The Role of p53-induced miR-145a in Senescence and Osteogenesis of Mesenchymal Stem Cells

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Abstract

**Background:** The osteogenic differentiation capacity of senescent bone marrow mesenchymal stem cells (MSCs) is diminished. However, little is known about the mechanisms. p53 not only regulates cellular senescence but also, as a negative regulator, is involved in bone formation. This study investigated the molecular mechanism of p53 in cellular senescence and osteogenesis.

**Methods:** The expression of p53 and its downstream gene p21 were measured during cellular senescence and osteogenesis differentiation of primary bone marrow MSCs. Then miR-145a was picked out from those p53-induced miRNAs as its expression change in bone marrow MSCs senescence and osteogenesis induced by H₂O₂ and BMP-9, respectively. The function of p53 induced miR-145a was analyzed by transfected with miRNA mimic in osteogenic differentiation of MSCs. Western blot and luciferase reporter assay were used for validating the target of miR-145a.

**Results:** p53, its downstream effector p21, and p53-induced miR-145a were significantly upregulated during primary MSCs senescence and osteogenesis. Overexpression of miR-145a promoted cellular senescence and inhibited osteogenic differentiation of MSCs. p53-induced miR-145a inhibited osteogenic differentiation by targeting core binding factor beta (Cbfb), and the restoration of Cbfb expression rescued the inhibitory effects of miRNA-145a on osteogenesis.

**Conclusions:** p53-induced miR-145a functions both in promoting senescence and inhibiting osteogenesis of MSCs, and the novel pathway p53/miR-145a/Cbfb in osteogenic differentiation of MSCs may represent new targets in the treatment of osteoporosis.

**Introduction**

The population ageing trend has led to an increasing number of osteoporosis patients worldwide[1]. Primary osteoporosis is a disease closely related to ageing. The body is composed of numerous cells, and the ageing of the body is first manifested in the ageing of cells. In turn, cell ageing in vitro may in a sense reflect the process of body ageing. Although ageing and senescence are not synonymous, there is a link between senescence and organismal ageing[2]. The fundamental cause of osteoporosis is the imbalance between bone formation and bone resorption, and bone formation is mainly mediated by bone marrow mesenchymal stem cells (MSCs). For aged MSCs, the ability to undergo
adipogenic differentiation was enhanced, while osteogenic differentiation was reduced, which led to a decrease in bone formation[3].

As an important tumour suppressor gene, p53 maintains the stability of the genome by causing cell cycle arrest and apoptosis under a variety of cell injury stress responses. At the same time, p53 is also closely related to ageing[4]. There are various ways to induce cellular senescence, including replicative senescence, oxidative stress, and oncogene-induced senescence, almost all of which involve changes in p53. Research indicated that the knock-in model of p53 showed obvious signs of ageing[5], and the deletion of an apoptosis regulator gene induced by p53 could save the loss of stem cells and decrease the signs of ageing, suggesting that the extensive apoptosis of stem cells is related to the senescence mediated by p53. In addition, p53 is involved in the regulation of bone formation[6]. Osteosclerosis has been detected in p53 knockout mice, and there is also evidence that p53 regulates osteoblast differentiation through transcription factors Runx2 and Osterix, which are involved in osteoblast differentiation and transformation and controlled by the BMP and IGF pathways. MDM2-deficient osteoblast progenitor cells have increased p53 activity and decreased level of Runx2[7]. In contrast, osteoprogenitor cells with p53 deletion showed increased proliferation and Runx2 expression, increased maturity of osteoblasts. These results indicate that p53 negatively regulates bone development and growth.

MicroRNAs (miRNAs) are a class of evolutionarily conserved, single-stranded non-coding RNA small molecules that exist in many organisms[8]. They are important posttranscriptional regulators of gene expression. MicroRNAs negatively regulate the translation of mRNAs by partial or complete base pairing to complementary sequences in the target mRNAs. It is understood that they may play a key role in regulating various biological processes, including development, cell differentiation, proliferation, and apoptosis. Through a large number of studies, researchers have confirmed that miRNAs play an important regulatory role in the process of bone metabolism. For example, in the initial stage of osteoblast differentiation, miRNA-29 activates the osteoblast differentiation signalling pathway by inhibiting the translation of multiple target genes, such as histone deacetylase 4 (HDAC4) [9]. Another study showed that miR-34a inhibits osteoclastogenesis by targeting transforming growth
factor-β-induced factor 2 (Tgif2). MiR-29 and miR-34a are the common transcription target of p53[10, 11]. With the help of miRNA, p53 indirectly regulates miRNA target genes through the transcriptional regulation of downstream miRNAs.

A remaining question is whether the downstream miRNAs of p53 are also involved in the regulation of cellular senescence and osteogenic differentiation of MSCs. In this study, we identified miR-145a, which was activated by p53 transcription, and studied its effects on the senescence and osteogenesis of MSCs.

**Materials And Methods**

**Cells**

For primary mouse bone marrow MSCs (mBMSCs) culture, bone marrow cells were isolated from femurs and tibias of 6-week-old C57BL/6 mice and cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin. The cell suspension was plated in 10-cm dish and incubated at 37°C in a humidity incubator with 5% CO2. The medium was changed every 3 days to clear non-adherent cells. When the cells reached 80%-90% confluence, MSCs were detached with 0.25% trypsin/1 nM EDTA (Hyclone, USA) and passaged. After passage, MSCs received routine characterization. Except for establishing in vitro replicative senescent cell model, mBMSCs at three to eight passages were used in this study. The detection of surface markers of mBMSCs was determined using flow cytometry. mBMSCs were incubated with anti-Sca-1, anti-CD44, anti-CD29, anti-CD45, or anti-CD34 for 30min. Then the samples were examined by flow cytometry.

C3H10T1/2 cells were maintained in DMEM containing 10% FBS, 1% penicillin and streptomycin.

**Osteogenic and Adipogenic Differentiation**

Osteogenic differentiation of mBMSCs and C3H10T1/2 cells were induced at approximately 80% confluence in osteogenic differentiation medium (ODM) containing 10% FBS complete high-glucose DMEM, 0.1 μM Dexamethasone, 10 mM β-Glycerophosphate and 50 μg/ml Ascorbic acid. ODM was changed every 3 days. Matrix mineralization or calcium depositions were examined with Alizarin Red staining at day 21. Cells were observed by inverted microscopy.
Adipogenic differentiation of mBMSCs was induced at approximately 80% confluence in adipogenic differentiation medium (ADM) containing 10% FBS complete hight-glucose DMEM, 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX), 1 μM Dexamethasone, 10 μM Insulin, and 200 μM Indomethacin. ADM was replaced every 3 days. Mature adipocytes or fat droplets formations were visualized by staining with Oil Red O solution. Cells were observed by inverted microscopy.

**Oil Red O, Alizarin Red, Alkaline phosphatase and SA-β-galactosidase staining**

For detection of lipid droplets, mBMSCs cultured in adipogenic medium for 2 weeks were fixed with 4% paraformaldehyde for 15 min and then stained with Oil Red O for 10 min at room temperature. Alizarin Red and ALP staining were performed according to a previously described procedure[12]. SA-β-galactosidase staining was performed with Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, USA) following the manufacturer's instruction. The blue-stained cells were regarded as positive cells.

**In vitro and in vivo senescent cell model**

Oxidative stress-induced senescence. mBMSCs were incubated with 50 μM H₂O₂ for 2 h. Then washing with PBS, cells were maintained in low-glucose DMEM for 24 hours and then harvested for mRNA and protein expression analyses.

Replicative senescence. An in vitro replicative senescent cell model was established using long-term cultured mBMSCs.

Since we had not isolated bone marrow MSCs from ageing mice successfully, in vivo senescent bone marrow MSCs were isolated from ageing Sprague Dawley (SD) rats (25-month-old).

**Transfection**

For miRNAs transfection, the functional role of miR-145a was verified by transfecting C3H10T1/2 cells with miR-145a mimic, and its negative controls (RiboBio, China) using the Lipofectamine2000 transfection agent (Invitrogen, USA) according to the manufacturer’s instructions.

For plasmids transfection, either empty vector pcDNA3.1 or pcDNA3.1 expressing mouse Cbfb (pcDNA3.1-Cbfb) were transiently transfected in C3H10T1/2 cells using the Lipofectamine2000 transfection agent.
**Gene expression analysis**

Total RNA was extracted with the RNAiso Plus reagent (Takara, Japan). For the mRNA analysis, total RNA was reverse-transcribed into complementary cDNA using the PrimeScript RT reagent Kit (Takara, Japan). For the miRNA analysis, the forward and reverse primers for miRNAs were designed by RiboBio Corporation (Guangzhou, China). Then, the total miRNA underwent polyA tailing and reverse transcription using the miDETECT A TrackTM miRNA qRT-PCR Starter Kit (RiboBio, China). All real-time PCR was conducted with the AB1 ViiA™ 7 Real-Time PCR System (Applied Biosystems) using the SYBR Green-based real-time detection method. The following thermal settings were used: 95°C for 30 seconds followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. The fold change was calculated with the $2^{ΔΔCT}$ method and normalized to the level of the housekeeping gene GAPDH or U6, respectively. The primer sequences used in this study are listed in Supplemental Table S1.

**Western Blot Analysis**

Whole-cell lysates were prepared on ice using cold RIPA lysis buffer (Beyotime, China) containing protease inhibitor (Thermo Fisher, USA). In brief, equal amounts of proteins (30 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. After incubation with the primary antibody at 4°C overnight, they were further immunoblotted with HRP-conjugated antibody at 37°C for 1 hour, developed with enhanced chemiluminescence (ECL) substrate (Millipore, USA) and chemiluminescence detection by ChemiDocTM MP Imaging System (Bio-Rad, United Kingdom). Primary antibodies and dilutions used were as follows: p53 (1:1000; Cell Signaling Technology); p21 (1:2000; Abcam); Runx2 (1:200; Santa Cruz Biotechnology); Cbfb (1:200; Santa Cruz Biotechnology); BMP9 (1:200; Santa Cruz Biotechnology); and GAPDH (1:2000; Beyotime Biotechnology).

**Luciferase assay**

Mouse wild type or mutant Cbfb 3′-UTR regions were chemically synthesized by Sangon Biotech (Shanghai, China) and cloned into psiCHECK2 luciferase reporter plasmids. The recombinant plasmids were respectively named as, psiCHECK2-Cbfb 3′ UTR-WT, psiCHECK2-Cbfb 3′ UTR-mut. HEK293T cells were seeded in 6-well plates. When the cells reached about 70% confluences, each recombinant
plasmid (2μg) and either the miR-145a mimic or the mimic negative control (mimic NC) were cotransfected into the cells with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's direction. After 48 h of transfection, firefly and Renilla luciferase activities were quantified using a dual luciferase assay system (Promega, USA).

**Statistical analysis**

All data are expressed as mean ± standard deviation (SD). Differences between two groups were evaluated by two-tailed Student’s t-test using GraphPad Prism 8 (GraphPad Software Inc., USA). All experiments were performed at least three times, and p<0.05 was considered statistically significant.

**Results**

**The characteristics of mBMSCs**

By passage 3, MSCs isolated from mouse bone marrow were mainly observed to be bipolar spindle-like cells (Supplemental Figure S1). When confluence was at 90%, the cells exhibited a spiral shape (Figure 1A). The isolated cells showed the potential to differentiate into adipogenic and osteogenic lineages after culture in induction media. Cells contained many Oil Red-O-positive lipid globules after 2 weeks of induction with ADM (Figure 1B). Similarly, calcium deposits stained by Alizarin Red were detected in mBMSCs induced for 3 weeks in ODM (Figure 1C). mBMSCs at passage 3 were strongly positive for mBMSCs markers, including CD29, CD44, and SCA-1, and negative for CD34 and CD45 (Figure 1D). In summary, our results demonstrated that the mBMSCs we used in our experiments were multipotent and highly pure.

**Changes in p53 and p21 expression in the process of mBMSCs senescence and osteogenic differentiation**

Previous studies showed that H₂O₂ induced cell premature senescence in MSCs after stimulation[13,14]. To induce the senescence of mBMSCs, we treated mBMSCs with 50 μM H₂O₂ and found that the senescence markers p53 and p21 were increased (Figure2A), and mBMSCs treated with 50 μM H₂O₂ displayed significantly higher senescence-associated β-galactosidase activity than the control (Supplemental Figure S2). Western blot results showed the same change (Figure 2B). Then, we examined the expression of p53 and p21 during the replicative senescence of mBMSCs by
continuous long-term passage[15] and found that p53 and p21 were significantly increased after passage 20 (P20) compared with the early phase passage 5 (P5) (Figure 2C, 2D). It is possible to conclude that p53 and p21 were upregulated in the process of cellular senescence induced by H₂O₂ or serial passage.

Then, we detected changes in p53 and p21 expression during osteogenic differentiation by inducing mBMSCs with Ad-BMP9 infection[16] and ODM. For Ad-BMP9 infection, we used a recombinant adenovirus expressing BMP9 and demonstrated that this recombinant adenovirus is capable of efficiently transducing mBMSCs (Supplemental Figure S3), and found p21 mRNA increased and p53 mRNA had no significant change 24 hours post-infection (Figure 2E); the change in protein levels was the same as the change in mRNA levels 48 hours post-infection, but the p53 level increased 24 hours post-infection and then decreased so that the p53 protein had no significant change between the control group (Ad-GFP) and the 48 hours post Ad-BMP9 infection group (Figure 2F). This may be due to the shorter half-life of wild-type p53 in normal cells[17]. For mBMSCs cultured in ODM, the p53 mRNA level decreased, while the protein level had no significant change after 14 days of culture in ODM; the p21 mRNA and protein levels increased, which coincided with the change in Runx2 (Figure 2G, H). These results showed that the expression of p53 and p21 had similar changes in the process of senescence and osteogenic differentiation.

Changes in the expression of p53-induced miRNAs during mBMSCs senescence and osteogenesis

To assess the expression levels of p53-induced miRNAs in the process of senescence and osteogenesis, we detected changes in miR-34a/b/c[18], miR-29[11], miR-145[19] and miR-192[20], all of which have been validated and are well-studied p53 downstream miRNAs. These p53-induced miRNAs were upregulated in senescent mBMSCs induced by H₂O₂, and miR-192 had the most obvious change (Figure 3A). Except for miR-29, other p53-induced miRNAs were upregulated after mBMSCs were infected with Ad-BMP9, and miR-145a had the largest fold-change (Figure 3B). Finally, we chose miR-145a as the target of interest of the p53-induced miRNAs that are involved in senescence and
osteogenic differentiation since we wanted to focus on the function of p53-induced miRNAs in osteogenic differentiation.

**miR-145a expression was upregulated during mBMSCs senescence and osteogenesis**

To confirm the results for miR-145a shown above, we detected the expression of miR-145a in the process of replicative senescence and found that miR-145a was upregulated significantly in the late passage cells (P20) (Figure 4A). Additionally, since bone marrow MSCs had been successfully isolated from rats, especially from the ageing rats (Supplemental Figure S4), we measured the expression changes of miR-145a in MSCs isolated from ageing rats (25 months old) compared with those isolated from young rats (2 months old) and found miR-145a was upregulated significantly (Figure 4B). Moreover, we observed, on the whole, miR-145a expression was upregulated in the process of osteogenesis induced by ODM (Figure 4C).

**Overexpression of miR-145a inhibited MSCs osteogenesis and promoted senescence**

miR-145a mimic was used to overexpress miR-145a in C3H10T1/2 cells, the murine mesenchymal stem cell line[21]. qRT-PCR analysis of miR-145a expression confirmed a significant increase in the miR-145a overexpression group (miR-145a mimic) compared with the control group (mimic negative control[mimic NC]) (Figure 5A). ALP staining showed that overexpression of miR-145a enhanced the osteogenic differentiation of mBMSCs cultured with ODM on day 7 (Figure 5C). The extracellular mineralization of C3H10T1/2 cells, as measured by Alizarin Red staining after 21 days in ODM culture displayed similar outcomes to those of ALP assays (Figure 5D). Overexpression of miR-145a significantly decreased the expression of osteogenesis-associated genes, including osterix, OCN and col1a1 (Figure 5E). Similarly, the osteogenesis-associated proteins Runx2 and ALP were decreased (Figure 5B). Moreover, C3H10T1/2 cells overexpressing miR-145a displayed significantly higher senescence-associated β-galactosidase activity than the control (Figure 5F).

**miR-145a inhibited MSCs osteogenesis by targeting Cbfβ**

Next, we examined the molecular bases behind by utilizing online databases (TargetScan and miRDB) to identify potential miR-145a target genes. The genes that overlapped in all two databases were selected to Gene Ontology (GO) analysis. Of all the predicted target genes, we found that Smad3,
Cbfb, Smad5, Ctnnbip1, which was highlighted with blue colour in Figure 6 A, are involved in osteoblast differentiation. Then we explore Smad3 and Cbfb, which had been verified as the target genes of miR-145a[22-24], given its reported role in altering bone formation and metabolism in other studies[25,26]. However, we cannot conclude that Smad3 is the target gene of miR-145a because the overexpression of miR-145a did not affect the mRNA and protein level of Smad3 in C3H10T1/2 cells (Supplemental Figure S5 A, B). Moreover, we observed that miRNA-145a mimic did not decrease the luciferase activities of wild type Smad3-3’UTR reporter vector (Supplemental Figure S5 C, D). Then we tested whether Cbfb is the target of miR-145a or not. An interaction between miR-145a and the complementary site within the 3’UTR of Cbfb (Figure 6 B) has been confirmed in other studies[22,27]. Figure 6 D shows that miR-145a targets Cbfb in C3H10T1/2 cells, given the significant reduction in Cbfb protein levels following miR-145a overexpression. No difference in Cbfb mRNA expression was found (Figure 6C), suggesting that miR-145a inhibits Cbfb at the level of translation in mBMSCs. Cbfb stabilizes Runx2 in osteoblasts by forming a complex[28]. Therefore, we expected that the suppression of Cbfb by miR-145a would disturb the function of Runx2 in osteogenesis. Indeed, we found a decrease in Runx2 protein levels following miR-145a overexpression in C3H10T1/2 cells (Figure 6 F). We performed a luciferase activity assay using a reporter plasmid, psiCHECK2, in which the Cbfb 3’-UTR was cloned into the luciferase gene. Overexpression of miR-145a significantly decreased the luciferase activity, whereas a mutation in the miR-145 binding site of the Cbfb 3’-UTR abrogated the response to miR-145 (Figure 6 E). Moreover, overexpression of Cbfb significantly reversed the inhibition of osteogenesis induced by miR-145a in C3H10T1/2 cells (Figure 6 F, G, H).

Discussion

Ageing is one of the most important risk factors for osteoporosis patients. With increasing age, the accumulation of aged MSCs in bone marrow tissue leads to the decreased ability of BMSCs to undergo osteogenic differentiation[3]. The p53/p21 pathway is one of the most important signalling pathways that mediate the ageing phenomenon of most cells[29]. In addition to regulating senescence, p53 negatively regulates osteogenic differentiation[30].
p53 functions as “the guardian of the genome” by activating or inhibiting the expression of a large
number of downstream genes, such as p21, Bcl-2, MDM2. However, with the discovery of miRNA, the p53/miRNAs/mRNAs axis was also found, whereby p53 indirectly regulates miRNA target genes through the transcriptional regulation of miRNAs, which enables the regulation scope of p53 to be more extensive and more precise[31, 32]. For example, p53 can directly activate the expression of MDM2, which functions in p53 ubiquitination and degradation. However, through the p53/miR-192/MDM2 axis, p53 can negatively regulate the expression of MDM2 by activating miR-192 expression, which makes the regulation more accurate[33]. In the regulation of osteogenic differentiation, p53 can directly inhibit osterix expression to negatively regulate osteogenic differentiation[34]. In addition, through the transcriptional activation of miR-34a, p53 inhibits the expression of Runx2 indirectly by the p53/miR-34/Runx2 axis[35], and by transcriptional inhibition of the miR-17-92 cluster, p53 indirectly increased the expression of Smurf1 by the p53/miR-17/Smurf1 axis to ultimately inhibit osteogenic differentiation[36].

By transcriptionally regulating the expression of miRNAs, p53 participates not only in the process of cellular senescence but also in osteogenic differentiation. we detected the common downstream miRNAs activated by p53 transcription in the process of senescence and osteogenic differentiation and found that miR-145a expression increased in the process of senescence, and it increased more significantly during mBMSCs osteogenic differentiation. The role of miR-145a in osteogenic differentiation has been reported previously. MiR-145a was decreased during osteogenic differentiation in human adipose-derived stem cells (ASCs), and miR-145 could suppress ASC osteogenic differentiation by suppressing FoxO1 directly[37]. Consistent with this finding, another study showed that miR-145a was decreased during osteogenic differentiation in the C2C12 and MC3T3-E1 cell lines and that it suppressed osteogenic differentiation by targeting sp7[38]. However, we found that the expression level of miR-145a increased during the osteogenic differentiation of mBMSCs. In other studies, similar results to our were reported that miR-145a was upregulated during the osteogenesis of human bone marrow-derived MSCs[39, 40]. The results of miR-145a expression during osteogenesis are not completely consistent, possibly due to different cell backgrounds. In addition to the target genes mentioned above (FoxO1 and SP7), Cbfb has also been reported as a
target gene of miR-145a[22, 27]. However, we found that in these studies, the 3’-UTR sequence inserted into the luciferase reporter used for target verification was too different from that of the normal occurring 3’-UTR of Cbfb, so we cloned the whole 3’-UTR of Cbfb and carried out the target verification assay again.

Some miRNAs were downregulated in the process of osteogenic differentiation, and their function was to inhibit osteogenic differentiation. This can be explained by the fact that after the osteogenic differentiation process was initiated, the expression of miRNAs decreased under the control of certain factors so that the differentiation could be successfully completed. For example, miR-103a was downregulated during cyclic mechanical stretch (CMS)-induced osteoblast differentiation, whereas its target gene Runx2 expression was upregulated in protein level, which helps keep osteoblast differentiation running smoothly[41]. However, in our research, we found that miR-145a expression increased in osteogenic differentiation, though it functions as an inhibitor of osteogenic differentiation. This may be due to the increase in p53 expression upstream, which leads to an increase in miR-145a downstream, thus inhibiting osteogenic differentiation. From this point of view, miR-145a is a mechanism for p53 to inhibit osteogenic differentiation.

The transcription factor p53 is involved in cellular senescence and osteogenic differentiation by upregulating the expression of miR-145a. The p53/miR-145a/Cbfb axis negatively regulates the osteogenic differentiation of mBMSCs. We found that the number of senescent cells increased after overexpression of miR-145a. However, our study only focused on the role of the p53/miR-145a/Cbfb axis in the osteogenic differentiation of mBMSCs, and its role in senescence needs further study.

Interestingly, Yi-Ping Li and colleagues have reported that the deletion of Cbfb can inhibit osteogenic differentiation and enhance lipogenic differentiation[42]. In accordance with this, the osteogenic differentiation ability of aged MSCs decreased, and the lipogenic differentiation ability increased. It can be speculated that Cbfb may also play a role in the senescence of MSCs, which is also the focus of our next work. Furthermore, it has been reported that p53 can directly bind to the Cbfb promoter region to activate its transcription[43] in acute myeloid leukemia cells. However, due to different cell backgrounds, we still need to verify this finding in MSCs.
Another problem that needs to be solved is why the expression of p53 increased in the process of osteogenic differentiation of mBMSCs. Several in vitro studies have confirmed the role of p53 as a negative regulator in MSCs differentiation pathways[44], which reflects the complexity of the underlying mechanisms. Therefore, the specific mechanism needs more in-depth study.

Conclusions
We found that p53-induced miR-145a promoted cellular senescence and inhibited the osteogenic differentiation of MSCs. The p53/miR-145a/Cbfb axis providing a new mechanism for p53 mediated inhibition of osteogenic differentiation, and hoping to provide a new target for the treatment of osteoporosis.

Abbreviations
miRNA microRNA
MSCs Mesenchymal stem cells
Cbfb Core binding factor beta
ODM Osteogenic differentiation medium
ADM Adipogenic differentiation medium
BMP9 Bone morphogenetic protein (BMP)-9

Declarations

Ethics declarations
All procedures involving experimental animals were performed following protocols approved by the Institutional Animal Care and Use Committee of Xinhua Hospital Affiliated to Shanghai Jiaotong University

Consent for publication
Not applicable.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests
Authors’ contributions

Yanhong Gao contributed to the study conception and design. Chao Xia, Tianyuan Jiang, Yonghui Wang, Xiaoting Chen, and Yan Hu performed the experiments. Chao Xia and Tianyuan Jiang created the graphs and wrote and revised the manuscript. All authors analyzed and interpreted experimental data. All authors read and approved the final manuscript.

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Figures

Figure 1

The multipotentiality and high purity of MSCs isolated from mouse bone marrow. Morphological appearance of third-passage mBMSCs (A). Adipogenic (B) and osteogenic (C) differentiation capacity of mBMSCs. mBMSCs surface markers evaluated through flow cytometric analysis (D)
Figure 2

A

B

C

D

E

F

G

H

Figure 2
Expression changes of p53 and p21 during cell senescence and osteogenic differentiation of mBMSCs. Expression of p53 and p21 determined by qRT-PCR (A) and western blot (B) analysis during cell senescence of mBMSCs induced by H2O2. Expression of p53 and p21 determined by qRT-PCR (C) and western blot (D) analysis in long-term cultured mBMSCs. Expression of BMP9, RUNX2, p53, and p21 measured by qRT-PCR in mBMSCs infected by Ad-BMP9 for 24 hours (E). Western blot analysis of protein expression of p53, p21, Runx2, BMP9 in mBMSCs infected by Ad-BMP9 for 1 day (1d) and 2 days (2d) (F). Expression of p53, p21, and Runx2 determined by qRT-PCR (G) and western blot (H) analysis in mBMSCs cultured in osteogenic differentiation medium (ODM) for 14 days (14d). Results are presented as the mean±SD (*, p<0.05; **, p<0.01; ***, p<0.001). Abbreviations: Runx2, Runt-related transcription factor 2; BMP9, bone morphogenetic protein 9.
Expression changes of p53-induced miRNAs during cell senescence and osteogenic differentiation of mBMSCs. Expression of miR-34a/b/c, miR-29, miR-192 and miR-145a measured by qRT-PCR in mBMSC treated with H2O2 (A) or infected by Ad-BMP9 (B). Results are presented as the mean±SD (*, p<0.05; **, p<0.01; ***, p<0.001).
Expression changes of miR-145a during cell senescence and osteogenic differentiation of mBMSCs. Expression of miR-145a determined by qRT-PCR in long-term cultured mBMSCs (A), in bone marrow MSCs isolated from ageing rats (B) and in mBMSCs cultured in ODM for 0 day (0D), 7 days (7D), 14 days (14D) and 21 days (21D) (C). Results are presented as the mean±SD (*, p<0.05; **, p<0.01; ***, p<0.001). Abbreviations: ODM, osteogenic differentiation medium.
The role of miR-145a in osteogenic differentiation and cell senescence of mBMSCs. miR-145a mimic was used to overexpress miR-145 in C3H10T1/2. qRT-PCR analysis of miR-145a expression on the third day (A) and expression of ALP and Runx2 measured by western blot on the seventh day (B) in C3H10T1/2 transfected with miR-145a mimic and cultured in ODM (B). Images of ALP staining on day 7 (C) and Alizarin Red staining on day 14 (D) of ODM induction in mBMSCs with miR-145a overexpression. C3H10T1/2 cells transfected with miR-145a mimic on day 2 were induced with 200 μM H2O2 for 2 hours and stained positive for SAβ-galactosidase 24 hours later (F). Results are presented as the mean±SD (*, p<0.05; **, p<0.01; ***., p<0.001). Abbreviations: OCN, Osteocalcin.
miR-145a inhibited osteogenic differentiation in C3H10T1/2 cells by targeting Cbfb. The potential miR-145a target genes predicted by TargetScan and miRDB (A). Putative miR-145a
binding site in Cbfb 3’-UTR (B). The mRNA level (C) and protein level (D) of Cbfb measured respectively by qRT-PCR and western blot in C3H10T1/2 overexpressed miR-145a. Luciferase reporter assay of Cbfb 3’-UTR (E) (Mean±SD, n=3 per group, * p < 0.05). Western blot analysis of the expression of Runx2 and Cbfb in C3H10T1/2 cells transfected with miR-145a mimic and pcDNA3.1-Cbfb and cultured in ODM on the day 3 (F) and ALP staining (G) and Alizarin Red staining (H) was carried out for these cells on day 7 and on day 21, respectively. Results are presented as the mean±SD (*, p<0.05).

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