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Bone marrow mesenchymal stem cells (BMSCs) exhibit an age-dependent reduction in osteogenesis that is accompanied by an increased propensity toward adipocyte differentiation. This switch increases adipocyte numbers and decreases the number of osteoblasts, contributing to age-related bone loss. Here, we found that the level of microRNA-188 (miR-188) is markedly higher in BMSCs from aged compared with young mice and humans. Compared with control mice, animals lacking miR-188 showed a substantial reduction of age-associated bone loss and fat accumulation in bone marrow. Conversely, mice with transgenic overexpression of miR-188 in osterix$^+$ osteoprogenitors had greater age-associated bone loss and fat accumulation in bone marrow relative to WT mice. Moreover, using an aptamer delivery system, we found that BMSC-specific overexpression of miR-188 in mice reduced bone formation and increased bone marrow fat accumulation. We identified histone deacetylase 9 (HDAC9) and RPTOR-independent companion of MTOR complex 2 (RICTOR) as the direct targets of miR-188. Notably, BMSC-specific inhibition of miR-188 by intra–bone marrow injection of aptamer-antagomiR-188 increased bone formation and decreased bone marrow fat accumulation in aged mice. Together, our results indicate that miR-188 is a key regulator of the age-related switch between osteogenesis and adipogenesis of BMSCs and may represent a potential therapeutic target for age-related bone loss.

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MicroRNA-188 regulates age-related switch between osteoblast and adipocyte differentiation

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Bone marrow mesenchymal stem cells (BMSCs) exhibit an age-dependent reduction in osteogenesis that is accompanied by an increased propensity toward adipocyte differentiation. This switch increases adipocyte numbers and decreases the number of osteoblasts, contributing to age-related bone loss. Here, we found that the level of microRNA-188 (miR-188) is markedly higher in BMSCs from aged compared with young mice and humans. Compared with control mice, animals lacking miR-188 showed a substantial reduction of age-associated bone loss and fat accumulation in bone marrow. Conversely, mice with transgenic overexpression of miR-188 in osterix+ osteoprogenitors had greater age-associated bone loss and fat accumulation in bone marrow relative to WT mice. Moreover, using an aptamer delivery system, we found that BMSC-specific overexpression of miR-188 in mice reduced bone formation and increased bone marrow fat accumulation. We identified histone deacetylase 9 (HDAC9) and RPTOR-independent companion of MTOR complex 2 (RICTOR) as the direct targets of miR-188. Notably, BMSC-specific inhibition of miR-188 by intra–bone marrow injection of aptamer-antagomiR-188 increased bone formation and decreased bone marrow fat accumulation in aged mice. Together, our results indicate that miR-188 is a key regulator of the age-related switch between osteogenesis and adipogenesis of BMSCs and may represent a potential therapeutic target for age-related bone loss.

Introduction

Bone marrow mesenchymal stem cells (BMSCs) have the potential to differentiate into various cell types, including adipocytes, chondrocytes, and osteoblasts (1, 2). Age-related osteoporosis is characterized by reduced bone formation and increased marrow fat accumulation (3–6). This age-related reduction in osteoblast activity is partly caused by the fact that BMSCs from elderly subjects have reduced capacity to differentiate into osteoblasts and increased capacity to differentiate into adipocytes (3, 4). However, the molecular mechanisms behind the shift from osteoblast to adipocyte differentiation in BMSCs remain elusive.

MicroRNAs (miRNAs) are a class of small (~22 nucleotides), single-stranded noncoding RNAs found in diverse organisms, which downregulate the expression of target genes by either mRNA degradation or translational inhibition (6, 7). Recently, several miRNAs were found to be involved in either osteogenesis or adipogenesis (8–12). However, most of these miRNAs have only been investigated in vitro, and their functional roles in the pathophysiological mechanisms responsible for age-related bone loss and accumulation of fat in bone marrow remain to be established. The roles of miRNAs in the age-related switch between osteoblast and adipocyte differentiation of BMSCs in bone marrow are also unclear.

Here, we identified a novel, highly expressed miRNA, miR-188, from the BMSCs of aged mice and human subjects. Our study demonstrated that miR-188 regulates BMSCs’ bifurcation into osteoblasts and adipocytes during aging. Knockout of Mir188 in mice reduced age-associated trabecular and cortical bone loss and marrow fat accumulation. Notably, intra–bone marrow injection of BMSC-targeting aptamer-antagomiR-188 stimulated trabecular and cortical-endosteal bone formation and decreased bone marrow fat accumulation in aged mice. Thus, our study provides a new mechanism and a novel therapeutic target for age-related bone loss.

Results

Aging induces miR-188 expression in BMSCs. Histological and immunohistochemical analyses of femora revealed increased number and area of adipocytes characterized by fat vacuoles in bone marrow and decreased number of osteocalcin-positive osteoblasts on the trabecular and endosteal bone surfaces in aged mice (18 months old) compared with young mice (3 months old) (Figure 1, A–D, and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI77716DS1). The increased bone marrow fat accumulation and decreased osteoblasts implied a switch from osteogenic differentiation to adipogenic differentiation of BMSCs. To determine the mechanism of the age-related differentiation switch of BMSCs, Sca-1+CD29+CD45+CD11b+ mesenchymal stem cells were sorted by FACS from bone marrow cells (13) of young and aged mice to identify dysregulated miRNAs by performing miRNA microarray analysis (Figure 1E). Among them, miR-188 showed the largest difference in expression between the 2 groups, being expressed approximately 30 times more highly in aged mice compared with young mice. The raw data of the microarray have been uploaded to GEO with the series record GSE57127 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57127).
The increased level of miR-188 was further confirmed by quantitative real-time RT-PCR (qRT-PCR) (Figure 1F).

We then isolated human BMSCs (defined as positive for STRO-1 and CD146 and negative for CD45) (14) from bone marrow cells by FACS. Notably, the miR-188 level in human BMSCs was positively correlated with age (Figure 1, G and H). These data suggest that miR-188 plays an important role in the aging process of BMSCs in both mouse and human.

**Figure 1. Aging induces miR-188 expression in BMSCs.** (A–D) Representative images of toluidine blue (T) staining (A, top) and osteocalcin (Ocn) immunohistochemical staining (A, bottom) and quantification of number and area (Ar) of adipocytes (B and C) and number of osteoblasts (D) in distal femora from 3-month-old and 18-month-old female C57BL/6 mice. Tb. N, trabecular number. Scale bars: 100 μm. n = 6 per group. (E) Microarray profiling results of deregulated miRNAs in BMSCs from young and aged mice. (F) qRT-PCR analysis of the levels of miR-188 expression in BMSCs derived from the mice at different ages. n = 6 per group. (G and H) Age-associated changes of miR-188 levels in BMSCs from 85 human females (G) and 85 males (H). Data shown as mean ± SD. *P < 0.05, **P < 0.01 (B–D, Student’s t test; F, ANOVA).

MiR-188 knockout mice show reduced age-associated bone loss and marrow fat accumulation. To investigate the role of miR-188 in vivo, we generated mir-188 knockout (Mir188−/−) mice via gene targeting mediated by transcription activator-like effector nuclease (TALEN) (Supplemental Figure 2, A and B, and ref. 15). The trabecular bone volume and number were higher and the trabecular separation was lower in the femora of aged (12 or 18 months old) Mir188−/− mice relative to their WT littermates (Figure 2, A–E).
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The cortical bone thickness was higher and the endosteal perimeter was lower in the femora of aged Mir188−/− mice compared with their WT littermates (Figure 2, F–H). The vertebral bone volume was also higher in aged Mir188−/− mice compared with their WT littermates (Figure 2, I and J). Accordingly, values of the trabecular maximum load and stiffness, which represent bone strength, were higher in the aged Mir188−/− mice than in their WT littermates (Figure 2, K and L). Calcein double labeling confirmed that aged Mir188−/− mice had significantly higher trabecular and endosteal bone formation rates (BFRs) compared with their WT littermates (Figure 3, A–D). However, no significant differences were observed between the young (3 months old) Mir188−/− mice and their WT littermates (Figure 2 and Figure 3, A–I).

Aged, but not young, Mir188−/− mice had significantly decreased number and area of adipocytes in the bone marrow (Figure 3, E–G) and higher number and surface of osteoblasts on the trabecular and endosteal bone surfaces (Figure 3, H and I, and Supplemental Figure 3, A and B) compared with their WT littermates. The number and surface of osteoclasts on the trabecular bone surface were not changed in Mir188−/− mice relative to their WT littermates (Supplemental Figure 3B). In vitro, BMSCs from Mir188−/− mice exhibited increased osteogenesis and decreased adipogenesis (Figure 3J). However, culture of bone marrow monocytes/macrophages showed that osteoclast formation was not affected (Supplemental Figure 3C).

Taken together, the results suggest that Mir188−/− mice have reduced age-associated bone loss and marrow fat accumulation. Transgenic expression of miR-188 in osterix+ osteoprogenitors leads to an accelerated bone marrow fat accumulation and bone loss. We then constructed transgenic mice overexpressing miR-188 in osterix+ osteoprogenitors to investigate whether overexpression of miR-188 in vivo would lead to bone loss and marrow fat accumulation. qRT-PCR revealed significantly higher levels of miR-188 expression in both line 1 and line 2 compared with that in WT controls (Supplemental Figure 4A). Both line 1 and line 2 (6 months of age) had lower trabecular bone volume of femur and vertebra relative to WT controls (Figure 4, A–E and I, and Supplemental Figure 4B). We chose the line 1 mice with higher miR88 expression (referred to as “transgenic mice” hereafter) for detailed analysis.

Figure 2. Mir188−/− mice exhibit reduced age-associated bone loss. (A–H) Representative microcomputed tomography (μCT) analysis of trabecular (B–E) and cortical (F–H) bone microarchitecture in femora from 3-, 12-, and 18-month-old WT and miR-188 knockout (Mir188−/−) mice. n = 6 per group. Tb. BV/TV, trabecular bone volume per tissue volume; Tb. N, trabecular thickness; Tb. Sp, trabecular separation; Ct. Th, cortical thickness; Ps. Pm, periosteal perimeter; Es. Pm, endosteal perimeter. (I and J) Representative μCT images (I) and quantification of the ratio of bone volume to tissue volume (J) of L4 vertebrae (Vt. BV/TV). n = 6 per group. (K and L) Three-point bending measurement of tibia maximum load (K) and stiffness (L). n = 5 per group. Data shown as mean ± SD. *P < 0.05, **P < 0.01 (Student’s t test).
The trabecular bone volume, number, and cortical bone thickness were lower, and the trabecular separation and endosteal perimeter were higher, in the femora of 6-month-old and 12-month-old transgenic mice relative to WT controls (Figure 4, A–H). The vertebral bone volume was also lower in miR-188 transgenic mice relative to WT controls (Figure 4). In addition, bone strength was lower in the miR-188 transgenic mice compared with WT controls (Figure 4, J and K). MiR-188 transgenic mice had significantly lower trabecular and endosteal BFRs relative to WT controls (Figure 4, L–N). Moreover, miR-188 transgenic mice had significantly higher number and area of adipocytes in the bone marrow and lower number and surface of osteoblasts on the trabecular and endosteal bone surfaces as compared with WT controls (Figure 4, O–R, and Supplemental Figure 3).

Figure 3. **Mir188** \(^{-/-}\) mice show higher osteoblastic bone formation and lower marrow fat accumulation in aged mice. (A) Representative images of calcein double labeling of trabecular (Tb), endosteal (Eb), and periosteal bone (Pb) with quantification (B–D) of bone formation rate per bone surface (BFR/BS) in femora of 18-month-old WT and **Mir188** \(^{-/-}\) mice. Scale bar: 50 μm. n = 6 per group. (E) Representative images of toluidine blue staining with quantification of number and area of adipocytes in distal femora (F and G). Scale bar: 100 μm. (H) Representative images of osteocalcin immunohistochemical staining with quantification of number of osteoblasts in distal femora (I). Scale bar: 100 μm. n = 6 per group. (J) Representative images of Oil Red O staining of lipids (top) and Alizarin Red S staining of matrix mineralization (bottom) in BMSCs from **Mir188** \(^{-/-}\) mice and WT mice cultured in adipogenesis induction medium for 14 days and osteogenesis induction medium for 21 days, respectively. Scale bar: 100 μm. Data are representative of 3 independent experiments. Data shown as mean ± SD. \(^*P<0.05\), \(^{**P<0.01}\) (Student’s t test).
significantly higher number and area of adipocytes in the bone marrow and lower number and surface of osteoblasts on the trabecular and endosteal bone surfaces (Figure 5, O–R, and Supplemental Figure 6, C and D) as compared with vehicle-treated mice. However, no differences of the osteoclast number and surface were observed between these 2 groups of mice (Supplemental Figure 6D).

These results reveal that mice with BMSC-specific overexpression of miR-188 have reduced bone formation and increased bone marrow fat.

MiR-188 promotes adipogenic differentiation of BMSCs. The qRT-PCR data showed that miR-188 expression increased gradually during adipogenesis of BMSCs (Figure 6A). To investigate the role of miR-188 during adipogenesis, BMSCs were transfected with the agomiR-188 or antagomiR-188 to overexpress or silence miR-188 (Figure 6B). The BMSCs were then cultured in adipogenesis induction medium. Overexpression of miR-188 facilitated lipid droplet formation in BMSCs induced by adipogenesis.
induction medium (Figure 6, C and D), accompanied by increased mRNA levels of peroxisome proliferator–activated receptor-γ (Pparg) and fatty acid binding protein 4 (Fabp4), 2 key markers of adipocyte differentiation (Figure 6, E and F). By contrast, silencing of miR-188 attenuated adipogenic differentiation of BMSCs (Figure 6, C and D) and inhibited Pparg and Fabp4 mRNA expression in BMSCs (Figure 6, E and F). The gene microarray analysis further confirmed that mRNA expression levels of Pparg and Fabp4, as well as other adipocyte differentiation markers (Igfbp2, Lpi, Mgst3), were lower in adipogenic induction medium–induced BMSCs of Mir188−/− mice as compared with their WT littermates (Figure 6G). The raw data of the microarray have been uploaded to GEO with the series record GSE63725 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63725). All of these results suggest that miR-188 promotes adipogenic differentiation of BMSCs. MiR-188 inhibits osteoblastic differentiation of BMSCs. The expression of miR-188 decreased gradually during osteogenesis, as assessed by qRT-PCR (Figure 7A). To investigate the role of miR-188 during osteoblastic differentiation, BMSCs were transfected with agomiR-188 or antagomiR-188 to overexpress or silence miR-188 and then cultured in osteogenesis induction medium. Overexpression of miR-188 inhibited, while silencing of miR-188 promoted, osteogenic differentiation of BMSCs measured by Alizarin Red staining (Figure 7, B–G). Consistently, osteoblast differentiation markers, alkaline phosphatase (ALP) activity and osteocalcin secretion, were lower in agomiR-188–transfected cells compared with control cells (Figure 7, D and E). Moreover, the mRNA level of the late-stage second osteoblast transcription factor osterix, but not the first osteoblast transcription factor Runx2, was inhibited by agomiR-188 (Figure 7, F and G). Conversely, antagomiR-188 transfection promoted osteogenic differentiation and increased osterix expression (Figure 7, B–G). Moreover, gene microarray analysis further confirmed that Alpl (ALP), Col1a (collagen type I α), Sp7 (osterix),
and other osteoblast differentiation markers were higher in osteoblastic induction medium–induced BMSCs of Mir188–/– mice as compared with their WT littermates (Figure 7H). The raw data of the microarray have been uploaded to GEO with the series record GSE63725 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63725). All of these results suggest that miR-188 inhibits osteogenic differentiation of BMSCs.

**MiR-188 directly targets HDAC9 and Rictor.** MiRNAs regulate the expression of mRNAs by binding to the 3′-untranslated regions (3′-UTRs) or amino acid coding sequences of target genes. We used miRanda (19), PicTar (20), and TargetScan (21) to predict the targets of miR-188. Among the predicted genes, we chose 6 for further analysis—histone deacetylase 9 (Hdac9), RPTOR-independent companion of MTOR complex 2 (Rictor), phosphatase and tensin homolog (Pten), zinc finger protein 281 (Znf281), GLIS family zinc finger 3 (Glis3), and ephrin B2 (Efnb2)—which had been reported to participate in bone metabolism (22–28). Overexpression or inhibition of miR-188 changed endogenous levels of HDAC9 and RICTOR protein, but not the others (Figure 8A). However, no changes of the mRNA levels of Hdac9 and Rictor were noted (Supplemental Figure 7).

Overexpression of miR-188 suppressed the luciferase activity of the Hdac9 or Rictor 3′-UTR reporter genes (Figure 8, C–E). Mutation of 2 nucleotides within the sequences of the putative target site in the 3′-UTR of Hdac9 or Rictor (MUT-pGL3-HDAC9, MUT1-pGL3-RICTOR, or MUT2-pGL3-RICTOR) abolished this repression, confirming the specificity of miR-188’s action (Figure 8, C–E).

We also detected the protein of HDAC9 or RICTOR in BMSCs of aged (18 months) and young (3 months) WT and Mir188–/– mice. HDAC9 and RICTOR protein levels were lower in 18-month-old WT mice compared with 3-month-old WT mice, while in Mir188–/– mice, and other osteoblast differentiation markers were higher in osteoblastic induction medium–induced BMSCs of Mir188–/– mice as compared with their WT littermates (Figure 7H). The raw data of the microarray have been uploaded to GEO with the series record GSE63725 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63725). All of these results suggest that miR-188 inhibits osteogenic differentiation of BMSCs.

Sequence analysis revealed 1 conserved binding site for miR-188 in the 3′-UTR of the HDAC9 gene (position 1914–1921) and 2 in the RICTOR gene (position 3761–3767, position 4112–4118) (Figure 8B). To investigate whether miR-188 directly targets HDAC9 and RICTOR, luciferase reporter constructs containing the predicted miRNA-binding site of HDAC9 and RICTOR (WT-pGL3-HDAC9, WT1-pGL3-RICTOR, or WT2-pGL3-RICTOR) were generated (Supplemental Table 1). We transfected WT-pGL3-HDAC9, WT1-pGL3-RICTOR, or WT2-pGL3-RICTOR with agomiR-188 or agomiR-NC into BMSCs and measured the effects of miR-188 on luciferase translation by the level of luciferase enzyme activity.
mice, HDAC9 and RICTOR protein levels were similar in young and aged mice (Figure 8F). However, HDAC9 and RICTOR protein levels were significantly lower in miR-188 transgenic mice compared with their WT littermates (Figure 8F).

Whether the miR-188 targets in HDAC9 and RICTOR are preserved in humans was also investigated. Sequence analysis revealed that binding sites for hsa-miR-188 in the 3′-UTR of human HDAC9 (position 1905–1912) and RICTOR (position 4344–4351) are conserved in mice (Supplemental Figure 8, A and B, and Supplemental Table 1). Luciferase reporter constructs containing the predicted miRNA-binding site of the human HDAC9 and RICTOR (WT-pGL3-hHDAC9 and WT-pGL3-hRictor) were generated (Supplemental Figure 8B) and then transfected into human BMSCs with agomiR-188 or agomiR-NC. Overexpression of miR-188 suppressed the luciferase activity of the human HDAC9 or RICTOR 3′-UTR reporter gene (Supplemental Figure 8, C and D). Mutation of the 2 nucleotides within the sequences of the putative target site in the 3′-UTR of human HDAC9 or RICTOR (MUT-pGL3-hHDAC9, MUT-pGL3-hRictor) abolished this repression, confirming the specificity of the action (Supplemental Figure 8, C and D). Furthermore, human BMSCs with transfection of agomiR-188 or antagoniR-188 showed that overexpression of miR-188 decreased endogenous HDAC9 or RICTOR protein while inhibition of miR-188 increased their protein levels (Supplemental Figure 8E).

These results demonstrate that HDAC9 and RICTOR are the targets of miR-188 in both mouse and human.

Injection of antagoniR-188 into bone marrow stimulates bone formation and decreases bone marrow fat in aged mice. To investigate the therapeutic effects of BMSC-specific inhibition of miR-188 on age-related osteoporosis, aptamer-antagomiR-188 was injected into the femoral bone marrow cavity of 15-month-old mice twice per month for 3 months. Intra–bone marrow injection of BMSC-targeting aptamer-antagomiR-188 significantly decreased the levels of miR-188 in BMSCs (Figure 9A). Aptamer-antagomiR-188 increased the trabecular bone volume, number, and cortical bone thickness, and decreased trabecular separation and the endosteal perimeter (Figure 9, B–I). Furthermore, bone strength increased in those mice that received aptamer-antagomiR-188 treatment (Fig-

Figure 7. MiR-188 inhibits osteogenic differentiation of BMSCs. (A) qRT-PCR analysis of the relative levels of miR-188 expression in BMSCs cultured in osteogenesis induction medium (300 ng/ml BMP-2, 50 μg/ml ascorbic acid, and 5 mM β-glycerolphosphate) for the days as indicated. n = 5 per group. (B and C) Representative images of Alizarin Red S staining (B) and quantitative analysis (C) of matrix mineralization in BMSCs transfected with agomiR-188, antagoniR-188, or their controls and cultured in osteogenesis induction medium for 21 days. n = 5 per group. Scale bar: 100 μm. (D and E) Analysis of ALP activity (D) and osteocalcin secretion (E) in BMSCs cultured in osteogenesis induction medium for 48 hours. (F and G) qRT-PCR analysis of the relative levels of osteventer (F) and Runx2 (G) mRNA expression in BMSCs cultured in osteogenesis induction medium for 48 hours. n = 5 per group. (H) Microarray profiling results of dysregulated osteogenic genes in WT and Mir188−/− mouse–derived BMSCs cultured in osteogenesis induction medium for 48 hours. Data shown as mean ± SD. *P < 0.05, **P < 0.01 (ANOVA).
related switch between osteoblast and adipocyte differentiation in bone marrow remain unclear. In this study, we revealed elevated miR-188 expression in BMSCs from aged mice and human subjects. Furthermore, Mir188–/– mice showed reduced age-associated bone loss and marrow fat accumulation. Osterix+ osteoprogenitor-specific miR-188 transgenic mice showed accelerated bone marrow fat accumulation and bone loss. These results reveal that miR-188 regulates the differentiation directions of BMSCs during aging and contributes to age-related bone loss.

Previously, it was reported that miR-188 regulates dendritic plasticity and synaptic transmission (29), and suppresses G1/S transition (30). However, there is no report on the role of miR-188 in the regulation of BMSC function. In this study, we defined a new mechanism whereby miR-188 regulates the BMSC switch in bone. MiRNAs mediate post-transcriptional gene silencing by base pairing to the complementary sites in the 3′-UTR of the target mRNA (31). We demonstrated that miR-188 directly targets HDAC9 and RICTOR mRNAs. HDAC9, which is dramatically downregulated during adipogenesis, inactivates PPARγ to repress adipogenesis and promote osteogenesis of BMSCs (22, 23). RICTOR, as a key component of mTORC2, which is a member of the mTOR complex implicated in resting cytoskeletal architecture, suppresses PPARγ activity and inhibits adipogenic differentia-
With the progressive aging of the general population, age-related bone loss becomes a growing public problem (32). A major requirement for the treatment of age-related bone loss is to identify anabolic agents that can increase bone formation and decrease fat accumulation in bone marrow via targeting BMSCs (33). In this study, we identified a new mechanism for developing this treatment. We used a BMSC-specific aptamer to deliver antagomiR-188 or agomiR-188 into mice BMSCs. Aptamers are single-stranded nucleic acid molecules that bind to targets via folding into a 3-dimensional structure with high affinity and selectivity (34, 35). Cell type-specific aptamers as drug delivery vehicles have been exploited to enhance the efficacy and safety of therapeutic drugs used in cancer (36, 37), HIV (38, 39), and eye-related diseases (40). Treatment with antagomiR-188 via a BMSC-specific aptamer increased bone

Figure 9. Injection of aptamer-antagomiR-188 into bone marrow stimulates bone formation and decreases marrow fat accumulation in aged mice. (A) qRT-PCR analysis of the levels of miR-188 expression in BMSCs of mice with BMSC-specific antagomiR-188 delivery. Aptamer-antagomiR-188 was injected into femoral bone marrow cavity of 15-month-old mice twice per month for 3 months. NC, negative control. (B–I) Representative μCT images (B) and quantitative μCT analysis of trabecular (C–F) and cortical (G–I) bone microarchitecture in femora from aptamer-treated mice. n = 10 per group. (J and K) Three-point bending measurement of femur maximum load (J) and stiffness (K). n = 5 per group. (L–N) Calcein double labeling–based quantification of bone formation rate per bone surface (BFR/BS) in femora. n = 5 per group. (O–R) Representative images of H&E staining (O, top) and osteocalcin immunohistochemical staining (O, bottom) and quantification of number and area of adipocytes (P and Q) and number of osteoblasts (R) in distal femora. Scale bars: 100 μm. n = 5 per group. Data shown as mean ± SD. *P < 0.05, **P < 0.01 (ANOVA).
formation and decreased bone marrow fat accumulation in aged mice, while treatment with agomiR-188 led to premature aging of bone in middle-aged mice, as evidenced by reduced bone formation and increased bone marrow fat. Currently, most drugs for osteoporosis that are available in the clinic inhibit bone resorption without increasing bone formation (41). Our results indicate that inhibition of miR-188 expression in BMSCs via an aptamer might be a new strategy to treat age-related bone loss and senile osteoporosis.

In conclusion, the current findings support the view that the age-related increase in miR-188 functions as a switch to regulate BMSC differentiation. The results reveal a new mechanism of age-related bone loss and identify a potential therapeutic target.

Methods

Construction of Mir188−/− mice using TALEN plasmids. The mir-188−/− specific TALEN plasmids were obtained from Beijing ComWin Biotech Co. Ltd. The target TALEN sequences of the Mir188 were: left, 5′-CCCCGCTCCCTCTCCTCCAG-3′, and right, 5′-CAGAGAGCTCACCTGTTTGC-3′. TALEN plasmids were digested by NotI restriction endonuclease. The digested plasmids were transcribed to mRNA in vitro using the mMESSAGE mMACHINE 17 Kit (Life Technologies), according to the manufacturer’s instructions. We purified the synthesized mRNAs using a MegaClear Kit (Life Technologies), according to the manufacturer’s instruction. The mRNAs were diluted to working concentration (50 ng/μl) in injection buffer (0.25 mM EDTA, 10 mM Tris, pH 7.4) treated with diethyl pyrocarbonate (Sigma-Aldrich).

C57BL/6 mice were used as embryo donors and were superovulated. The TALEN mRNAs were injected into the cytoplasm of pronuclear-stage fertilized eggs, which were obtained from oviducts after superovulated female C57BL/6 mice were mated to C57BL/6 stud males. The injected zygotes were cultured in M2 medium (Sigma-Aldrich) at 37°C for 24 hours. Zygotes that developed into the 2-cell stage were selected to transfer into the oviducts of pseudopregnant inbred (ICR) female mice.

Genomic DNA was extracted from tail tips. For genotyping of miR-188, PCR was carried out using the following primers (synthesized by Sangon Biotech): forward, 5′-TCTTGCGCCAGATGTTGTG-3′, and reverse, 5′-AGGGAGTTCAAAGGCAGCATG-3′. To determine the accuracy of PCR, we collected the PCR products from agarose gel to use as templates for sequencing.

Generation of osterix osteoprogenitor-specific mir-188 transgenic mice. To generate osterix osteoprogenitor-specific mir-188 transgenic (mir-188-Tg) mice, a plasmid containing the osterix promoter to drive gene expression was constructed. First, we cloned the mouse pre-miR-188 cDNA (synthesized by Genscript Co.) into the SalI-EcoRI site in a plasmid containing the osterix promoter, resulting in osterix-pre-miR-188 vector. The plasmid (osterix-pre-miR-188) was then transfected into BMSCs using Lipofectamine 2000 (Invitrogen). Empty vector was also transfected into BMSCs as a control. qRT-PCR was used to detect the expression of miR-188. The fragments of the osterix-pre-miR-188 were then purified and microinjected into C57BL/6J F1 mouse oocytes, and the oocytes were then surgically transferred into pseudopregnant C57BL/6J dams. Two lines with high levels of miR-188 expression were selected from 5 transgenic founders and bred in C57BL/6 strain for 6 generations to obtain offspring with a defined genetic background. One line with a sevenfold overexpression of miR-188 was extensively studied. The WT mice were used as controls.

All mice were maintained in the specific pathogen-free facility of the Laboratory Animal Research Center at Central South University.

BMSC isolation and culture. For mouse BMSC isolation, bone marrow cells were flushed from femora of female mice and incubated for 20 minutes at 4°C with PE-, FITC-, peridinin chlorophyll protein-, and allophycocyanin-conjugated antibodies that recognized mouse Sca-1 (108108; BioLegend), CD29 (102206; BioLegend), CD45 (103132; BioLegend), and CD11b (101226; BioLegend). For human BMSC isolation, human bone marrow cells were incubated with PE-, allophycocyanin-, and PE-conjugated antibodies that recognized human STRO-1 (catalog 340106; BioLegend), CD45 (catalog 304012; BioLegend), and CD146 (catalog 361008; BioLegend) at 4°C for 30 minutes. Acquisition was performed on a FACS Aria model (BD Biosciences), and the analysis was performed using FACS DIVE software version 6.1.3 (BD Biosciences).

The sorted mouse CD29 Sca-1 CD45 CD11b− BMSCs and human CD45 STRO-1 CD45− BMSCs were cultured for 1–2 weeks to reach 80%–85% confluence. Then, first-passage BMSCs were detached and seeded in culture flasks for enrichment of cell populations. As second-passage BMSCs reached confluence after approximately 1 week, they were subcultured. Only third-passage BMSCs were subjected to induction of adipogenic and osteogenic differentiation, and transfection of plasmids.

MiRNA microarray assay. Small RNAs were isolated from the total RNA of BMSCs from young (3 months old) and aged (18 months old) female C57BL/6 mice, and then labeled with Cy3. The Oebiotech Company performed the miRNA microarray assay. The fragmentation mixtures were hybridized to an Agilent-046065 Mouse miRNA Microarray V19.0 8×60K (Agilent). Feature Extraction software 10.7.1.1 (Agilent) analyzed the scanned images using default parameters to obtain background subtracted and spatially detrended processed signal intensities as the raw data. Raw data were normalized in a quantile algorithm with Genespring 12.0 (Agilent). Probes for which at least 100% of samples in any 1 condition out of 2 conditions had flags in “Detected” were maintained. The raw data of the microarray have been uploaded to GEO with the series record GSE57127.

Gene chip microarray assay. RNAs were isolated from WT and Mir188−/− mouse–derived BMSCs cultured with adipogenesis induction medium or osteogenesis induction medium for 48 hours. The RNAs were sent to the UCLA Clinical Microarray Core to perform the gene chip microarray assay. The fragmentation mixtures were hybridized to an Affymetrix mouse 1.0 gene ST array. Robust multiarray average was used to perform the data normalization. Principal component analysis in Partek Genomics Suite was used to perform the cluster analysis of different samples. ANOVA was used to identify the differential expression genes. The raw data of the microarray have been uploaded to GEO with the SuperSeries record GSE63725.

Histochemistry analysis. Histochemistry analysis was performed as described previously (42, 43). Briefly, femora were harvested from mice after euthanasia, fixed in 10% formalin for 24 hours, and decalcified in 10% EDTA for 14 days, before being embedded in paraffin. Four-micrometer-thick longitudinally oriented bone sections were stained with H&E, TRAP, and toluidine blue to quantify number and surface of osteoblasts, number and surface of osteoclasts, and number and area of adipocytes, respectively.

OsteoMeasureXP Software (OsteoMetrics Inc.) performed histomorphometric measurements of 2-dimensional parameters of the
trabecular bones. To label the mineralization fronts, the mice were injected with 25 mg/kg calcine at 8 and 2 days before euthanasia. The femora were fixed in 70% ethanol and dehydrated in increasing concentrations of ethanol, and the undecalcified bones were embedded in methyl methacrylate. Serial 5-μm sections of the femur were made using a microtome. The parameters obtained for the bone formation were bone formation rate per bone surface, osteoblast surface per bone surface, and osteoblast number per bone perimeter. The parameters measured for bone resorption were osteoclast surface per bone surface and osteoclast number per bone perimeter.

**Immunohistochemical staining.** Immunohistochemical staining was performed as described previously (44, 45). Briefly, bone sections were processed for antigen retrieval by digestion with 0.05% trypsin was performed as described previously (44, 45). Briefly, bone sections were processed for antigen retrieval by digestion with 0.05% trypsin at 37°C for 15 minutes, and then incubated with primary antibody against osteocalcin (catalog M173; Takara) overnight at 4°C. Subsequently, an HRP-streptavidin detection system (Dako) was used to detect the immunoactivity, followed by counterstaining with hematoxylin (Sigma-Alrich). Sections incubated with polyclonal rabbit IgG (R&D Systems Inc.) served as negative controls.

**Identification of an aptamer using cell-SELEX procedure.** The selection technology to generate aptamers is called systematic evolution of ligands by exponential enrichment (SELEX) (16–18). The library of synthetic DNAs comprised a 40-base central random sequence flanked by primer sites on either side (5′-GGAATTCAGTCGGA-3′). Four-nanomole single-stranded DNA (ssDNA) pools were denatured by heating to 80°C for 10 minutes in a selection buffer containing 50 mM Tris-HCl and 1 mM MgCl₂, and 0.1% NaCl, and then renatured at 0°C for 10 minutes. The mouse BMSCs, monocytes/macrophages, or preosteoclasts (10⁶ cells for the first round and 10⁵ cells for the further rounds) were incubated with the ssDNA at 37°C for 30 minutes in selection buffer. Partitioning of bound and unbound ssDNA sequences was done by centrifugation. After centrifugation and washing 3 times with 1 ml selection buffer (with 0.2% BSA), cell-bound ssDNAs were amplified by PCR. Aptamers obtained from the tenth round of selection were PCR-amplified using unmodified primers and cloned into E. coli using the TA Cloning Kit (Promega Corp.).

**Construction of BMSC-specific aptamer delivery system.** The BMSC-specific aptamer was synthesized by Genscript Co. AgomiR-188, antagomiR-188, and their respective negative controls (NCs) were synthesized by Ribobio Co.

We mixed 1 part by volume of a polyethyleneimine (PEI) solution (100 μg/ml, pH 6.0) with 6 parts by volume of a 4.2-μM sodium citrate to form the PEI-citrate core structure (nanocore). Then, we added 3 parts by volume of synthetic BMSC aptamers (50 nM) and agomiR-188 (1 μM) or antagomiR-188 (1 μM) to the nanocore for 5 minutes of reaction to assemble the nanocomplex.

Mice received either 40 μl of an agomiR-188 nanocomplex or an antagomiR-188 nanocomplex, or a comparable volume of PBS via periostral injection into medullary cavity of femur twice per month for 3 months.

**mRNA 3′-UTR cloning and luciferase reporter assay.** For functional analysis of miR-188, the segments of the mouse and human HDAC9 and Rictor 3′-UTR, including the predicted miR-188–binding site, were PCR-amplified. The PCR products were purified and inserted into the XbaI-FseI site immediately downstream of the stop codon in the pGL3 control luciferase reporter vector (Promega Corp.), resulting in mouse and human WT-pGL3-HDAC9, WT1-pGL3-RICTOR, or WT2-pGL3-RICTOR. The HDAC9 and RICTOR mutants for the miR-188 seed regions were prepared using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) to get mouse and human MUT-pGL3-HDAC9, MUT1-pGL3-RICTOR, or MUT2-pGL3-RICTOR. Mouse and human BMSCs were transfected with either WT or mutant pGL3 construct, the pRL-TK renilla luciferase plasmid (Promega Corp.), and agomiR-188 or agomiR-NC for 48 hours using Lipofectamine 2000 (Invitrogen). The dual luciferase reporter assay system (Promega Corp.) was used to quantify luminescent signal using a luminometer (Glomax; Promega Corp.). Each value from the firefly luciferase assay was normalized to the renilla luciferase value from the cotransfected phRL-null vector (Promega Corp.). The nucleotide sequences of primers for WT and mutant reporter plasmids are shown in Supplemental Table 2.

**Adipogenic differentiation assay.** To induce adipogenic differentiation of BMSCs in vitro, BMSCs were cultured in 6-well plates at 2.5 × 10⁵ cells per well with adipogenesis induction medium (α-MEM containing 10% FCS, 0.5 mM 3-isobutyl-1-methylxanthine, 5 μg/ml insulin, and 1 μM dexamethasone) for 14 days. Culture medium was changed every second day. We performed Oil Red O staining to detect mature adipocytes in cultures with adipogenesis induction.

**Osteogenic differentiation and mineralization assay.** To induce osteoblastic differentiation, BMSCs were cultured in 24-well plates at 5 × 10⁴ cells per well with osteogenesis induction medium (300 ng/ml BMP-2, 50 μg/ml ascorbic acid, and 5 mM β-glycerophosphate) for 48 hours. Then, the cell lysates were homogenized for ALP activity assay by spectrophotometric measurement of p-nitrophenol release using an enzymatic colorimetric ALP Kit (Roche). Culture media were collected for assessment of secreted osteocalcin levels using a specific immunoaassay kit (DiaSorin).

To induce osteoblastic mineralization, BMSCs were cultured in 6-well plates at 2.5 × 10⁵ cells per well with osteogenesis induction medium, as described above, for 21 days. Then, cells were stained with 2% Alizarin Red S (Sigma-Aldrich) at pH 4.2 to evaluate the cell matrix mineralization. A Diaphot Inverted Microscope and Camera System (Nikon) was used for imaging. Alizarin Red S released from the cell matrix into the cetyl-pyridinium chloride solution was quantified by spectrophotometry at 540 nm.

To normalize protein expression to total cellular protein, a fraction of the lysate solution was subjected to the Bradford assay.

**Preosteoclast and osteoclast differentiation.** Monocytes and macrophages were harvested from bone marrow of 6-month-old female WT and Mir188⁻/⁻ mice by flushing of the femur and tibia marrow space.
Flushed bone marrow cells were cultured on Petri dishes in α-MEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 30 ng/ml M-CSF (R&D Systems Inc.) overnight. After discarding of the adherent cells, floating cells were incubated with M-CSF (30 ng/ml) to obtain monocytes and macrophages. Monocytes and macrophages were further cultured in 24-well plates (1×10^5 cells per well) with 30 ng/ml M-CSF and 60 ng/ml RANKL (PeproTech) for 3 days to obtain preosteoclasts. Alternatively, we incubated monocytes and macrophages with 30 ng/ml M-CSF and 200 ng/ml RANKL for 8 days to obtain fully mature multinucleated osteoclasts. TRAP activities of the preosteoclasts and osteoclasts were detected using a commercial kit (Sigma-Aldrich).

qRT-PCR analysis. We performed qRT-PCR using a Roche Molecular Light Cycler as previously described (46, 47). Total RNA from tissues or cultured cells was isolated using the TRizol reagent (Invitrogen), and reverse transcription was performed using 1 μg total RNA and SuperScript II (Invitrogen). Amplification reactions were set up in 25-μl reaction volumes containing SYBR Green PCR Master Mix (PE Applied Biosystems) and amplification primers. A 1-μl volume of cDNA was used in each amplification reaction. Primer sequences are listed in Supplemental Table 3 and Supplemental Table 4.

Statistics. Data are presented as mean ± SD. For comparisons of 2 groups, 2-tailed Student’s t test was used. Comparisons of multiple groups were made using 1-way ANOVA. All experiments were repeated at least 3 times, and representative experiments are shown. Differences were considered significant at *P* < 0.05.

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