Conditional Disruption of miR17~92 in Osteoclasts Led to Activation of Osteoclasts and Loss of Trabecular Bone in Part Through Suppression of the miR17-Mediated Downregulation of Protein-Tyrosine Phosphatase-oc in Mice

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ABSTRACT

This study sought to understand the regulation of an osteoclastic protein-tyrosine phosphatase (PTP-oc), a positive regulator of osteoclast activity. Our past studies suggested that PTP-oc is regulated posttranscriptionally. The 3'-untranslated region (UTR) of PTP-oc mRNA contains a target site for miR17. During osteoclastic differentiation, there was an inverse relationship between the cellular levels of miR17 (expressed as one of the six cluster genes of miR17/C2492) and PTP-oc mRNA. Overexpression of pre-miR17/C2492 in mouse osteoclast precursors reduced PTP-oc mRNA level and the size of the derived osteoclasts, whereas deletion of miR17 or inhibition of miR17 resulted in the formation of larger osteoclasts containing more nuclei that expressed higher PTP-oc mRNA levels and created larger resorption pits. Thus, PTP-oc-mediated osteoclast activation is modulated in part by miR17/C2492, particularly miR17. The miR17~92 osteoclast conditional knockout (cKO) mutants, generated by breeding miR17~92loxp/loxp mice with Ctsk-Cre mice, had lower trabecular bone volume/total volume (Tb.BV/TV), trabecular bone mineral density (Tb.BMD), trabecular connectivity density (Tb.Conn-Dens), trabecular number (Tb.N), and trabecular thickness (Tb.Th), but larger trabecular separation (Tb.Sp), and greater bone resorption without a change in bone formation compared to littermate controls. The cKO marrow-derived osteoclasts were twice as large, contained twice as many nuclei, and produced twice as large resorption pits as osteoclasts of littermate controls. The expression of genes associated with osteoclast activation was increased in cKO osteoclasts, suggesting that deletion of miR17~92 in osteoclasts promotes osteoclast activation. The cKO osteoblasts did not show differences in cellular miR17 level, alkaline phosphatase activity, and bone nodule formation ability. In conclusion, miR17-92 negatively regulates the osteoclast activity, in part via the miR17-mediated suppression of PTP-oc in osteoclasts. © 2017 American Society for Bone and Mineral Research.

KEY WORDS: OSTEOCLASTS; CELL/TISSUE SIGNALING; GENETIC ANIMAL MODELS; MOLECULAR PATHWAYS—REMODELING; EPIGENETICS

Introduction

Bone resorption is essential for all aspects of bone physiology, and is determined by the formation, activity, and survival of mature osteoclasts. The most prevalent disease associated with an abnormality in bone resorption is osteoporosis. Understanding the molecular mechanism(s) regulating not only the formation but also the activation of osteoclasts is needed in order to fully understand the pathophysiology of the various subtypes of osteoporosis. Over the past two decades, enormous advances have been made in our understanding of the regulation of osteoclastogenesis at the molecular level, but much less is known about the molecular mechanisms controlling the activation of osteoclasts.

We have previously obtained compelling in vitro and in vivo evidence that a structurally unique, nonreceptor, transmembrane protein-tyrosine phosphatase (PTP) in osteoclasts, which we termed PTP-oc,11 is a potent positive regulator of osteoclast activation.12,23 The molecular mechanism of PTP-oc to regulate osteoclast activation is highly complex. On the one hand, PTP-oc acts through direct dephosphorylation of the inhibitory phosphorytrosine-527 residue (pY527) of Src to activate its protein-tyrosine kinase (PTK) activity.24-80 This in turn promotes: (i) the Src PTK-dependent activation of the JNK2/NFκB pathway.
to enhance osteoclast survival[4]; (ii) the Src PTK-dependent phosphorylation of pY759-β3-integrin to activate the β3-integrin signaling[8]; (iii) the Src PTK-dependent phosphorylation of pY525/526 of Syk to stimulate immunoreceptor tyrosine-based activation motif (ITAM) signaling, which acts in concert with the β3-integrin signaling to further promote osteoclast adhesion, spreading, and cytoskeleton reorganization[13,18–100]; and (iv) the Src PTK-dependent phosphorylation and inactivation of Shp1, a key mediator of immunoreceptor tyrosine-based inhibitory motif (ITIM) signaling, which is a negative regulatory mediator of osteoclast differentiation and activity[11], leads to suppression of the inhibitory ITIM/Shp1 signaling in osteoclasts, resulting in further activation of osteoclasts.

Additionally, PTP-oc also acts in a Src PTK–independent manner to inactivate the forward signaling of EphA4, which is a potent negative regulatory mechanism for osteoclast activation[12], through dephosphorylation of several key phosphotyrosine residues at the juxtamembrane domain of EphA4[13]. This results in further activation of osteoclasts. The foregoing findings bring forth an intriguing concept that PTP-oc enhances activation of osteoclasts, not only by activating stimulatory pathways (i.e., JNK2/NFκB, β3-integrin, and ITAM/Syk signaling), but also by suppressing inhibitory pathways (i.e., ITIM/Shp1 and EphA4 signaling) in both Src PTK–dependent and Src PTK–independent manners.

Knowledge of how PTP-oc is regulated is important, not only with respect to the understanding of the physiological or pathological function of this osteoclastic enzyme in bone-wasting diseases, but may also allow future development of rational basis for PTP-oc–based therapies for these diseases. In this regard, we found that certain resorption activators (e.g., PTH, prostaglandin E2 [PGE2], IL-1, or 1,25(OH)2D3) increased PTP-oc expression of PTP-oc, activated osteoclasts, and increased bone resorption.

Materials and Methods

Materials

Culture media were purchased from Life Technologies (Grand Island, NY, USA) and fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT, USA) or Atlantic Biologicals (Flowery Branch, GA, USA). Tissue culture supplies were from Falcon (Oxnard, CA, USA). The enhanced chemiluminescence detection kit for Western blots was obtained from Millipore (Billerica, MA, USA). Recombinant soluble receptor activator of NFκB ligand (RANKL) and macrophage colony stimulating factor (m-CSF) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and CallBiochem (San Diego, CA, USA), respectively. The anti-pY173-Vav3 and anti-Vav3 antibodies were purchased from BioSource International (Camarillo, CA, USA), or Santa Cruz Biotechnology. Ads-CMV-Cre or Ads-CMV-eGFP adenoviral vectors were produced by Baylor College of Medicine (Houston, TX, USA). All other reagents were obtained from either Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Los Angeles, CA, USA).

Animals

Breeding pairs of homozygous miR17–92loxp/loxp mice (Mir17MT.173/J, Jackson Laboratory, Bar Harbor, ME, USA; stock # 008458) were provided to us by Dr. Chandrasekhar Kesavan of our Musculoskeletal Disease Center. The miR17–92 osteoclast conditional knockout (cKO) mice were generated by breeding miR17–92loxp/loxp mice (in C57Bl/6 genetic background) with Ctsk-Cre transgenic mice (also in C57Bl/6 genetic background) obtained from Dr. Laurie Glumcher of the Harvard Medical School. The schematic representation of the breeding protocol is outlined in Supporting Fig. 1. This breeding protocol should yield 25% cKO mutants (Ctsk-Cre/+ /miR17–92loxp/loxp), 50% phenotypically normal littermates (Ctsk-Cre/+ /miR17–92loxp/loxp or Ctsk-Cre/+ /miR17–92loxp/loxp) and 25% heterozygotes (Ctsk-Cre/+ /miR17–92loxp/loxp). The phenotypically normal littermates were used as the controls for comparison. Mice were housed in groups of four mice per cage, fed a regular Envigo/Harlan Teklad 7001 rodent diet (Placentia, CA, USA), and in a 12-hour light/12-hour dark cycle.

All animal protocols were reviewed and approved by the Animal Care and Use Committee of the Jerry L. Pettis Memorial VA Medical Center.

Genotyping assay

Tail vertebrae tissue (2 to 3 mm) was taken from each pup at weaning under anesthesia, and was digested overnight using the DNeasy kit (Qiagen, San Diego, CA, USA). The quality and quantity of genomic DNA were analyzed by the 260 nm/280 nm absorbance ratio.

Genotyping of miR17–92loxp/loxp mice was performed according to the PCR-based genotyping assay recommended by the Jackson Laboratory. The homozygous loxp+/− mutants yielded a single PCR product of 289 bp, whereas the littermate controls produced a single PCR product of 255 bp. Heterozygous mutants gave both PCR bands. The Ctsk-Cre transgenic mice were also genotyped with a PCR-based assay[16,17] using the following set of primers: (i) 5′-TTATTCCTTCCGCCAGTAG-3′; (ii) 5′-TGCTGTTATACGCTTCTG-3′; and (iii) 5′-TACGTCTTGGGCAGCCCG-3′. The PCR reaction mix was first hot-started at 94°C for 1.5 min, followed by 35 cycles of denaturation at 95°C for 30 s, extension 54°C for 1 min, and renaturation at 72°C for 1 min. The reaction was ended with 2 min at 72°C. The WT littermates showed a single product of 135 bp, whereas the Ctsk-Cre transgenic mice showed a product of 300 bp with or without the 135-bp product band. Homozygous osteoclast miR17–92 cKO mutants were those mice homozygous for miR17–92loxp/loxp and positive for Ctsk-Cre. Heterozygous mutants were those mice heterozygous for miR17–92loxp/loxp and positive for Ctsk-Cre, whereas littermate controls would be
either homozygous or heterozygous for miR17−92loxP/loxP but negative for Ctsk-Cre (Supporting Fig. 1).

Cell cultures

Primary marrow-derived osteoclasts were generated from bone marrow osteoclast precursors of 8-week-old to 10-week-old miR17−/− cKO mice or littermate controls according to the procedure described. The average size of the derived tartrate-resistant acid phosphatase (TRAP)-positive, multinucleated (more than two nuclei) osteoclasts was determined at magnification ×4 using the OsteoMeasure system (OsteoMetrics, Decatur, GA, USA), and individual osteoclasts were counted per frame for the entirety of each 24-well area by an investigator who did not know the identity of the treatment groups. The number of nuclei was counted by tabulation for each osteoclast and segmented into groups of a factor of five.

Primary osteoblasts were isolated from calvaria of 7-week-old to 10-week-old cKO mutants or littermate controls by collagenase digestion as described and were maintained in DMEM supplemented with 10% FBS and antibiotics. Cells at passages 1 to 3 were used in this study.

Generation of pLL-pre-miR17−92 expression plasmid construct

To generate pre-miR17−92 expression construct, the DNA fragment containing the miR17−92 precursor sequence that comprises the stem-loop structure and 120-bp upstream and 120-bp downstream flanking regions was PCR-amplified with C57BL/6J mouse genomic DNA as the template. It was then subcloned into Hpal and XhoI sites of the pLL3.7 vector (Addgene, Cambridge, MA, USA). The ligated product was transformed into Escherichia coli XL2 blue (Agilent Technologies, Santa Clara, CA, USA) and the colonies containing the expression construct were selected by ampicillin resistance and confirmation for the presence of miR17 sequence by DNA sequencing.

To transfect primary marrow osteoclast precursors, cells were treated with 0.6 μg of either pLL3.7 plasmid (empty vector) or pLL-pre-miR17−92 construct in the presence of the Effectene Transfection Reagent (Qiagen, Valencia, CA, USA). After 5 days of the RANKL and m-CSF treatment, total RNAs were extracted from transfected cells using miRNeasy kit (Qiagen). The PTP-oc mRNA (normalized against Ppia (cyclophilin) mRNA) and miR17 (normalized against U6 RNA levels) were determined as described in the quantitative reverse transcription PCR assays section.

Quantitative reverse transcription PCR assays

The cellular mRNA levels of PTP-oc, Cre, and those genes typically associated with osteoclastogenesis or those associated with osteoclast activation were measured by qPCR and normalized against the housekeeping gene, Ppia mRNA, as described. The primer sequences of each test gene are shown in the Supporting Table 1. The data (normalized against Ppia mRNA) were analyzed with Opticon Monitor Software 2.0 (Bio-Rad Laboratories, Hercules, CA, USA). The relative fold change was calculated by the threshold cycle (ΔCt) method.

Cellular miR17 assay

The cellular levels of miR17 (or miR19a) were measured as follows: a miR17 (or miR19a) TaqMan-based specific PCR primer with a stem-loop sequence for reverse transcription (RT) was synthesized by Applied Biosystems (Foster City, CA, USA). Ten nanograms (10 ng) of total RNAs were used in each RT reaction. Reactions were incubated at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. The reverse-transcribed cDNA was used as template in qPCR reaction using the TaqMan Small RNA Assay kit (Applied Biosystems). Samples were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative level miR17 was normalized against the U6 gene. All reactions were run in duplicate. Results are reported as fold of untreated controls.

The assay for the binding site for miR17 on the 3′-untranslated region of PTP-oc mRNA

Briefly, total RNAs were isolated from primary osteoclasts, and cDNA was prepared by RT-PCR. A 1.2-kb fragment corresponding to the entire 3′-untranslated region (3′UTR) domain of PTP-oc mRNA was PCR-amplified using the cDNA as the template with the following primer set: forward primer, 5′-ATCGTTCAAGCGGATCCTCCTCCACGAGT-3′ and reverse primer, 5′-ATCGTTCAAGCGGACCGACCGAGAATCTCCGTGCG-3′. The underlined sequences are the restriction site for Sgf I and Not I, respectively. The PCR product was then cloned into the Sgf I and Not I sites of the luciferase reporter gene-expressing pMIR target vector (OriGene Technologies, Inc., Rockville, MD, USA). The gel-purified ligated product was then transformed into the E. coli DH5α strain (Zymo Research, Irvine, CA, USA). The positive clones containing the expression construct were selected by kanamycin resistance. The plasmid inserts of several positive clones were then isolated and the presence of the 3′UTR domain of the PTP-oc mRNA was confirmed by sequencing. A clone containing the correct sequence of the 3′UTR of PTP-oc mRNA was expanded. The plasmid was isolated and transfected into HEK293T cells in the presence or absence of 2.5 μM of the locked nucleic acid (LNA)-based miR17 mimic (has-miRNA-17-5p miRCURY LNA) or the miRCURY LNA negative control (both from Exiqon, Woburn, MA, USA) using the Effectene transfection reagent (Qiagen). After 24 hours, the cells were lysed and the luciferase reporter gene activity was determined with a commercial luciferase assay kit (Promega Luciferase Assay Systems kit).

Resorption pit formation assay

The resorption pit formation assay was performed as described. Because targeted overexpression of PTP-oc(2) or deficient EphA4 expression(12) in osteoclast precursors did not significantly alter the number of mature osteoclasts formed in response to the RANKL/m-CSF treatment, we focused on measurements of the average pit area per resorption pit (determined by dividing total pit area by the number of pits measured), which was reported as an index of the average bone resorption activity per osteoclast. Accordingly, the total area of ~50 resorption pits was determined with the OsteoMeasure system by an investigator who did not know the identity of the treatment group.

Immunofluorescent staining of the actin ring of osteoclasts

Very briefly, marrow-derived osteoclasts derived from cKO or littermate control mice, plated on uncoated glass slides, were paraformaldehyde-fixed and permeabilized with Triton X-100. The cells were then incubated with FITC-phalloidin at 37°C in 1% DMSO in PBS. The actin rings of the stained cells were visualized under a fluorescent microscope.
Mineralized nodule formation assay
Briefly, bone marrow cells of 8-week-old to 10-week-old cKO or littermate controls were flushed out of long bones and were cultured in α-MEM containing 10% FBS, 50 μM penicillin/streptomycin, and 25 μM amphotericin B for 24 hours. The adherent cells were further cultured for 48 to 96 hours until confluent. The attached marrow stromal cells were isolated by trypsin, were plated at 1 × 10⁵ cells per well in six-well plates, and incubated with α-MEM containing 15% FBS until 90% confluent. 10 mM of β-glycerophosphate and 50 μg/mL ascorbic acid were then added for 28 days. Mineralized nodules were stained with Alizarin red and the number of mineralized nodules per well were counted under microscope.

The alkaline phosphatase activity (normalized against protein content) of cultured osteoblasts was measured as described.[119]

Western immunoblot assays
Relative levels of pY173-Vav3 and total Vav3 were determined by Western immunoblots as described,[5,6] using a polyclonal antibody against pY173 residue of Vav3 and a polyclonal anti-Vav3 antibody, respectively.

Peripheral quantitative computed tomography bone parameter measurements
The peripheral quantitative computed tomography (pQCT) scanning was performed on the femur of 8-week-old mice as described,[20] using a Stratec XCT 960 M pQCT (Norland Medical Systems, Madison, WI, USA). Trabecular bone mineral content (BMC) and bone mineral density (BMD) were determined with the threshold setting of 230 to 630 mg/cm². A threshold setting of 630 mg/cm² was used to determine cortical bone parameters. The length of each femur was determined with a digital caliper. All measurements were performed without the knowledge of the identity of the treatment group.

Micro-computed tomography bone parameter measurements
The three-dimensional bone phenotype was assessed on the right femur by micro-computed tomography (μCT) using a Scanco vivaCT40 μCT scanner (Scanco Medical, Brüttsisellen, Switzerland) as described.[2] Trabecular measurements were performed at the secondary spongiosa of distal femur (at a site that was 10% of the full length of the femur from the distal end). Accordingly, a region of 0.8 mm in thickness at 10% of the full length from the distal end of each femur was scanned. The trabecular masks were defined in a semiautomatic manner, starting from the outer mask of the femur and application of 15 erosion cycles to ensure that no cortex was included in the measurement. This approach would adjust for the ∼3% shorter femur in miR17−/− cKO mutants.

Bone histomorphometry
Static bone resorption and dynamic bone histomorphometric parameters were measured at the secondary spongiosa of the femur as described.[119]

Statistical analysis
Results are shown as mean ± SE. Statistical significance was determined with one-way ANOVA followed by the Tukey post hoc test using the Systat 11 statistic software (Systat Software, Inc., Richmond, CA, USA). The difference was considered significant when p < 0.05.

Results
PTP-oc expression in osteoclasts is negatively regulated by miR17
We inspected the 3’UTR of both the murine and human PTP-oc mRNA for potential miRNA target sites using the online TargetScan database (Whitehead Institute for Biomedical Research, Cambridge, MA. USA; http://www.targetscan.org/vert_71/), and identified three conserved putative target sites for miRNAs. They are located at: (i) position 204–211 for miR-133a/133b; (ii) position 325–332 for miR25/32/92/363/367; and (iii) position 900–906 for miR17/20/93/106 (Fig. 1A). We focused on miR17 because the mRNA of a structurally-related PTP (PTPn) was previously shown to be negatively regulated by miR17.[15] To confirm that the 3’UTR of PTP-oc indeed contains a functional miR17 target site, we cloned the 3’UTR domain of the PTP-oc mRNA into the pMir Target Report Vector, which was then transfected into HEK293T cells. (HEK293T cells were used instead of osteoclastic cells, because HEK293T cells are readily transfecatable, whereas the transfection efficiency of osteoclastic cells is notoriously low). Figure 1B shows that treatment with 3 μM of the LNA-based miR17 mimic, but not the negative control mimic, suppressed the expression of the luciferase reporter gene activity by ∼80%, confirming the presence of a target site for miRNA17 in the 3’UTR region of the murine PTP-oc mRNA.

We next tested whether miR17 expression in osteoclastic cells is associated with a corresponding change in PTP-oc expression during the RANKL/m-CSF-induced osteoclast formation. At 0, 3, and 6 days of the RANKL/m-CSF treatment of primary mouse marrow osteoclast precursors, the PTP-oc mRNA level was increased progressively. Conversely, there was a time-dependent decrease in the cellular miR17 level (Fig. 1C). Notably, there is an inverse relationship between the cellular level of miR17 and that of PTP-oc mRNA (Fig. 1D), suggesting a negative regulatory relationship between PTP-oc expression and miR17 expression in osteoclasts.

We have previously shown that certain resorptive cytokines (eg, PTH, 1,25(OH)₂D₃, PGE₂, and IL-1α, but not RANKL, IL-6, or TNF-α) upregulated the expression levels of PTP-oc mRNA and protein in osteoclasts.[5,6] Thus, we tested whether the miR17 expression in osteoclasts is correspondingly regulated by these resorption cytokines. As shown in Fig. 1E, IL-1α and 1,25 (OH)₂D₃ each reduced the miR17 level, whereas IL-6 or TNF-α had no significant effect. These findings are consistent with the premise that certain resorption cytokines upregulate expression of PTP-oc in part through suppression of the miR17 expression.

To evaluate whether overexpression of miR17 in osteoclasts would suppress the PTP-oc signaling, osteoclast precursors were transfected with a pLL3.7-based pre-miR17−/− expression plasmid or the empty pLL3.7 plasmid (control) vector. The transfected cells were treated with RANKL/m-CSF for 5 days to allow differentiation into osteoclasts. The pre-miR17−/− plasmid-transfected osteoclasts showed a 2.5-fold increase in cellular miR17 level compared with control osteoclasts, indicating a successful transfection. This overexpression was accompanied by a ∼50% decrease in cellular PTP-oc mRNA levels (Fig. 2A). Overexpression (or activation) of the PTP-oc signaling in
osteoclasts has been shown to increase the cellular pY173-Vav3 level, which is a key component of the PTP-oc/β3-integrin signaling.\textsuperscript{(8,12)} Thus, we compared the average cell size and cellular pY173-Vav3 level in osteoclasts derived from pre-miR17/C2492-transfected cells with corresponding controls. The average cell size was 43% ± 8% smaller (p < 0.05) and the cellular pY173-Vav3 level was 52% ± 17% lower (p < 0.05) in the pre-miR17–92 plasmid-transfected osteoclasts (Fig. 2B). Accordingly, overexpression of miR17–92 in osteoclasts appears to suppress PTP-oc expression and its signaling.

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**Fig. 1.** The 3'UTR region of the murine and human PTP-oc mRNA contains three putative miRNA target sequences (A), particularly that for miR17 (B), existence of a negative relationship between miR17 and PTP-oc expression during RANKL-induced osteoclastogenesis (C, D), and regulation of miR17 expression in osteoclasts by certain resorption cytokines (E). (A) The location of putative miRNA target sequences on the 3'UTR of PTP-oc mRNA predicted by the TargetScan software program. (B) Treatment of the 3'UTR of the PTP-oc mRNA containing luciferase reporter gene expression plasmid by miR17 mimic suppressed the expression of luciferase. (C, D) Marrow osteoclast precursors (n = 3 per group) were treated with RANKL/m-CSF for 0, 3, or 6 days. (C) The relative levels of PTP-oc mRNA and miR17 are shown as fold of controls (mean ± SE). *p < 0.05. (D) An inverse correlation between PTP-oc mRNA level and miR17 levels. (E) Marrow osteoclast precursors were treated with RANKL/m-CSF for 6 days. The resulting osteoclasts were treated with 10 nM IL-1α, 10 nM 1,25(OH)\textsubscript{2}D\textsubscript{3}, 10 nM IL-6, 10 nM TNF-α, or solvent for 24 hours. The cellular level of miR17 is reported as relative fold of the miR17 level in untreated control osteoclasts (mean ± SE, n = 3) (p = N.S. means p > 0.05). In C and E, the dashed lines indicate the respective levels of PTP-oc mRNA and miR17 of untreated cells at each test time point. N.S. = not significant.
To rule out the possibility of off-target effects of overexpression of mir17–92, we examined whether deficient expression of mir17–92 in osteoclasts would increase PTP-oc expression level, cell size, number of nuclei, and bone resorption activity of osteoclasts in vitro. We transduced marrow osteoclast precursors of mir17–92flox/flox mice with an adenoviral vector expressing either Cre (Ad5-CMV-Cre) or GFP (Ad5-CMV-eGFP). At 4 days posttransduction, the cellular mir17 levels of Ad5-CMV-Cre–transduced cells were ~45% of that of Ad5-GFP-treated control cells (Fig. 2C). The osteoclasts derived from Ad5-Cre–transduced precursors showed 86% ± 32% increase in cellular PTP-oc mRNA levels (Fig. 2C), which was 35% to 40% larger in the average cell size with an average of 75% more nuclei (Fig. 2D), and created approximately twofold larger resorption pits (Fig. 2E) than the Ad5-CMV-GFP–transduced control osteoclasts.

To evaluate if the observed effects of Ad5-CMV-Cre–mediated deletion of mir17–92 were due to deficient mir17 expression and not to other members of the mir17–92 cluster gene, we transfected osteoclast precursors with 25 pmol/mL of the LNA antisense-based inhibitor for mouse mir17-5p, mir19a-5p (another member of the mir17–92 cluster gene—included for comparison), or control LNA using Lipofectamine for 4 hours. Treatment with these antisense inhibitors reduced the cellular levels of mir17 or mir19a each to ~60% of the control level at 24 hours (Fig. 2F). Inhibition of mir17, but not that of mir19a, yielded osteoclasts approximately twice as large as control-LNA oligonucleotide-treated cells (Fig. 2G). These and foregoing findings support the contention that mir17 (and not mir19a) is a negative epigenetic regulator of PTP-oc expression and PTP-oc signaling in osteoclasts. We did not test the other members of

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Fig. 2. Overexpression of mir17–92 (A, B) or suppression of mir17–92 expression by gene deletion (C–E) or antisense oligonucleotides (F, G) in osteoclasts altered PTP-oc expression, average cell size, number of nuclei, and resorption activity of osteoclasts. (A) The cellular level of mir17 and PTP-oc mRNA in the pLL-pre-mir17–92-transfected cells and the empty vector–transfected control cells (mean ± SE, n = 6 for each). (B) A representative photomicrograph of empty vector control–treated or pLL-pre-mir17–92–treated osteoclasts. (bottom left) The relative average size of mir17–92–overexpressing osteoclasts and control osteoclasts. Results are shown as % of the average size of littermate control osteoclasts (mean ± SE, n = 3 per group). (bottom right) The relative cellular level pY173-Vav/total Vav3 (right) of the respective control. N.S. overexpressing osteoclasts and control osteoclasts. Results are shown as % of the average size of littermate control osteoclasts (mean ± SE, n = 4 per group). In C–E, osteoclast precursors derived from mir17–92flox/flox mice were transduced with either Ad5-CMV-GFP (control cells) or Ad5-CMV-Cre (mir17–92-deficient cells). (C) The cellular levels of mir17 (left) and PTP-oc mRNA (right) in mir17–92-deficient and control osteoclasts (mean ± SE, n = 4 per group). (D) (top) A representative photomicrograph of control or mir17–92-deficient osteoclasts. (bottom left) The average cell size of the mir17–92-deficient and control osteoclasts. (bottom right) The average number of nuclei per osteoclasts of mir17–92-deficient and control osteoclasts (mean ± SE, n = 4 per group in both panels). (E) Average resorption pit size per pit determined on ~50 pits per dentine slice (mean ± SE, n = 4–5 per group). In F and G, marrow osteoclast precursors were transfected with 25 pmol/mL of a mouse mir17-5p LNA-modified antisense-based inhibitor (mirCURY LNA, with a fully phosphorothioate-modified backbone), a mouse mir19a-5p or control oligonucleotide for 4 hours with Lipofectamine. (F) The cellular levels of mir17 or mir19a, respectively, in the marrow-derived osteoclasts (mean ± SE, n = 3). (G) Effects of each miRNA inhibitor on the average size of the derived osteoclasts. (top) The representative photomicrograph of TRAP-stained osteoclasts of each group. (bottom) The relative cell size of derived osteoclasts from each treatment group (mean ± SE, n = 3 per group). The dashed lines represent the 100% of each respective control. N.S. = not significant.
the mir17~92 cluster gene (ie, mir18, mir19b, mir20, and mir92). Because TargetScan analysis reveals that the 3'UTR domain of PTP-oc mRNA also contains potential target sites for mir20 and mir92, it is conceivable that the inhibition of these two miRNAs might yield similar enhancing effects on PTP-oc expression and osteoclast activation.

Characterization of bone phenotypes of mir17~92 osteoclast cKO mutant mice

To test whether deficient expression of mir17~92 in osteoclasts would lead to activation of osteoclasts and bone loss in vivo, we generated a colony of mir17~92 osteoclast cKO mutant mice by crossing mir17~92lox/lox mice with Ctsk-Cre mice. Cathepsin K is generally recognized as a marker of mature osteoclasts, which is believed to be expressed in low levels in osteoclast precursors. We evaluated whether the proximal promoter of Ctsk that drives the Cre recombinase expression in Ctsk-Cre transgenic mice is also specific for mature osteoclasts but not their precursors. (The proximal promoter of Ctsk may have different cell-type specificity, because the native promoter contains much more regulatory elements than just the proximal promoter.) Thus, we measured by qPCR the relative expression level of Cre mRNA in osteoclast precursors of Ctsk-Cre transgenic mice during the course of m-CSF/RANKL-mediated osteoclast differentiation. The undifferentiated osteoclast precursors (without the RANKL/m-CSF treatment) expressed almost undetectable levels of Cre mRNA (ie, ΔCt = ~35 cycles in qPCR). As the precursors differentiated to osteoclasts during the 6 days of RANKL/m-CSF treatment, there was a highly significant (p < 0.001, ANOVA) time-dependent increase in the expression level of Cre mRNA in osteoclasts starting from day 1 of the RANKL treatment (Supporting Fig. 2). It is noteworthy that the Cre expression level after 2 or 3 days of the RANKL treatment was clearly detectable (top panel) and could be considered substantial: Each was 80-fold to 160-fold of that of the basal level. Thus, it is conceivable that there might be sufficient Cre expression even at the early stages of osteoclastic differentiation to mediate the deletion of the mir17~92 gene.

The level of mir17 and mir19a in marrow-derived cKO osteoclasts was each reduced to ~30% of those of marrow-derived osteoclasts generated from littermate controls (Supporting Fig. 3A), confirming that the mir17~92 gene was indeed significantly knocked down in osteoclastic cells of these mice. These cKO marrow-derived osteoclasts also showed an approximately twofold increase in PTP-oc mRNA compared to control osteoclasts (Supporting Fig. 3B).

The body weight of 8-week-old male or female cKO mutants was not different from that of littermate controls of corresponding gender, but the femur length of cKO mutants of either gender was shorter significantly (p < 0.05) by 3% to 4% (Supporting Table 2). The pQCT measurements reveal that mir17~92 cKO mutants of both genders exhibited ~20% and 10% to 24% reduction in trabecular bone mineral content (Tb.BMC) and trabecular bone mineral density (Tb.BMD), respectively, along with ~7% increase in the endosteal circumference in the femur compared to femurs of corresponding littermate controls (Supporting Table 2). The cortical BMC (Cb.BMC) of either gender was lower by ~10%, but there were no significant differences in cortical BMD (Ct.BMD) or cortical thickness (Ct.Th) between cKO mutants and corresponding littermate controls. There was also no difference in the periosteal circumference at mid-shaft. Accordingly, deficient expression of mir17~92 in osteoclasts yielded a similar bone phenotype in male and female mutant mice.

We also performed μCT analysis to further characterize the bone phenotype of the mutant mice (Fig. 3). The male mir17~92 cKO mutant mice had significantly lower trabecular bone volume/total volume (Tb.BV/Tv), trabecular bone mineral density (Tb.BMD), trabecular connectivity density (Tb.Conn-Dens), trabecular number (Tb.N), and trabecular thickness (Tb.Th), as well as a significant increase in trabecular separation (Tb.Sp). In contrast, the cortical bone volume/total volume (Ct.BV/Tv), cortical bone mineral density (Ct.BMD), cortical thickness (Ct.Th), and cortical porosity of the cKO mutants at mid-shaft were mostly not different from those of bones of littermate controls. Thus, the reduction in bone mass in mir17~92 cKO mutant mice was much larger in trabecular bone than in cortical bone. The female mutant mice showed very similar μCT bone phenotype (Fig. 3).

Characterization of osteoclast phenotype of mir17~92 osteoclast cKO mutant mice

We also characterized the marrow-derived osteoclasts of the cKO mutants. The total number of TRAP-positive, multinucleated osteoclasts formed in vivo from cKO marrow precursors was ~20% less than those formed from the same number of precursors of littermate controls (Fig. 4A), but this decrease did not reach statistical significance. Because there was no increase in the number of osteoclasts, it follows that deficient expression of mir17~92 in osteoclastic cells may not promote osteoclastogenesis. The average cell size and the average number of nuclei of osteoclasts from cKO mutants were each approximately twice as large as that of WT osteoclasts (Fig. 4B). These findings suggest that, similar to overexpression of PTP-oc12 or deficient Epha4 expression,12 deficient mir17~92 expression appears to enhance fusion of osteoclast precursors to form larger osteoclasts with more nuclei. The importance of osteoclast fusion in osteoclast activation is well documented by the strong correlation between bone resorption activity and the number of nuclei of osteoclasts in vivo.22,23 Consistent with the greater functional activity, the average pit size created by each cKO osteoclast in the pit formation assay was also twice as large as that formed by osteoclasts of littermate controls (Fig. 4C). Because activation of the PTP-oc/β3-integrin signaling enhances osteoclast activity in part through cytoskeletal reorganization, polarization, and construction of sealing zone and ruffled borders,8 we also immunofluorescent-stained osteoclasts of cKO mutants and those of littermate controls for the actin ring and found that the fluorescent staining of the actin ring of cKO osteoclasts was more pronounced and more intense than littermate control osteoclasts (Fig. 4D).

We further evaluated whether deficient mir17~92 expression in osteoclast precursors would affect osteoclastogenesis, indirectly, by comparing the relative expression levels of known genes associated with osteoclastogenesis in osteoclasts derived from cKO or WT mice. The expression level of several genes known to be associated with osteoclastogenesis (Rank, Traf6, Acp5, Mif, and Nfatc1) were significantly increased (Supporting Fig. 4).
Lack of effects of Ctsk-Cre-mediated deletion of miR17~/C24~92 in growth plate cartilage development

There is recent evidence that the Ctsk promoter used to drive Cre in these transgenic mice might not be entirely specific to osteoclastic cells. We also found that Ctsk is not strictly specific for osteoclastic cells, because substantial expression of Cre was found in the cartilage and the brain, as well as a much lower level in the liver of the cKO mouse (Fig. 5A). However, there was no detectable Cre expression in skeletal muscle or osteoblasts. To further investigate the specificity of the Ctsk promoter in the context of bone, we performed an immunohistochemical staining for the Cre recombinase expression on longitudinal thin sections of the femur of a Ctsk-Cre transgenic mice (Fig. 5B). Although strong immunohistochemical staining of Cre (brownish color) was noted in osteoclasts in both the trabecular and cortical bones, no detectable immunostaining was seen in osteoblasts or in osteocytes.

Because of the Cre expression seen in cartilage, we evaluated if Ctsk-Cre-mediated deletion of miR17~/C24~92 would affect the growth plate cartilage development, as the femurs of these cKO mice were 3% to 4% shorter than those of littermate controls.

Figure 5C shows the Ctsk-Cre-mediated deletion of miR17~/C24~92 had no discernible effects on the lengths of the growth plate, proliferative zone, or hypertrophic zone. Thus, despite a 3% to 4% reduction in femur length, deficient miR17~/C24~92 in chondrocytes in cKO mice may have only negligible effects on the growth plate development.

Lack of effects of Ctsk-Cre-mediated deletion of miR17~/C24~92 on osteoblast functions in vitro and bone formation in vivo

Because miR-17 promotes osteoblast differentiation of mesenchymal stem cells (MSCs) through downregulation of Smurf1, and because recent studies show evidence for transdifferentiation of chondrocytes into osteoblasts, we cannot ignore the possibility that the Ctsk-Cre-mediated deletion of miR17~/C24~92 could still have indirect effects on osteoblast functions and bone formation, even though there was no detectable Cre expression in osteoblasts of the Ctsk-Cre transgenic mice (Fig. 5A, B). To test this possibility, we first measured miR17 level in isolated osteoblasts from three pairs of cKO mice and littermate controls and found no significant
differences (94%±5% of WT controls, p = not significant, Fig. 6A), confirming that Ctsk-Cre–mediated deletion did not delete the miR17–92 gene in osteoblasts. We next determined the effect of the co-culture of cKO osteoclast precursors with osteoblasts of littermate controls and vice versa on the generation of large osteoclasts with more nuclei. Large osteoclasts with more nuclei were formed only when cKO osteoclast precursors were co-cultured with either osteoblasts from littermate control or cKO osteoblasts (Fig. 6B), indicating that the phenotype of larger osteoclasts was due to deletion of miR17–92 in osteoclast precursors. Finally, we compared the differentiation status of cKO osteoblasts with littermate control osteoblasts by comparing cellular alkaline phosphatase (ALP) activity (Fig. 6C) and bone nodule–forming ability (Fig. 4).

Fig. 4. Marrow-derived osteoclast precursors of miR17–92 cKO mutants yielded larger osteoclasts (A) with more nuclei (B), which have greater bone resorption activity (C), and formed more intense actin ring structure (D) than osteoclast precursors of littermate controls in response to RANKL/m-CSF in vitro. (A) (top) A representative photomicrograph of marrow-derived multinucleated osteoclasts of cKO mutants and littermate controls. Scale bars = 20 μm. (bottom left) The total number of osteoclasts (two or more nuclei) derived from 5000 precursor cells. (bottom right) The relative average cell size of the derived osteoclasts. Results are shown as % of the average size of control osteoclasts (mean ± SE, n = 3 to 4 per group in both left and right panels). The dashed line indicates the 100% of the relative average cell size of the derived control osteoclasts. (B) The average number of nuclei of the marrow-derived cKO osteoclasts and WT osteoclasts (mean ± SE, n = 4 per group). (C) Resorption pit formation assay was performed with marrow-derived osteoclasts from 4 littermate controls and 5 miR17–92 cKO mutant mice. Results are shown as average resorption pit size per pit determined on ~50 pits per dentine slice (mean ± SE). (D) A representative photomicrograph of the immunofluorescently-stained actin ring of osteoclasts derived from littermate control mice (top) or from cKO mutants (bottom) plated on uncoated glass slides visualized under a fluorescent microscope. Scale bars = 20 μm. N.S. = not significant.

Fig. 5. Tissue-specificity of the Ctsk promoter (A), absence of immunohistochemical staining of Cre recombinase in osteoblasts and osteocytes in the Ctsk-Cre transgenic mice (B), and the lack of an effect of Ctsk-Cre-mediated disruption of miR17–92 on growth plate cartilage development (C). (A) Cre recombinase expression was measured in various cells and tissues of a cKO mouse. Briefly, total RNA was isolated from osteoclasts, osteoblasts, articular cartilage, brain, liver, skeletal muscle, and xiphoid cartilage. Cre mRNA and Gapdh mRNA (as control for RNA loading) were detected by PCR. (B) A representative immunohistochemical staining of Cre protein on a longitudinal section of growth plate and trabecular bone at the metaphysis (top and middle panels) and cortical bone on the diaphysis at a site immediately underneath the secondary spongiosa (bottom panel) of the distal femur of a Ctsk-Cre transgenic mouse. Top is the negative control without the antibody against Cre. The positive cells for Cre expression were stained brown. The slides were counterstained in blue with hematoxylin. (C) The length of the entire growth plate, proliferative zone, and hypertrophic zone were measured on Goldner’s-stained longitudinal sections of the distal femurs of 8-week-old male cKO mutants and WT mice (mean ± SE, n = 5 per group). N.S. = not significant.
of their marrow stromal cells (Fig. 6D), and we detected no obvious difference in either parameter.

To assess the effect of Ctsk-Cre-mediated deletion of miR17~92 on the bone formation in vivo, we performed dynamic bone formation histomorphometry on cKO and littermate control mice, and found no significant difference in mineral apposition rate (MAR) or in bone formation rate per bone surface (BFR/B.Pm) between the two mouse strains (Table 1). Conversely, each measured static bone resorption parameter of cKO mutants was significantly greater than those of age- and gender-matched littermate controls. Thus, deficient expression of miR17~92 increased bone resorption but had no effects on bone formation in vivo.

Discussion

There is indisputable evidence that miRNAs are key epigenetic regulators of most signaling pathways and cellular processes in various cell types, tissues, and organs, and osteoclasts are no exception. In this report, we provide compelling in vitro and in vivo evidence that miR17~92, particularly miR17, is a negative regulator of the osteoclast activity and bone resorption. Our findings that miR17~92 cKO mutants had highly active osteoclasts and substantial loss of trabecular bone along with marked increase in osteoclastic resorption, that conditional deletion of miR17~92 in osteoclasts resulted in significant upregulation of genes associated with osteoclastic resorption, that conditional deletion of miR17~92 in osteoclasts resulted in significant upregulation of genes associated with osteoclast activation but not those genes implicated in osteoclastogenesis, and that miR17~92 cKO marrow-derived osteoclast precursors did not yield more osteoclasts than littermate control osteoclast precursors in response to RANKL/m-CSF suggest that miR17~92 may not be a regulator of osteoclastogenesis. On the other hand, the miR17~92 cKO mutants showed significant increases in the number of osteoclasts per bone surface (N.Oc/B.Pm) and osteoclast surface per bone surface (Oc.S/BS). These findings are not compatible with our tentative conclusion that miR17~92 is not a regulator of osteoclastogenesis. In addition, cathepsin K,
miR17 inhibits osteoclast activation through suppression of PTP-oc

Table 1. Bone Formation Histomorphometry Parameters of 8-Week-Old Homozygous Osteoclast miR17−92 cKO Mice With Corresponding Littermate Controls

| Parameters                        | Osteoclast miR17−92 cKO | WT littermates |
|-----------------------------------|-------------------------|----------------|
| Dynamic bone formation histomorphometric parameters |                         |                |
| B.Pm (mm)                         | 8.49 ± 1.60             | 5.71 ± 1.42    |
| MAR (μm/day)                      | 1.68 ± 0.26             | 1.58 ± 0.09    |
| BFR/B.Pm                          | 0.60 ± 0.16             | 0.63 ± 0.06    |
| (mm² × 10⁻³/day/mm²)              |                         |                |
| Static bone resorption histomorphometric parameters |                         |                |
| N.Oc (#)                          | 119.7 ± 12.6            | 92.1 ± 5.4     |
| Oc.Pm (mm)                        | 4.32 ± 0.58             | 2.13 ± 0.17    |
| OcS/BS (%)                        | 35.31 ± 1.14***         | 22.8 ± 0.40    |
| N.Oc/T.Ar (1/mm²)                 | 79.61 ± 7.85            | 56.3 ± 6.26    |
| N.Oc/B.Pm (1/mm)                  | 10.64 ± 0.42**          | 8.32 ± 0.21    |

Values are mean ± SE. Dynamic bone formation parameters were done in male cKO and littermate controls (n = 3–5 mice per group), whereas static bone resorption parameters were performed in female cKO and littermate controls (n = 3–4 mice per group).

B.Pm = bone surface; MAR = mineral apposition rate; BFR = bone formation rate; N.Oc = number of osteoclasts; Oc.Pm = osteoclast surface; Oc.S/BS = osteoclast surface per bone surface; N.Oc/T.Ar = osteoclast number per tissue area; and N.Oc/B.Pm = osteoclast number per bone surface.

*p < 0.05; ** p < 0.01; *** p < 0.001, when compared with corresponding littermate controls.

The context of osteoclasts, is recognized as a marker for mature osteoclasts and is not expressed in less differentiated osteoclast cells. Thus, the use of the Ctsk promoter may not result in the deletion of miR17−92 in undifferentiated osteoclasts, and as such may not allow definitive conclusion about osteoclastogenesis. Consequently, much additional work is needed to resolve this issue. Regardless of whether miR17−92 is a regulator of osteoclastogenesis, it is clear that miR17−92 is a potent negative regulator of osteoclastic activity and bone resorption.

This study also offers strong, albeit circumstantial, in vitro evidence that the suppressive action of miR17−92 on osteoclast activation is in part due to the miR17-mediated suppression of PTP-oc expression and signaling. That the bone and osteoclast phenotypes of miR17−92 osteoclast cKO mutants are very similar to that of mutant mice with targeted overexpression of PTP-oc in osteoclasts(2) further suggests an inverse regulatory relationship between miR17−92 and PTP-oc in osteoclast activation. However, it is interesting to note that, unlike miR17−92 cKO mutants (this study) and EphA4 null mice(12), which showed the osteopenic and activated osteoclast phenotypes in both male and female mutant mice, transgenic overexpression of PTP-oc in cells of osteoclastic lineage, using the TRAP exon-1c promoter, yielded the osteoclast phenotype only in male mutant mice(2). One of the possible explanations for the apparent gender differences between miR17−92 cKO mutants and PTP-oc overexpression mutants is that miR17−92 may act through other genes to regulate osteoclastic resorption, which may contribute to the gender differences between the two mouse strains. Another potential explanation is that because the overexpression of PTP-oc in osteoclasts of the PTP-oc transgenic mice was driven by a relatively weak TRAP exon-1c promoter, the relative low overexpression levels of PTP-oc (ie, approximately twofold increase), along with the apparent negative interaction between the estrogen receptor signaling and the PTP-oc-Src signaling may result in a subtle (less significant) osteoclast phenotype in female as opposed to male mutant mice(2).

Our future studies will address this important apparent discrepancy.

Three intriguing findings of this study may be relevant to the potential mechanism by which miR17−92 may act to regulate the activation process of mature osteoclasts. First, similar to activation of the PTP-oc signaling in osteoclasts—either through overexpression of PTP-oc(2) or deletion of EphA4(12)—deletion of miR17−92 increased osteoclast fusion. This conclusion is based on the fact that deficient expression of miR17−92 increased their average cell size and number of nuclei per cell of the osteoclasts, and deletion of miR17−92 in osteoclast precursors not only did not increase but even reduced the total number of osteoclasts formed in response to RANKL/m-CSF.

Second, osteoclast-stimulatory transmembrane protein (OC-Stamp)(30–32) (and the related dendritic cell-specific transmembrane protein DC-Stamp)(13,14), d2 isoform of vascular (H+) ATPase V0 domain (ATP6v0d2),(34,35) and osteoclast-associated receptor (Oscar)(36) have been demonstrated to be essential genes for osteoclast function. It is intriguing that the expression of these osteoclast fusion genes (OC-Stamp, ATP6v0d2, and Oscar) was not affected significantly by deficient expression of miR17−92. Because the expression of DC-Stamp, OC-Stamp, ATP6v0d2, and perhaps also Oscar, in osteoclasts is regulated by NFATc1,(31,34) and because the expression of NFATc1 was not altered by deficient expression of miR17−92, it is not entirely surprising that disruption of miR17−92 in osteoclasts did not have a significant effect on the expression levels of these genes. Nevertheless, the lack of increases in the expression of these osteoclast fusion genes raises the interesting possibility that miR17−92 modulates osteoclast fusion via mechanisms that are independent of or downstream to these osteoclast fusion genes.

There is increasing evidence that activation of Rac1 and/or Rac2 in osteoclasts may play an essential role in osteoclast fusion.(37–39) Rac1/Rac2 activation (through PTP-oc-dependent β3-integrin-mediated activation of Vav3) is a key downstream component of the PTP-oc/EphA4 pathway in osteoclasts.(12,13) In this study, overexpression of miR17−92 significantly suppressed the activation level of Vav3 in osteoclasts (Fig. 2B). It is possible that miR17−92 may regulate osteoclast fusion in part through changes in the levels of activated Rac1/Rac2 via modulation of the activation status of Vav3. This interesting alternative mechanism has merits and will be addressed in our future studies.

The third intriguing finding is that deficient expression of miR17−92 in osteoclasts also significantly upregulated the expression of β3-integrin, Mmp3, and Mmp9. The PTP-oc signaling has been shown to enhance osteoclast activation in part through enhancing the β3-integrin signaling pathway.(2,8) Activation of the β3-integrin signaling in osteoclasts triggers the cytoskeletal reorganization, formation of ruffled border and sealing zone, increased osteoclast migration and osteoclast adhesion to resorbing bone surface, and the release of degradative enzymes, including Mmp3 and Mmp9; all of which are required for activation of the osteoclast. Thus, it is highly likely that miR17−92 may also modulate osteoclast activity through the PTP-oc–induced Src-mediated activation of the
osteoblasts of the cKO mutant mice. However, recent studies developed.

This study, however, has an important caveat. As reported by Yang and colleagues, we also found that the Ctsk promoter is not strictly specific for osteoclasts, because substantial Ctsk promoter-driven Cre expression is found in the cartilage (chondrocytes). The likely scenario that the Ctsk-Cre-mediated deletion of miR17−92 would also delete this cluster gene in chondrocytes could be a significant confounding issue with respect to endochondral bone formation and the overall skeletal development. However, despite the 3% to 4% reduction in the femur length in the cKO mutants, we found no significant differences in the total growth plate length, the length of the proliferative zone, or the length of the hypertrophic zone between the cKO mutants and age- and gender-matched WT littermates. We also did not find notable gross anatomy differences in the various types of cartilage or the key internal organs in the cKO mutants. Thus, we conclude that deficient expression of miR17−92 in chondrocytes has only marginal effects on developmental bone growth and the overall cartilage development.

We did not find any detectable Cre mRNA expression or any significant difference in the expression level of miR17 in the osteoblasts of the cKO mutant mice. However, recent studies have provided evidence for the transdifferentiation of chondrocytes into osteoblasts. Thus, we cannot overlook the possibility that deletion of miR17−92 in chondrocytes could indirectly affect the differentiation and function of osteoblasts as well as bone formation. On the other hand, our findings that (i) the basal alkaline phosphatase activity of cKO osteoblasts was not different from that of WT osteoblasts, (ii) the bone nodule formation ability of cKO marrow stromal cells was also not different from that of WT marrow stromal cells, and (iii) there were no significant differences in the dynamic bone formation histomorphometric parameters between cKO mice and WT littermates, do not support this possibility. Accordingly, we conclude that the Ctsk-Cre-mediated disruption of miR17−92 did not affect the differentiation and function of osteoblasts nor that it affected bone formation.

Under normal conditions, bone resorption is tightly coupled to bone formation, in that an increase in bone resorption is usually followed by an increase in bone formation of equal magnitude. Thus, it is intriguing that the increase in bone resorption in miR17−92 cKO mice was not accompanied by a corresponding increase in bone formation. However, it should be noted that this apparent uncoupling of bone formation to bone resorption is not unique to these miR17−92 cKO mutant mice, because other transgenic mouse strains, eg, PTP-oc transgenic mice, EphA4 null mice, Shp1 null mice, and Claudin-18 null mice, also showed an increase in bone resorption without a change in bone formation. Nevertheless, the mechanistic cause(s) for the apparent uncoupling of bone formation to bone resorption in miR17−92 cKO mutant mice is unclear at this time, and our future work will address the mechanisms contributing to the uncoupling of bone formation to resorption in these mutant mice.

In conclusion, this study provides the first compelling evidence that miR17−92, particularly miR17, is a potent negative regulator of osteoclast activation in part through its suppression of the expression of PTP-oc and its signaling pathway. Our ultimate objective of this research is to develop a novel and effective antiresorptive therapy that would target the miR17/PTP-oc/EphA4 regulatory axis of osteoclast activation. In this regard, the miR17 would appear to be an appealing drug target, as miR17 mimics can be developed into novel and effective antiresorptive therapies. Although the current miRNA-based therapeutic technology has a number of challenges limiting its therapeutic utility, we are hopeful that the challenges of the miRNA-based therapeutic technology will soon be overcome and that the miR17-based therapies for osteoporosis and related bone-wasting diseases will become feasible.

Disclosures

All authors state that they have no conflicts of interest.

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