METTL14 mediates m^{6}a modification on osteogenic proliferation and differentiation of bone marrow mesenchymal stem cells by regulating the processing of pri-miR-873

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Abstract. N^6-methyl-adenosine (m^{6}a) is involved in the occurrence and development of various diseases such as autogenic immune disease and tumors. Methyltransferases regulate primary (pri)-microRNA (miRNA/miR) processing by mediating m6a modifications, consequently affecting pathological processes including immune-related diseases by regulating both innate and adaptive immune cells. However, the roles of m^{6}a on the biological functions of bone marrow mesenchymal stem cells (BMSCs) remain to be elucidated. The relative expression levels of methyltransferase-like 14 (METTL14) and other methyltransferases, demethylases, and miR-873 in bone samples from patients with osteoporosis and from normal individuals were measured by reverse transcription-quantitative PCR. Cell Counting Kit-8 assay was used to examine the proliferation of BMSCs. Co-immunoprecipitation (Co-IP) was used to investigate the binding of METTL14 to DiGeorge syndrome critical region 8 (DGCR8). RNA immunoprecipitation (RIP) was used to examine the binding of METTL14 to pri-miR-873. METTL14 and m^{6}a modifications were highly detected in patients with osteoporosis compared with the controls. Co-IP results indicated that silencing of METTL14 reduced METTL14 and m^{6}a modification levels in BMSCs. Downregulation of METTL14 significantly promoted the proliferation of BMSCs. RIP results suggested that METTL14/m^{6}a methylation modification promoted the processing of pri-miR-873 by binding to DGCR8 in BMSCs. Furthermore, overexpression of miR-873 inhibited the proliferation of BMSCs. The results also showed that miR-873 mimics significantly inhibited the proliferation in small interfering (si)-METTL14 transfected BMSCs; however, miR-873 inhibitors markedly promoted the proliferation of si-METTL14 transfected BMSCs. METTL14 and m^{6}a modifications were upregulated in osteoporosis samples. METTL14 promoted the processing of pri-miR-873 into mature miR-873 by regulating m^{6}a modification. Furthermore, overexpression of miR-873 significantly inhibited the proliferation of BMSCs. Therefore, the METTL14/m^{6}a/miR-873 axis may be a potential target for the treatment of osteoporosis.

Introduction

Osteoporosis is a progressive bone disease characterized by low bone mass and the prevalence of osteoporosis, based on 343,704 participants from 37 countries, was 19.7% (1,2). It can result in reduced bone strength, consequently increasing the risk of fracture. Osteoporosis is age-related and increasingly recognized as a major public health concern (3). The consequences of fracture can be serious, leading to morbidity and mortality of the patients, and osteoporotic fracture also increases the risk of subsequent fracture, but the effect of these subsequent fractures on mortality risk has not been systematically studied (4). Osteoporosis is a multifactorial disease caused by endogenous and exogenous factors such as age, low body mass, diseases disturbing the bone metabolism, long term medication and lack of physical exercise. Reduction in endogenous bone marrow only mesenchymal stem cells (BMSCs), such as proliferation and differentiation, is considered as one of the causes. The activity of BMSCs and their microenvironment may be disturbed in patients with osteoporosis (5,6).

N^6-methyl adenosine (m^{6}a) is an RNA modification method widely found in eukaryotic mRNAs and long non-coding RNAs (7). The level of RNA m^{6}a modification is regulated by both methyltransferase and demethylase. m^{6}a shows dynamic changes in different tissues and pathological processes (7). It participates in the formation and development of various diseases (8-10). For example, Clancy *et al* (9) found that knocking down the expression of fat mass and obesity

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Abbreviations: BMSCs, bone marrow mesenchymal stem cells; DGCR8, DiGeorge syndrome critical region 8; m^{6}a, N^6-methyl-adenosine; METTL14, methyltransferase-like 14; miR/miRNA, microRNA

Key words: METTL14, m^{6}a modifications, BMSCs, miR-873, osteoporosis

associated (FTO) in adipocytes significantly increased the expression level of m^{6}a. FTO was negatively correlated with m^{6}a expression levels. m^{6}a is involved in precursor cell differentiation by regulating the runt-related transcription factor (RUNX)1T1 shearing process. Baylin *et al* (8) verified that the AlkB homolog 5, RNA demethylase (ALKBH5)-m^{6}a-p53 signaling pathway served an important role in spermatogenesis and apoptosis in mouse testis tissue and sperm cells; Alkbh5 deficiency leads to aberrant spermatogenesis and apoptosis in mouse testes. ALKBH5 has been shown to regulate homeobox protein NANOG (NANOG) protein and mRNA expression in breast stem cells by m^{6}a modification (10). Next-generation sequencing was used to elucidate the human epigenome and the dysregulation in diseases (8); the association between epigenetic abnormalities and mutations in genes regulating DNA methylation has been revealed (8). Epigenetic alterations are novel targets in the development of specific biomarkers of diseases. However, the effects of m^{6}a modification on osteoporosis remain to be elucidated.

Methyltransferase-like 14 (METTL14) is the central component of the m^{6}a methylated transferase complex, which is involved in the dynamic reversible process of m^{6}a modification. METTL14 catalyzes m^{6}a methylation on mRNA or non-coding RNA to regulate gene expression and cell phenotypes (11). The present study found that methyltransferase-like 14 (METTL14) and m^{6}a modification levels were significantly upregulated in osteoporosis samples. METTL14 regulated the proliferation of BMSCs through the m^{6}a/miR-873 signaling pathway. Therefore, these findings may provide guidance for novel treatment of osteoporosis.

Materials and methods

Patient tissues and cell cultures. Bone samples were obtained from patients (male:female=1:1; age range 50-75) who underwent hip replacement in Tangdu Hospital, Air Force Military Medical University, Xi'an, Shaanxi; the cohort included 36 patients with osteoporosis and 36 healthy donors. Patients were recruited between September 2018 and March 2021. There was no significant difference in age and sex between the two groups. Normal donors with normal bone density had a T-score ≥-1.0 and patients with osteoporosis had a T score ≤-2.5 (3). Informed consent forms were signed by the patients and healthy donors, and all research protocols were reviewed and approved by the Medical Ethics Committee of Tangdu Hospital (approval no. 202109616). The present study was performed following the guidelines of the Declaration of Helsinki. BMSCs were isolated from femoral neck fracture when the patients or healthy donors underwent hip replacement. Cells were cultured with 1X mesenchymal stem cell medium (MSCM) + L-glutamine (Beijing Yuhengfeng Technology Co., Ltd.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and cultured at 37˚C in a 5% CO_2 incubator. When cells reached 90% confluence, they were passaged by washing with PBS, followed by dissociation with 0.25% Trypsin and 0.02% EDTA.

Reverse transcription-quantitative PCR (RT-qPCR) and cell transfection. Total RNA was extracted from patient samples and BMSCs (1x10^6), and cDNA was synthesized using a PrimeScript 1st strand cDNA Synthesis kit (Takara Bio, Inc.). RNA extraction, cDNA synthesis and qPCR were performed according to the manufacturer's protocols. NanoDrop 2000 (Thermo Fisher Scientific, Inc.) was used to measure the level and quality of RNA. The Power SYBRGreen PCR Master Mix (Thermo Fisher Scientific, Inc.) was used for qPCR. The forward and reverse primer pairs were as follows: Proliferating cell nuclear antigen (PCNA), Forward 5'-TGCTCTGAGGTACCTGAACT-3' and Reverse 5'-TGCTTCCTCATCTTCAATCT-3'; osteocalcin, Forward 5'-CGCTACCTGTATCAATGGCTGG-3' and Reverse 5'-CTCCTGAAAGCCGATGTGGTCA-3'; bone γ-carboxyglutamate protein 2 (Bglap2), Forward 5'-TAGTGAACAGACTCCGGCGCT-3' and Reverse 5'-TGTAGGCGGTCTTCAAGCCAT-3'; Sp7, Forward 5'-GGCTTTTCTGCGGCAAGAGGTT-3' and Reverse 5'-CGCTGATGTTTGCTCAAGTGGTC-3'; runt-related transcription factor 2 (Runx2), Forward 5'-TAAAGTGACAGTGGACGGTCCC-3' and Reverse 5'-TGCGCCCTAAATCACTGAGG-3'; sterol regulatory element-binding transcription factor 1 (SREBP1), Forward 5'-GACGGGGATCCCTCAGCTCAGAGCCGTGGT-3' and Reverse 5'-GACGGCAAGCTTTTAGCTTTTGTGAGCGGCATTTC-3'; adiponectin, Forward 5'-CAGGCCGTGATGGCAGAGATG-3' and Reverse 5'-GGTTTCACCGATGTCTCCCTTAG-3'; CCATT/enhancer binding protein α (C/EBPα), Forward 5'-CCACGCCTGTCCTTAGAAAG-3' and Reverse 5'-CAGTTTTTCCAATGTCACCCCTAC-3'; peroxisome proliferator-activated receptor γ (PPARγ), Forward 5'-AGCCTGCGAAAGCCTTTTGGTG-3' and Reverse 5'-GGCTTCACATTCAGCAAACCTGG-3'; METLL14 Forward 5'-CTGAAAGTGCCGACAGCATTGG-3' and Reverse 5'-CTCTCCTTCATCCAGATACTTACG-3'; METLL3 Forward 5'-AGCCTTCTGAACCAACAGTCC-3' and Reverse 5'-CCGACCTCGAGAGCGAAAT-3'; WT1-associated protein, Forward 5'-GCAACAACAGCAGGAGTCTGCA-3' and Reverse 5'-CTGCTGGACTTGCTTGAGGTAC-3'; METTL4 Forward 5'- CTTGGTCTGTGGAGGTAGTTGC-3' and Reverse 5'-CCAGTATAAGACCTTCGTAGGGC-3'; ALKBH5 Forward 5'-GCCTATTCGGGTGTCGGAAC-3' and Reverse 5'-CTGAGGCCGTATGCAGTGAG-3'; FTO Forward 5'-ACTTGGCTCCCTTATCTGACC-3' and Reverse 5'- TGTGCAGTGTGAGAAAGGCTT-3'; YTH m^{6}a RNA-binding protein 2, Forward 5'-GGTTCTGTGCATCAAAAGGATGG-3' and 5'-CCAAAGAATAGGAAAAGCCAATGG-3'; GAPDH, Forward 5'-CAGTGCCAGCCTCGTCCCGTAGA-3' and Reverse 5'-CTGCAAATGGCAGCCCTGGTGAC-3'. The $2^{-\Delta\Delta Cq}$ method was used to calculate all experimental data (11). The program used for qPCR was: 95˚C for 5 min, 45 cycles of 95˚C for 15 sec, 60˚C for 20 sec and 72˚C for 10 sec.

The construction and design of small interfering (si)RNA targeting METTL14 (si-METTL14; 5'-GCAGCACCUCGGUCAUUUA-3') and the negative control (si-NC; 5'-AAGCTTCATAAGGCGCATAGC-3'), miR-873 mimic (5'-GCAGGAACUUGUGAGUCUCCU-3') and the control (5'-UUCUCCGAACGUGUCACGUTT-3'), miR-873 inhibitor (5'-AGGAGACUCACAAGUUCCUGCTT-3') and the control (5'-CAGUACUUUUGUGUAGUACAA-3') were completed by Shanghai GenePharma Co., Ltd. Briefly, 25 nM plasmids were used for each transfection. Transfections of 5x10^5 cells were performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher

Scientific, Inc.) at 37˚C for 12 h. At 12 h post-transfection, culture medium was replenished with fresh culture medium containing 10% FBS. Each experiment was performed three times.

Cell Counting Kit-8 (CCK-8) assay. BMSCs in the logarithmic growth phase ($5x10^4$/well)were seeded into a 96-well culture plate. In brief, cell viability was determined on days 1, 2, 3 and 4; 10 μl CCK-8 solution (Dojindo Laboratories, Inc.) was added to the cells, and the cells were incubated at 37˚C for additional 2 h. Subsequently, the absorbance at 450 nm was detected using a microplate reader (680; Bio-Rad Laboratories, Inc.).

Western blotting. The total protein samples were extracted from transfected cells using RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China). A BCA kit (Pierce; Thermo Fisher Scientific, Inc.) was used to determine the protein concentration. Equal amounts (~30 μg) of samples were separated using 10% SDS-PAGE gels, and protein were then transferred to nitrocellulose membrane (MilliporeSigma). The membranes were blocked with PBS containing 5% non-fat milk for 1 h at room temperature, followed with incubation with primary antibodies against PCNA (1:1,000; cat. no. ab92552; Abcam), osteocalcin (1:2,000; cat. no. sc-376835; Santa Cruz), Bglap (1:1,000; cat. no. MBS3223783; MyBioSource, Inc.), Sp7 (1:2,000; cat. no. ab209484; Abcam), Runx2 (1:2,000; cat. no. ab92336; Abcam), SREBP1 (1:1,000; cat. no. ab28481; Abcam), adiponectin (1:2,000; cat. no. ab181281; Abcam), C/EBPα, (1:2,000; cat. no. ab40761; Abcam), PPARγ (1:2,000; cat. no. ab59256; Abcam) or β-actin (1:1,000; cat. no. ab8226; Abcam) in a cold room at 4˚C overnight. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated horse anti-mouse (1:2,000; cat. no. 7076; Cell Signaling Technology, Inc.) or goat anti-rabbit IgG (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.) at room temperature for 1 h. Protein bands were visualized using an ECL kit (Pierce; Thermo Fisher Scientific, Inc). The density of bands was semi-quantified using ImageJ (version 1.44; National Institutes of Health).

Cell cycle distribution. Cells were seeded onto 6-well plates ($4x10^5$ cells/well). The cell suspension was spun down using a low-speed centrifugation (120 x g at 4˚C for 5 min. Cell pellets were washed and re-suspended in PBS, fixed with pre-chilled ethanol (70%) and left at 4˚C for two days. Prior to being subjected to flow cytometry, cells were lysed using ice-cold methanol, centrifuged at 180 x g at 4˚C for 5 min and then re-suspended in propidium iodide (PI; MilliporeSigma) staining buffer containing PI (50 μl/ml) and RNase A (250 μl/ml). Cell cycle distribution was evaluated using a flow cytometer (BD Biosciences), and the results were analyzed by FlowJo version 7.6 software (FlowJo LLC).

Osteogenic differentiation of BMSCs. For osteogenic differentiation of BMSCs, when the cells reached 70% confluence, the culture medium was replenished with fresh medium containing L-DMEM, 10% FBS, 100 nM dexamethasone, 10 mM β-glycerophosphate and 250 mM L-ascorbic acid (Gibco; Thermo Fisher Scientific, Inc.). The cells were cultured for 22 days at 37˚C and the medium was replenished every two days. Subsequently, cells were fixed using 4% paraformaldehyde at room temperature for 30 min and stained using 0.1% Alizarin Red S (cat. no. A5533; MilliporeSigma) at room temperature until obvious calcium deposits appeared.

Adipogenic differentiation of BMSCs. BMSCs were seeded in a 6-well plate ($4x10^5$ cells/well). When the cells reached 70% confluence, they were incubated with adipocyte-inducing medium comprising L-DMEM, 10% FBS, 1.0 mmol/l dexamethasone, 0.5 mmol/l isobutyl-methylxanthine and 10 mg/l insulin (all from MilliporeSigma) at 37˚C for 12 days. Cells were then stained with 0.3% Oil Red O (cat. no. O8018; Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 1 h.

Co-immunoprecipitation (Co-IP). A Pierce Co-Immunoprecipitation kit (Thermo Fisher Scientific) was used. Cell lysates were incubated with protein A agarose with gentle shaking at 4˚C for 10 min. To remove non-specific binding, the samples were centrifuged at 4˚C at 1,000 x g for 15 min. The lysates with equal amounts (1 mg) of total protein were incubated with 2 μg rabbit anti-METTL14 antibody (cat. no. HPA038002; MilliporeSigma) and a negative control rabbit IgG antibody at 4˚C overnight. Subsequently, the samples were incubated with protein A/G agarose at 4˚C for another 2 h. After centrifugation at 1,000 x g at 4˚C for 10 min, the agarose was rinsed using cold lysis buffer, and proteins were eluted using Laemmli buffer (Bio-Rad Laboratories, Inc.) and subjected to 10% SDS-PAGE. The mutual binding between different proteins by the complexes bound after immunoprecipitation was detected by western blotting analysis according to the aforementioned protocol.

RNA m^6a RIP. Total RNA was extracted from $1x10^6$ isolated BMSCs of osteoporosis group and controls following the transfection with si-METTL14 or si-NC. m^6a -IP-qPCR was performed following a protocol with minor modifications. Total RNAs were extracted using TRIzol® (Thermo Fisher Scientific, Inc.) and purified through GenElute mRNA Miniprep kit (MilliporeSigma). All RNAs were placed in a sonicator at 30 Hz for 3 min at room temperature and treated with DNase I; the samples were sonicated for 10 sec. In total, 5 μg of purified mRNAs were fragmented into 200-300 nt fragments by incubation in RNA fragmentation reagent (Thermo Fisher Scientific, Inc.) at 94˚C for 30 sec and then stopped with stop solution. Of the fragmented mRNAs, 10% were kept as input. The remaining mRNAs were incubated with 6.25 μg m^6a-specific antibody (cat. no. 202003; Synaptic Systems GmbH) or rabbit IgG (Beyotime Institute of Biotechnology) in IP buffer [50 mM Tris-HCl (pH 7.4), 750 mM NaCl, 0.5% Igepal CA-630, 0.4 U/μl RNasin (Promega Corporation)] for 2 h at 4˚C. Then the mixture was incubated with 25 μl Dynabeads Protein A (Thermo Fisher Scientific, Inc.) for another 2 h at 4˚C The antibodies were bound to the magnetic beads and incubated in 100 μl RIP Wash buffer (Sigma Aldrich) at room temperature for 30 min. After extensive washing, the bound mRNAs were eluted with 6.7 mM N6-methyladenosine (Sigma) in IP buffer and then recovered with ethanol precipitation. The immunoprecipitated mRNAs and input mRNAs

were processed as in RT-qPCR aforementioned to evaluate the relative expression levels of primary (pri)-miRNA.

Biotin-labeled pri-let-7e probe was generated by *in vitro* transcription followed by biotin-labeling (Thermo Fisher Scientific, Inc.). A total of 1 pmol of biotin-labeled RNA probes was incubated with increasing concentrations of purified recombinant protein expressed in *E. coli* in reaction buffer [20 mM HEPES-KOH (pH 8.0), 150 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% (w/v) Triton X-100] at 4˚C for 1 h. After the reaction, the samples mixed with loading buffer [10 mM Tris-HCl, 3% (w/v) sucrose and dyes] were loaded on 8% (w/v) native gel and run at a constant voltage of 100 V. After electrophoresis, the probe was transferred onto hybond-N+ membrane and detected using Chemiluminescent Nucleic Acid Detection Module Kit (Thermo Fisher Scientific, Inc.).

cDNA was synthesized using a PrimeScript 1st strand cDNA Synthesis kit (Takara Bio, Inc.). RNA extraction, cDNA synthesis and qPCR were performed according to the manufacturer's protocols. NanoDrop 2000 (Thermo Fisher Scientific, Inc.) was used to measure the level and quality of RNA. The Power SYBRGreen PCR Master Mix (Thermo Fisher Scientific, Inc.) was used for qPCR. The forward and reverse primer pairs were as follows: miR-873, Forward 5'-GCA GGAACUUGUGAGUCUCCU-3' and Reverse 5'-AGGAGA CUCACAAGUUCCUGC-3'; let-7e Forward 5-'GGGTGA GGTAGGAGGTTGT-3' and Reverse 5'-CAGTGCGTGTCG TGGAGT-3'; GAPDH, Forward 5'-CAGTGCCAGCCTCGT CCCGTAGA-3' and Reverse 5'-CTGCAAATGGCA GCC CTGGTGAC-3'. The $2^{-\Delta\Delta Cq}$ method was used to calculate all experimental data (12). The program used for qPCR was: 95˚C for 5 min, 45 cycles of 95˚C for 15 sec, 60˚C for 20 sec and 72˚C for 10 sec.

Statistical analysis. SPSS v15.0 statistical software (SPSS, Inc.) was used to analyze the experimental data. All data are expressed as the mean ± SD. Differences between and among groups were respectively analyzed using t-test or one-way ANOVA followed by Tukey's post-hoc test. To investigate the linear correlations between two variables, Pearson's correlation test was performed. $P<0.05$ was considered to indicate a statistically significant difference.

Results

m^{6}a modification levels are significantly upregulated in osteoporosis samples and downregulated METTL14 significantly inhibits m^{6}a modification levels in BMSCs. The present study compared the m^{6}a modification levels in osteoporotic and healthy bone samples using RT-qPCR, and the results indicated that the relative levels of m^{6}a modification in osteoporosis tissues were significantly higher compared with the controls (Fig. 1A). To further explore the cause of the higher levels of m^{6}a modification, the relative levels of methyltransferases and demethylases in the two groups were compared. The data revealed that the relative content of METTL14 in osteoporosis samples was notably higher than other enzymes such as METTL3, WTAP, METTL4,ALKBH5, FTO and YTHDF2 compared with normal bone samples (Fig. 1B). Furthermore, the relative expression levels of m^{6}a and METTL14 in BMSCs were significantly increased in osteoporosis BMSCs (Fig. 1C and D). In addition, the protein levels of METTL14 were increased in osteoporosis BMSCs (Fig. 1E). The m^{6}a content in total RNA and expression of METTL14 was reduced in cells treated with si-METTL14 (Fig. 1F and G). Co-IP experiments were used to further verify the relationship between METTL14 and m^{6}a. The results showed that METTL14 could bind to the DiGeorge syndrome critical region 8 (DGCR8) protein in BMSCs. Following treatment with Rnase, the binding effects between METTL14 and DGCR8 was inhibited (Fig. 1H). Silencing of METTL14 significantly reduced the binding effects of METTL14 and DGCR8 and the level of m^{6}a modification (Fig. 1I).

Silencing of METTL14 promotes the proliferation and differentiation of BMSCs. To explore the effects of METTL14 silencing, BMSCs were transfected with si-METTL14 and cultured for 3 days. As a result, the proliferation of BMSCs was significantly improved compared with si-NC group (Fig. 2A). The mRNA and protein expression levels of cell proliferation marker PCNA were examined by RT-qPCR and western blotting, respectively, and the data show that the expression of PCNA in BMSCs was significantly increased following transfection with si-METTL14 (Fig. 2B and C). Flow cytometry was used to detect the effects of METTL14 on the cell cycle of BMSCs. Following the transfection with si-METTL14, the proportion of cells at S phase was increased significantly in BMSCs compared with sh-NC, whereas the percentage of cells at the G_0/G_1 phase were markedly decreased (Fig. 2D-F). In addition, the results of Alizarin Red S and Oil Red O staining indicated that knockdown of METTL14 promoted the differentiation of BMSCs (Fig. 2G and I). Consistent with these findings, the expression levels of corresponding differentiation markers osteocalcin, Bglap2, Sp7, Runx2, SREBP1, Adiponectin, C/EBPa and PPARr were also increased in cells treated with si-METTL14 (Fig. 2H and J).

METTL14/m^{6}a modification promotes processing of pri-miR-873 by binding to DGCR8 in BMSCs. A study has shown that m^{6}a modification is involved in the processing of pri-miRNA and promotes the conversion of pri-miRNA into mature miRNA (8). A previous study reported that, miR-873-3p targets HDAC4 to stimulate matrix metalloproteinase-13 expression upon parathyroid hormone exposure in rat osteoblasts (13). Furthermore, miR-873 could affect the proliferation and migration of different types of cells (14-16). The results of the present study showed that the levels of miR-873 were significantly upregulated in osteoporosis samples ($r=0.589$; Fig. 3A). The expression of METTL14 and miR-873 were positively correlated in osteoporosis samples using Pearson's correlation test (Fig. 3B). Following transfection with si-METTL14, miR-873 was significantly reduced in BMSCs (Fig. 3C), whereas the levels of pri-miR-873 were significantly increased (Fig. 3D). RIP was used to verify the relationship between METTL14/m^{6}a modification and pri-miR-873. The results suggested that in BMSCs transfected with si-METTL14, the content of pri-miR-873 bound to DGCR8 was significantly reduced (Fig. 3E). Similarly, the content of pri-miR-873 modified by m^{6}a was also markedly decreased after the transfection with si-METTL14 (Fig. 3F).

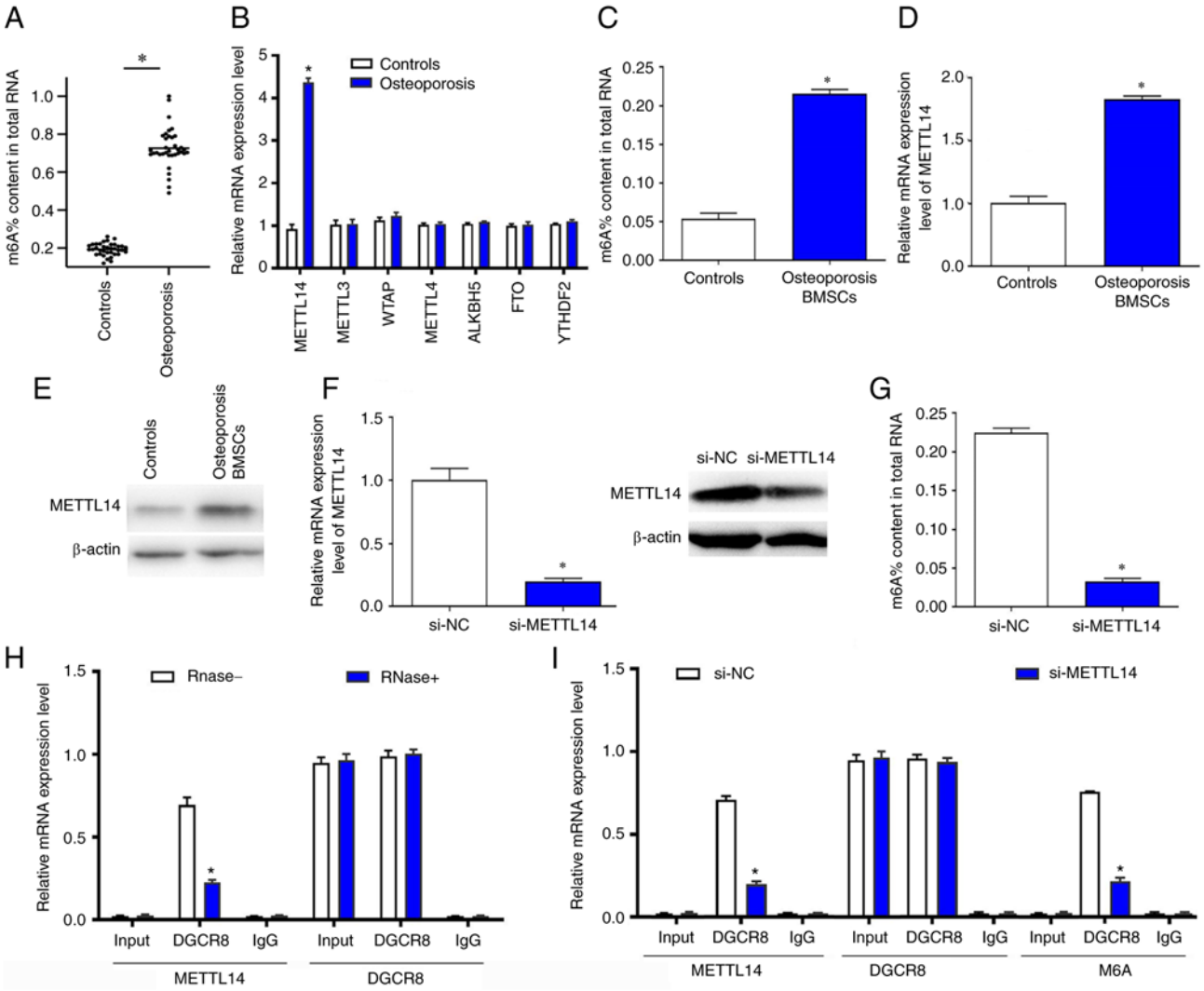

Figure 1. m^{6}a modification levels were significantly overexpressed in patients with osteoporosis and downregulated METTL14 significantly inhibited m^{6}a modification levels in BMSCs. (A) m^{6}a content in healthy controls and patients with osteoporosis was detected by RT-qPCR assay. (B) METTL14 and other methyltransferase and demethylase mRNA expression levels in the two experimental groups were measured using RT-qPCR. (C) m^{6}a content in total RNA of osteoporosis BMSCs after transfection with si-NC and si-METTL14 were detected. (D) METTL14 relative expression levels were evaluated in BMSCs. (E) The protein expression levels of METTL14 was evaluated in osteoporosis BMSCs by western blotting. (F) The mRNA levels of METTL14 was decreased in cells transfected with si-METTL14. (G) The protein levels of METTL14 and m^{6}a% content in total RNA was reduced in cells treated with si-METTL14. (H) The relationship between METTL14 and DGCR8 was verified by Co-IP assay. (I) Co-IP was used to explore the relationship between METTL14 and m6a modification. The data are presented as the mean ± SD; *P<0.05 vs. control BMSCs or si-NC. ALKBH5, AlkB homolog 5, RNA demethylase; BMSCs, bone marrow mesenchymal stem cells; Co-IP, co-immunoprecipitation; DGCR8, DiGeorge syndrome critical region 8; FTO, fat mass and obesity associated; m^{6}a, n6-methyl-adenosine; METTL14, methyltransferase-like 14; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering RNA.

Overexpression of miR-873 significantly inhibits BMSCs cell proliferation. To investigate the effects of miR-873 on BMSCs, gain- and loss-of function experiments were performed. The transfection efficiencies of miR-873 inhibitors and mimics were confirmed by RT-qPCR (Fig. 4A). CCK-8 assays were used to examine proliferation. The results showed that the proliferation of BMSCs after transfection with miR-873-mimics was significantly lower compared with the miR-NC group at the 72 h timepoint (Fig. 4B). Following overexpression of miR-873, the mRNA and protein expression levels of PCNA in BMSCs were markedly reduced compared with miR-NC group (Fig. 4C). Flow cytometry analysis indicated that the percentage of cells in the S phase was markedly decreased in BMSCs transfected with miR-873-mimics compared with miR-NC, whereas the percentage of cells in the G_0/G_1 phase was significantly increased (Fig. 4D-F). Furthermore, the results of Alizarin Red S and Oil Red O staining revealed that overexpression of miR-873 markedly inhibited the differentiation of BMSCs (Fig. 4G and I). Consistent with these findings, the mRNA and protein levels of corresponding differentiation markers osteocalcin, Bglap2, Sp7, Runx2, SREBP1, adiponectin, C/EBPa and PPARr were also reduced in cells treated with miR-873 mimics (Fig. 4H and J).

METTL14/miR-873 signaling is involved in the regulation of BMSC proliferation. To further explore the effects of the METTL14/miR-873 signaling on BMSCs, cells were transfected with si-NC, si-METTL14 or si-METTL14 + miR-873

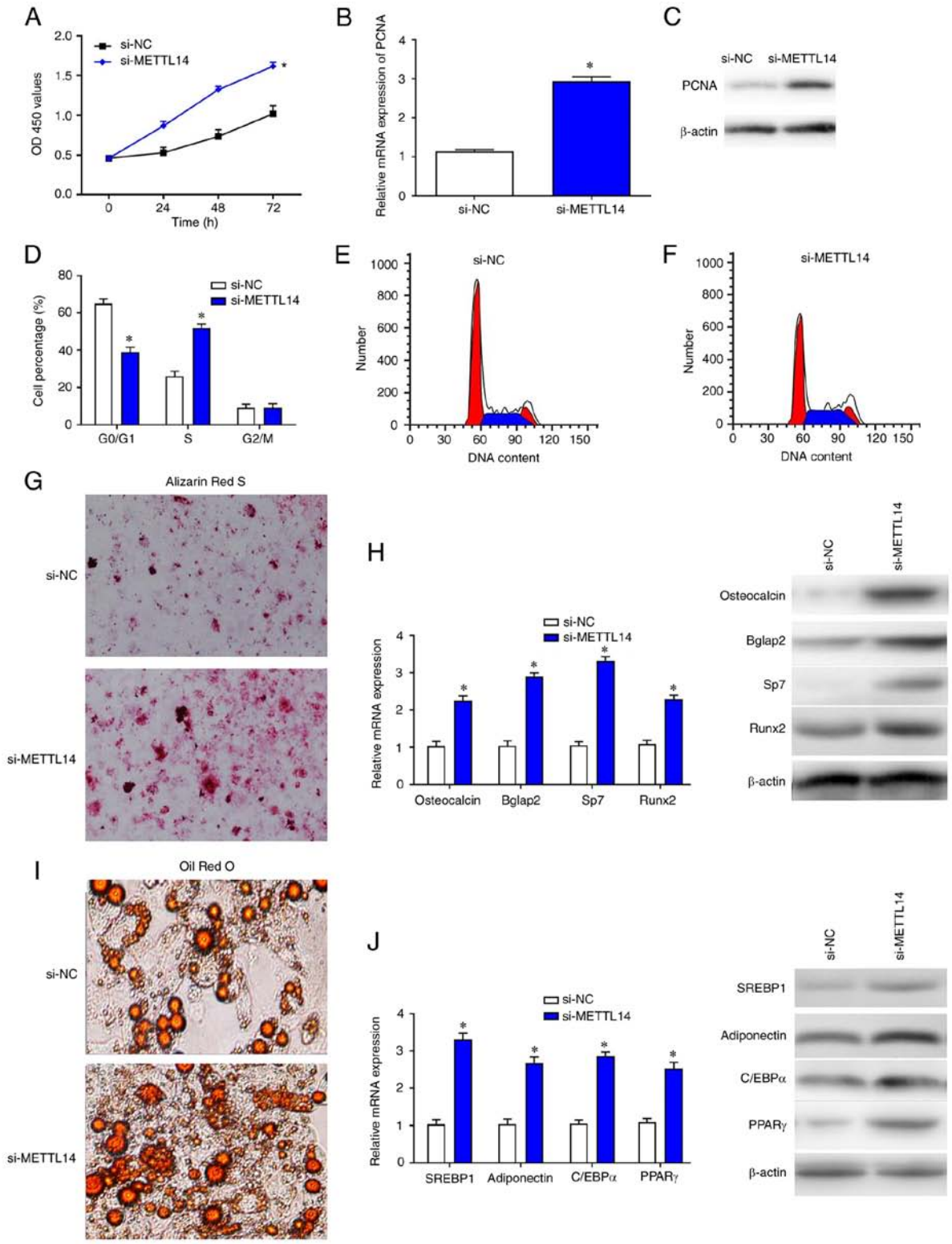

Figure 2. METTL14 downregulation promotes BMSC proliferation. (A) Cell Counting Kit-8 assay was used to examine the proliferative ability of BMSCs after transfection with si-NC and si-METTL14. (B) Reverse transcription-quantitative PCR and (C) western blotting were used to measure the expression levels of PCNA in transfected cells. (D-F) Flow cytometry was used to detect the percentage of BMSCs at different phases of the cell cycle. (G and I) The differentiation of cells was enhanced after the treatment with si-METTL14 (magnification, x200). (H and I) The expression of differential-associated markers were increased in cells transfected with si_METTL14. The data are presented as the mean ± SD; *P<0.05 vs. si-NC. BMSCs, bone marrow mesenchymal stem cells; C/EBPα, CCATT/enhancer binding protein α; METTL14, methyltransferase-like 14; NC, negative control; PCNA, proliferating cell nuclear antigen; PPARγ, peroxisome proliferator-activated receptor γ; RunX, runt-related transcription factor; si, small interfering RNA; SREBP1, sterol regulatory element binding protein 1.

mimics and cultured for 3 days. The results revealed that the proliferative ability and PCNA mRNA expression levels, (Fig. 5A-C) in cells co-transfected with si-METTL14 + miR-873 mimics were significantly reduced compared with those transfected with si-METTL14 alone. In addition, there were fewer cells at the G_0/G_1 stage and more cells at S stage

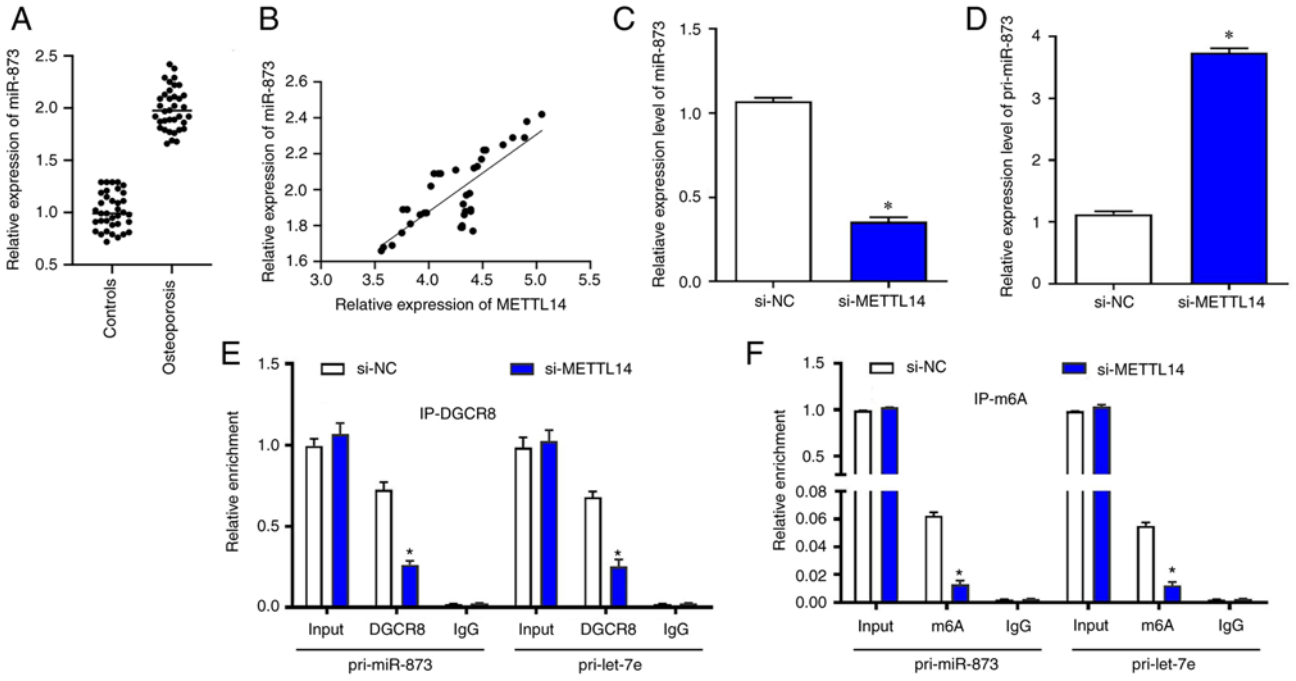

Figure 3. METTL14/m^{6}a modification promotes pri-miR-873 processing by binding to DGCR8. (A) The relative expression levels of miR-873 in control and osteoporosis groups were detected by reverse transcription-quantitative PCR in BMSCs. (B) The expression of METTL14 and miR-873 were positively associated in osteoporosis samples. Relative expression levels of (C) miR-873 and (D) pri-miR-873 were measured in transfected cells. (E) The content of pri-miR-873 bound to DGCR8 was measured by RIP in BMSCs. (F) The content of m6a-modified pri-miR-873 was detected by RIP in BMSCs. The data are presented as the mean ± SD; *P<0.05 vs. si_NC. BMSCs, bone marrow mesenchymal stem cells; DGCR8, DiGeorge syndrome critical region 8; m^{6}a, N^6-methyl-adenosine; METTL14, methyltransferase-like 14; miR, microRNA; pri-, primary; RIP, RNA immunoprecipitation.

after the transfection with si-METTL14, which was reversed by the treatment with miR-873 mimics (Fig. 5C). However, proliferation and PCNA expression were markedly increased in cells co-transfected with si-METTL14 + miR-873 inhibitors compared with those transfected with si-METTL14 alone (Fig. 5D-F). In addition, there were fewer cells at G_0/G_1 stage and more cells at S stage after the transfection with si-METTL14, which was strengthened after the treatment with miR-873 inhibitors (Fig. 5F). The expression of corresponding differentiation markers osteocalcin, Bglap2, Sp7, Runx2, SREBP1, adioponectin, C/EBPa and PPARr were enhanced in cells treated the si-METTL14, and these effects were reversed by miR-873 mimics co-transfection and strengthened by miR-873 inhibitors (Fig. 5G).

Discussion

Osteoporosis is a multifactorial bone disease and is characterized by loss of bone mass and reduced bone strength (1). The consequences of osteoporosis-related fracture can be life threatening (2). Impaired proliferation and differentiation in endogenous BMSCs is considered as one of the causes of osteoporosis (3).

RNA m^{6}a modification was first reported in 1974 (13). Subsequent research found that RNA m^{6}a modification widely exists in mouse bovine and rabbit zygotes and exhibits dynamic and reversible changes in different developmental stages and different tissues (7). RNA m^{6}a modification is jointly regulated by methyltransferases including METTL3, METTL14, WT1-associated protein and KIAA1429, and by demethylases such as FTO and ALKBH5 (17-21). The DGCR8 gene encodes a subunit of the microprocessor complex that mediates the biogenesis of miRNAs from the pri-miRNA transcript (9,10). It has also been reported that METTL14/m^{6}a modification can regulate the processing of pri-miR-126 by binding to DGCR8 protein and serve an important role in tumor metastasis (18). Previous studies have shown that m^{6}a modification can mark pri-miRNA molecules and recognize DGCR8 molecules in a METTL3/m^{6}a-dependent manner, thereby participating in the maturation of pri-miRNAs and leading to differential expression of miRNAs in various biological processes (19,20).

The present study found that the level of m^{6}a modification was significantly higher in osteoporosis samples compared with controls, and that METTL14 expression levels in the osteoporosis group were markedly upregulated compared with other methyltransferases and demethylases. The results of Co-IP indicated that METTL14 could bind to DGCR8 in BMSCs. Silencing of METTL14 not only reduced the METTL14 expression but also decreased the level of m^{6}a modification. These findings suggested that METTL14 regulated the m^{6}a methylation modification by binding to DGCR8. Furthermore, knockdown of METTL14 significantly promoted the proliferation of BMSCs, increased the proportion of cells at S phase and decreased the percentage of cells at G_0/G_1 phase. These results indicated that METTL14 may affect the proliferation of BMSCs. However, the detailed mechanisms remain unclear and require further investigation. Consistent with the present findings, METTL14 could regulate the proliferation of tumor cells (11). Furthermore, METTL14 suppressed the metastatic potential of hepatocellular carcinoma by modulating N6-methyladenosine-dependent primary microRNA processing (19).

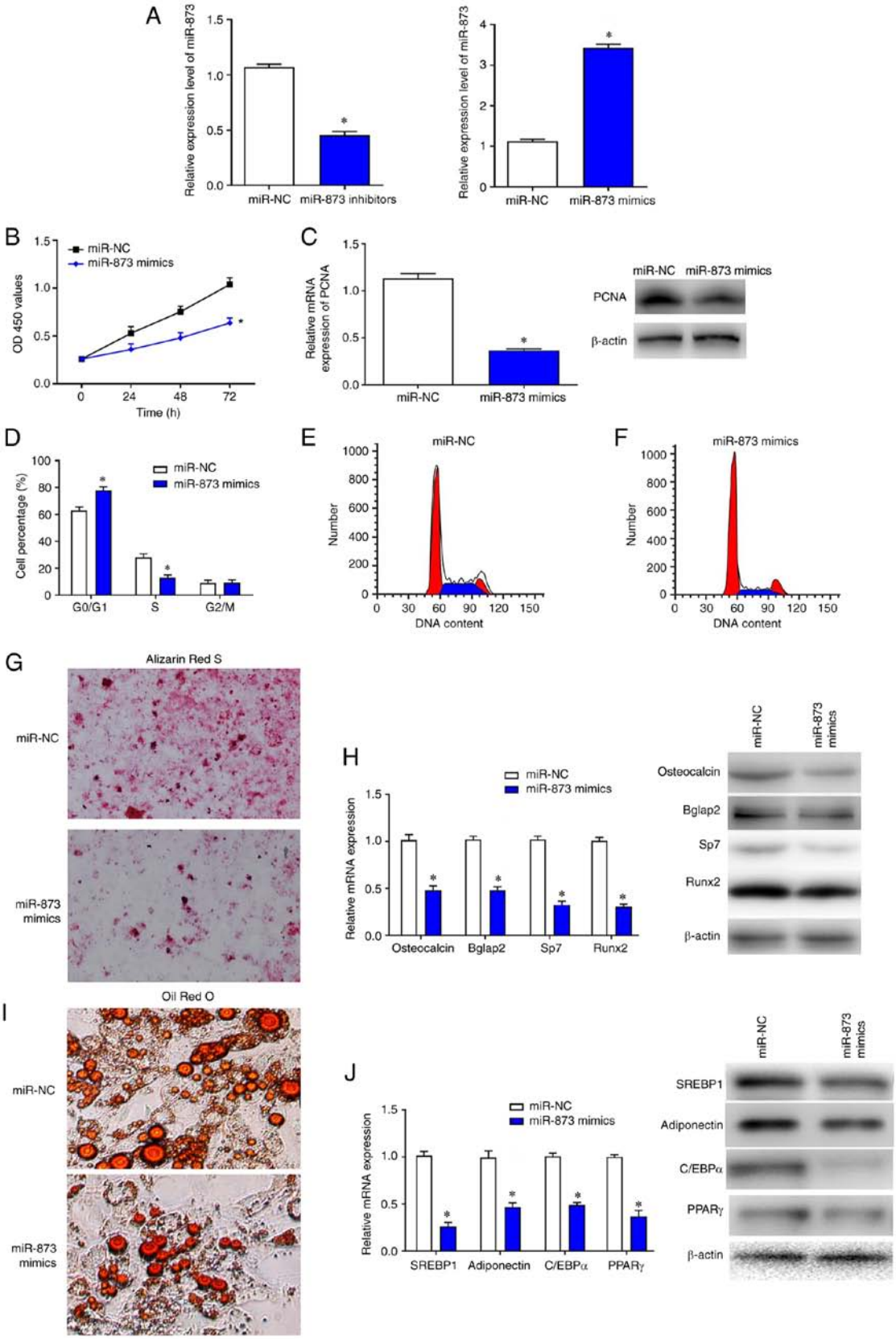

Figure 4. Overexpression of miR-873 significantly inhibits BMSC proliferation. (A) The transfection efficiencies of miR-873 inhibitors and mimics were confirmed by RT-qPCR. (B) The proliferation of BMSCs was detected by Cell Counting Kit-8 assay. (C) RT-qPCR and western blotting were used to measure the relative expression levels of PCNA mRNA and protein, respectively. (D) Flow cytometry was used to evaluate the proportion of BMSCs at different phases. (E and F) The DNA contents in miR-NC and miR-873 mimics transfected cells were evaluated. (G and I) The differentiation of cells was inhibited by the transfection with miR-873 mimics (magnification, x200). (H and J) Western blots revealed the expression of differentiation-related markers were reduced in miR-873 mimic transfected cells. The data are presented as the mean ± SD; *P<0.05 vs. miR-NC. Bglap2, bone γ-carboxyglutamate protein 2; BMSCs, bone marrow mesenchymal stem cells; C/EBPα, CCATT/enhancer binding protein α; miR, microRNA; NC, negative control; OD, optical density; PCNA, proliferating cell nuclear antigen; PPARγ, peroxisome proliferator-activated receptor γ; RT-qPCR, reverse transcription-quantitative PCR; RunX, runt-related transcription factor; SREBP1, sterol regulatory element binding protein 1.

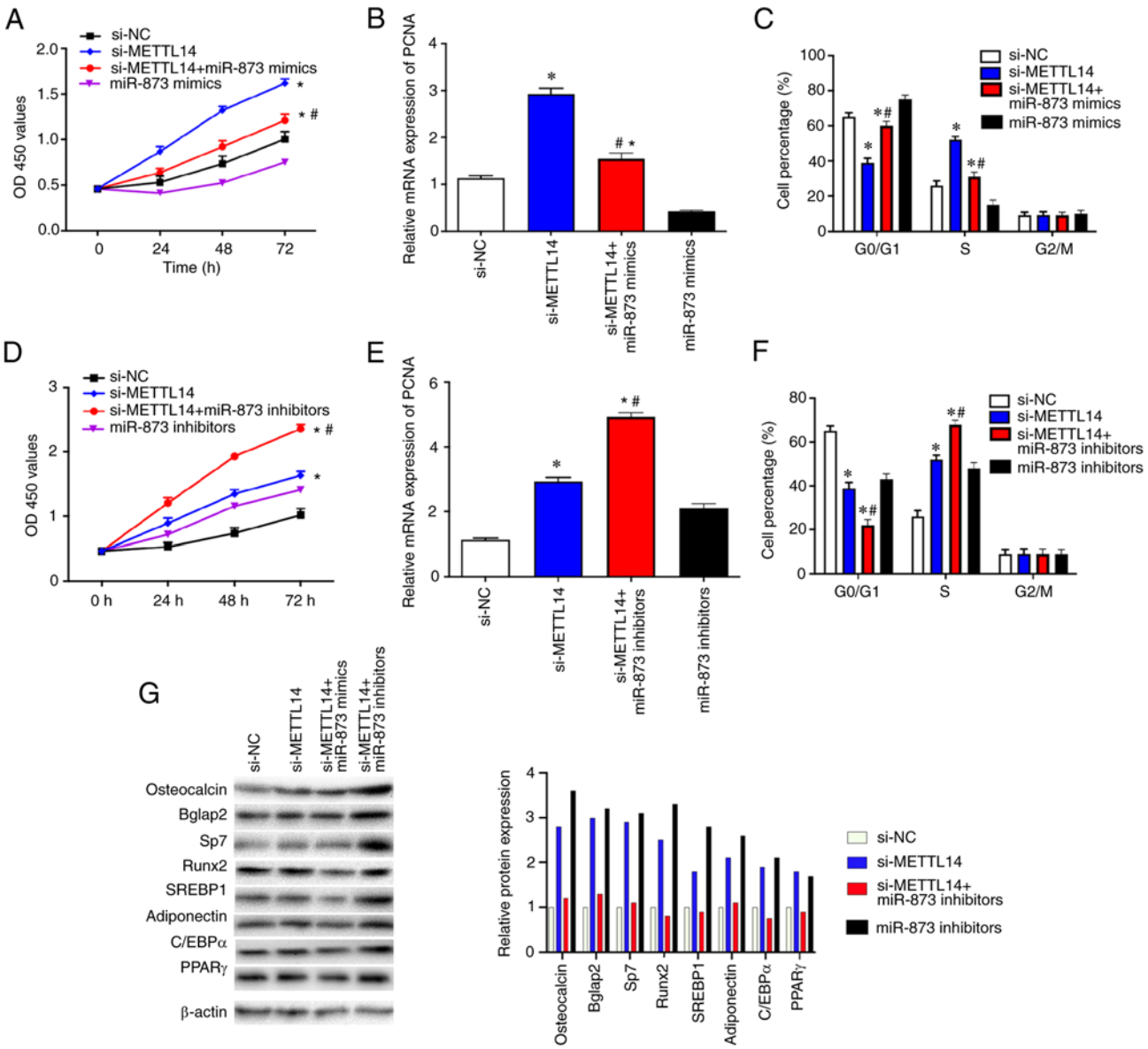

Figure 5. METTL14/miR-873 axis serves an important role in the proliferation of BMSCs. (A) CCK-8 assay was used to examine the proliferation of BMSCs transfected with si-NC, si-METTL14 or si-METTL14 + miR-873 mimics. (B) RT-qPCR was used to determine the mRNA expression levels of PCNA in transfected BMSCs. (C) Flow cytometry was used to investigate the cell cycle in transfected BMSCs. (D) CCK-8 assay was used to detect the proliferation of BMSCs after the transfection with si-NC, si-METTL14, or si-METTL14 + miR-873 inhibitors. (E) RT-qPCR was used to measure the PCNA mRNA expression levels in transfected BMSCs. (F) Flow cytometry was used to investigate the cell cycle of transfected BMSCs. (G) The expression of corresponding differentiation markers osteocalcin, Bglap2, Sp7, Runx2, SREBP1, adioponectin, C/EBPa and PPARr were examined by western blotting. The data are presented as the mean ± SD; *P<0.05 vs. si-NC; #P<0.05 vs. si-METLL14. BMSCs, bone marrow mesenchymal stem cells; C/EBPα, CCATT/enhancer binding protein α; METTL14, methyltransferase-like 14; miR, microRNA; NC, negative control; OD, optical density; PCNA, proliferating cell nuclear antigen; PPARγ, peroxisome proliferator-activated receptor γ; RNA; RT-qPCR, reverse transcription-quantitative PCR; RunX, runt-related transcription factor; si, small interfering SREBP1, sterol regulatory element binding protein 1.

miR-873 is an endogenous, non-coding single-stranded RNA molecule that is involved in the occurrence and development of various diseases. Gao *et al* (14) found that miR-873 was highly expressed in lung adenocarcinoma cell lines and tissues, and the overexpression of miR-873 could significantly facilitate the proliferation and migration of these cells. miR-873 is also found to inhibit colorectal cancer cell by directly targeting TAB1 and TRAF5 (15). Recently, it has been reported that miR-873 is significantly upregulated in congenital heart disease tissues and can significantly inhibit the proliferation of H9C2 cardiomyocytes (16).

The present study found that miR-873 was significantly overexpressed in patients with osteoporosis. Silencing of METTL14 significantly reduced the levels of pri-miR-873 bound to DGCR8. Knockdown of METTL14 also decreased the expression of pri-miR-873 modified by m^6a in BMSCs. Furthermore, overexpression of miR-873 inhibited the proliferation of BMSCs, reduced the proportion of cells at S phase and increased the percentage of cells at G_0/G_1 phase. Further experiments revealed that miR-873 mimics significantly inhibited the upregulated proliferation of BMSCs caused by si-METTL14. In addition, miR-873 inhibitors further promoted the proliferation of BMSCs transfected with si-METTL14.

These data suggested that METTL14 may regulate the proliferation of BMSCs through m^{6}a/miR-873. Consistent with the present findings, previous studies indicate that miR-873 is involved in the regulation of different types of cells (14-16). However, there are some limitations in the present study. For example, other YTH protein family members could also serve a role in this process to recognize the m^{6}a modification.

In conclusion, the results of the present study showed that METTL14 and m^{6}a modification levels were significantly higher in bone samples from patients with osteoporosis compared with normal individuals. METTL14 promoted the processing of pri-miR-873 into mature miR-873 by mediating m^{6}a modification, thereby inhibiting the proliferation of BMSCs. Thus, METTL14/m^{6}a/miR-873 axis may be a novel candidate for the treatment of osteoporosis. However, the treatment of osteoporosis may need to be developed by the promotion of osteoblasts by BMSCs and the inhibition of osteoclast differentiation; these issues should be addressed in a future study.

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Availability of data and materials

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

Authors' contributions

BL initiated this study. XD, BL, JZ, XL, KY, KR, XZ, XB and WG conducted the experiments and data analyses. XD and BL confirm the authenticity of all the raw data. All authors drafted the manuscript and all authors read and approved the final manuscript.

Ethics approval and consent to participate

Informed consents were signed by the patients and healthy donors, and all research protocols were reviewed and approved by the Medical Ethics Committee of Tangdu Hospital (approval no. 202109616).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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