Melatonin Enhances Osteoblastogenesis from Senescent Mesenchymal Stem Cells via MMSET Mediated Chromatin Remodeling

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Abstract

Large numbers of elderly people have aging-associated osteoporosis, but efficient approaches to ameliorate bone loss are limited due to our poor understanding of the underlying mechanisms. In this study, we found that melatonin levels in bone marrow decreased with age, and melatonin primarily enhanced the osteogenic potential of mesenchymal stem cells (MSCs) derived from elderly donors compared with fetal- or young adult-derived MSCs. Mechanistic studies indicated melatonin treatment alleviated the senescence-related hypermethylation of the MMSET promoter, leading to elevated expression of the histone methyltransferase NSD2, and promoted the histone H3 dimethylation modification at lysine 36 of the osteogenic genes RUNX2 and SP7/OSTERIX as a consequence. MMSET depletion partially abolished the effects of melatonin on osteogenesis in senescent MSCs in vitro. Moreover, melatonin treatment promoted bone formation and alleviated the progression of osteoporosis in a mouse model of aging. Clinically, severity of senile osteoporosis (SOP) in patients was associated with melatonin levels in bone marrow plasma and the MMSET expression in MSCs, and melatonin treatment enhanced osteoblastogenesis from MSCs derived from SOP patients. Our study discovered a previously unreported epigenetic regulatory role for melatonin in alleviating MSC senescence and suggests that melatonin may be a potent agent for preventing aging-associated osteoporosis.

Keywords: melatonin; mesenchymal stem cells; osteoporosis; senescence; MMSET
Introduction

Melatonin is a neurohormone synthesized and secreted predominantly by the pineal gland under the rhythmic control of the suprachiasmatic nucleus and the light/dark cycle (1, 2). Previous studies have shown that melatonin is a key molecule in a wide variety of physiological and pathological processes due to the diverse expression of melatonin and its receptors (3, 4). Many of the effects of melatonin are mediated directly through membrane-bound melatonin receptors or indirectly via nuclear orphan receptors of the RORα/RZR family (5). Accumulating evidence has also indicated that melatonin is involved in bone remolding, osteoporosis, osseointegration of dental implants, and dentine formation (6, 7). The aging-related reduction of melatonin levels has been shown to be a crucial factor in bone loss and osteoporosis with aging (8). Osteoporosis is a debilitating chronic disease marked by decreased bone density and strength, resulting in fragile bones (9). The loss of bone among the elderly occurs silently and progressively, without obvious symptoms until a painful fracture occurs. Therefore, serum melatonin levels might be utilized as a biomarker for the early monitoring and prevention of osteoporosis, and a better understanding of the functional machinery of melatonin will benefit the application of melatonin in alleviating the aging-related progression of osteoporosis (8).
Mesenchymal stem cells (MSCs) in bone marrow are multipotent stromal cells with the ability to differentiate into a variety of osteogenic, chondrogenic, adipogenic, or myogenic lineages\(^\text{(10)}\). Melatonin can modulate multiple signals to drive the commitment and differentiation of MSCs into osteoblasts\(^\text{(11,12)}\). Increased oxidative stress and cell injury with aging are causal factors of reduced osteogenesis by MSCs.

Numerous studies have confirmed that melatonin can promote osteoblast-like cell proliferation, enhance the expression of type I collagen and bone marker proteins, and facilitate the formation of a mineralized matrix\(^\text{(13,14)}\). A study also suggested that melatonin exerts suppressive effects on osteoclasts via the upregulation of calcitonin secretion by osteocytes\(^\text{(15)}\). Mechanistically, through binding to the MT2 receptor, melatonin elevates the gene expression of bone morphogenetic protein 2 (BMP2), BMP6, alkaline phosphatase (ALP), osteocalcin, and osteoprotegerin to favor osteogenesis, and simultaneously suppresses the receptor activator of NF-κB ligand pathway to attenuate osteolysis\(^\text{(8)}\). Intriguingly, osteoblasts from MT2\(^{-/-}\) mice exhibit intrinsic defects in differentiation and mineralization compared with their wild-type counterparts, and the mutant cells fail to respond to melatonin\(^\text{(16)}\). However, despite these known phenotypes and functions of melatonin on osteoblastogenesis, the substantial molecular regulatory mechanisms, especially the epigenetic machinery, are still not well elucidated.

Osteoblasts are bone-forming cells derived from MSCs, and the stemness and differentiation properties of MSCs have been shown to decline with age and cellular
senescence(17). Nevertheless, an in-depth understanding of the mechanisms involved in cellular senescence remains elusive, due to the highly intrinsic heterogeneity and complicated genetic or epigenetic regulatory processes in MSCs. Melatonin is an effective agent for the alleviation of apoptotic factors to protect MSCs from cell injury(18). A series of studies conducted by SH Lee and colleagues have demonstrated that melatonin treatment enhances kidney-derived MSC proliferation and prevents cell senescence, probably by upregulating PPARγ, via the PrPC-dependent enhancement of mitochondrial function, or by exosomes carrying microRNAs(18-20). A study showed that melatonin can restore the osteoporosis-impaired osteogenic potential of bone marrow-derived MSCs by preserving SIRT1-mediated intracellular antioxidation(21). Moreover, incubation of bone marrow-derived MSCs with melatonin predominantly enhances the expression of BCL2, but decreases the expression of BAX, to protect MSCs from apoptosis(22). Despite these findings, it is unknown whether the beneficial effect of melatonin on maintaining MSC regeneration is based on an aging-associated mechanism. Thus, a thorough understanding of the molecular processes controlling MSC senescence is crucial for identifying the drivers and effectors of age-associated MSC dysfunction and to guide the translational application of MSCs in the clinical setting. In this study, we examined the gene expression profiles of human bone marrow MSCs derived from fetal, young adult, and elderly donors to screen for aging-related genes. We discovered a previously unreported phenotype in MSCs derived from elderly donors, but not from fetal or young adult donors, were more sensitive to melatonin stimulation, at least partially
through the alleviation of DNA methylation on the promoter of the histone methyltransferase *MMSET* gene. Importantly, melatonin levels in bone marrow plasma were correlated with progression of senile osteoporosis in clinic. Mechanistically, *MMSET* upregulation facilitated the expression of the osteogenic genes *RUNX2* and *SP7/OSTERIX* by modulating the levels of histone 3 dimethylation at lysine 36, and the beneficial effect of melatonin against bone loss was confirmed in a mouse model of aging. Thus, our study is the first to report the in-depth epigenetic regulatory mechanism of melatonin on the senescence and osteoblastogenesis-related properties of MSCs.

**Methods**

**Ethic approval**

This study was approved by the Ethic Committee of Tianjin Medical University (No. TMUhMEC2018014), and all the protocols were conformed to the Ethical Guidelines of the World Medical Association Declaration of Helsinki. Signed informed consent was obtained from all participating individuals prior to participation in the study. Animal studies were approved by the Committee on Animal Research and Ethics of Tianjin Medical University (No. TMUaMEC2018001), and the Animal Experiments Ethics Committee of the Fifth Central Hospital of Tianjin (No. TJWZX2018047). All protocols conformed to the Guidelines for Ethical Conduct in the Care and Use of Nonhuman Animals in Research.
Isolation and culture of bone marrow-derived MSCs

For MSCs isolation from young (n=12, 17-44 years old, median=25, 10 males and 2 females) and elderly healthy donors (n=12, 65-82 years old, median=68.5, 10 males and 2 females) or osteoporosis patients (n=12, 74-90 years old, median=79, all male), 3-5 mL of bone marrow biopsies were diluted up to 10 mL with high-glucose Dulbecco's modified Eagle medium (DMEM) (Gibco, Life Technologies, Carlsbad, CA, USA) and gently loaded onto the top of 10 mL Ficoll Paque Plus (GE Healthcare, Wauwatosa, Wisconsin, USA) and cells were then fractionated on a lymphoprep density gradient by centrifugation at 800×g for 25 minutes at room temperature with the acceleration at 1. After centrifuge, interfacial mononuclear cells were collected, and washed with phosphatebuffered saline (PBS) at 300× g for 10 minutes at room temperature twice, resuspended in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco, Life Technologies, Carlsbad, CA, USA), 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 2 mM L-glutamine (Gibco, Life Technologies, Carlsbad, CA, USA), seeded, and incubated at 37°C/5% CO2. For MSCs isolation from femur of human fetuses (n=12, 16-22 weeks, median=18.5, 7 males and 5 females), bone marrow was cultured directly in culture media. After 48 hours, nonadherent cells were removed by changing the medium. Thereafter, the medium was changed every two days. When the cells reached 85%-95% confluence, they were trypsinized, counted, and plated again. Cells from passages 3-6 were used for the experiments.
Isolation of mitochondria from MSC cells

Mitochondrial mRNA was isolated accordingly to previous report(23). Briefly, MSC cells were digested with Trypsin-EDTA Solution and washed twice with precooled PBS. The cells were suspended in the mitochondrial isolation buffer (Mitochondria Isolation Kit for Cultured Cells, Beyotime, C3601) and placed on ice for 15 minutes, homogenized for 15 times, then centrifuged at 600g for 10 minutes at 4°C. The supernatant was further centrifuged at 11,000 × g for 10 min at 4°C, and the precipitated mitochondria were used for mitochondrial RNA extraction assay using Trizol (Life Technologies, South San Francisco, CA USA).

Transfection, virus package and infection

Transient transfections to HEK293T cells were performed using polyethyleneimine (PEI) (ThermoFisher Scientific, Carlsbad, CA, USA) in the OPTI-MEM medium (Life Technologies, Carlsbad, CA, USA) with a ratio of 1: 4 to 1: 6 of DNA: PEI. Viral particles were produced by HEK293T cells in a 10 cm dish transfected with 4 μg PMD2.G and 6 μg psPAX2 packaging plasmids (Addgene, Watertown, MA, USA), together with 8 μg lentiviral expressing vectors encoding target genes, including pCMV3-C-HA-MMSET, pLKO.1 vector encoding shRNAs targeting MMSET gene or DNMT3b gene. Supernatant carrying the viral particles was harvested 60 hours after transfection and concentrated to 100× volume by Poly (ethylene glycol) 8,000 (Sigma-Aldrich, St. Louis, MS, USA).
For viral infection, $2 \times 10^5$ MSCs were seeded in 1 mL new complete media for 6 hours and then added 50 μL viral concentration and 8 μg/mL polybrene, and cells were spin at 1800 rpm for 45 minutes at 20°C. 12 hours after spinfection, the medium was changed and cells were cultured for another 48 hours until further management.

**Senescence β-Galactosidase Staining**

MSCs were cultured in 6-well plate for indicated time, then culture medium were removed and washed once with 2 mL of 1× PBS. After cells were fixed with 1 mL of fixative solution for 15 min at room temperature and washed three times with 2 mL of 1× PBS, 1 mL of the staining solution was added to incubate cells at 37°C for hours until chromogenic reaction achieved.

**Osteogenesis induction in vitro**

MSCs were cultured in a 6-well plate with complete medium. After reaching 80% confluence, the medium was changed to osteogenic differentiation medium in presence or absence of 1 μmol/L melatonin for 14 days with a medium change every 3 days. The osteogenic differentiation medium was composed of high-glucose DMEM, penicillin (100 U/mL), streptomycin (100 μg/mL), dexamethasone (0.1 μmol/L), 10% FBS, ascorbic acid (50 μg/mL), and β-glycerol phosphate (10 mmol/L). Osteogenic differentiation was detected by Alizarin Red S staining or alkaline phosphatase assay (ALP).
**Alizarin Red S staining quantitation assay**

The culture medium was aspirated and the cells were washed three times with PBS. Then, the cells were fixed with fresh 70% ethanol for 60 minutes at 4°C or fresh 95% ethanol for 10 minutes at room temperature, washed three times with PBS, and stained with 40 mM Alizarin Red S solution, pH 4.2 (Sigma-Aldrich, St. Louis, MS, USA) for 5-10 minutes at room temperature. Removed the Alizarin Red S solution and wash it with PBS to stop the color reaction. The images were captured with a visible light microscope. To quantify the Alizarin Red staining intensity, stained cells were incubated with a 10% (w/v) solution of cetyl pyridinium chloride (Sigma-Aldrich, St. Louis, MS, USA) in 10 mmol/L Na-phosphate buffer (pH 7.0) for 15 minutes at room temperature, then 200 μL of supernatant was transferred to a 96-well plate and read by measuring the absorbance at 562 nm with a microplate reader. Each experiment was performed in triplicate.

**Alkaline phosphatase assay and quantification**

Osteogenic differentiation was detected by Alkaline Phosphatase staining. Briefly, the culture medium was aspirated and the cells were fixed with 10% neutral formalin buffer for 10 minutes, washed three times with 1×PBS, and stained with alkaline phosphatase dyeing working solution (Beyotime) at room temperature in dark for 10 minutes or longer, until the color developed to the desired depth. Removed the dyeing working solution and wash it with deionized water for 1-2 times to stop the color reaction. The images were captured with a visible light microscope. To quantify the ALP activity in control and
osteoblast-differentiated MSCs, we used the Alkaline Phosphatase Assay Kit (Colorimetric) (BioVision) with modified protocols. Cells were cultured in under normal or osteogenic induction conditions. On day 7, wells were rinsed once with PBS and were fixed using 3.7% formaldehyde in 90% ethanol for 30 seconds at room temperature; then fixative was removed and 50 µL of p-nitrophenyl phosphate solution was added to each well and incubated for 60 minutes in the dark at room temperature until a clear yellow color developed. Reaction was subsequently stopped by adding 20 µL of stop solution. Optical density was then measured at 405nm using a SpectraMax/M5 fluorescence spectrophotometer plate reader. The data were then analyzed by evaluating the increase in ALP expression of the treated samples compared to the untreated. Each experiment was performed in triplicate.

**ELISA**

Melatonin level in bone marrow plasma was measured using a human MT(Melatonin) ELISA Kit (Elabscience, Cat: E-EL-H2016c, Wuhan, China) accordingly. Briefly, bone marrow plasma from young (n=15, aging 17-45 years, median =30, 11 males and 4 females) and elderly donors (n=24, aging 56-84 years, median=65.5, 15 males and 9 females) were diluted at 1:5 with sample dilution buffer and added into the plate with primary antibody for incubation at 37°C for 45 min. Afterward, secondary antibody were prepared accordingly and added into samples at 37°C for 30 min. Then, the substrate was
added to develop the signal for detection. Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

**Quantitative reverse transcription polymerase chain reaction (qRT-PCR)**

Total RNA was extracted from cells in different treatment groups using Trizol (Life Technologies, South San Francisco, CA USA) and then converted to cDNA using the 5× All-In-One reverse transcription MasterMix (abm, Vancouver, Canada) according to the manufacturer's instructions. qRT-PCR was performed on a QuantStudio 3 Real-Time PCR System (Applied Biosystems) using EvaGreen 2× qPCR MasterMix (abm, Vancouver, Canada). Expression levels of the following genes were analyzed: *AANAT, HIOMT, RUNX2, COL1A1, OPN, SP7, BGLAP, MT1, MT2, DNMT1, DNMT3a, DNMT3b* and *MMSET*. The expression level of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene served as a reference. The Ct value of the GAPDH was subtracted from the Ct value of the target gene ($\Delta$Ct), and the average $\Delta$Ct value of the triplicates was recorded. The relative expression levels of each gene were determined using the $2^{-\Delta\Delta Ct}$ method.

**Western blotting**

Protein lysates were prepared in RIPA-buffer (50 mM Tris-Hcl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.5% sodium deoxycholate, 1% NP-40, 1 mM sodium ovanadate, 10 μg/mL aprotinin, 1 mM phenylmethanesulfonyl fluoride, and 10 μg/mL leupeptin) supplemented with complete protease inhibitors (Roche, Indianapolis, IN, USA). The
protein concentration was determined using the BCA protein assay kit (ThermoFisher Scientific, Carlsbad, CA, USA). Cell lysate (50 μg) was separated by electrophoresis on SDS-PAGE gel and transferred to nitrocellulose membranes (Pall Corporation, Washington, NY, USA). Membranes were blocked with 5% non-fat milk for 1 hour at room temperature and probed overnight at 4°C with specific antibodies. Antibodies used in this study were listed in the supplementary resources. Membranes were washed three times in PBST the next day, then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature, washed three times with PBST and finally bands were visualized using an enhanced chemiluminescence system (Millipore, Los Angeles, CA USA). The representative Western blot images for at least three independent experiments shown in the figures have been cropped and auto contrasted.

**Chromatin-immunoprecipitation (ChIP), ChIP-qPCR and ChIP-sequencing (ChIP-seq)**

20 million cells were washed in PBS and cross-linked with 1% formaldehyde for 10 minutes at room temperature and then quenched by addition of glycine (125 mM final concentration) for 5 minutes. For Nuclei isolation, cells were resuspended in cell lysis buffer (50 mM Tris, pH 8.0, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100), incubated the tube on ice for 20 minutes to swell. Harvested the nuclei by centrifugation at 2000 g for 5 minutes at 4°C resuspended in 1 mL ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) and incubated on ice for 10
minutes. Chromatin was fragmented to 200-500 bp using 10 cycles using the Vibra-Cell Ultrasonic Liquid Processors (SONICS, Newtown, CT, USA). For each IP, chromatin was immunoprecipitated with 2 µg of antibody in IP dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0) at 4°C overnight. Chromatin was precleared for 2 hours each with protein G agarose beads (Cell Signaling Technology, Danvers, MA, USA) before immunoprecipitation. The immunoprecipitated material was washed, once in TSE I buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS), once in TSE II buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, pH 8.0, 500 mM NaCl, 1% Triton X-100, 0.1% SDS), once in LiCl buffer (10 mM Tris-HCl, pH 8.1, 250 mM LiCl, 1% deoxycholate, 1% NP40, 1mM EDTA) and once in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0) before elution in elution buffer (100 mM NaHCO3, 1% SDS). The samples were removed from beads, reversed cross-linked overnight at 65°C and DNA was isolated using QIAquick PCR Purification Kit (QIAGEN, Germantown, MD, USA). Precipitated DNA was analyzed by qPCR or high-throughput sequencing (Novogene Co., Ltd.). Antibodies used in this study were listed in the supplementary resources.

**Gene expression microarray**

Total RNA was extracted from MSC cells of different age groups using TRIzol reagent (Life Technologies, South San Francisco, CA USA) according to the manufacturer's instructions. Quality of the purified RNA was tested on Agilent 2100 Bio analyzer
Libraries for cluster generation and DNA sequencing were prepared following an adapted method from BGISEQ-500 platform. The low quality reads (more than 20% of the bases qualities are lower than 10) were filtered to get the clean reads. Then those clean reads were assembled into Unigenes, followed with Unigene functional annotation, SSR detection and calculate the Unigene expression levels and SNPs of each sample. Finally, DEGs (differential expressed genes) were identified between samples and do clustering analysis and functional annotations.

**Reduced representation bisulfite sequencing (RRBS)**

DNA was extracted from MSC cells of different age groups using Roche kit according to the manufacturer's instructions. Digesting DNA using the *MspI* restriction enzyme, which cuts DNA at its recognition site (C ↓ CGG) independent of the CpG methylation status, then, repairing the end and ligating adapters for Illumina sequencing, selecting gel-based DNA fragments with insert sizes ranging from 160 bp to 400 bp, bisulfite treated two successive rounds, after which we observed 98% converted cytosines outside the CpGs, the bisulfite-converted library was used to PCR amplification for 20 cycles, finally, single-read sequencing for 76 cycles using an Illumina Genome Analyzer II.

**Animal experiment and bone morphology in vivo**

Aging C57 mice (18 months old) and adult C57 mice (2 months old) were blindly randomized to mock group and melatonin treatment group, and treated with vehicle or 10 mg/kg melatonin respectively by subcutaneous injection for 10 weeks, twice a week.
weeks before termination, calcein (0.5 mg/mice, i.p., biw × 2) was intraperitoneally injected for the last 2 weeks, twice a week. To detect the fluorescence intensity in mouse femur, bone tissues were resin-embedded for hard tissue slides cutting with a 50 μm thickness, and pictures were taken with a fluorescent microscope.

To assess the in vivo bone morphology, high-resolution X-ray microtomography was performed on mice femur with the SkyScan 1276 microtomograph (BrukermicroCT, Kontich, Belgium). After segmentation, the 3D models were constructed with the CtAn software (release 2.5, Skyscan). 3D measurements were obtained with the CtAn software (release 2.5, Skyscan). Trabecular bone analysis was performed on the femur body.

**Statistical analysis**

Data were shown as mean ± SD for at least three independent experiments. Differences between groups were determined using paired two-tailed Student’s t-test or two-way ANOVA. The Least Significant Difference (LSD) was used for post hoc analysis when treatments are less than 2, and the Tukey’s Honest Significant Difference test as the post hoc tests when treatments are >3. Pearson correlation test was used to determine the correlations between gene expressions, and survival analysis was done by GraphPad Prism 5.0. A P value less than 0.05 was considered statistically significant. *, P < 0.05; **, P < 0.01, compared with the controls, respectively.

**Results**
Melatonin levels in bone marrow decrease with aging

Because melatonin is an important endocrine hormone regulating osteogenesis and bone homeostasis, we measured its levels in bone marrow plasma from donors of different ages using an enzyme-linked immunosorbent assay. The amount of melatonin in bone marrow decreased in an age-associated manner, as the average level of melatonin in donors aged under 45 years was approximately 400 pg/mL, but it was less than 250 pg/mL in donors over 60 years old (Figure 1a). Meanwhile, we assessed the expression of melatonin receptors in bone marrow-derived MSCs isolated from fetal, young adult, and elderly donors using real-time PCR but did not find significant changes in MT1 or MT2 expression (Figure 1b, 1c). To clarify the impact of synthetic enzymes in melatonin synthesis, we isolated total mRNA or mitochondrial mRNA of MSCs derived from fetal, young adult, and elderly donors, and detected the expressions of two key rate-limiting enzymes, arylalkylamine-N-acetyltransferase (AANAT) and hydroxyindole-O-methyltransferase (HIOMT)(24). Total mRNA levels of AANAT and HIOMT were declined at different ranges, but mitochondrial mRNAs were significantly suppressed in MSCs from the old donors, suggesting a possible reason for melatonin attenuation in bone marrow (Figure 1d, 1e). At the same time, the primary characteristic of senescent cells, the activity of lysosomal β-galactosidase, increased gradually in MSCs from the fetal, the young and the old donors (Figure 1f, 1g). Seemingly, melatonin levels in bone marrow have a close link with aging-associated osteoporosis.
The expression of MMSET is suppressed in senescent MSCs

To screen for differentially expressed genes related to aging, we compared the differences in the gene expression profiles of bone marrow MSCs derived from fetal, young adult, and elderly donors, using gene chip microarray analysis. Multiple comparisons between the three groups of MSCs showed that there was a larger number of differentially expressed genes between the fetal and elderly groups than between the other groups, with 1835 genes with a greater than 2-fold change in expression; comparisons between the fetal and young adult groups and between elderly and young adult groups identified 994 and 325 genes, respectively (Figure 2a). Among the 41 genes that overlapped in all three comparisons, we found that the expression of some genes known to correlate with stemness, such as ATP binding cassette subfamily G member 2 (ABCG2), insulin receptor substrate 1 (IRS1), and suppressor of cytokine signaling 1 (SOCS1), together with MMSET, was greatly downregulated in senescent MSCs (Figure 2b). Real-time PCR confirmed that MMSET was maintained at a relatively high level in MSCs from fetal and young adult donors, while its expression was dramatically reduced in senescent MSCs from elderly donors (Figure 2c). Western blot analysis further revealed that MMSET protein expression was significantly decreased in senescent MSCs (Figure 2d). Taken together, these data indicated that MMSET may be an important factor in aging-associated osteoporosis.
Osteogenic potential of senescent MSCs declines in parallel with MMSET downregulation

MSCs isolated from the different age groups were cultured in osteoblast differentiation medium for 14 days. ALP activity was investigated and Alizarin Red S staining was performed to assess capacity for osteoblastogenesis. We found that senescence led to the impaired osteogenic potential of bone marrow-derived MSCs, as indicated by decreased ALP activity and mineralization staining (Figure 3a). Quantification of ALP activity and the number of mineralized nodules also confirmed the gradual decline of osteogenic potential with the senescence of MSCs (Figure 3b, 3c). Meanwhile, the expression of osteogenic markers, including bone gamma-carboxyglutamate protein (BGLAP), osteopontin (OPN), and type I collagen alpha 1 (COL1A1), was reduced in senescent MSCs (Figure 3d). Remarkably, association analysis indicated a strong correlation between ALP levels and MMSET expression (Figure 3e), as well as between RUNX2 and MMSET expression (Figure 3f). Interestingly, MSCs from elderly donors exhibited higher ALP activity and mineralized nodule formation after the induction of osteogenesis if they possessed higher levels of MMSET expression (Figure 3g, 3h, 3i).

Melatonin stimulates MMSET expression in senescent MSCs

To explore the relationship between melatonin and MMSET, we treated cultured MSCs from donors of different ages with exogenous melatonin (1 µM) and then examined
**MMSET** expression. We found that MSCs from elderly donors exhibited higher sensitivity to melatonin than those from young adults, but no significant change was observed when compared with fetal MSCs (**Figure 4a**). Western blot analysis was performed using three primary cells of MSCs derived from donors of each age group, and the results further confirmed the downregulation of MMSET expression in senescent MSCs and revealed that senescent MSCs were more sensitive to melatonin (1 µM) treatment (**Figure 4b**). Consistently, exogenous melatonin (1 µM) significantly ameliorated the repressive status of H3K36me2 in MSCs from elderly donors, which is the catalytic target of MMSET (**Figure 4c**). To further ascertain the role of melatonin in the osteogenic differentiation of aged MSCs, we examined the expression of the osteogenic markers *RUNX2* and *SP7/OSTERIX*. Their expression was elevated in senescent MSCs in a dose-dependent manner after treatment with melatonin for 24 h (**Figure 4d**). These data indicated that MMSET upregulation is a marker of melatonin-mediated osteogenesis in senescent MSCs. Since MMSET is a histone methyltransferase that catalyses H3K36me2, and H3K36 methylation has long been implicated in promoting gene transcription(25), we sought to screen genes regulated by H3K36me2 under the stimulation of melatonin using chromatin immunoprecipitation sequencing (ChIP-seq). No large scale genome-wide changes in H3K36me2 occupancy upon melatonin treatment were observed, however the promoter and other regions including the 3’-and 5’-untranslated region (UTR), non-coding regions were slightly increased by 1%, and intergenic region decreased by 2% (**Figure 4e**). Melatonin
treatment slightly increased the genome-wide average H3K36me2 signals (Figure 4f), and track profiles indicated that the change in H3K36me2 was occurring in promoter, enhancer, and gene body regions, as seen by the representative tracks of the RUNX2 and SP7 genes (Figure 4g).

**MMSET favors osteogenic differentiation**

Previous studies have identified MMSET as a histone methyltransferase that can methylate histones H3 and H4. We found that the repressive status of H3K36me2 was ameliorated and the activated status of H3K27me3 was reduced in MSCs from elderly donors if they expressed MMSET at a high level (Figure 5a), suggesting that elevated MMSET expression is a marker of melatonin-mediated osteogenesis in senescent MSCs. To further ascertain the role of MMSET in the osteogenic differentiation of aged MSCs, we manipulated *MMSET* expression using a lentivirus carrying short hairpin RNA (shRNA) targeting *MMSET* or with *MMSET* overexpression vectors. *MMSET* knockdown led to a decrease in H3K36me2 levels, whereas MMSET overexpression led to an increase in H3K36me2 levels (Figure 5b). MSCs with MMSET overexpression displayed a stronger potential for osteogenesis. After osteogenic induction, these cells exhibited higher levels of ALP activity, increased numbers of mineralized nodules, and enhanced expression of osteogenic markers compared with their vector control counterparts (Figure 5c, 5d). In contrast, MSCs with MMSET knockdown exhibited lower levels of ALP activity and decreased numbers of mineralized nodules (Figure 5e).
Silencing of *MMSET* impaired the expression of *RUNX2* and *SP7/OSTERIX* induced by melatonin treatment (Figure 5f). ChIP-qPCR analysis showed that melatonin treatment enhanced the recruitment of H3K36me2 to *RUNX2* and *SP7/OSTERIX* promoters in a dose-dependent manner (Figure 5g), and the recruitment of H3K36me2 to *RUNX2* and *SP7/OSTERIX* promoters was correspondingly altered when *MMSET* was knocked down or overexpressed compared with the control groups (Figure 5h). Importantly, a high concentration of melatonin failed to enhance the recruitment of H3K36me2 to *RUNX2* and *SP7/OSTERIX* promoters after *MMSET* silencing (Figure 5i). Collectively, these data showed that melatonin favored osteogenic differentiation via the MMSET-mediated modification of H3K36me2 on the promoters of osteogenic driver genes.

*Melatonin alleviates DNA methylation of the MMSET promoter*

Subsequently, we wished to identify the mechanism underlying the downregulation of *MMSET* in senescent MSCs. We analyzed the genome-wide methylation profiles of cultured MSCs from donors of different ages using reduced representation bisulfite sequencing (RRBS). Based on our data regarding the differentially methylated regions (DMRs) of osteogenesis gene promoters in each genomic feature group, we performed a relative enrichment analysis to identify the changes in the methylation of genes in MSCs from the different age groups to elaborate the relationship between senescence and the methylation status of genes. Genomic distribution of DMRs according to the genomic features in the RRBS data on the different MSC groups showed that the methylation
levels on CpG islands (CGis) increased with aging, but there were no significant changes in the gene body, 3’-untranslated region (UTR), 5’-UTR, exons, and intergenic and intronic regions (**Figure 1a**). By using the UCSC Genome Browser with the human *MMSET* gene assembly, we found a 1805-bp DNA sequence with a high GC percentage (76.5%) containing 261 CGis in the *MMSET* promoter, and the DNA methylation status within the CGis increased gradually in MSCs from young adult and elderly donors compared with those from fetal donors (**Figure 6a**).

A previous study indicated that the expression of DNMT1 and DNMT3a decreases in aged cells, whereas the expression of DNMT3b mRNA and protein increases steadily.(26) In the present study, we also observed that melatonin (1 µM) treatment effectively decreased *DNMT3b* expression but had no significant effect on *DNMT1* and *DNMT3a* levels (**Figure 6b**). Indeed, in MSCs from elderly donors, there was a negative correlation between MMSET and DNMT3b expression (**Figure 6c**). Consequently, treatment of senescent MSCs with the methyltransferase inhibitor 5-aza to induce DNA demethylation increased MMSET levels (**Figure 6d**). To further ascertain the relationship between DNMT3b and MMSET in aged MSCs, we knocked down *DNMT3b* expression using shRNA (**Figure 2a**). DNMT3b silencing directly rescued the expression of MMSET in aged MSCs, but it did not have an obvious effect on MMSET expression in MSCs treated with an increasing concentration of melatonin (**Figure 6e**). Notably, only when DNMT3b expression was rescued in the DNMT3b-knockdown MSCs, but not
vector control, could restore the upregulation of MMSET and the osteogenic RUNX2 and
SP7 expressions upon melatonin treatment (Figure 6f), consequentially enhancing the
MSC derived osteogenesis reflected by significantly augmented Alizarin Red staining
and ALP activity (Figure 6g and 6h, S Figure 2 b and 2c). Taken together, our results
suggest that the decrease of MMSET levels in senescent MSCs may due to
DNMT3b-mediated promoter methylation. Melatonin treatment can modify the
epigenetic status of the MMSET promoter via DNMT3b-mediated promoter methylation.

Melatonin facilitates the osteogenesis of MSCs derived from aged mice via MMSET

We verified the relationship between melatonin and MMSET in aged mice. We first
evaluated the effects of melatonin on osteoblast-mediated bone formation in vivo. Aged
mice (18 months old) and young adult mice (2 months old) were treated with vehicle or
melatonin by subcutaneous injection for 8 weeks, and then injected intraperitoneally with
calcein for 2 weeks (Figure 7a). We also assessed the osteogenic potential of MSCs from
melatonin-treated aged and young adult mice. MSCs from aged mice clearly exhibited
decreased osteogenic differentiation, as evidenced by lower levels of ALP activity and
reduced numbers of mineralized nodules (Figure 7b); nevertheless, ALP activity and
matrix mineralization intensity were effectively increased in the melatonin-treated group
compared with the DMSO-treated control group in aged mice (Figure 7c, 7d).
Meanwhile, the decreased expression of MMSET in MSCs from aged mice was
significantly increased by melatonin treatment, but those from adult mice demonstrated a
limited enhancement (Figure 7e). Subsequently, the in vivo bone formation rate per bone
surface (BFR/BS) by calcein staining and undecalcified bone sections were imaged and
analyzed. The results showed that the bone formation rate was increased in the aged
mouse group compared with the young adult mouse group under the same treatment
conditions (Figure 7f, 7g). A micro-CT assay showed that melatonin significantly
increased the bone mass of aged mice and improved the femoral trabecular
microstructure (Figure 7h, 7i). Overall, these data from aged mice also supported the
hypothesis that melatonin can ameliorate osteogenesis and bone mass of aged mice, and
this effect may depend on the induction of MMSET expression.

Melatonin treatment recovers the osteogenic potential of MSCs derived from patients
with senile osteoporosis

Finally, we investigated the association between MMSET and senile osteoporosis (SOP)
in the clinical setting. MSCs were collected from bone biopsy samples from healthy
elderly individuals and patients with SOP and every donor was examined by radiography
(Figure 8a). We found that MMSET expression was significantly lower in bone marrow
MSCs from donors with SOP than in those from age-matched control donors (Figure 8b).
There was a positive correlation between bone mineral density, as measured with dual
energy X-ray absorptiometry, and melatonin levels in bone marrow in patients with SOP
(Figure 8c), and between bone mineral density and MMSET expression (Figure 8d).
Furthermore, MMSET expression showed a very strong correlation with melatonin levels
in bone marrow (Figure 8e). Accordingly, melatonin (1 µM) treatment enhanced MMSET expression in MSCs derived from patients with SOP (Figure 8f), and improved their differentiation efficiency after osteogenic induction, as evidenced by higher levels of ALP activity, increased numbers of mineralized nodules (Figure 8g, 8h), and upregulated RUNX2 and SP7/OSTERIX expression (Figure 8i). These data strongly suggest that MMSET downregulation is a characteristic of aging-associated osteoporosis and imply that melatonin may be an efficient therapeutic agent for patients with SOP.

Discussion

The composition, structure, and function of bone deteriorate as a consequence of aging, thereby increasing the risk of osteoporosis in elderly individuals. In this study, we report that melatonin levels in bone marrow decrease in an aging-related manner, and treatment with melatonin can reverse the impaired osteogenic potential of senescent MSCs through an epigenetic regulatory mechanism on the histone methyltransferase MMSET (Figure 9). Our study therefore supports the use of melatonin as a potent therapeutic agent for the prevention of aging-associated osteoporosis.

Melatonin is a hormone that is secreted mainly from the pineal gland during darkness and plays a vital role in circadian rhythms(27). Bone marrow in pinealectomized rats can still produce high levels of melatonin, suggesting a function for melatonin in regulating the bone marrow microenvironment(28). Melatonin is known to modulate bone formation
and osteoblast differentiation of bone marrow-derived MSCs(12). Studies have revealed that melatonin can boost osteoblast differentiation by upregulating osterix protein stability and expression(14), reduce autophagy in high glucose-cultured osteoblasts, and alleviate diabetes-induced osteoporosis by suppressing the ERK pathway(29). In addition, melatonin restores the osteoporosis-impaired osteogenic potential of bone marrow-derived MSCs by preserving SIRT1-mediated intracellular antioxidation(30), and SPRY4 may be partially responsible for the melatonin-mediated osteogenesis of bone marrow-derived MSCs(31). However, it is generally unknown how melatonin promotes the osteogenic potential of bone marrow-derived MSCs undergoing aging. In this study, we found that melatonin in bone marrow was declined with aging, probably due to downregulation of the key rate-limiting enzymes AANAT and HIOMT in mitochondrion, which is consistent with a recent study indicating that AANAT could accelerate aging in a knockout mice model(32). Our study also suggests MMSET (also known as WHSC1 or NSD2) is an important factor mediating the effects of melatonin. Melatonin treatment effectively improved MMSET expression and enhanced the H3K36me2 levels of osteogenic genes in senescent MSCs. As a consequence, ALP activity, matrix mineralization, and the expression of osteogenic markers were increased in the melatonin treatment group. By contrast, RNA interference-mediated MMSET knockdown attenuated melatonin-reversed osteogenic differentiation. MMSET is a histone methyltransferase that can methylate histones H3 and H4. MMSET overexpression correlates with an increase in H3K36me2 and a decrease in H3K27me3 across the genome, leading to a
looser chromatin structure. MMSET is frequently overexpressed in patients with multiple myeloma and its methyltransferase activity is crucial for clonogenicity(33). However, the relationship between MMSET with aging and osteoporosis has not been reported previously, and this is the first description of the importance of this mediator in MSC senescence.

Our results established a link between MMSET expression and aging. We identified about 40 genes whose expressions were altered during the senescence of MSC, most of that are stemness-related or senescence-related, such as CCDC28B, SLC19A1, SOX9, ABCG2, NANOG, RUNX2, and CDKN2A as expected. The results also revealed that MMSET was greatly decreased in senescent MSCs derived from the bone marrow of elderly donors. Western blot analysis further confirmed that MMSET downregulation was accompanied by decreased levels of H3K36me2 in senescent MSCs. The osteogenic potential of senescent MSCs greatly declined in parallel with MMSET downregulation. Additionally, MMSET ablation effectively abolished the melatonin-enhanced osteogenic differentiation of senescent MSCs. Thus, MMSET downregulation seems to be an important characteristic of senescent MSCs, and it weakens the osteogenic differentiation potential of MSCs. Given that MMSET is a histone methyltransferase, we speculate that it affects osteogenic differentiation via epigenetic mechanisms. Ample evidences have indicated that H3K36me2 regulates transcription activity around the TSS region, such as antagonizing Polycomb Repressive Complex 2 (PRC2)-mediated transcription silencing
through H3K27me3, and acts as a safeguard to ensure transcription fidelity (34).

Therefore, melatonin meliorates bone loss through enhancing transcription activities of

RUNX2 and SP7 mediated by MMSET, probably through modifying the histone

H3K36me2 around the TSS region of osteogenic gene promoter. However, other

cis-elements or enhancer located far away from the TSS region should be considered. As

expected, MMSET overexpression led to an increase of H3K36me2 levels on the

promoters of osteogenic RUNX2 and SP7/OSTERIX genes before the induction of

osteogenesis. These results strongly indicate that MMSET may render the differentiation

propensity of MSCs toward osteoblasts via an epigenetic mechanism. Although our study

suggested MMSET is a critical responder to melatonin treatment in ameliorating the loss

of bone, it is probably only one part of the consequence of senescence and one that could

be of general relevance, since the loss of expression resulted in a general impact on loss

of bone. Other genes playing important roles in osteogenesis, or an integrated role under

melatonin stimulation should be considered.

Senile osteoporosis arises from multiple age related events associated with the aberrant

expression of multiple genes, and accumulating evidence links aging-associated

osteoporosis to epigenetic alterations (35). Epigenetic mechanisms are very important in

the acquisition of cell fate in stem cells. It is possible that aging triggers epigenetic

alterations, which bias the differentiation potential of bone marrow-derived MSCs. When

young well-differentiated cells enter into senescence, there is a drift in DNA methylation.
A previous study revealed that the abundance of DNMT1 and DNMT3a decreases in senescent cells, whereas DNMT3b expression increases steadily\(^{(26)}\). In fact, DNA methylation is well known for gene silencing of \textit{BMP2}, because higher CpG methylation in the \textit{BMP2} promoter is found in osteoporotic individuals compared with healthy adults\(^{(36)}\). Given that BMP2 is a pivotal molecule modulating bone formation, aberrant methylation in its promoter region may result in impeded osteogenesis\(^{(36)}\). Our research indicates that the decrease of MMSET may be a consequence of aging due to DNA methylation of the \textit{MMSET} promoter. Analysis of the underlying mechanism showed that the decrease of MMSET in senescent MSCs may be due to DNMT3b-mediated promoter methylation, and treatment with melatonin effectively decreased the expression of \textit{DNMT3b} to enhance MMSET expression. Dr. Reiter has proposed that melatonin exerts DNMT inhibitory effects either by masking DNMT target sequences or by blocking the active site of the enzyme\(^{(37)}\). Our study investigation also suggests that melatonin suppresses the expression of NDMT3b, thereby attenuates the DNA methylation status on \textit{MMSET} promoter, consequentially promotes the MMSET transcription activity. However, the detailed working machinery of melatonin on DNMT3b suppression has not been clarified in the current study and needs further investigation.

Taken together, our study identified MMSET as a modulator of melatonin-mediated osteogenesis. Melatonin modifies the DNA methylation of the \textit{MMSET} promoter in bone marrow-derived MSCs to promote bone formation. Thus, this study highlights the
importance of melatonin-MMSET pathway in aging-associated osteoporosis.

Nevertheless, the current study focuses on restoring and accentuating bone formation using melatonin, but the in vivo bone dynamics comprise osteoblast-derived bone formation and osteoclast-driven resorption(38). Thus, apart from the assessment of bone formation in response to melatonin, the residual components of bone have not been investigated in this study, although recent reports have revealed inhibitory effects of melatonin on osteoclastogenesis(39,40). In addition, melatonin has additional effects unconducive to the treatment of age-related bone loss, whether it is amenable to therapeutic purpose remains further investigation.
Data availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. The ChIP-seq data was deposited at the Gene Expression Omnibus database under accession number GSE158786. Requests for any materials in this study should be directed to Zhiqiang Liu and obtained through an MTA.

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to the conception and design of the experiments, drafting of the manuscript, and critical
review of the manuscript.

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FIGURE 1  Melatonin in bone marrow decreases with aging. (a) Melatonin levels in the bone marrow plasma of donors under 45 years old (n=15) or over 60 years old (n=24) measured by ELISA. mRNA levels of MT1 (b), MT2 (c), AANAT and HIOMT (d) in total cell lysate (e) or mitochondrion of bone marrow MSCs derived from aborted fetuses (<22 weeks, n=12), young adults (16-45 years, n=12), and the old (> 60 years, n=12). n.s., no significance. (f) Representative images of activity of lysosomal β-galactosidase in MSCs from donors with different ages. Scale bar, 50 μm. (g) Quantification of the percentage of β-galactosidase staining positive MSCs in three groups (n=12). For the
ELISA assay, each sample was triplicated; for the quantification of \(\beta\)-galactosidase staining, 9 random vision field with 200\(\times\) magnification were analyzed.

FIGURE 2  Gene expression profile assay identifies MMSET downregulation in senescent MSCs. (a) Venn diagram shows the number of differently expressed genes in three comparisons of three groups. (b) Heat map shows hierarchical clustering of altered genes in MSCs derived from donors of different ages. Fc, fold change; cut off: Fc > 2 and \(P < 0.01\). (c) mRNA levels of \(MMSET\) in MSCs derived from donors of different ages (n = 12 with each detection triplicated). (d) Representative protein levels of MMSET in MSCs from the three groups. All Western blot results represents a representative example of three independently performed studies, each yielding similar results.
FIGURE 3  Osteogenic potential of senescent MSCs declines in parallel to MMSET downregulation. (a) Representative images of alkaline phosphatase assay (ALP) staining and Alizarin Red S assay for MSCs from fetal, young or old donors cultured with osteogenic media for 14 days. Scale bar, 100 μm. (b) and (c) Quantification of Alizarin Red S staining and ALP staining of 12 donors in each group (n = 12 with each detection triplicated). (d) Osteogenic marker genes expressions in MSCs from different groups cultured with osteogenic media for 14 days (n=3 with each detection triplicated). **, P < 0.01. (e) Correlation of ALP levels and MMSET expression in MSCs of different groups (n = 36). (f) Correlation of RUNX2 and MMSET expression in MSCs of different age.
groups (n = 36). (g) Representative of MMSET protein level in senescent MSCs (n = 12). (h) Representative images of ALP staining and Alizarin Red S assay in senescent MSCs with different MMSET levels. (i) Quantification of ALP staining and Alizarin Red staining (n=12 with each sample triplic). All Western blot results represents a representative example of three independently performed studies, each yielding similar results.

**FIGURE 4** Melatonin stimulates MMSET expression in senescent MSCs. (a) mRNA level of MMSET of MSCs from donors of different ages treated with melatonin (n = 12 with each sample triplicated). n.s., no significance. (b) The MMSET expression in three primary MSCs from donors of different ages treated with melatonin. (c) The
H3K36me2 levels in three primary MSCs from donors of different ages treated with melatonin. (d) mRNA levels of RUNX2 and SP7/OSTERIX in MSCs treated with increasing dosage of melatonin for 24 hours (n = 3 with each detection triplicated). *, P < 0.05; **, P < 0.01. (e) Pie chart for ChIP-seq profile to show percentage of genes enriched by H3K36me2 in MSC cells treated with vehicle or 1 µM melatonin (n = 3); (f) Changes of H3K36me2 density around the TSS region of genes. (g) Representative ChIP-seq profiles for H3K36me2 occupancy at the promoter regions of RUNX2 and SP7/OSTERIX genes. All Western blot results represents a representative example of three independently performed studies, each yielding similar results.
FIGURE 5  MMSET favors osteogenic differentiation. (a) Levels of MMSET, H3K36me2, and H3K27me3 in MSCs (n=6). (b) Expression of MMSET and the corresponding H3K36me2 level in MSCs with MMSET knockdown (upper panel) or overexpression (lower panel). (c) The representative images (upper panel) and quantification (lower panel) of ALP and Alizarin Red S assay in MSCs with MMSET overexpression (n = 8). (d) Expressions of RUNX2 and SP7/OSTERIX in MSCs with MMSET overexpression (n = 3 with each detection triplicated). (e) The representative images (upper panel) and quantification (lower panel) of ALP and Alizarin Red S assay in MSCs with MMSET silencing (n = 8). (f) RUNX2 and SP7/OSTERIX expressions in MSCs with MMSET silencing treated with increasing amount of melatonin for 24 hours (n = 3 with each detection triplicated). *, P < 0.05; **, P < 0.01. g, h and i, ChIP-qPCR analysis showed the recruitment of H3K36me2 on RUNX2 and SP7 promoter in MSCs treated with increasing dosage of melatonin for 24 hours (g), with overexpressed MMSET (h), or with MMSET silencing and then treated with increasing dosage of melatonin for 24 hours (i). The values represent mean ± standard error of the mean (SEM) (n = 3 with each detection triplicated). n.s., no significance. **, P < 0.01. All Western blot results represents a representative example of three independently performed studies, each yielding similar results.
FIGURE 6 Melatonin alleviates DNA methylation of MMSET promoter. (a) Methylation signatures at an island on MMSET gene promoter in MSCs from different ages groups. (b) Expressions of DNMTs of senescent MSCs treated with 1 μM melatonin for 24 hours determined by real time PCR (n = 6). (c) Expressions of MMSET and DNMT3b in senescent MSCs (n = 6). (d) MMSET expression in 3 senescent MSCs treated with 10 μM 5-aza. (e) Expression of MMSET in MSCs with DNMT3b knockdown (KD) and treated with increasing dosage of melatonin for 24 hours. (f) DNMT3b, MMSET, RUNX2 and SP7 expression in the DNMT3b-knockdown MSCs with DNMT3b overexpression and treated with vehicle or 1 μM melatonin. Representative images of Alizarin Red S assay (g) and ALP staining (h) in the
DNMT3b-knockdown MSCs with DNMT3b overexpression and treated with vehicle or 1 µM melatonin cultured with osteogenic media for 14 days. All Western blot results represents a representative example of three independently performed studies, each yielding similar results.

**FIGURE 7** Melatonin facilitates osteogenesis of MSCs from aging mice via MMSET. (a) Treatment schedule and experiment design in aging mice and adult mice. (b) Representative images of ALP and Alizarin Red S staining of MSCs cultured with osteogenic media for 14 days from aging and young mice in presence with vehicle or melatonin. Quantification of ALP (c) and Alizarin Red S staining (d) of MSCs from aging
and young mice groups treated with vehicle or melatonin (n = 10). (e) Representative MMSET expression in MSCs from aging and young mice treated with vehicle or melatonin for 24 hours. (f) The representative images of calcein stained femur bone from aging and young mice treated with vehicle or melatonin for 10 weeks. Quantification of bone formation rate (BFR/BS) (g), the percentage of bone volume to total volume (BV/TV) (h) and number of bone trabecula (i) in femurs of aging and young mice treated with vehicle or melatonin (n = 8). n.s., no significance. All Western blot results represents a representative example of three independently performed studies, each yielding similar results.
FIGURE 8  Melatonin treatment recovers osteogenic potential of MSCs from patients with senile osteoporosis. (a) Radiographic images of healthy control (H Ctrl) and patients with senile osteoporosis (Pt). Red arrows indicate the typical area with osteoporosis. (b) MMSET expression in MSCs isolated from healthy control and patients with senile osteoporosis (n = 18 with each detection triplicated). Correlation of bone mass density (BMD) with melatonin level in bone marrow plasma (c), and MMSET expression in MSCs from senile osteoporosis patients (d) (3 independent reads for n = 12 patients). (e) Correlation of MMSET expression in MSCs and melatonin level in bone marrow plasma of patients with senile osteoporosis (3 independent reads for n = 12 patients). (f) MMSET expression in 12 MSCs from patients with senile osteoporosis treated with 1 μM melatonin for 24 hours (n = 3 with each detection triplicated). **, P < 0.01. (g) The representative images of ALP and Alizarin Red S staining in MSCs from donors with senile osteoporosis treated with osteogenic media for 14 days in presence of DMSO vehicle or melatonin. (h) Quantification of ALP and Alizarin Red S staining for MSCs under osteogenesis induction and in presence of DMSO vehicle or melatonin (3 independent reads for n = 6 MSCs). (i) mRNA levels of RUNX2 and SP7/OSTERIX in MSCs under osteogenesis induction and in presence of DMSO vehicle or melatonin (n = 3 with each detection triplicated). **, P < 0.01.
FIGURE 9 Proposed schematic diagram for mechanisms of promoting osteoblastogenesis from senescent BMSCs by melatonin: melatonin alleviates the senescence-related hypermethylation of the MMSET promoter, upregulates expression of the histone methyltransferase NSD2; NSD2 promotes the histone H3 dimethylation modification at lysine 36 of the osteogenic genes RUNX2 and SP7/OSTERIX as a result, and consequentially enhances osteoblastogenesis from BMSCs.