Supplemental Methods

Cell culture

Primary mouse calvarial osteoblasts were isolated as previously described (1). Briefly, calvaria from newborn C57BL/6 mice were collected and sequentially rinsed in HBSS and serum-free _-MEM (Sigma Chemical Co.). Calvaria were digested into a single cell suspension in serum-free _-MEM containing 2 mg/ml collagenase and 0.25% trypsin. Cells were washed and plated at flask. These cultured osteoblasts were characterized and displayed an osteoblastic phenotype as assessed by their cAMP responsiveness to parathyroid hormone (PTH), expression of ALP and osteocalcin expression, as well as the capacity to form mineralized bone nodules as previously described (2, 3). The cells were incubated in _-MEM (Gibco-BRL Co.) supplemented with 5% FBS, 100U/ml penicillin and 100µg/ml streptomycin. Cells were maintained in a humidified 5% CO2 atmosphere at 37°C. For small RNA isolation and cloning, osteoblasts were plated at 1_10^6 cells/$25cm^2$ flask, and cultured in 10% FBS and 50 _g/ml ascorbic acid for 9 days, after which osteoblasts were harvested for small RNA isolation and cloning.

Osteoclasts were prepared from C57BL/6 mice as d previously escribed (4). Briefly, primary mouse osteoblasts were prepared from calvariae of newborn C57BL/6 mice and bone marrow cells prepared from tibiae of 8-week-old male C57BL/6 mice. Osteoblasts (2_10^6 cells/dish) and bone marrow cells (2_10^7 cells/dish) were co-cultured in 100-mm tissue culture dishes containing _-MEM (Gibco) with 10% FBS and 1,25(OH)2 vitamin D3 (10^{-8} M) for 7 days with a medium change every 2 days. After 7 days of culture, each dish usually yieled $2\text{-}4\_10^4$ osteoclasts. The dishes were then treated with 0.001% Pronase and 0.02% EDTA in PBS for 10 min to remove osteoblasts. More than 99% of the adherent cells prepared were TRAP-positive and multinucleated. These cells were incubated for 2 h with _-MEM containing 10% FBS.

The mouse stromal cell ST2 was obtained from RIKEN BioResource Center (BRC). Cells were cultured in _-MEM (Gibco), supplemented with 10% FBS, 100U/ml penicillin and 100µg/ml streptomycin at 37°C with 5% CO2. Human 293 cells were purchased from the American Type Culture Collection (ATCC). Cells were cultured in MEM (Gibco) supplemented with 10% FBS, 100U/ml penicillin and 100µg/ml streptomycin at 37°C with 5% CO2.

BMSCs were obtained from 4- to 6-week-old C57BL/6 mice as previously described (5). Briefly,

mice were euthanized and both femur and tibia were excised aseptically and external soft tissues were discarded. The epiphyses of each bone were removed with a razor blade, and the marrow was flushed from the diaphysis with growth medium containing _-MEM (Invitrogen) supplemented with 10% FBS, 1% penicillin, streptomycin and 0.1% fungizone. The cell suspension was prepared by repeatedly aspirating the bone marrow cells through a 20 gauge needle. The cells were seeded in 10-cm tissue culture dishes (1-2_10^6 cells/dish) and grown in the growth medium in a humidified atmosphere of 5% CO2 at 37°C. Medium was changed every 2-3 days to remove non-adherent cells, and adherent cells were cultured until confluent. At confluence, BMSCs were passaged after digestion with 0.25% trypsin/1 mM EDTA.

Osteoblastic differentiation was induced by changing to media containing 10% FBS supplemented with 300 ng/ml BMP2 (Peprotech).

Bioinformatic analysis

DNA sequences were analyzed to locate small RNA sequences in the cloning vector. Each RNA sequence was subjected to BLAST analysis against the mouse genome. To identify miRNAs, all small RNAs were initially searched in the miRBase (6-8). If a small RNA completely or partially matched any registered miRNA from different organisms and its size ranged from 19 to 24 nt, we classified it as a miRNA. Secondary structures of RNA precursors were predicted using a fragment of ~200 bp genomic sequence flanking the small RNA at both the 5_ and 3_ ends and the mfold program (9). If a small RNA was 19–24 nt long, its precursor sequence could form a stem–loop structure, and it had not been registered in the miRBase, we classified it as a novel miRNA. Genes encoding these miRNAs were then located on chromosomes.

ALP activity and osteocalcin secretion assay

Cells were grown to confluence in 24-well plates. The cells were then washed with PBS and scraped into a solution containing 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl, 1% Triton X-100, 0.02% NaN3, and 1 μg/ml aprotinin. The lysates were homogenized, then ALP activity was assayed by spectrophotometric measurement of p-nitrophenol release at 37°C. To normalize protein expression to total cellular protein, a fraction of the lysate solution was used in a Bradford protein assay.

Osteocalcin released into the culture media was measured using a specific radioimmunoassay kit (DiaSorin). To normalize protein expression to total cellular protein, a fraction of the lysate solution was

used in a Bradford protein assay.

Measurement of mineralized matrix formation

After stable transfection with pre-miR-2861 or miR-C, ST2 cells were cultured in _-MEM medium containing 10% FBS, 300 ng/ml BMP2, 50 _g/ml ascorbic acid and 5mM _-glycerolphosphate for 20 days. Mineralized matrix revealed by Alizarin Red S staining (10). Briefly, cells were fixed in 70% ethanol for 1 h at room temperature and stained with 40mM Alizarin Red S for 10min. Cell preparations were washed with PBS to eliminate nonspecific staining. The stained matrix was assessed using a Nikon Diaphot inverted microscope and photographed using a Nikon camera (Nikon). Alizarin Red S staining was released from cell matrix by incubation in cetyl-pyridinium chloride, and the amount of released dye was quantified by spectrophotometry at 540 nm.

Calcium accumulation in cell layer

After transfection with anti-miR-2861 or anti-miR-C, ST2 cells were cultured in _-MEM medium containing 10% FBS, 300 ng/ml BMP2, 50 _g/ml ascorbic acid and 5mM _-glycerolphosphate for 4 days. The cells were washed three times with Ca^{2+}- and Mg^{2+}-free PBS and incubated overnight at room temperature in 0.6 N HCl with gentle shaking. Total calcium deposition was quantified with cresolphthalein complexone (Sigma calcium assay kit 587-A, Sigma) spectrophotometrically at 575 nm.

Northern Blot

Total RNA was extracted with Trizol reagent (Invitrogen). Northern blot was performed as previously described (11). Briefly, 20 _g RNA was separated on a 15% urea-PAGE gel with 0.5_TBE and transferred to a Hybond-N+ nylon membrane (Amersham) using a semi-dry transfer cell (Bio-Rad). Hybridization was performed according to a standard protocol. ^{32}P-labeled oligonucleotide probes complementary to mature miR-2861 were used in the hybridization. U6 served as the loading control. The blots were stripped and re-hybridized with ^{32}P-labeled oligonucleotide probes for U6. The probe sequence of U6 was 5_-ATATGGAACGCTTCACGAATT-3_.

qRT-PCR analysis

qRT-PCR was performed as as previously described (12, 13) using Roche Molecular Light Cycler (Roche). Total RNA from cultured cells or tissues was isolated using Trizol reagent (Invitrogen), and reverse transcription was performed using 1.0μg total RNA and SuperScript II (Invitrogen). Amplification

reactions were set up in 25_l reaction volumes containing amplification primers and SYBR Green PCR Master Mix (PE Applied Biosystems). One μl of cDNA was used in each amplification reaction. Preliminary experiments were carried out for primer concentration optimization. Primer sequences for mouse type II Runx2 (14), HDAC5, ALP, osteocalcin and TRAP are listed in Supplementary Table 6.

Amplifications were performed in parallel with calibration curves in triplicate for each analysis. Each sample was analyzed six times during each experiment. The experiments were carried out at least twice. Amplification data were analyzed using the Sequence Detector System Software (PE Applied Biosystems). Relative quantification was calculated by normalizing the test crossing thresholds (Ct) with the _-actin amplified control Ct. The results were normalized to _-actin.

Western blot

To investigate Runx2 and HDAC5 protein expression levels in cells and bones, Western blot was performed. Protein concentrations were determined using a Bradford protein assay. 100μg of protein from each sample was loaded onto a 7.5% polyacrylamide gel. After electrophoresis, the SDS-PAGE separated proteins were transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% nonfat milk in PBS and then incubated with anti-Runx2(Santa Cruz), anti-HDAC5 (Santa Cruz), anti-Myc (Santa Cruz) or _-actin (Abcam) in PBS for 3 h The membrane was then reprobed with appropriate secondary antibodies conjugated with horseradish peroxidase for 1 h. Blots were processed using an ECL kit (Santa Cruz) and exposed to film.

Plasmid Constructs

To express miR-2861 in ST2 cells, the pSilencer vector (Ambion) was constructed by inserting an 82-bp genomic sequence of miR-2861 precursor (pre-miR-2861). The oligonucleotides used are listed in Supplemental Table 7. The oligos were annealed and subsequently ligated with T4 DNA ligase (Invitrogen) into the pSilencer 4.1-CMV vector expressing the puromycin cassette.

For functional analysis of miR-2861, a segment of the mouse HDAC5 CDS was PCR amplified from mouse genomic DNA. The product was then inserted into the XbaI-FseI site immediately downstream of the stop codon in the pGL3-Control Firefly Luciferase reporter vector (Promega), resulting in WT-pGL3-HDAC5. The QuickChange site-directed mutagenesis kit (Stratagene) was used to introduce two point mutations into the seed region of WT-pGL3-HDAC5, resulting in MUT-pGL3-HDAC5. The human WT HDAC5 CDS luciferase expression vector containing the putative target site of miR-2861 was constructed

according to the method by which WT-pGL3-HDAC5 was produced. Plasmid DNA was sequenced to ensure authenticity. The sequences of the PCR and mutagenic primers are given in Supplemental Table 8.

To create WT pre-miR-2861 (pre-miR-2861-C) and mutant pre-miR-2861 (pre-miR-2861-G) expression vectors, we firstt constructed WT and mutant pre-miR-2861 cloning vectors. The genomic fragment containing the miR-2861 precursor and its flanking regions was amplified from normal human genomic DNA. The PCR product was cloned in the pCR2.1 vector according to the manufacturer's protocol. Positive clones were selected by restriction digestion using BamHI and XhoI and confirmed by DNA sequencing. After WT cloning, the QuikChange site-directed mutagenesis kit (Stratagene) was used to generate the G mutation in the pre-miR-2861 by PCR using the WT pre-miR-2861 construct as the template. Finally, the WT and mutant pre-miR-2861 were subcloned into the mammalian expression vector pCDNA3.1 (+) (Invitrogen) between BamHI and XhoI restriction sites using a rapid DNA ligation kit (Roche), resulting in pcDNA3-miR-2861-C and pcDNA3-miR-2861-G. The primer sequences are given in Supplemental Table 8.

The CDS of mouse HDAC5 was amplified from total RNA extracted from ST2 cells by reverse transcription-PCR (RT-PCR) using primers designed according to the 5_ and 3_ UTR regions of the mouse HDAC5 cDNA sequence (Supplemental Table 9). The QuikChange site-directed mutagenesis kit (Stratagene) was used to generate the two mutations in HDAC5 (mutant HDAC5) by PCR using the WT HDAC5 construct as the template. The introduced mutations did not result in amino acid changes in the HDAC5 protein. Finally, the WT and mutant HDAC5 were cloned into the pCDNA3.1 (+) expression vector (Invitrogen) at XbaI/KpnI sites.

The full-length type II Runx2 cDNA was generated by RT-PCR amplification (Supplemental Table 9) from total RNA derived from BMP2-induced ST2 cells. The resulting product was cloned into the pCR2.1 vector and sequenced to confirm the amplification of the full-length cDNA. The Runx2-KR-240/245/365/366 mutant plasmids were constructed using the QuikChange Site-Directed Mutagenesis kit (Stratagene) using type II Runx2 cDNA as a template according to the manufacturer's manual. The sequences of mutagenic primers are shown in Supplemental Table 9. Each mutant was sequenced to confirm that only the desired mutation occurred during the manipulations. Thereafter, the Runx2 full-length type II isoform and Runx2-KR-240/245/365/366 mutant were tagged with Myc in a CMV promoter-derived mammalian expression vector (pCS4–3Myc).

Micro-CT analysis

The right femur of each mouse was fixed with 4% paraformaldehyde for 24 h and subsequently washed with 10% saccharose solution; 12 h later, micro-CT scanning was performed using the GE explore Locus SP system (GE Healthcare Company) (15). This machine is a cone-bean scanning system to examine small animal specimens in vitro. The scanning protocol was set at X-ray energy settings of 80 kv and 80 _A, and the sample was scanned over 1 entire 360° rotation, with an exposure time of 3000 ms/frame. An isotopic resolution of 7_7_7 _m voxel size that displayed the microstructure of the mouse proximal femur was selected, and the angle of increment around the sample was set to 0.4°, which resulted in the acquisition of 900 2D images. X-ray fluoroscopy was performed to correct the placement of each sample within the sample holder and to ensure that the whole sample was included within the scanning field. A modified Feldkamp cone-beam algorithm was used to reconstruct the 2D projections into a 3D volume. Several structural parameters of the proximal femur, including the trabecular bone volume (BV/TV: %), thickness (Tb.Th: _m), trabecular number (Tb.N: mm^{-1}), and trabecular bone separation (Tb.Sp: _m) were obtained by micro-CT.

Histomorphometric Analysis

For histological analyses, the mice were injected with 25 mg/kg calcein at 8 and 2 d before euthanasia. The femurs were fixed in 70% ethanol, dehydrated in increasing concentrations of ethanol, and the undecalcified bones were embedded in methyl methacrylate. Serial 5-_m sections in the proximal region of the femur were made using a microtome. The parameters obtained for the bone formation were BFR/BS, MAR, Ob.S/BS and N.Ob/B.Pm. The parameters measured for bone resorption were Oc.S/BS and N.Oc/B.Pm.

Bone biomechanical measurements

Bone biomechanical testing was performed as previously described (16). Briefly, after DXA scanning, the left femur was used for three-point bend,which was performed using a computer-controlled mechanical testing machine (WDW3100, Changchun, China) equipped with a 500 N M-SI sensor (Celtron) under the following conditions: sample space, 9 mm and plunger speed, 1.8 mm/min. The load–deformation curve was plotted, and the bone biomechanical index, including the ultimate load, ultimate stress, elastic modulus, and stiff index, was calculated based on this curve.

Biochemistry

Blood and urine samples were collected between 7:00 A.M and 9:00 A.M. after fasting overnight, and the blood samples were allowed to clot. The samples were then centrifuged, divided into aliquots and stored at _70°C until assayed. Serum calcium, phosphate, and alkaline phosphatase as well as urine calcium and creatinine were measured with Hitachi 7170 automatic biochemical analyzer (HITACHI). Serum intact PTH was measured with the Bayer intact PTH assay using the ADVIA Centaur® system (Bayer). Serum 25–hydroxyl vitamin D was measured with an ELISA kit (Biomedica Medizinprodukte). The serum concentration of bone alkaline phosphatase (BAP), a marker of bone formation, was measured with an ELISA kit (BAP from MetraTM BAP EIA kit, Quidel Corporation,). As a marker of bone resorption, serum cross-linked N-telopeptides of type I collagen (NTX) were measured using an ELISA kit (Osteomark, Ostex, Inc.). The intra- and inter-assay coefficients of variation were less than 5.0% and 7.3% for 25–hydroxyl vitamin D, 4.8 and 6.6% for BAP, and 5.5 and 7.8% for NTX respectively.

Mouse serum B-ALP and TRAP-5b were also detected by performing ELISA with commercial kits (from ADL and SBA Sciences (Finland), respectively). The intra- and inter-assay coefficients of variation were less than 7.5% and 5.2% for B-ALP and 5.0% and 6.6% for TRAP-5b, respectively. The absorbance was read on a _Quant™ microplate spectrophotometer (Bio-Tek).

Supplemental References

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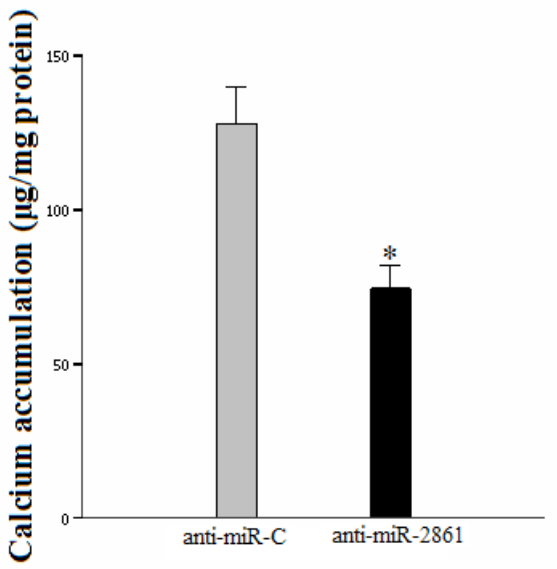
Supplemental Figure 1 Inhibition of miR-2861 reduces calcium accumulation in mouse ST2 cells. BMP2-induced ST2 cells were transfected with anti-miR-2861 or anti-miR-C, and then cultured in mineralization medium. After 5 days of culture, total calcium accumulation was quantified using a calcium assay. The bar represents the mean ± SD (*P < 0.05, vs. anti-miR-C, n = 5).

Supplemental Figure 1

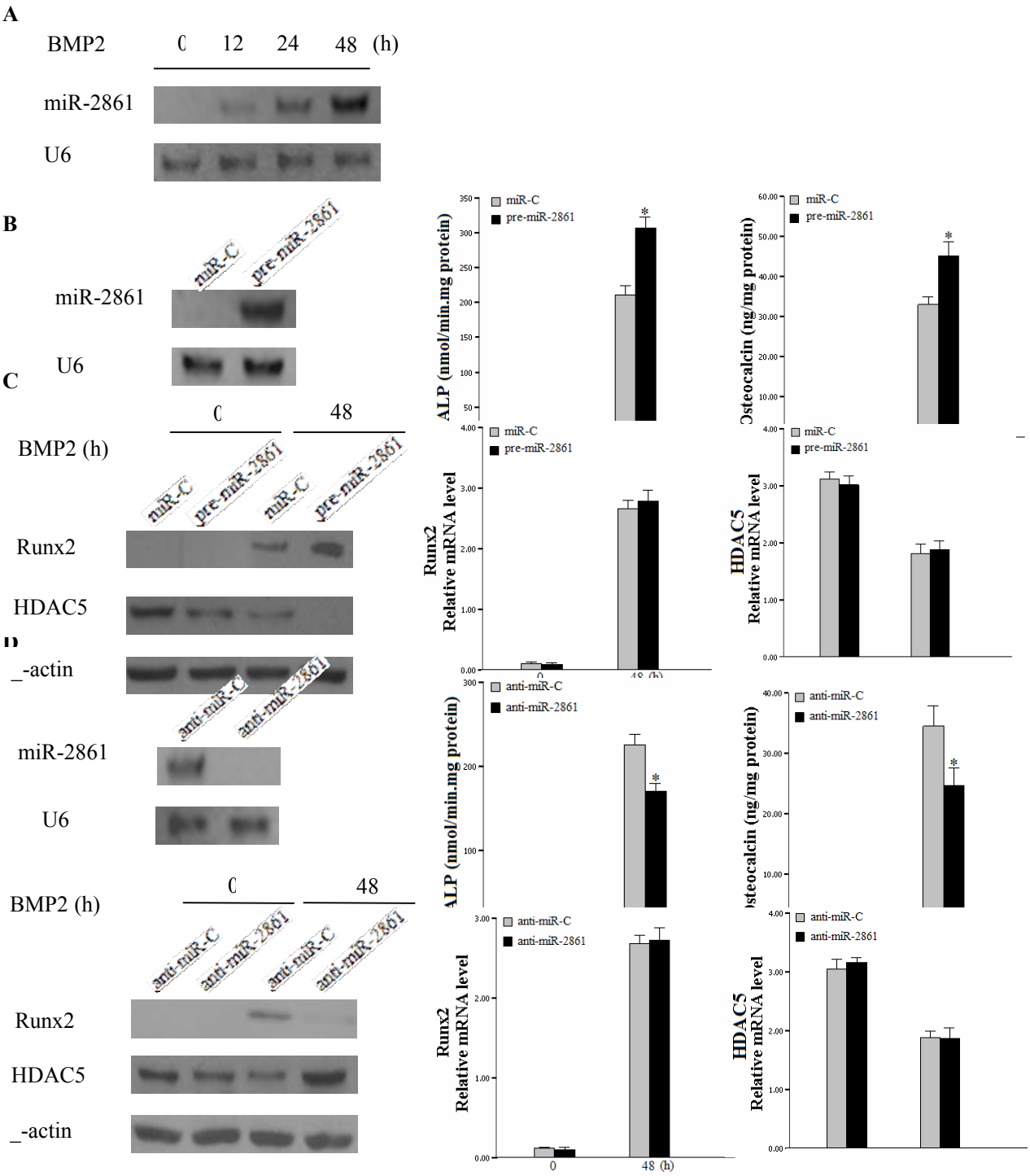


Supplemental Figure 2 miR-2861 promotes BMP2-induced mouse BMSCs osteogenic differentiation.

(A) Northern blot analysis of miR-2861 levels in BMSCs after transfection with pre-miR-2861 or miR-C. U6 was used as a loading control. The blots were stripped and re-hybridized with ^{32}P-labeled oligonucleotide probes for U6. **(B)** Overexpression of miR-2861 enhanced BMP2-induced BMSC osteogenic differentiation. BMSCs were transfected with pre-miR-2861 or miR-C for 12 h, then treated with BMP2 for 48 h. ALP activity and osteocalcin secretion were determined. The bar represents the mean ± SD (*P < 0.05, vs. miR-C, n = 5). **(C)** Levels of Runx2 and HDAC5 mRNA were determined by qRT-PCR and given as fold induction relative to control. Runx2 and HDAC5 protein expression levels were determined using western blot and expressed as densitometry of Runx2/_-actin. The bar represents the mean ± SD (*P < 0.05, vs. miR-C, n = 3). **(D)** Inhibition of miR-2861 attenuated BMP2-induced

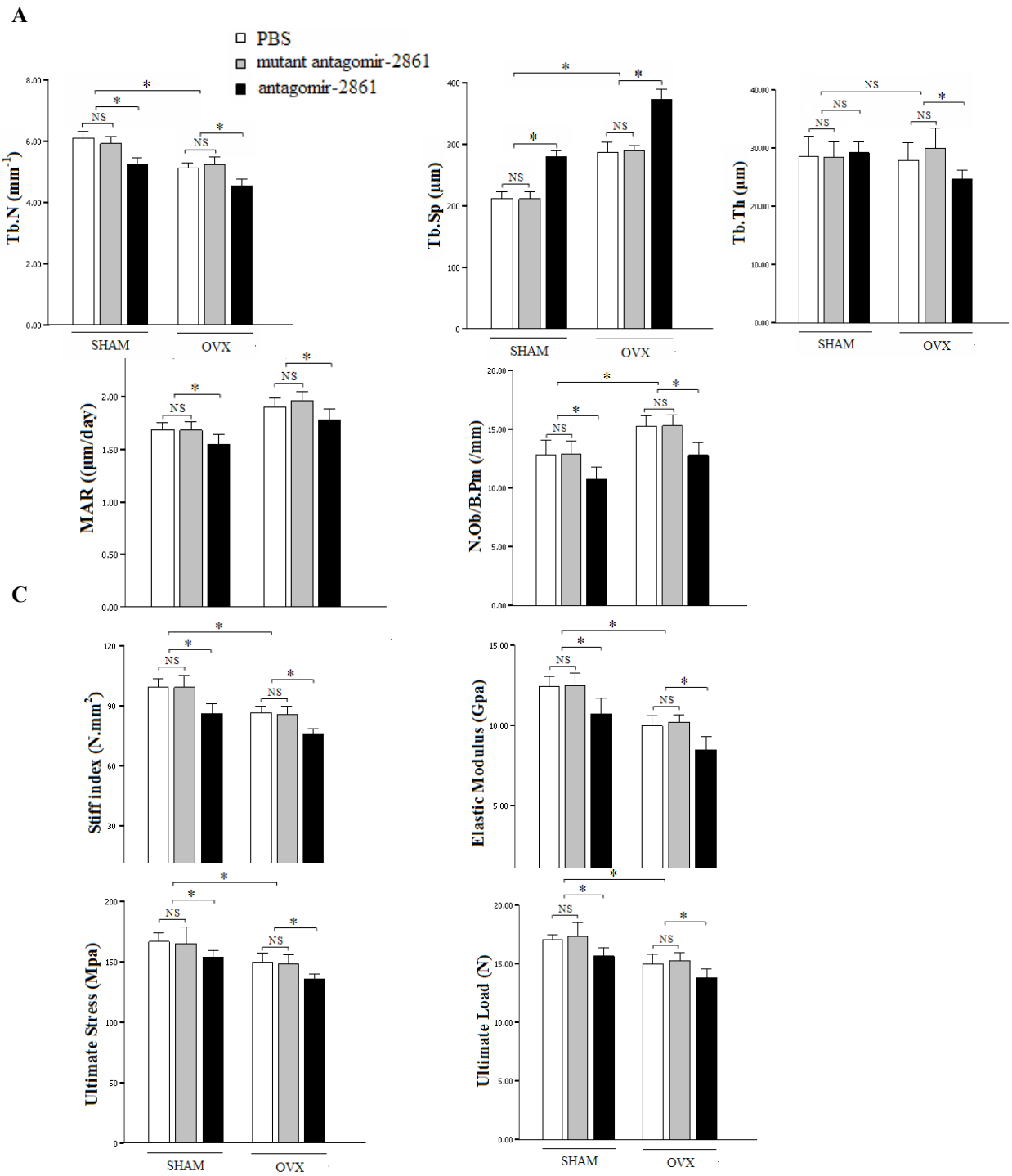
osteogenic differentiation. BMSCs were treated with BMP2 and transient transfected with anti-miR-2861 or anti-miR-C. Northern blot identified that the miR-2861 inhibitor inactivated miR-2861 in BMP2-induced BMSCs. ALP activity and osteocalcin secretion were assessed at 48 h as described in B. Runx2 and HDAC5 mRNA and protein expression were measured as described in C.

Supplemental Figure 2



Supplemental Figure 3 Antagomir-2861-treated mice display decreased bone mass and reduced osteoblast activity. (A) Microstructural analyses of the femur from treated SHAM and OVX mice, as measured by micro-CT. trabecular separation (Tb.Sp), trabecular number (Tb.N) and trabecular thickness (Tb.Th). **(B)** Bone histological analysis of femurs. MAR, mineral appositional rate; BS, bone surface. N.Ob/B.Pm, number of osteoblasts per bone perimeter. **(C)** Changes in the biomechanical parameters of the femur. Data are mean ± SD of 8 mice per group. **P* < 0.05.

Supplemental Figure 3



Supplemental Table 1 Summary of miRNA information

Name	Sequence (5' to 3')	Conservation	Location
mmu-miR-2861	GGGGCCUGGCGGCGGGCGG	H.s[a],M.m[b]	Ch[c]2
mmu-let-7a	UGAGGUAGUAGGUUGUAUAGUU	H.s,M.m	Ch13
mmu-let-7b	UGAGGUAGUAGGUUGUGUGGUU	H.s,M.m	Ch15
mmu-let-7c	UGAGGUAGUAGGUUGUAUGGUU	H.s,M.m	Ch16
mmu-let-7d	AGAGGUAGUAGGUUGCAUAGUU	H.s,M.m	Ch13
mmu-let-7e	UGAGGUAGGAGGUUGUAUAGUU	H.s,M.m	Ch17
mmu-let-7f	UGAGGUAGUAGAUUGUAUAGUU	H.s,M.m	Ch13
mmu-let-7g	UGAGGUAGUAGUUUGUACAGUU	H.s,M.m	Ch9
mmu-let-7i	UGAGGUAGUAGUUUGUGCUGUU	H.s,M.m	Ch10
mmu-miR-103	AGCAGCAUUGUACAGGGCUAUGA	H.s,M.m	Ch11,2
mmu-miR-106b	UAAAGUGCUGACAGUGCAGAU	H.s,M.m	Ch5
mmu-miR-107	AGCAGCAUUGUACAGGGCUAUCA	H.s,M.m	Ch19
mmu-miR-1196	AAAUCUACCUGCCUCUGCCU	M.m	Ch14
mmu-miR-1224	GUGAGGACUGGGGAGGUGGAG	H.s,M.m	Ch16
mmu-miR-125a-5p	UCCCUGAGACCCUUUAACCUGUGA	H.s,M.m	Ch17
mmu-miR-125b-5p	UCCCUGAGACCCUAACUUGUGA	M.m	Ch16
mmu-miR-140*	UACCACAGGGUAGAACCACGG	H.s,M.m	Ch8
mmu-miR-143	UGAGAUGAAGCACUGUAGCUC	H.s,M.m	Ch18
mmu-miR-145	GUCCAGUUUUCCCAGGAAUCCCU	H.s,M.m	Ch18
mmu-miR-151-5p	UCGAGGAGCUCACAGUCUAGU	H.s,M.m	Ch15
mmu-miR-15b	UAGCAGCACAUCAUGGUUUACA	H.s,M.m	Ch3
mmu-miR-16	UAGCAGCACGUAAAUAUUGGCG	H.s,M.m	Ch14,3
mmu-miR-17	CAAAGUGCUUACAGUGCAGGUAG	H.s,M.m	Ch 14
mmu-miR-181a	AACAUUCAACGCUGUCGGUGAGU	H.s,M.m	Ch1,9
mmu-miR-182	UUUGGCAAUGGUAGAACUCACACCG	H.s,M.m	Ch6
mmu-miR-183	UAUGGCACUGGUAGAAUUCACU	H.s,M.m	Ch6
mmu-miR-185	UGGAGAGAAAGGCAGUUCCUGA	H.s,M.m	Ch16
mmu-miR-191	CAACGGAAUCCCAAAAGCAGCUG	H.s,M.m	Ch9
mmu-miR-196a	UAGGUAGUUUCAUGUUGUUGGG	H.s,M.m	Ch11,15
mmu-miR-199a-3p	ACAGUAGUCUGCACAUUGGUUA	H.s,M.m	Ch9,1
mmu-miR-206	UGGAAUGUAAGGAAGUGUGUGG	H.s,M.m	Ch1
mmu-miR-20a	UAAAGUGCUUAUAGUGCAGGUAG	H.s,M.m	Ch14
mmu-miR-21	UAGCUUAUCAGACUGAUGUUGA	H.s,M.m	Ch11
mmu-miR-214	ACAGCAGGCACAGACAGGCAGU	H.s,M.m	Ch1
mmu-miR-221	AGCUACAUUGUCUGCUGGGUUUC	H.s,M.m	ChX
mmu-miR-222	AGCUACAUCUGGCUACUGGGU	H.s,M.m	ChX
mmu-miR-23a	AUCACAUUGCCAGGGAUUUCC	H.s,M.m	Ch8
mmu-miR-23b	AUCACAUUGCCAGGGAUUACC	H.s,M.m	Ch13
mmu-miR-24	UGGCUCAGUUCAGCAGGAACAG	H.s,M.m	Ch13,8
mmu-miR-25	CAUUGCACUUGUCUCGGUCUGA	H.s,M.m	Ch5

mmu-miR-26a	UUCAAGUAAUCCAGGAUAGGCU	H.s,M.m	Ch9,10
mmu-miR-26b	UUCAAGUAAUUCAGGAUAGGU	H.s,M.m	Ch1
mmu-miR-27a	UUCACAGUGGCUAAGUUCCGC	H.s,M.m	Ch8
mmu-miR-27b	UUCACAGUGGCUAAGUUCUGC	H.s,M.m	Ch13
mmu-miR-29a	UAGCACCAUCUGAAAUCGGUUA	H.s,M.m	Ch6
mmu-miR-30c	UGUAAACAUCCUACACUCUCAGC	H.s,M.m	Ch4,1
mmu-miR-30d	UGUAAACAUCCCCGACUGGAAG	H.s,M.m	Ch15
mmu-miR-31	AGGCAAGAUGCUGGCAUAGCUG	H.s,M.m	Ch4
mmu-miR-320	AAAAGCUGGGUUGAGAGGGCGA	H.s,M.m	Ch 14
mmu-miR-34b-3p	AAUCACUAACUCCACUGCCAUC	H.s,M.m	Ch9
mmu-miR-34c*	AAUCACUAACCACACAGCCAGG	H.s,M.m	Ch9
mmu-miR-351	UCCCUGAGGAGCCCUUUGAGCCUG	M.m	ChX
mmu-miR-361	UUAUCAGAAUCUCCAGGGGUAC	H.s,M.m	ChX
mmu-miR-378	ACUGGACUUGGAGUCAGAAGG	H.s,M.m	Ch18
mmu-miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU	H.s,M.m	Ch11
mmu-miR-574-5p	UGAGUGUGUGUGUGUGAGUGUGU	H.s,M.m	Ch5
mmu-miR-669c	AUAGUUGUGUGUGGAUGUGUGU	M.m	Ch2
mmu-miR-674	GCACUGAGAUGGGAGUGGUGUA	H.s,M.m	Ch2
mmu-miR-690	AAAGGCUAGGCUCACAACCAAA	M.m	Ch16
mmu-miR-698	CAUUCUCGUUUCCUUCCCU	M.m	Ch4
mmu-miR-709	GGAGGCAGAGGCAGGAGGA	M.m	Ch8
mmu-miR-720	AUCUCGCUGGGGCCUCCA	H.s,M.m	Ch3
mmu-miR-7a	UGGAAGACUAGUGAUUUUGUUGU	M.m	Ch13,7
mmu-miR-92a	UAUUGCACUUGUCCCGGCCUG	H.s,M.m	Ch14,X
mmu-miR-92b	UAUUGCACUCGUCCCGGCCUCC	H.s,M.m	Ch3
mmu-miR-93	CAAAGUGCUGUUCGUGCAGGUAG	H.s,M.m	Ch5
mmu-miR-99b	CACCCGUAGAACCGACCUUGCG	H.s,M.m	Ch17

Sequences listed represent the full length sequence of each miRNA identified in this study. Sequence conservation between human and mouse are indicated.
[a]Homo sapiens.
[b]Mus musculus.
[c]Chromosome.

Supplemental Table 2 Characteristics in patients with heterozygous pre-miR-2861 mutation

	Sex	Ag	Years afte menopaus	BMD lumbar spine (T score)	BMD Femoral neck (T score)
OP7'father	M	47	-	-2.6	-2.7
OP7'mother	F	46	2	-2.9	-2.8
OP7' paternal aunt	F	50	5	-2.7	-2.8
OP7' paternal grandfather	M	69	-	-2.9	-2.9
OP7' maternal grandfather	M	68	-	-3.0	-2.8

Notes: M=Male_F=Female.

Supplemental Table 3 The values of bone turnover biochemical markers for normal children (5-18 years old, 176 female and 181 male) according to Tanner stage

		n	Serum BAP (U/L)		Serum NTX (nmol BCE)		Urinary Ca/Cr ratio (mg/mg)	
			Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
Children:	Female	176						
	Male	181						
Tanner_e	Female	30	76±7	56-89	106±9	86-123	0.36±0.06	<0.60
	Male	33	73±11	50-93	105±13	88-126	0.37±0.05	<0.63
Tanner_e	Female	39	130±13	103-167	113±10	93-143	0.33±0.05	<0.56
	Male	33	136±15	101-172	116±16	90-150	0.33±0.06	<0.59
Tanner_e	Female	41	153±16	116-198	109±8	87-126	0.29±0.03	<0.50
	Male	39	160±16	113-198	112±13	81-133	0.30±0.02	<0.53
Tanner_e	Female	36	50±10	33-77	41±6	23-59	0.16±0.03	<0.36
	Male	40	69±10	39-81	66±9	33-83	0.20±0.03	<0.41
Tanner_e	Female	30	26±3	10-43	13±3	6-27	0.07±0.02	<0.15
	Male	36	33±7	13-47	19±9	7-35	0.10±0.03	<0.20

Supplemental Table 4 The values of bone turnover biochemical markers in patients with heterozygous pre-miR-2861 mutation and controls

	Subject with mutation	Normal control
Serum BAP (U/L)	19.6±2.3*	30.1 ± 3.3
Serum NTX (nmol BCE)	19.9±2.1	18.0 ± 4.3
Urinary Ca/Cr ratio (mg/mg)	0.07±0.02	0.08±0.03

Note: The control included 10 normal women (Mean age, 50 years), and 10 normal men (Mean age, 53 years).

* $P<0.05$, compared with the control.

Supplemental Table 5 Nucleotide sequences used in miRNA cloning

Name	Sequence (5_ to 3_)	
5_ RNA dapter	GACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA	cDNA library
RT primer	CGAATTCTAGAGCTCGAGGCAGGCGACATG(T)25VN	cDNA library
RT-1	GACTGGAGCACGAGGACACTGA	Cloning
RT-1r	CGAATTCTAGAGCTCGAGGCAGG	Cloning
pcDNA	GCAGAGCTCTCTGGCTAAC	CPCR
BGHr	CTAGAAGGCACAGTCGAGG	CPCR
RTQ-UNIr	CGAATTCTAGAGCTCGAGGCAGG	PCR

Supplemental Table 6 Nucleotide sequences of primers used for quantitative RT-PCR detection

Gene	Acc. No	Primer sequence	Size
Type II Runx2	NM_009820	F: 5_-AGCCTCTTCAGCGCAGTGAC-3_	20
		R: 5_-CTGGTGCTCGGATCCCAA-3_	18
HDAC5	NM_001077696	F: 5_-TGTCACCGCCAGATGTTTTG-3_	20
		R: 5_-TGAGCAGAGCCGAGACACAG-3_	20
ALP	NM_007431	F: 5_-TTGTGCCAGAGAAAGAGA-3_	18
		R: 5_-GTTTCAGGGCATTTTTCAAGGT-3_	22
Osteocalcin	NM_001032298	F: 5_-CTGACAAAGCCTTCATGTCCAA-3_	22
		R: 5_-GCGCCGGAGTCTGTTCACTA-3_	20
TRAP	NM_007388	F: 5_-GGCCGGCCACTACCCCATCT-3_	20
		R: 5_-CACCGTAGCGACAAGCAGGACTCT-3_	24

Note: F, forward primer; R, reverse primer; Acc. No, Genbank accession numbers; Size, primer size.

Supplemental Table 7 Nucleotide sequences of primers for pre-miRNA reporter plasmids

Pre-miRNA	Primer sequences
pre-miR-2861	primer1: 5_-GATCCGCTCCTCTGCAGCTCCGGCTCCCCCTGGCCTCCCGGGAACTA CAAGTCCCAGGGGGCCTGGCGGCGGGCGGAGACCGGAAGAGGGAAA-3_
	primer2: 5_-AGCTTTTCCCTCTTCCGGTCTCCGCCCGCCGCCAGGCCCCCTGGGAC TTGTAGTTCCCGGGAGGCCAGGGGGAGCCGGAGCTGCAGAGGAGCG-3_

Supplemental Table 8 Nucleotide sequences of primers for WT and mutant reporter plasmids.

Gene	Acc. No	Primer sequence	Size
WT HDAC5	NM_001077696	F: 5_-GGCTCTAGATCACCAACTCGCACCTCA-3_	27
		R:5_-GGCCGGCCGCTTACCCACCGTCCTCAT-3_	27
mutant HDAC5		F: 5_-CAACACAGCAGGAGGCTGAGAGGCAAGCGCTTCAGTCCC-3_	39
		R:5_-GGGACTGAAGCGCTTGCCTCTCAGCCTCCTGCTGTGTTG-3_	39
Human WT HDAC5	NM_001015053	F:5_-GGCTCTAGACGGTCACTGTCACCAACTCA-3_	29
		R:5_-GGCCGGCCTCTGCTTCTCCAGGAACTGC-3_	28
WT pre-miR-2861		F:5_-AGTCGGCTCTTCCCGTCTCG-3_	20
		R:5_-CTCCTCACTTGGGCGTCCCT-3_	20
mutant pre-miR-2861		F:5_-CCCCTGGCCTGTCGGGAACTACAAGTC-3_	27
		R:5_-GACTTGTAGTTCCCGACAGGCCAGGGG-3_	27

Notes: F, forward primer; R, reverse primer; Acc. No, Genbank accession numbers; Size, primer size.

Supplemental Table 9 Nucleotide sequences of primers for full length WT and mutant HDAC5 CDS and type II Runx2

Gene	Acc. No	Primer sequence	Size
WT HDAC5 CDS	NM_001077696	F: 5_-CCCCCTCCCGTCCCAGCCCCCAACGTCAGC-3_	30
		R: 5_-ATGAAGCCCAAAGGGATGGGGGCCAGGGTG-3_	30
mutant HDAC5 CDS		F: 5_-CAACACAGCAGGAGGCTGAGAGGCAAGCGCTTCAGTCCC-3_	39
		R: 5_-GGGACTGAAGCGCTTGCCTCTCAGCCTCCTGCTGTGTTG-3_	39
Type II Runx2	NM_009820	F: 5_-ATGGCGTCAAACAGCCTCTTCAGCGCAG-3_	28
		R: 5_-TCAATATGGCCGCCAAACAGACTCATCC-3_	28
Runx2 K240R mutant		F: 5_-CCAAGAAGGCACAGACAGAGGCTTGATGACTC-3_	32
		R: 5_-GAGTCATCAAGCCTCTGTCTGTGCCTTCTTGG-3_	32
Runx2 K245R mutant		F: 5_-TGATGACTCTAGACCTAGTTTGTTCTCTGATCGCC-3_	35
		R: 5_-GGCGATCAGAGAACAAACTAGGTCTAGAGTCATCA-3_	35
Runx2 K265R mutant		F: 5_-CCTTCCTCTCTCAGTAGGAAGAGCCAGGC-3_	29
		R: 5_-GCCTGGCTCTTCCTACTGAGAGAGGAAGG-3_	29
Runx2 K266R mutant		F: 5_-CCTCTCTCAGTAAGAGGAGCCAGGCAGG-3_	28
		R: 5_-CCTGCCTGGCTCCTCTTACTGAGAGAGG-3_	28

Notes: F, forward primer; R, reverse primer; Acc. No, Genbank accession numbers; Size, primer size.