

Inhibition of Circulating miR-194-5p Reverses Osteoporosis through Wnt5a/ β -Catenin-Dependent Induction of Osteogenic Differentiation

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Mesenchymal stem cells (MSCs) critically contribute to bone formation, and proper induction of osteogenic differentiation can lead to an increase in bone mass. In the present study, we reported that an increased miR-194-5p level in plasma is inversely related to the degree of bone formation in osteoporosis patients. We also noted that increased miR-194-5p in the MSCs of ovariectomized (OVX) mice and agomiR-194-5p manipulation of miR-194-5p significantly suppressed bone formation, both in aged and OVX mice. Furthermore, our *in vitro* study showed that overexpression of miR-194-5p suppresses osteogenic differentiation, as evidenced by the decreased bone formation marker genes and matrix mineralization. The luciferase assay indicated that Wnt family member 5a (Wnt5a) is a target gene of miR-194-5p that positively regulates osteogenic differentiation. Collectively, these data indicated that miR-194-5p inhibition may be a potential strategy for osteoporosis prevention.

INTRODUCTION

Osteoporosis is a chronic systemic disease characterized by decreased mass and microarchitectural deterioration of bone. It is mainly seen in postmenopausal women and with aging, and, hence, with the aging population, the socioeconomic burden of osteoporosis is on the rise.¹ Osteoporosis patients have increased bone fragility and fracture susceptibility, which are associated with significant mortality and poor quality of life.² Although various drugs have been developed to inhibit bone resorption and increase bone formation, their side effects limit their long-term administration.³ Therefore, further research to elucidate the molecular and cellular factors in play is necessary.

Bone mesenchymal stem cell (BMSC) differentiation plays a major role in the maintenance of normal bone homeostasis.⁴ Accumulating evidence has suggested that induction of adipocyte differentiation impairs bone formation, while increased osteogenic differentiation is essential for bone mass increase.⁵⁻⁷ Therefore, a clear understanding of MSC fate regulation is required. Recent studies have focused on investigating the regulation of extrinsic and intrinsic regulators on

MSC fate. For example, activation of the histone demethylation protein lysine demethylase 4B (KDM4B) induces osteogenic differentiation of MSCs.⁸ Although the molecular mechanism of MSC differentiation has been extensively studied, the role of microRNA (miRNA) control in MSC commitment, and consequently the connection between miRNAs and osteoporosis, is yet to be clarified. miR-194-5p, a vertebrate-specific miRNA, mainly regulates proliferation and energy production in tumor cells. For example, miR-194-5p promotes breast cancer cell proliferation, migration, and invasion.⁹ However, overexpression of miR-194-5p suppresses the migration and invasion ability of colorectal cancer cells.¹⁰ Recent studies have indicated that miR-194-5p is involved in the differentiation of myoblast and hematopoietic progenitors,^{11,12} which highly suggests that miR-194-5p could modulate cell differentiation. Previous studies have reported that miR-194-5p is upregulated in women with osteoporosis, which may be a negative regulator for osteoporosis.¹³ It has been widely accepted that enhanced osteogenic differentiation of MSCs is essential to protect against osteoporosis. However, whether miR-194-5p affects osteoporosis progression via regulating MSCs differentiation and its underlying mechanism still needs further investigation.

Therefore, in the present study, we demonstrate that circulating miR-194-5p suppresses the osteogenic differentiation of MSCs. Injection of miR-194-5p directly into the bone marrow reduces osteoblast differentiation, thereby promoting osteoporosis. Furthermore, the underlying mechanism of miR-194-5p-mediated MSCs differentiation was investigated.

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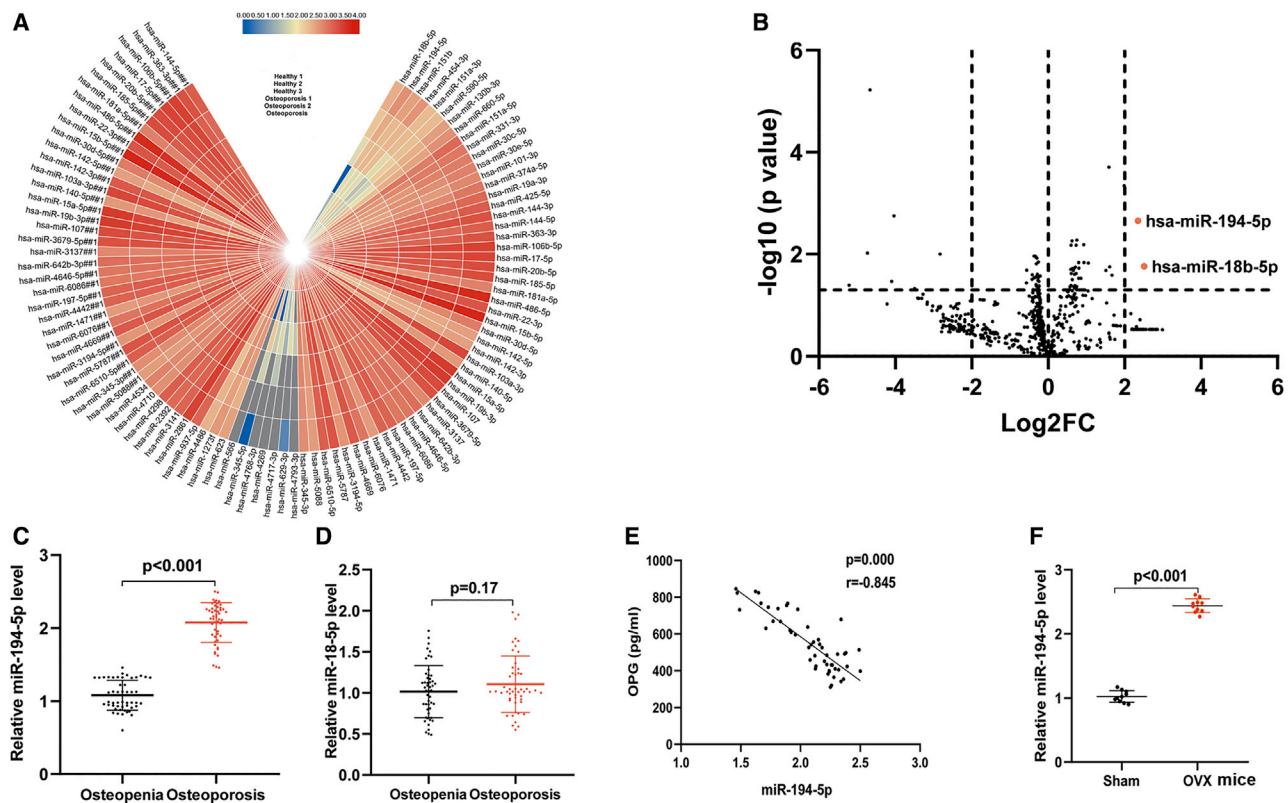


Figure 1. miR-194-5p Is Highly Increased in Osteoporosis Patients and OVX Mice

(A) A heatmap of the 50 most upregulated and most downregulated miRNAs was constructed. (B) The total dysregulated miRNAs were visualized using a volcano plot. (C and D) The level of miR-194-5p (C) and miR-18-5p (D) in the serum of healthy volunteers and osteoporosis volunteers was evaluated using quantitative real-time PCR. $n = 50$. (E) Association between serum miR-194-5p and bone formation marker (OPG) in osteoporosis patients. $n = 50$. (F) The miR-194-5p level in the bone MSCs of control (sham) and OVX mice was assessed using quantitative real-time PCR. $n = 10$.

RESULTS

miR-194-5p Is Highly Increased in Osteoporotic Patients and Ovariectomized (OVX) Mice

To investigate serum miRNA expression in osteoporosis, we obtained miRNA microarray expression profiling data from the Gene Expression Omnibus (GEO) databases (GEO: GSE64433). Subsequently, the miRNAs of healthy and osteoporotic patients were analyzed with a GEO2R tool, and the 50 most upregulated and 50 most downregulated miRNAs were constructed using a heatmap (Figure 1A). Total miRNA dysregulation was visualized using a volcano plot (Figure 1B). As shown in Figure 1B, both miR-194-5p and miR-18-5p levels were significantly increased in the osteoporosis serum samples in comparison to the healthy serum samples ($p < 0.05$, \log_2 fold change [FC] > 3). To further confirm the level of miR-194-5p and miR-18-5p in serum, we collected blood samples from 50 healthy and 50 osteoporotic patients. As shown in Figure 1C, the miR-194-5p level was significantly higher in the plasma of osteoporotic patients in comparison to the healthy volunteers. However, no significant difference was observed in the miR-18-5p level between the two groups (Figure 1D). A strong negative association between serum miR-194-5p level and osteoprotegerin (OPG) concentration was also observed in the osteoporotic

patients (Figure 1E). Subsequently, we assessed the miR-194-5p level in the bone MSCs of sham and OVX mice. Our data suggested that the miR-194-5p level was significantly increased in the MSCs of OVX mice in comparison to sham mice (Figure 1F). Collectively, these data suggest that an elevated miR-194-5p level is closely linked to osteoporosis.

miR-194-5p Suppresses Osteoblast Differentiation and Decreases Bone Mass *In Vivo*

To determine whether increased miR-194-5p *in vivo* affects bone homeostasis in adult mice, 12-month-old male mice were treated with phosphate-buffered saline (PBS) or miR-194-5p injected into the femoral bone marrow. Three months later, micro-computed tomography (micro-CT) analysis of the femur revealed that miR-194-5p injection significantly decreased trabecular bone volume/tissue volume (BV/TV), trabecular numbers (Tb.N.), and trabecular thickness (Tb.Th.) and increased trabecular separation (Tb.Sp.) (Figures 2A–2E). The trabecular and cortical bone mass was clearly decreased in the miR-194-5p treatment group, as evidenced by lower cortical thickness (Ct.Th.) and bone mineral density (BMD) (Figures 2F and 2G). In agreement with the above results, decreased bone area

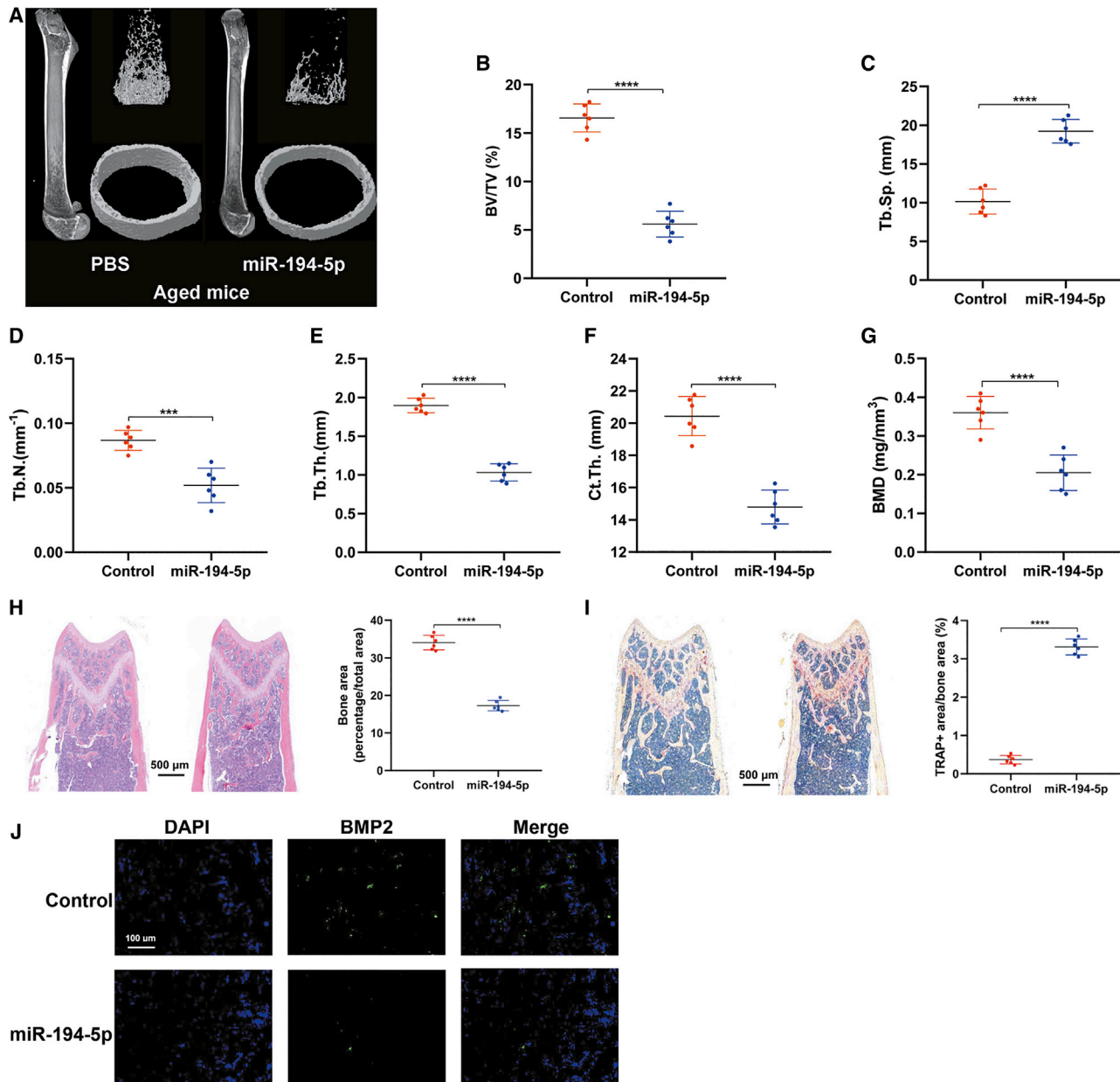


Figure 2. Overexpression of miR-194-5p Decreases Bone Mass and Promotes Osteoporosis in Aged Mice

(A) Representative images of the femur of aged male mice. (B–E) Quantification of trabecular BV/TV (B), Tb.Sp. (C), Tb.N. (D), and Tb.Th. (E) of mice from the two groups. (F and G) Quantification of trabecular Ct.Th. (F) and BMD (G) of mice from the two groups. (H) H&E staining of the femoral metaphysis and quantification of the bone area. (I) TRAP staining of the femoral metaphysis and quantification of the TRAP-positive area. (J) Representative images of BMP2-positive osteoblasts on the surface of trabecular bone. $n = 6$. *** $p < 0.01$, **** $p < 0.0001$.

with increased tartrate-resistant acid phosphatase (TRAP)-positive area was observed in the miR-194-5p treatment group in comparison to the control group (Figures 2H and 2I). Notably, miR-194-5p significantly decreased the bone morphogenetic protein 2 (BMP2)-positive osteoblast number when compared to control mice (Figure 2J). Collectively, these data suggest that miR-194-5p promotes osteoporosis progression in aged mice.

miR-194-5p Induces Bone Mass Loss in OVX Mice

To test the effect of miR-194-5p on osteoporosis, we treated OVX mice with miR-194-5p. After 3 months of administration, the miR-194-5p treatment group showed lower BV/TV, Tb.N., and Tb.Th. and higher Tb.Sp (Figures 3A–3E). In agreement with the above results, the Ct.Th. and BMD levels were significantly decreased in the miR-194-5p treatment group (Figures 3F and 3G). Importantly, the

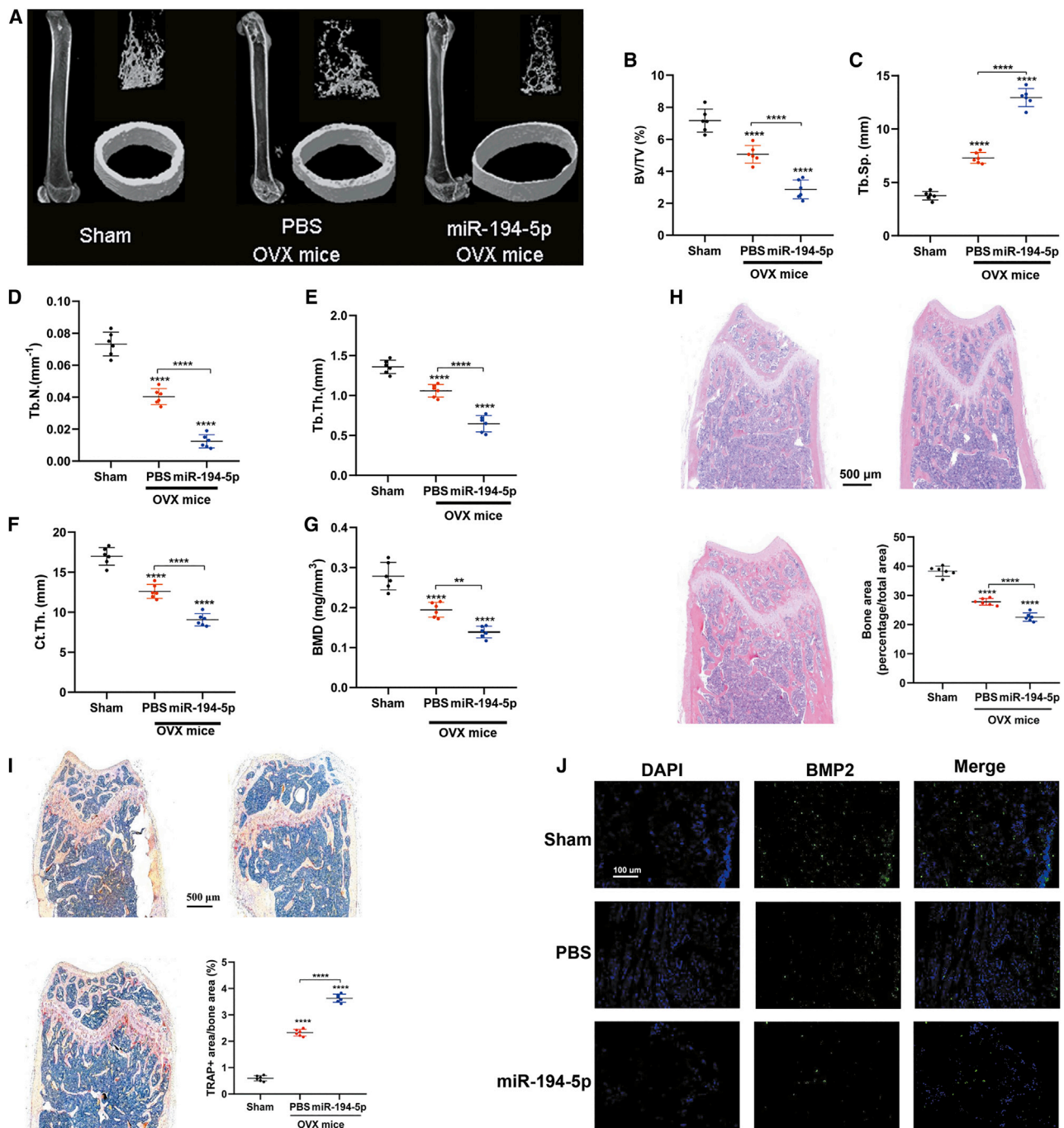


Figure 3. miR-194-5p Induce Bone Mass Loss in OVX Mice

(A) Representative images of the femur of OVX mice. (B–E) Quantification of trabecular BV/TV (B), Tb.Sp. (C), Tb.N. (D), and Tb.Th. (E) of mice from the three groups. (F and G) Quantification of trabecular Ct.Th. (F) and BMD (G) of mice from the three groups. (H) Representative images of H&E staining of the femoral metaphysis and quantification of the bone area. (I) Representative images of TRAP staining of the femoral metaphysis and quantification of the TRAP-positive area. (J) Representative images of BMP2-positive osteoblasts on the surface of trabecular bone. n = 6. *p < 0.05, **p < 0.01, ****p < 0.0001.

decreased bone area and TRAP-positive area also demonstrated the negative effect of miR-194-5p in osteoporosis (Figures 3H and 3I). This result was further supported by a decrease in BMP2-positive os-

teoblasts on the surface of trabecular bone (Figure 3J). Collectively, these data indicated that miR-194-5p contributes to the progression of osteoporosis in OVX mice.

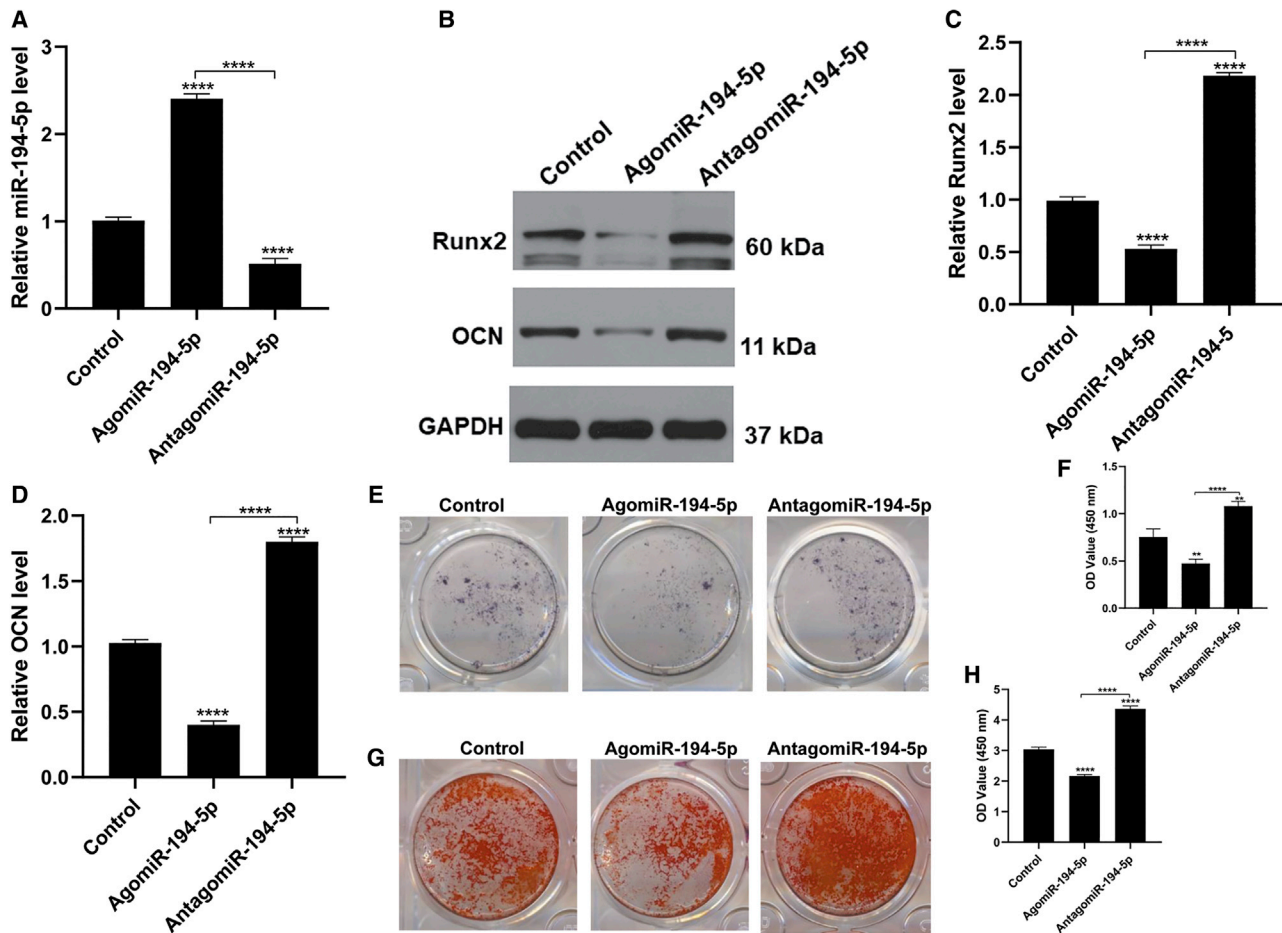


Figure 4. miR-194-5p Suppresses Osteogenic Differentiation of MSCs

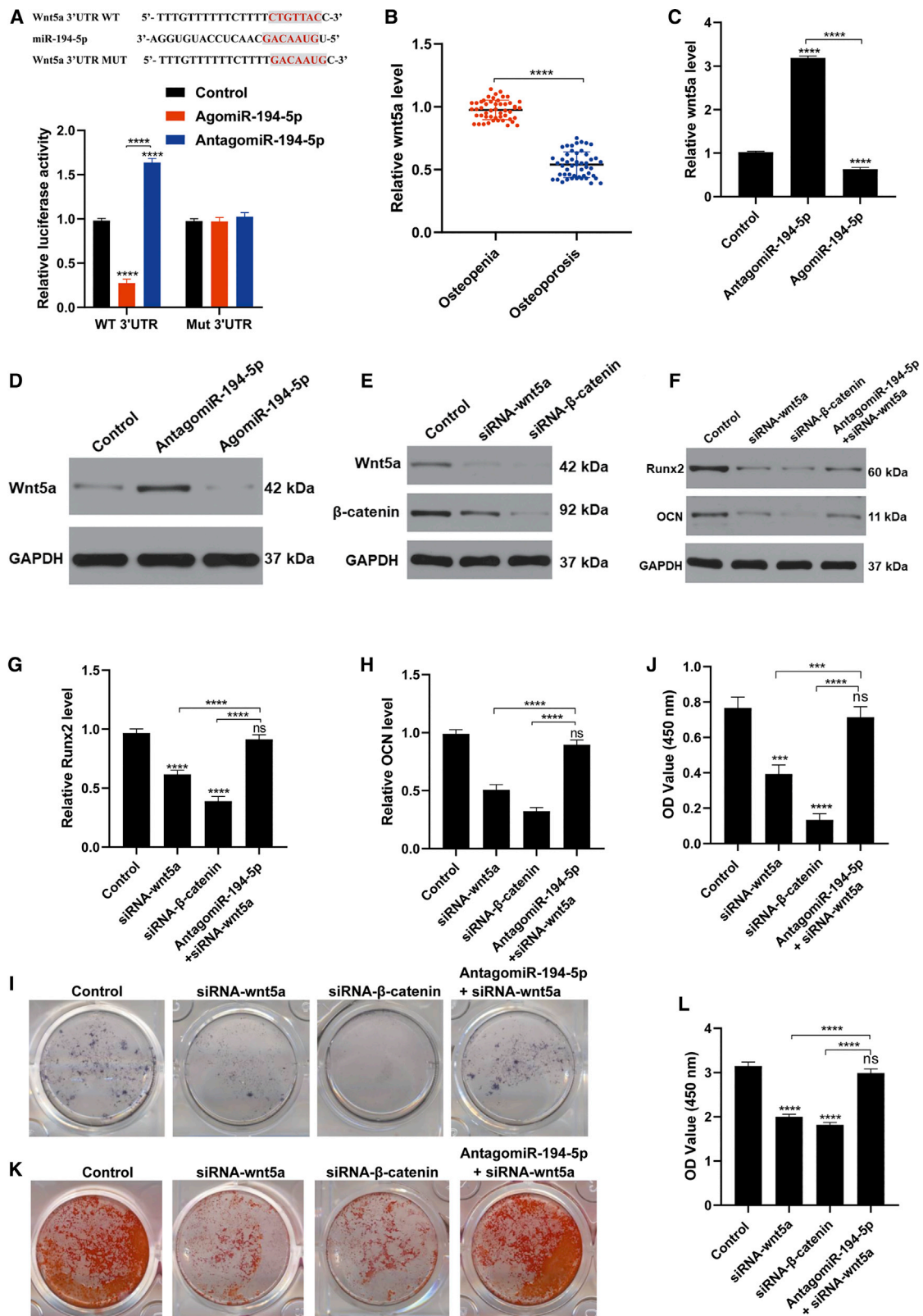
(A) Quantitative real-time PCR showed that miR-194-5p expression was increased with agomiR-194-5p treatment. (B) Western blot showed that the osteogenic-related markers Runx2 and OCN were decreased with agomiR-194-5p treatment. (C and D) Quantitative real-time PCR showed that the osteogenic-related markers Runx2 (C) and OCN (D) were decreased with agomiR-194-5p treatment. (E) ALP staining of MSCs after treatment with the indicated agents for 3 days. (F) Quantification of ALP staining among the three groups. (G) Alizarin red staining of MSCs 21 days after the indicated treatment. (H) Quantification of alizarin red staining of MSCs on day 21. All data are expressed as mean \pm SD. *** p < 0.001, **** p < 0.0001.

miR-194-5p Inhibits Osteogenic Differentiation *In Vitro*

As miR-194-5p overexpression impairs bone mass and decreases osteoblasts *in vivo*, we hypothesized that miR-194-5p is involved in MSC differentiation. Therefore, to investigate whether miR-194-5p treatment can inhibit osteogenic differentiation we measured osteogenic-related genes, and performed alkaline phosphatase (ALP) and alizarin red staining. We found that agomiR-194-5p treatment significantly increased the level of miR-194-5p in MSCs, while antagomiR-194-5p treatment decreased the level of miR-194-5p (Figure 4A). Osteogenic-related gene expression was significantly suppressed with agomiR-194-5p treatment, evidenced through decreased Run family transcription factor 2 (Runx2) and osteocalcin (OCN) levels (Figures 4B–4D). In agreement with the above results, diminished ALP activity and mineralization capacity were observed in the agomiR-194-5p group (Figures 4E–4H). Collectively, these results demonstrate that miR-194-5p is a key regulator of osteogenic differentiation.

Wnt5a/ β -Catenin Signaling Is Essential for miR-194-5p-Mediated Inhibition of Osteogenic Differentiation

To investigate the molecular mechanisms of miR-194-5p-mediated osteogenic differentiation, we screened the miRDB database (<http://mirdb.org/>) to identify potential targets. Previous studies reported that the Wnt signaling pathway is closely associated to bone formation and osteoporosis;¹⁴ hence, we hypothesize that Wnt family member 5a (Wnt5a) may be a potential target of miR-194-5p. To identify the relationship between miR-194-5p and Wnt5a, we used a wild-type (WT) Wnt5a 3' UTR or a mutant-type (Mut) Wnt5a 3' UTR construct fused to a luciferase reporter. The results showed that agomiR-194-5p substantially suppressed WT Wnt5a 3' UTR reporter activity, while antagomiR-194-5p increased WT Wnt5a 3' UTR reporter activity. However, both agomiR-194-5p and antagomiR-194-5p had no impact on Mut Wnt5a 3' UTR reporter activity (Figure 5A). The quantitative real-time PCR results showed that the serum level



(legend on next page)

of Wnt5a in the osteopenia group was significantly higher than that in the osteoporosis group (Figure 5B). Next, we performed an *in vitro* study to test whether miR-194-5p affects the Wnt/ β -catenin signaling pathway. When MSCs were transfected with the miRNA construct for 24 h, lower relative Wnt5a levels in the agomiR-194-5p group were noted, compared with the other groups (Figures 5C and 5D). MSCs were treated with Wnt5a and small interfering RNA (siRNA) β -catenin to verify the role of the Wnt/ β -catenin signaling pathway during MSC differentiation. We demonstrated that Wnt5a and β -catenin levels were markedly decreased after siRNA-Wnt5a or siRNA- β -catenin treatment (Figure 5E). Inhibition of either Wnt5a or β -catenin significantly reduced osteogenic differentiation, evidenced through decreased osteogenic markers (Figures 5F–5H), diminished ALP activity, and decreased mineralization capacity (Figures 5I–5L). Not surprisingly, miR-194-5p inhibition (antagomiR-194-5p) partly rescued the negative effect of siRNA-Wnt5a on osteogenic differentiation. Taken together, these data suggest that the Wnt5a/ β -catenin signaling pathway is involved in the osteogenic differentiation through interaction with miR-194-5p.

DISCUSSION

A shift in BMSC lineage is a hallmark of osteoporosis, but the underlying mechanism of the MSC differentiation remains poorly understood. In the present study, we provide evidence for the role of circulating miR-194-5p in MSC differentiation and osteoporosis progression.

The role of miRNAs has been well established in the physiological and pathological processes of disease, and dysregulated miRNAs are thought to be main contributors in osteoporosis.¹⁵ Specifically, elevated miR-182 has been shown to increase bone resorption and induce osteoporosis.¹⁶ Conversely, miR-34a is protective in osteoporosis.¹⁷ In the present study, we identified that the miR-194-5p level is markedly increased in the serum of osteoporosis patients, a level negatively correlated with the bone formation marker OPG. Our result was consistent with previous studies that identified miR-194-5p as a potential biomarker for postmenopausal osteoporosis.¹⁸ Thus, elevated miR-194-5p levels are likely to contribute to the development of osteoporosis.

Prior studies have reported that miR-194-5p regulates the progression of various cancers.^{19,20} However, its mechanism in osteoporosis remains elusive. Osteoporosis is a complex pathophysiological process determined by the viability of different cells, including osteoblasts, osteoclasts, and MSCs.²¹ Based on the elevated miR-194-5p level in the MSCs of OVX mice, a new connection between miR-194-5p and MSCs has become clear, specifically that miR-194-5p suppresses the capacity of

osteogenic differentiation. Furthermore, a decrease in BMP2-positive osteoblasts seen in the trabecular bone of aged and OVX mice after miR-194-5p treatment highlights the negative effect of miR-194-5p.

It is well established that the canonical Wnt pathway governs processes such as proliferation, migration, and differentiation. The significance of Wnt/ β -catenin signaling in osteogenic differentiation has been well documented.^{22,23} In the present study, we identified that the Wnt signaling pathway is involved in miR-194-5p-mediated osteogenic differentiation. We then offer evidence that miR-194-5p inhibits Wnt5a expression, thereby suppressing Wnt/ β -catenin pathway activation. Previous studies have suggested that Wnt activation is necessary for MSC differentiation.²⁴ In line with prior studies, we found that inhibition of the Wnt pathway through treatment with siRNA-Wnt5a-conditioned medium markedly inhibits osteogenic differentiation. miR-194-5p may regulate multiple targets, with Wnt5a being just one of the main targets that affects MSC differentiation. Therefore, further studies are necessary to investigate the underlying mechanism of miR-194-5p on MSC differentiation.

Although multiple miRNAs have been reported to be involved in osteoporosis, the origin of such dysregulated miRNAs remains unclear. With aging, many molecules are released from senescent cells and transported in the blood or to other cells. Previous studies have suggested that dying cells can transfer miR-194-5p into residual repopulating cells.²⁵ We infer that miR-194-5p may be the metabolites of senescent cells, which could further affect the metabolic processes of other normal cells. Better understanding of the mechanisms of MSC differentiation may shed new light on osteoporosis and other bone mass loss-related diseases. In the current study, we demonstrated that miR-194-5p regulates MSC differentiation through inhibition of the Wnt/ β -catenin pathway. However, the pathogenesis of osteoporosis is complex, and the effect of miR-194-5p on other cell types, including osteoblasts and osteoclasts, requires further investigation.

Our findings establish a new mechanism of miR-194-5p in MSC differentiation. Circulating miR-194-5p suppresses Wnt5a expression, thereby inhibiting Wnt/ β -catenin activation and in turn suppressing osteogenic differentiation and inducing osteoporosis *in vivo* (Figure 6). Thus, therapeutic interventions targeting miR-194-5p offer great promise in the treatment of osteoporosis.

MATERIALS AND METHODS

Statement of Human and Animal Rights

Blood was collected from 50 healthy and 50 osteoporosis volunteers. All clinical procedures were approved by the Committee on Clinical

Figure 5. Wnt5a/ β -Catenin Signaling Is Involved in miR-194-5p-Mediated Osteogenic Differentiation

(A) Effect of miR-194-5p on the luciferase activity of WT Wnt5a 3' UTR or Mut Wnt5a 3' UTR after treatment with agomiR-194-5p or antagomiR-194-5p in MSC cells. (B) The level of Wnt5a in the serum of healthy volunteers and osteoporosis volunteers was evaluated using quantitative real-time PCR. n = 50, (C and D) The Wnt5a levels were evaluated by quantitative real-time PCR (C) and western blot (D) after antagomiR-194-5p and agomiR-194-5p treatment. (E) The Wnt5a and β -catenin levels were assessed by western blot after indicated treatment. (F–H) Western blot (F) and quantitative real-time PCR were used to evaluate the osteogenic-related markers Runx2 (G) and OCN (H). (I) ALP staining of MSCs after indicated treatment for 3 days. (J) Quantification of ALP staining of MSCs on day 3. (K) Alizarin red staining of MSCs after 21 days following indicated treatment. (L) Quantification of alizarin red staining of MSCs on day 21. The data are expressed as mean \pm SD. *p < 0.05, **p < 0.01, ***p < 0.001.

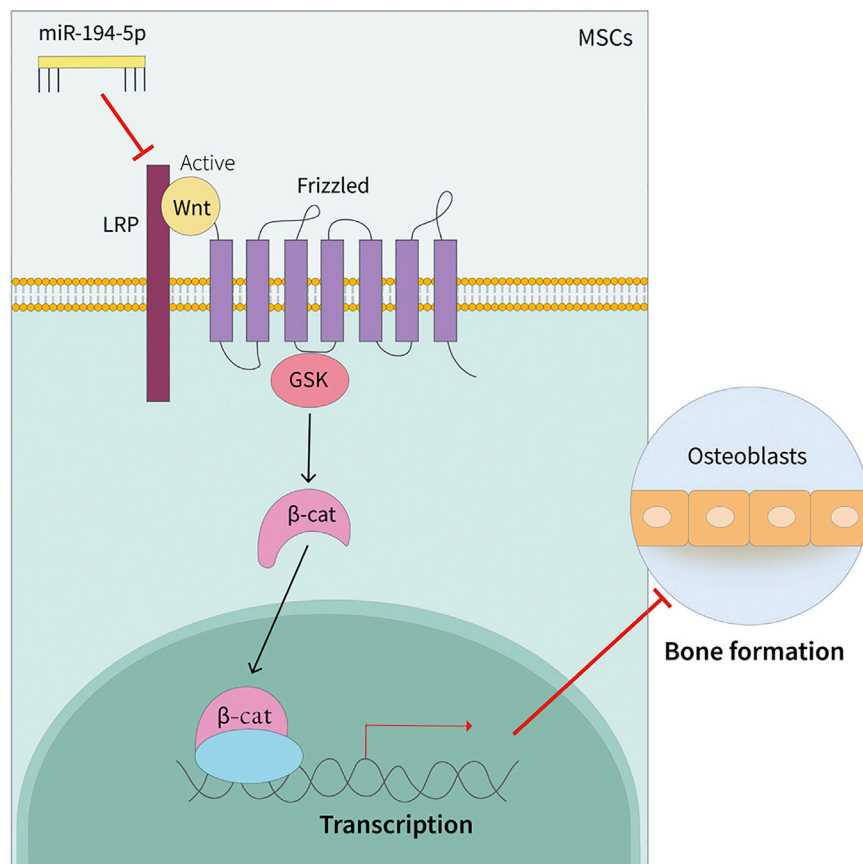


Figure 6. Diagrammatic Sketch of miR-194-5p-Medicated Osteoblast Differentiation

Circulating miR-194-5p is transported to the bone marrow, where it is taken up by MSCs. Subsequently, miR-194-5p suppresses Wnt/ β -catenin pathways, resulting in osteogenic differentiation inhibition.

(50 nM) using Lipofectamine 3000 (Invitrogen, USA). All experiments were repeated at least three times independently.

Osteogenic Differentiation and Staining

For osteogenic differentiation of MSCs, cells were incubated in an osteogenic induction medium (Cyagen Biosciences, USA). After induction for 3 days, quantitative ALP staining was performed according to the manufacturer's instructions. After induction for 21 days, BMSCs were stained using alizarin red at room temperature for 10 min, followed by rinsing with PBS.

Western Blot

Total protein was collected from cells using a lysis buffer. Protein concentration was evaluated using a bicinchoninic acid (BCA) kit. Ten micrograms of protein was separated using a 10% SDS-PAGE gel, followed by transfer to a polyvinylidene fluoride (PVDF) membrane. The membranes were incubated with primary

antibodies at 4°C overnight, followed by a 1-h incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature. The primary antibodies used were as follows: anti-Wnt5a (1:500, Abcam, MA, USA, #ab229200), anti- β -catenin (1:500, Abcam, MA, USA, #ab16051), anti-Runx2 (1:500, Abcam, MA, USA, #ab23981), anti-OCN (1:500, Abcam, USA, #ab93876), and anti-GAPDH (1:10,000, Abcam, USA, #ab37168). All experiments were performed in triplicate.

Ethics in the Union Hospital (Wuhan, China). All samples were obtained with signed informed consent. Detailed information on the volunteers is listed in Table S1. C57BL/6 mice were purchased from the Center of Experimental Animal, Tongji Medical College, Huazhong University of Science and Technology. All animal protocols were in compliance with the Ethics Committee of the Institutional Animal Care and Use Committee at Tongji Medical College, Huazhong University of Science and Technology.

Cell Culture and Transfection

6- to 8-week-old male C57BL/6 mice were sacrificed and the femurs and tibias were dissected. The bone marrow was flushed with PBS, followed by centrifugation at $300 \times g$ and suspension in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) with 1% penicillin/streptomycin (P/S). Cells were incubated in a humidified incubator at 37°C with 5% CO₂. Before transfection, cells were seeded at 50% confluency in DMEM containing 10% FBS for 24 h.

The control siRNA (#37007), mouse Wnt5a siRNA (#sc-41113), and mouse β -catenin siRNA (#29210) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). AgomiR-194-5p and antago-miR-194-5p were purchased from GenePharma (Shanghai, China). Cells were transfected with miR-194-5p (200 μ m) or siRNA

Quantitative Real-Time PCR Analysis

Total RNA was isolated from cells using TRIzol (Invitrogen, USA). For mRNA, the equivalent of 1 μ g of total RNA was reverse transcribed in a 12- μ L reaction containing 1 \times mRNA-specific reverse transcriptase (RT) primers (1 μ L), total RNA (1 μ g), 10 mM deoxyribonucleotide triphosphate (dNTP) mix (1 μ L), and distilled water. The above solution was mixed at 65°C for 5 min and quick-chilled on ice. Subsequently, 5 \times first-strand buffer (4 μ L), 0.1 M DTT (2 μ L), and RNaseOUT recombinant ribonuclease inhibitor (1 μ L) were added to the above solution. The solution was incubated at 37°C for 2 min, followed by adding 1 μ L of Moloney murine leukemia virus (M-MLV) RT and incubating the solution at 25°C for 10 min and 37°C for 50 min. This was followed by heat inactivation for 15 min at 70°C. Amplification reactions were performed using Fast-Start Universal SYBR Green Master (Rox) (Roche) with 1 μ L of RT

under optimized conditions at 95°C for 5 s, 58°C for 30 s, and 72°C for 30 s for 40 cycles, followed by a melting curve analysis. For miRNA extraction, the miRNeasy serum kit (QIAGEN, CA, USA) was used according to the manufacturer's instructions. The miR-194-5p level was measured using an all-in-one miRNA quantitative real-time PCR detection kit (GeneCopoeia, MD, USA) according to the manufacturer's instruction. PCRs were performed and GAPDH and U6 were selected as the reference gene for mRNA and miRNA, respectively. The comparative Ct method ($2^{-\Delta\Delta C_t}$) was used to calculate the results. The sequences of the primers are listed in Table S2. Each sample was analyzed five times during each experiment. The experiments were carried out at least three times.

Enzyme-Linked Immunosorbent Assay (ELISA)

The plasma of patients was collected and the OPG level was assessed using a human OPG kit purchased from MultiSciences (Hangzhou, China, #70-EK11342) according to the manufacturer's instructions.

Animal Experiments

Ten-month-old C57BL/6 mice were randomly divided into two groups. The control group received 30 μ L of PBS twice monthly for 3 months through periosteal injection into the bone marrow cavity of the femur. The experimental group received agomiR-194-5p (20 μ mol/L in 100 μ L of PBS) twice monthly for 3 months through periosteal injection into the bone marrow cavity of the femur. Eight-week-old C57BL/6 mice were randomly divided into a sham or an OVX group. The sham group underwent fat tissue excision near the ovaries, and the OVX group underwent bilateral ovariectomy surgery. Four weeks later, when the osteoporotic mouse model was established, the sham group received 30 μ L of PBS, and the OVX group received 30 μ L of agomiR-194-5p (20 μ mol/L in 100 μ L of PBS) twice monthly for 3 months. All mice were sacrificed after 3 months, and the femurs were collected for further analysis (n = 6 per group).

Micro-CT Analysis

The femurs of mice were collected for micro-CT scanning (Bruker SkyScan 1176 scanner mCT system). Bones were scanned at a high resolution with an energy of 37 kV and 121 mA. The Bruker mCT evaluation software was used to construct and analyze the 3D images of the bone. To evaluate the bone structure, we collected the following parameters: BV/TV (%), Tb.Th., Tb.N., Tb.Sp., Ct.Th., and BMD.

Hematoxylin and Eosin (H&E) Staining and TRAP Staining

The femurs were harvested from mice after the experiments and fixed in 10% neutral buffered formalin for 24 h. The samples were then decalcified and sectioned into 4- μ m-thick slices for further analysis. For H&E staining, the sections were deparaffinized in xylene and rehydrated through a graded series of ethanol. The sections were stained with hematoxylin for 5 min and eosin for 1 min. TRAP staining was performed according to the instructions of the TRAP kit (Sigma-Aldrich, St. Louis, MO, USA). Histological analyses of H&E-stained and TRAP-stained samples were performed with ImageJ software.

Immunofluorescence Staining

For bone sample immunofluorescence staining, the slides were deparaffinized for antigen retrieval. Next, the slides were permeabilized with 0.1% Triton X-100 and blocked with 5% normal goat serum. The slides were incubated with primary antibodies against BMP2 (1:1,000, Abcam) at 4°C overnight, followed by incubation with the specified secondary antibodies. The nuclei were counterstained with DAPI. Images were obtained with a fluorescence microscope in five random fields of each group.

Microarray Procedure

The miRNA-sequencing data (GEO: GSE64433) were obtained from the GEO database, which included whole-blood samples from three healthy volunteers and three osteoporosis volunteers. The differentially expressed miRNAs were analyzed using the online tool GEO2R. A volcano plot was constructed to illustrate all differential miRNAs, and a heatmap was used to exhibit the 50 most upregulated miRNAs and 50 most downregulated miRNAs using TBtools (<http://www.tbtools.com/>).

Statistical Analysis

All data were expressed as mean \pm SD. The Student's unpaired t test was performed to compare the difference between two groups. Comparison between three or more groups was performed using one-way analysis of variance (ANOVA) with Tukey's post hoc test. A p value <0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omtn.2020.07.023>.

AUTHOR CONTRIBUTIONS

B.M. and G.L. conceived and designed the experiments. Y.X., C.Y., L.C., H.X., L.H., and Y.H. performed the experiments. F.C., Y.S. and W.Z. performed statistical analyses. B.M., A.C.P. and G.L. wrote and edited the paper.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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OMTN, Volume 21

Supplemental Information

Inhibition of Circulating miR-194-5p Reverses

Osteoporosis through Wnt5a/ β -Catenin-

Dependent Induction of Osteogenic Differentiation

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Inhibition of circulating miR-194-5p reverses osteoporosis through Wnt5a/ β -catenin signaling-dependent induction of osteogenic differentiation

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Table S1. Information of osteopenia volunteers and osteoporosis volunteers. (Osteopenia, $-2.5 \leq$ Spine T-score ≤ -1.0 ; Osteoporosis, Spine T-score < -2.5).

Volunteer	Age (Year)	Spine T-score	Volunteer	Age (Year)	Spine T-score
Osteopenia 1	63	-1.6	Osteoporosis 1	60	-2.8
Osteopenia 2	67	-1.9	Osteoporosis 2	66	-3.1
Osteopenia 3	58	-1.5	Osteoporosis 3	69	-3.2
Osteopenia 4	54	-1.1	Osteoporosis 4	63	-2.6
Osteopenia 5	56	-1.2	Osteoporosis 5	58	-2.8
Osteopenia 6	66	-1.7	Osteoporosis 6	69	-3.4
Osteopenia 7	52	-1.0	Osteoporosis 7	67	-2.8
Osteopenia 8	71	-1.8	Osteoporosis 8	65	-2.7
Osteopenia 9	63	-1.4	Osteoporosis 9	64	-3.1
Osteopenia 10	68	-1.6	Osteoporosis 10	73	-3.8
Osteopenia 11	65	-1.9	Osteoporosis 11	69	-3.6

Osteopenia 12	66	-2.1	Osteoporosis 12	68	-3.1
Osteopenia 13	59	-1.5	Osteoporosis 13	64	-2.9
Osteopenia 14	62	-1.3	Osteoporosis 14	59	-2.8
Osteopenia 15	57	-1.4	Osteoporosis 15	57	-3.2
Osteopenia 16	68	-1.8	Osteoporosis 16	53	-3.5
Osteopenia 17	65	-1.5	Osteoporosis 17	60	-3.5
Osteopenia 18	62	-1.2	Osteoporosis 18	59	-3.1
Osteopenia 19	60	-1.1	Osteoporosis 19	55	-3.3
Osteopenia 20	64	-1.5	Osteoporosis 20	53	-2.8
Osteopenia 21	58	-1.1	Osteoporosis 21	68	-3.9
Osteopenia 22	62	-1.3	Osteoporosis 22	74	-4.2
Osteopenia 23	66	-1.2	Osteoporosis 23	66	-4.0
Osteopenia 24	69	-1.8	Osteoporosis 24	65	-3.2
Osteopenia 25	57	-1.5	Osteoporosis 25	60	-3.9
Osteopenia 26	56	-1.1	Osteoporosis 26	68	-2.5
Osteopenia 27	63	-1.9	Osteoporosis 27	61	-3.6
Osteopenia 28	52	-1.4	Osteoporosis 28	72	-3.9
Osteopenia 29	54	-1.3	Osteoporosis 29	70	-3.5
Osteopenia 30	50	-1.1	Osteoporosis 30	57	-2.9
Osteopenia 31	59	-1.7	Osteoporosis 31	61	-2.7
Osteopenia 32	62	-1.5	Osteoporosis 32	64	-2.6
Osteopenia 33	61	-1.4	Osteoporosis 33	65	-3.1
Osteopenia 34	60	-1.8	Osteoporosis 34	60	-2.6
Osteopenia 35	68	-1.5	Osteoporosis 35	62	-3.2
Osteopenia 36	64	-1.9	Osteoporosis 36	67	-3.6
Osteopenia 37	62	-1.8	Osteoporosis 37	69	-3.4
Osteopenia 38	66	-1.6	Osteoporosis 38	65	-2.8
Osteopenia 39	59	-1.2	Osteoporosis 39	58	-3.2
Osteopenia 40	57	-1.1	Osteoporosis 40	63	-3.6
Osteopenia 41	53	-1.1	Osteoporosis 41	54	-2.7
Osteopenia 42	58	-1.5	Osteoporosis 42	58	-2.6
Osteopenia 43	59	-2.1	Osteoporosis 43	55	-2.6
Osteopenia 44	62	-1.8	Osteoporosis 44	60	-2.8
Osteopenia 45	64	-2.2	Osteoporosis 45	63	-3.2
Osteopenia 46	68	-2.4	Osteoporosis 46	59	-2.9
Osteopenia 47	53	-1.5	Osteoporosis 47	57	-3.5
Osteopenia 48	56	-1.6	Osteoporosis 48	68	-3.1
Osteopenia 49	58	-1.5	Osteoporosis 49	63	-2.8
Osteopenia 50	64	-1.3	Osteoporosis 50	66	-2.9

Table S2. miRNA and mRNA Primer Sequences.

miRNA or Gene name	Primer Sequence (5' to 3')
mmu-miR-194-5p-Forward	GCGGCGGTGTAACAGAACTCC
mmu-miR-194-5p-Reverse	ATCCAGTGCAGGGTCCGAGG
hsa-miR-194-5p-Forward	CTAGTACCTAGAGGAACCTTTGAAGACTGTT ACAGCTCAGCA
has-miR-194-5p-Reverse	AGCTTGCTGAGCTGTAACAGTCTTCAAAGGT TCCTCTAGGTA
mmu-Runx2-Forward	CGCCACCACTCACTACCACAC
mmu-Runx2-Reverse	TGGATTTAATAGCGTGCTGCC
mmu-OCN-Forward	TTCTGCTCACTCTGCTGACCC
mmu-OCN-Reverse	CTGATAGCTCGTCACAAGCAGG
U6-Forward	GTGGACCGCACAAAGCTCGCT
U6-Reverse	TTGTTGAACGGCACTGTGTATAGCA
GAPDH-Forward	TGAAGGGTGGAGCCAAAAG
GAPDH-Reverse	AGTCTTCTGGGTGGCAGTGAT