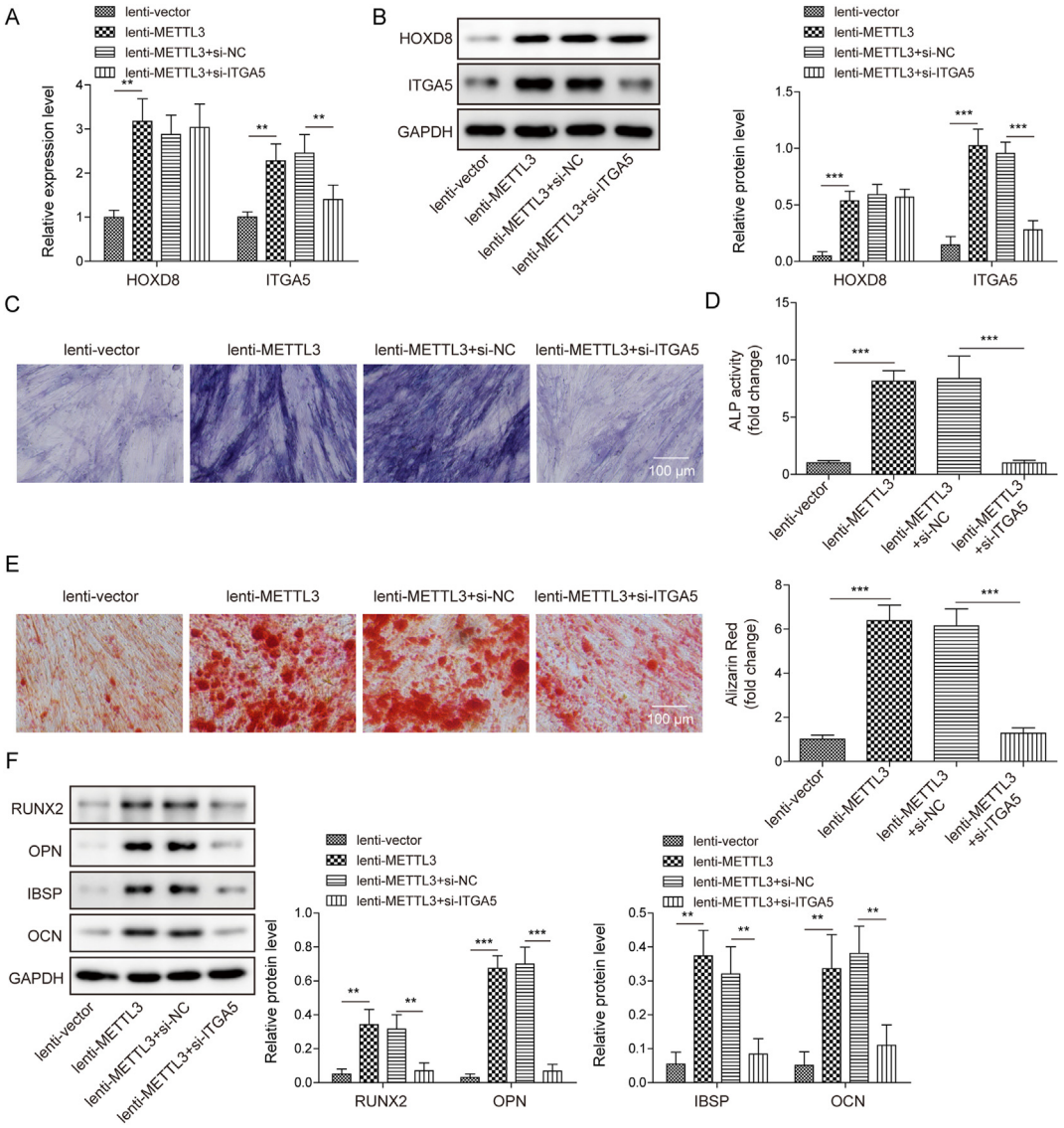

Fig. 5. METTL3 augmented ITGA5 expression via HOXD8 to enhance the osteogenic differentiation of CPT MSCs. CPT MSCs were infected with lenti-vector or lenti-METTL3 and transfected with si-NC or si-ITGA5. (A) qRT-PCR investigated the expression levels of HOXD8 and ITGA5. (B) Western blot detected the protein abundance of HOXD8 and ITGA5. (C) ALP staining was conducted by ALP Detection Kit (scale bar = 100 μm). (D) ALP activity was detected by ALP Assay Kit. (E) Osteogenic differentiation was assessed by ARS staining (scale bar = 100 μm). (F) Western blot detected RUNX2, OPN, IBSP, and OCN protein abundance. Each experiment was repeated in triplicates. Experimental data are indicated as means ± SD, n = 3. $^{**}P < 0.01$; $^{***}P < 0.001$.

osteogenic differentiation, which was consistent with the previous study in bone marrow stem cells. It originally supplemented the function of METTL3 in the osteogenic differentiation of CPT MSCs. Moreover, we found that HOXD8 was a key promoter of osteogenic differentiation in MSCs in previous studies [14,16]. We first verified that HOXD8 posed the m^6A modification, and the modification levels declined in CPT MSCs. Further, this study originally validated that METTL3 could bind with the HOXD8. And the overexpressed METTL3 enhanced the HOXD8 expression and promoted its RNA stability in CPT MSCs, which was also first demonstrated in this study. It clarified that METTL3 overexpression could promote the osteogenic differentiation of CPT MSCs and stabilize HOXD8 mRNA in an m^6A-dependent manner. This experimental evidence offered a specific interaction mechanism between them in the CPT MSCs' osteogenic differentiation process.

ITGA5 is one of the Integrin family members, exerting the regulator of signaling transduction of cells and cellular matrix. As reported, ITGA5 overexpression can promote osteoblasts proliferation, bone formation, and tibial compression reactions [27]. The overexpressed ITGA5 has been associated with enhanced osteogenic differentiation and bone regeneration of bone marrow-derived MSCs [28]. Our study found that ITGA5 expression was significantly lower in CPT MSCs than in normal iliac periosteum-derived MSCs. Furthermore, ITGA5 overexpression promoted osteogenic differentiation and marker protein expression. This founding was consistent with the previous founding, in which the overexpressed ITGA5 enhanced the osteogenic or osteoblast differentiation of MSCs that derived from bone marrow [29]. It provided an original cellular target of osteogenic differentiation in CPT clinical therapy. Additionally, we first validated that HOXD8 transcriptionally activated ITGA5 to enhance its expression in CPT MSCs, supplementing the regulatory mechanism of HOXD8 in the osteogenic differentiation process [14]. At the same time, this study proved that METTL3 augmented osteogenic differentiation by

promoting ITGA5's transcriptional activation via HOXD8. This study demonstrated the role and function of the METTL3/HOXD8/ITGA5 axis in CPT MSCs.

Overall, this research revealed that METTL3 augmented the MSCs' osteogenic differentiation by enhancing the HOXD8/ITGA5 axis in CPT. While the identified mechanisms still need to be validated in an *in vivo* model. Collectively, our experimental data uncovered the function and mechanism of METTL3 in the osteogenic differentiation of CPT MSCs, and the METTL3/HOXD8/ITGA5 axis could be utilized as the cellular target to enrich the current clinical treatment of CPT.

Ethics approval and consent to participate

All procedures in our work were kept in line with the approved principles from the Medical Ethics Committee of The Children's Hospital of Hunan Province (KYSQ2023-126).

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Authors' contributions

Weihua Ye: Conceptualization; Data Curation; Writing - Original Draft; Project administration; Zheng Liu: Methodology; Validation; Yaoxi Liu: Formal analysis; Investigation; Han Xiao: Visualization; Qian Tan: Resources; An Yan: Supervision; Guanghui Zhu: Writing - Review & Editing; Funding acquisition.

Consent for publication

The informed consent was obtained from study participants.

Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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