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# Osteoclast-Specific Dicer Gene Deficiency Suppresses Osteoclastic Bone Resorption

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## ABSTRACT

Osteoclasts are unique cells that resorb bone, and are involved in not only bone remodeling but also pathological bone loss such as osteoporosis and rheumatoid arthritis. The regulation of osteoclasts is based on a number of molecules but full details of these molecules have not yet been understood. MicroRNAs are produced by Dicer cleavage an emerging regulatory system for cell and tissue function. Here, we examine the effects of Dicer deficiency in osteoclasts on osteoclastic activity and bone mass in vivo. We specifically knocked out Dicer in osteoclasts by crossing Dicer flox mice with cathepsin K-Cre knock-in mice. Dicer deficiency in osteoclasts decreased the number of osteoclasts (N.Oc/BS) and osteoclast surface (Oc.S/BS) in vivo. Intrinsically, Dicer deficiency in osteoclasts suppressed the levels of TRAP positive multinucleated cell development in culture and also reduced NFATc1 and TRAP gene expression. MicroRNA analysis indicated that expression of miR-155 was suppressed by RANKL treatment in Dicer deficient cells. Dicer deficiency in osteoclasts suppressed osteoblastic activity in vivo including mineral apposition rate (MAR) and bone formation rate (BFR) and also suppressed expression of genes encoding type I collagen, osteocalcin, Runx2, and Efnb2 in vivo. Dicer deficiency in osteoclasts increased the levels of bone mass indicating that the Dicer deficiency-induced osteoclastic suppression was dominant over Dicer deficiency-induced osteoblastic suppression. On the other hand, conditional Dicer deletion in osteoblasts by using 2.3 kb type I collagen-Cre did not affect bone mass. These results indicate that Dicer in osteoclasts controls activity of bone resorption in vivo. J. Cell. Biochem. 109: 866–875, 2010. 

**KEY WORDS:** DICER; OSTEOCLASTS; CATHEPSIN K; microRNA

Osteoclasts are unique cells that resorb bone [Horowitz et al., 2001; Harada and Rodan, 2003; Nakamura et al., 2003; Sims et al., 2004; Martin and Sims, 2005; Martin et al., 2006; Datta et al., 2008; McGrath et al., 2008] and deregulation of these cells leads to either failed or increased bone resorption as observed in osteoporosis and rheumatoid arthritis, [Grigoriadis et al., 1994; Marzia et al., 2000; Schaller et al., 2004; Zhao et al., 2006]. Recent analyses of osteoclasts point to the importance of several pathways

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of regulation [Takayanagi, 2007]. First is cytokine signaling molecules between osteoclasts and osteoblasts such as RANKL. RANKL controls its downstream events involving c-fos, NFATc1 as well as co-receptors and calcineurin. Second is integrin mediated signaling and third is nuclear receptors such as estrogen receptors. Estrogen receptors are present in osteoclasts and play a pivotal role in the regulation of bone resorption. These signals are modulated by a number of molecules, and other types of controlling pathways that are not yet understood may still exist.

MicroRNAs are an emerging regulatory system in physiological and pathological events [Bernstein et al., 2003; Cobb et al., 2005; Harfe et al., 2005; Kanellopoulou et al., 2005; Muljo et al., 2005; Andl et al., 2006; Cobb et al., 2006; Harris et al., 2006; Yi et al., 2006; Murchison et al., 2007; O'Rourke et al., 2007; Schaefer et al., 2007; Thai et al., 2007; Chen et al., 2008; Kobayashi et al., 2008]. MicroRNAs are produced through several steps of modification including the cleavage by a key protein called Dicer. Upon removal of Dicer, microRNA production is downregulated [Harfe et al., 2005]. We examined the role of Dicer specifically in osteoclasts to explore its function in the regulation of bone metabolism. As Dicer null mice are embryonic lethal [Bernstein et al., 2003], these mice cannot be used to examine microRNA function in osteoclasts during the remodeling events taking place in adult bone. Therefore, we deleted the Dicer gene specifically in osteoclasts by using a conditional floxed allele of Dicer [Harfe et al., 2005] and the cathepsin K cre-system [Nakamura et al., 2007].

## MATERIALS AND METHODS

### ANIMALS

Dicer floxed mice were generated as described previously [Harfe et al., 2005]. As a deleter mouse, we used cathepsin K-Cre knock in mice [Nakamura et al., 2007]. To generate the osteoclast-specific Dicer-deficient mice, we crossed cathepsin K Cre knock in mice with Dicer flox mice. Cre positive Dicer hetero-flox mice were further crossed with Dicer flox mice, and Cre positive Dicer floxed mice and Cre negative Dicer floxed mice were used for the analysis. As another type of mice, 2.3 kb collagen type I promoter cre-mice were used to knock out Dicer in osteoblasts [Dacquin et al., 2002]. Seven-week-old male mice were used for the analysis. Mice were housed under controlled conditions at 24°C on a 12-h light and 12-h dark cycle. All animal experiments were approved by the animal welfare committee of Tokyo Medical and Dental University.

### THREE-DIMENSIONAL (3D) MICRO X-RAY COMPUTED TOMOGRAPHY (μCT) ANALYSIS

3D-μCT images were obtained using equipment, Scan-Xmate-E090 (Comscan Tecno, Sagamihara, Japan) and computer software, Tri/3D-Bon (Ratoc System Engineering, Tokyo, Japan). In the trabecular bone analysis, bone volume/tissue volume (BV/TV), trabecular number (Tb.N), and trabecular thickness (Tb.Th) were examined in the secondary trabecular bone area of distal femora. In wild-type (WT) mice, trabecular bone area for the analysis was set from the closer margins at 0.2 mm to the further margins at 1.2 mm from the growth plate. In cKO mice, the area was adjusted by shorter mean bone length, correcting the influence of different femur length; closer and further margins were set at 0.18 and 1.08 mm, respectively. In cortical bone analysis, axial images at femoral mid shaft were analyzed to calculate periosteal and endosteal perimeters, cortical thickness (Ct.Th), bone area (B.Ar), and cortical area (Ct.Ar).

### BONE HISTOMORPHOMETRIC ANALYSIS

Calcein labeling was conducted to estimate the levels of newly formed bone within a unit time period. Calcein (Kanto Chemical, Tokyo, Japan) (10 mg/kg body weight) was injected intraperioneally 2 and 4 days before sacrifice. Femora fixed in 4% paraformaldehyde were rinsed in 0.1 M phosphate buffer, and embedded in 4% carboxymethylcellulose. Sagittal cryo-sections were prepared in 5 μm thickness, and the calcein signals were captured by fluorescent microscopy. Single labeled bone surface (sLS), double labeled bone surface (dLS), and total bone surface (BS) were separately measured. Mineralizing surface (MS) per BS was calculated as (dLS + sLS/2)/BS. The distance between parallel calcein lines was measured to yield mineral apposition rate (MAR (μm/day)). Bone formation rate (BFR) was calculated as MAR multiplied by MS/BS. Histomorphometric analysis was performed by focusing the same area as analyzed in μCT analysis. Osteoclast number per bone surface (N.S/BS, N/mm) and osteoclasts surface per bone surface (Oc.S/BS, %) were analyzed by TRAP staining in sagittal sections of tibiae. Tibiae were fixed in 4% paraformaldehyde, decalcified in 15% EDTA, and embedded in paraffin blocks. Osteoclast number per bone surface (N.Oc/BS, N/mm) and osteoclast surface per bone surface (Oc.S/BS, %) were analyzed by tartrate-resistant acid phosphatase (TRAP) staining on the 7-μm thick sagittal sections, as described previously [Mizoguchi et al., 2008]. TRAP positive cells containing one or more nuclei and sitting on the surface of the trabeculae were defined as osteoclasts. One section per animal was analyzed for these parameters.

### TERMINAL DEOXYNUCLEOTIDYLTRANSFERASE-MEDIATED UTP END LABELING (TUNEL) ASSAY.

TUNEL assay was conducted according to the instruction of in situ apoptosis detection kit (Takara Bio, Shiga, Japan). Briefly, decalcified sections were incubated with 15 μg/ml proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% $H_2O_2$ for 5 min at room temperature and then washed with PBS. Sections were immersed in terminal deoxynucleotidyltransferase (TdT) buffer containing deoxynucleotidyltransferase and biotinylated dUTP, incubated in a humid atmosphere at 37°C for 90 min, and then washed with PBS. The sections were incubated at room temperature for 30 min with anti-FITC horseradish peroxidase-conjugated antibody, and the signals were visualized with diaminobenzidine (DAKO, glostrup, Denmark). This was followed by the TRAP staining as described above.

### RT-PCR ANALYSIS

For RT-PCR analysis, dissected humeri were kept in "RNA later" (Sigma, MO) for about 1 week at −20°C. Bone was immersed in Trizol reagent (Invitrogen, CA), and were homogenized using Polytron PT3100 homogenizer (Kinematica AG, Lucerne,

Switzerland). RNA was extracted according to manufacturer's protocol. Reverse transcription (RT) was carried out using 1 μg total RNA, 150 ng random primers (Invitrogen), 0.125 mM deoxynucleotide triphosphate mix (Takara Bio), 10 mM dTT and 200 U of superscript II reverse transcriptase (Invitrogen) in 20 μl volume. Quantitative real-time PCR analysis was carried out using iCycler (Bio-Rad, CA) and iQ5 data analyzing software. The reaction was performed in a 25 μl reaction mixture containing 2 μl of cDNA samples, 5 μM of sense and antisense primers, and 12.5 μl of iQ SYBR Green Supermix (Bio-Rad). The primer sequences were as follows: *Runx2*, forward, 5′-TGG CTT GGG TTT CAG GTT AGG G-3′, reverse, 5′-TCG GTT TCT TAG GGT CTT GGA GTG-3′; *Col1a1*, forward, 5′-CTG ACT GGA AGA GCG GAG AG-3′, reverse, 5'-GCA CAG ACG GCT GAG TAG G-3'; *Osteocalcin*, forward, 5′-ACC TTA TTG CCC TCC TGC TT-3′ reverse, 5′-GCG CTC TGT CTC TCT GAC CT-3′; *Cathepsin K*, forward, 5′-TTC TCC TCT CGT TGG TGC TT-3′, reverse, 5′-AAA AAT GCC CTG TTG TGT CC-3′; *Trap*, forward, 5′-TGG CTG AGG AAG TCA TCT GAG TTG-3′, reverse, 5′-GAC CAC CTT GGC AAT GTC TCT G-3′; *Rankl*, forward, 5′-CCT GAG GCC CAG CCA TTT-3′, reverse, 5′-CTT GGC CCA GCC TCG AT-3′; osteoprotegerin (*Opg*), forward, 5′-TAC CTG GAG ATC GAA TTC TGC TT-3, reverse, 5′-CCA TCT GGA CAT TTT TTG CAA A-3′; *Nfatc1*, forward, 5′-AGC CCA AGT CTC ACC ACA GG-3′, reverse, 5′-CAG CCG TCC CAA TGA ACA GC-3′; and glyceraldehyde-3-phosphate dehydrogenase, forward, 5′-AGA AGG TGG TGA AGC AGG CAT C-3′, reverse, 5′-CGA AGG TGG AAG AGT GGG AGT TG-3′. The PCR conditions were set as 40 cycles of [95°C for 15 s, 55°C for 30 s, and 72°C for 30 s]. For the quantitative real-time PCR analysis of the *Efnb2* and miR-155, Taq Man Gene Expression Assay (Applied Biosystems, CA) and TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) were used and PCR was conducted as manufactures protocol. The mRNA expression levels of genes of interest were evaluated by calculating the ratios against *Gapdh* expression levels.

### IN VITRO OSTEOCLAST FORMATION AND MINERALIZED NODULE FORMATION

Bone marrow cells were harvested from the tibia. Bone marrow cells were plated in $1 \times 10^5$ cells/cm$^2$, and cultured in α-minimal essential medium (Invitrogen) with 10% fetal bovine serum (Invitrogen) containing 10 ng/ml M-CSF (R&D, MN). After 2 days, adherent cells were used as bone marrow macrophages (BMMs). BMMs were further cultured in the presence of 100 ng/ml soluble RANKL (Peprotech, London, UK) and 10 ng/ml M-CSF. Three days after RANKL stimulation, TRAP positive multinucleated cells were counted as osteoclasts.

For mineralized nodule formation, bone marrow cells were plated at $1 \times 10^6$ cells/cm$^2$ to 12 well plates, and were cultured in α-minimal essential medium with 10% fetal bovine serum containing 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate. The cultures were stained with alizarin red solution on day 21. The area of mineralised nodules was measured with ImageJ analysis program.

### MICRORNA ARRAY ANALYSIS

BMMs were cultured with 10 ng/ml M-CSF (control) in the presence or absence of 100 ng/ml RANKL for 24 h, and total RNA was isolated by MiRVana MiRNA Isolation kit (Ambion). Cy3-labeled RNA from control BMMs and Cy5-labeled RNA from RANKL treated BMMs were mixed and hybridization to the MiRNA array was conducted using *MiR*Vana$^{TM}$ MiRNA Bioarray system Ver.2 (Ambion). Data was analyzed by microarray data analysis tool software (Figen, Nagoya, Japan).

### FLOW CYTOMETRY

Bone marrow cells from WT and cKO mice were incubated with anti-CD16/32 antibody (clone 93, eBioscience) to block non-specific binding of antibodies. After washing with 2% FBS/PBS, the cells were stained with allophycocyanin-conjugated anti-c-kit (clone ACK2), phycoerythrin-conjugated anti-c-fms (clone AFS98), and fluorescein isothiocyanate-conjugated anti-CD11b (clone M1/70). The cells were analyzed by FACSCalibur (BD Biosience) and CellQuest software.

### STATISTICAL EVALUATIONS

All the numeral data in the results were presented as mean values ± standard deviations (SD). Statistical analysis was performed based on Student's *t*-test or Welch's *t*-test according to the results of *F*-test. Difference was judged to be statistically significant when $P$ values were $<0.05$.

## Results

### DICER DEFICIENCY IN OSTEOCLASTS INCREASES BONE MASS

To investigate the role of Dicer in osteoclasts, we crossed Dicer floxed mice with cathepsin K cre knock-in mice. Mice with Dicer deficiency in osteoclasts (hereafter, cKO mutant) were born normally without exhibiting any significant abnormalities in skeletal patterning. 3D μCT analysis revealed that Dicer deficiency in osteoclasts resulted in more crowdedness of trabecular bone pattern compared to WT (Fig. 1A). Quantification of the 3D-μCT data indicated that Dicer deficiency in osteoclasts increased the trabecular bone mass compared to WT mice (BV/TV) (Fig. 1B). Similarly, Dicer deficiency in osteoclasts increased trabecular thickness (Fig. 1C). The growth of the mutant mice was retarded compared to WT (Fig. 1D,E).

### DICER DEFICIENCY IN OSTEOCLASTS SUPPRESSES BONE-RESORPTION IN VIVO

Histological sections of the femora of Dicer deficient cKO mice revealed sparsity of TRAP positive cells compared to WT (Fig. 2A). Stromal cellularity was similar in the two genotypes. Quantification showed that Dicer deficiency in osteoclasts reduced the levels of osteoclast surface (Oc.S/BS) (Fig. 2B) and also those of osteoclast number (N. Oc/BS) (Fig. 2C). These results indicated that Dicer deficiency in osteoclasts suppressed the levels of bone resorption activity in vivo.

### DICER DEFICIENCY IN OSTEOCLASTS SUPPRESSES OSTEOCLASTOGENESIS IN CULTURE

To address intrinsic effects of Dicer deficiency in osteoclasts, we cultured bone marrow derived cells from cKO mutant mice in the

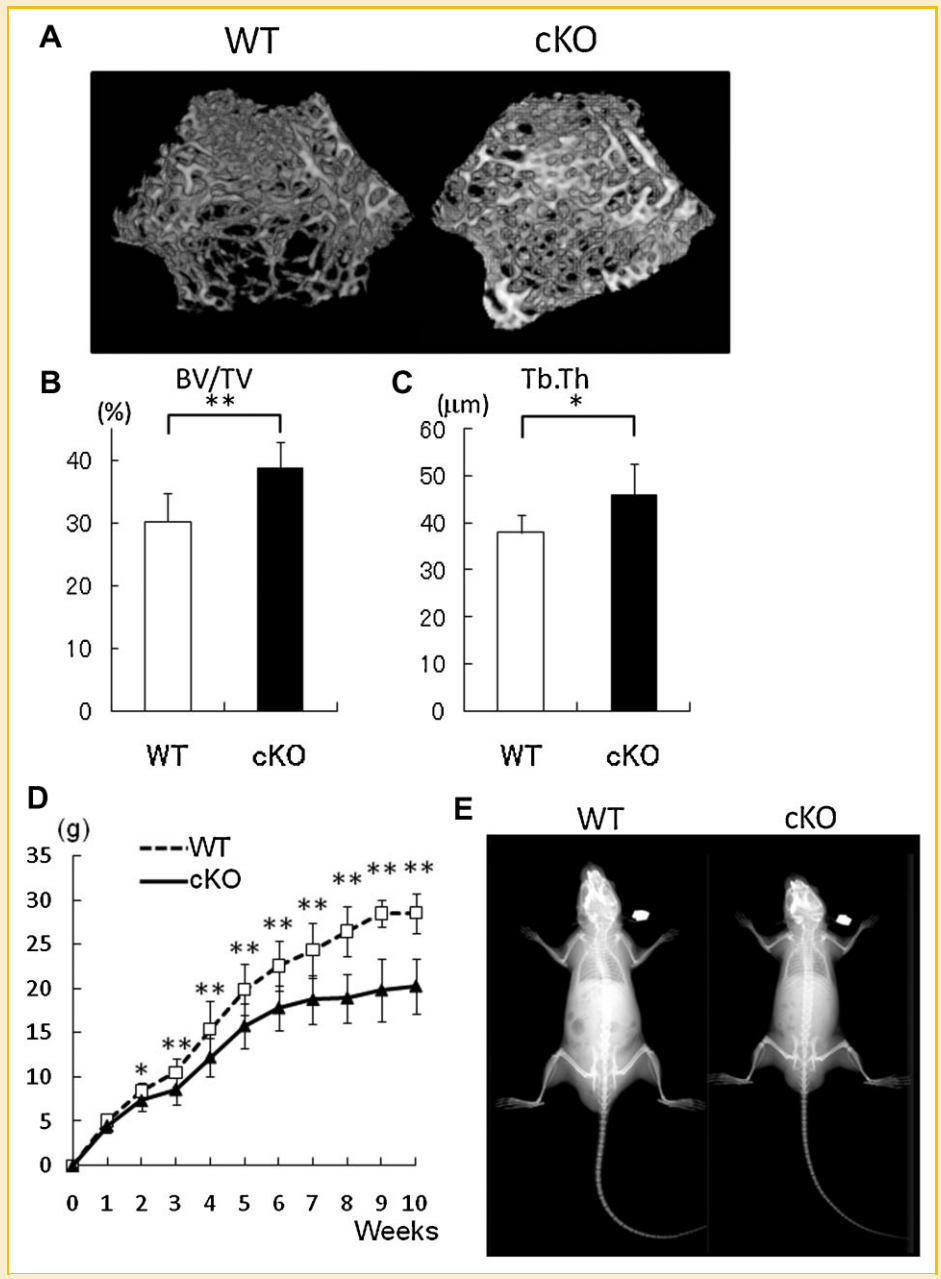


Fig. 1. Dicer deficiency in osteoclasts increases bone volume. 3D-μCT images of the secondary trabecular regions of distal metaphyses of the femora (A). Dicer deficiency in osteoclasts increased the levels of bone volume/tissue volume (BV/TV) (B), and trabecular thickness (Tb.Th) (C). Wild-type (WT); mice with Dicer deficiency in osteoclasts (cKO). The growth curve of the mice (D) and X-ray pictures (E). Asterisks indicate that the difference is statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$).

presence of M-CSF to obtain bone marrow derived macrophages (BMMs) and subjected these BMMs to the treatment with RANKL, an osteoclastogenic cytokine. Bone marrow cells differentiated into TRAP positive multinucleated osteoclasts. The size of TRAP-positive cells in Dicer deficient bone marrow cell cultures tended to be smaller than WT cells (Fig. 3A). Quantification of the number of TRAP positive multinucleated cells revealed that Dicer deficiency in osteoclasts suppressed RANKL-induced development of TRAP positive cells in culture (Fig. 3B). Thus, Dicer intrinsically control the development of osteoclasts.

### DICER DEFICIENCY IN OSTEOCLASTS SUPPRESSES EXPRESSION LEVELS OF OSTEOCLASTIC PHENOTYPE-RELATED GENES

RT-PCR analysis on the RNA extracted from adult long bone was performed to examine the effects of Dicer deficiency in osteoclasts on the expression levels of genes encoding-proteins related to osteoclast phenotype. Dicer deficiency in osteoclasts suppressed the expression levels of *Trap* mRNA compared to control mice (Fig. 4A). With respect to *Nfatc1*, the master regularoty gene for osteoclasts, Dicer deficiency in osteoclasts suppressed mRNA levels slightly (Fig. 4B). These observations indicated that Dicer deficiency

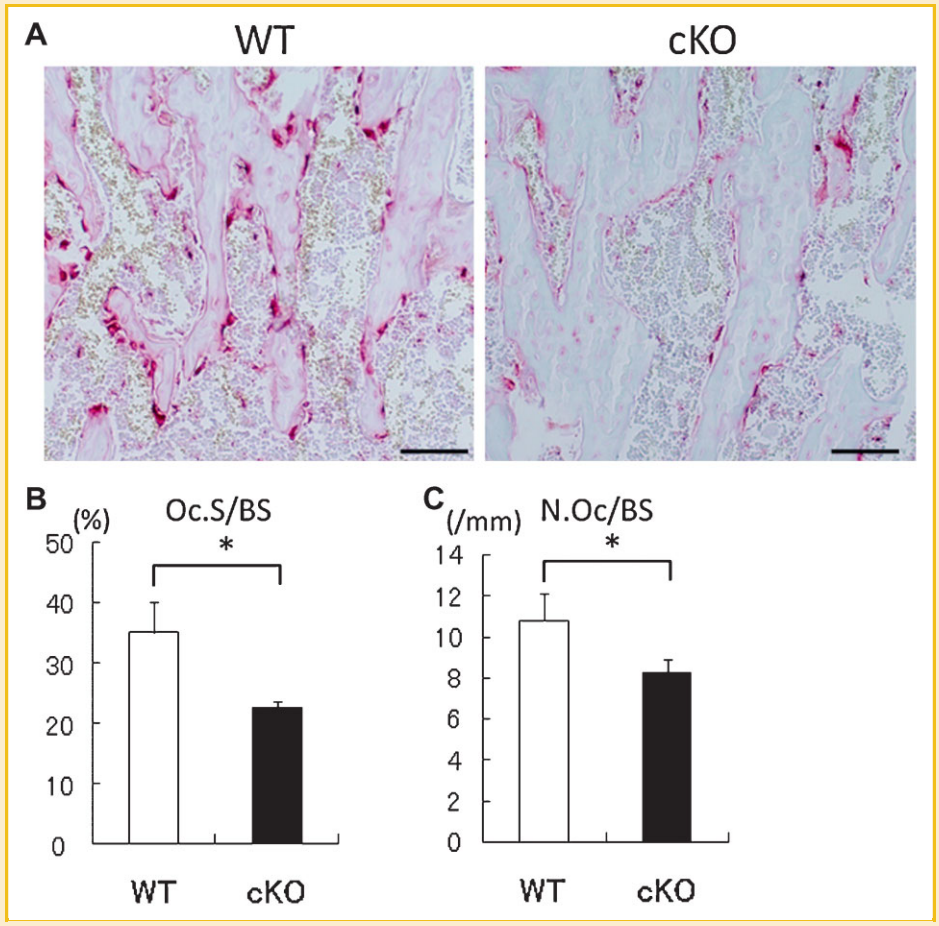


Fig. 2. Dicer deficiency in osteoclasts suppresses the levels of osteoclastic parameters in vivo. TRAP stained sections of the secondary trabecular regions of the femora (A). Histomorphometric parameters of bone resorption. Osteoclast surface/bone surface (Oc.S/BS) (B). Osteoclast number/bone surface (N.Oc/BS) (C). Bars represent 100 μm. Wild-type (WT); mice with Dicer deficiency in osteoclasts (cKO). Asterisk indicates that the difference is statistically significant ($P < 0.05$).

suppressed the expression of genes encoding the regulatory transcription factor for osteoclast development and its downstream target genes. Ephrin proteins have been suggested to link osteoclastic and osteoblastic activities [Zhao et al., 2006]. We therefore examined expression of ephrinB2 (*Efnb2*). Dicer deficiency in osteoclasts suppressed *Efnb2* expression in the bone of mice compared to WT (Fig. 4C).

### DICER DEFICIENCY IN OSTEOCLASTS DID NOT ALTER THE FRACTION SIZE OF OSTEOCLAST PROGENITOR CELLS OR APOPTOSIS OF OSTEOCLASTS

To examine whether decreased number of osteoclasts occurred due to decreased number of pre-osteoclasts, bone marrow cells from WT and cKO mice were subjected to flow-cytometric analysis. We defined pre-osteoclasts as c-kit positive, c-fms positive, and CD11b low cells. Such fraction of osteoclast precursors in total bone marrow appeared to be similar in WT and cKO mice (Fig. 5).

As precursor cell population sizes were similar, we then examined whether osteoclast-specific Dicer deficiency affect the apoptosis of osteoclasts. TUNEL staining was conducted using sagittal sections of femora. However, we detected no significant difference in the levels of TUNEL positive osteoclasts between WT and cKO mice (data not shown). These results suggest that the decreased number of osteoclasts in Dicer-cKO mice might be caused by reasons other than the decrease in population sizes of pre-osteoclasts or the increase in apoptosis of osteoclasts, but impairment of differentiation process.

### RANKL STIMULATION OF BMMS RESULTS IN SMALL ALTERATION OF THE miRNA EXPRESSION PROFILES

The phenotype observed in Dicer-deficient mice could be caused by impaired miRNA expressions during osteoclastogenesis. To identify the candidate miRNAs involved in the changes in osteoclastogenesis, we conducted miRNA array analysis. BMMs were cultured in the presence of 10 ng/ml M-CSF with or without 100 ng/ml RANKL for 24 h. These cells were subjected to miRNA expression profile analysis. At 24 h time point, morphological appearances of RANKL-treated cells were similar to those in control cells, whereas mRNA expression of *Trap* was already increased (data not shown). Relatively high expression levels were observed with respect to more than 100 miRNAs in RANKL treated and control cells (Fig. 6A). Total of 662-miRNAs were analyzed. Largest effects of RANKL were

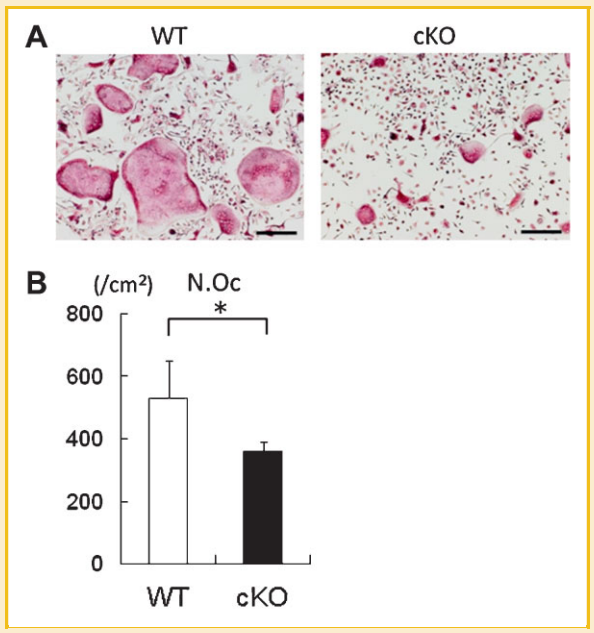


Fig. 3. Dicer deficiency in osteoclasts suppresses osteoclastogenesis in vitro. In vitro osteoclastogenesis assay was performed with bone marrow cells using 10 ng/ml M-CSF and 100 ng/ml RANKL. TRAP positive multinucleated osteoclastic cells were visualized under the microscope; scale bars represent 200 μm (A,B). Wild-type (WT); mice with Dicer deficiency in osteoclasts (cKO). Asterisk indicates that the difference is statistically significant ($P < 0.05$).

observed in miR-302c whose expression was up-regulated about 1.78-fold in microRNA array analysis. However, real-time PCR analysis revealed that the baseline expression level of miR-302c was relatively low. Our results suggest that the causative miRNAs may not exhibit changes large enough to be detectable in our microarray assay.

## MICRORNA-155 ANALYSES IN DICER DEFICIENT CELLS

As Dicer deficiency suppressed osteoclastogenesis in culture, we sought to examine candidate miRNAs responsible for the suppression of osteoclastogenesis. To this end, we focused on microRNA 155 (miR-155), which has been implicated in macrophage function [O'Connell et al., 2007]. Computer-based analyses indicated that miR-155 is a possible regulator of SHIP that inhibits osteoclastogenesis previously [Takeshita et al., 2002]. As a baseline, miR-155 levels in BMMs from Dicer deficient mice were similar to those from WT. RANKL treatment did not significantly alter miR-155 levels in WT. In contrast, RANKL treatment suppressed miR-155 levels in BMMs from Dicer deficient mice (Fig. 6B). These data suggest that Dicer deficiency suppressed miR-155 upon RANKL treatment to possibly increase SHIP, an inhibitor for osteoclasts.

## DICER DEFICIENCY IN OSTEOCLASTS SUPPRESSES OSTEOBLASTIC ACTIVITY IN VIVO

To examine whether Dicer deficiency in osteoclasts may influence coupling events in bone turnover, dynamic bone formation parameters were analyzed to assess osteoblastic activities in vivo. Dicer deficiency in osteoclasts suppressed the levels of in vivo bone

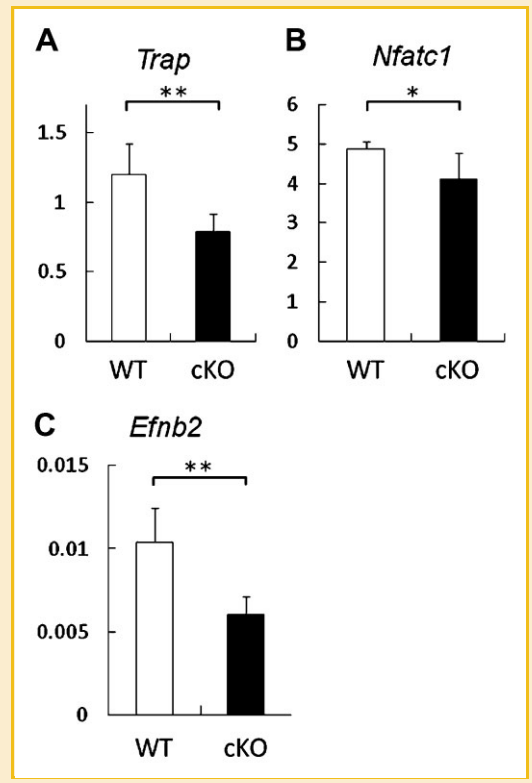


Fig. 4. Dicer deficiency in osteoclasts suppressed the levels of osteoclast phenotype-related gene expression. Messenger RNA expression levels of genes encoding proteins related to osteoclastic phenotype. A: Tartrate-resistant acid phosphatase (Trap), B: Nfatc1 and C: EfnB2. Values were normalized against *Gapdh* expression in bone. Analysis was based on real-time RT-PCR. Wild-type (WT); mice with Dicer deficiency in osteoclasts (cKO). Asterisks indicate that the difference is statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$).

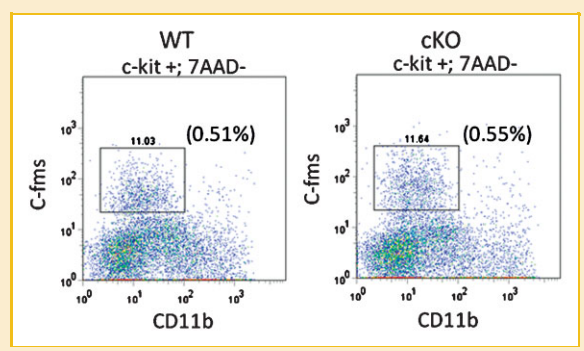


Fig. 5. Osteoclast-specific Dicer deficiency does not alter the fraction size of osteoclast progenitor cells. Flow-cytometric analysis was performed on bone marrow cells from 6-week-old mice, using antibodies directed against c-fms, CD11b and c-Kit as surface markers. Fraction of osteoclast precursor cells was comparable between WT and cKO mice, as indicated by the representative scatter plots (n = 3). Wild-type (WT); mice with Dicer deficiency in osteoclasts (cKO).

formation parameters including mineralized surface (MS/BS) (Fig. 7A,B) and MAR (μm/day) (Fig. 7C). As a secondary parameter of bone formation in histomorphometry, Dicer deficiency in osteoclasts reduced BFR ($\mu m^2$/day/μm) (Fig. 7D). These observations

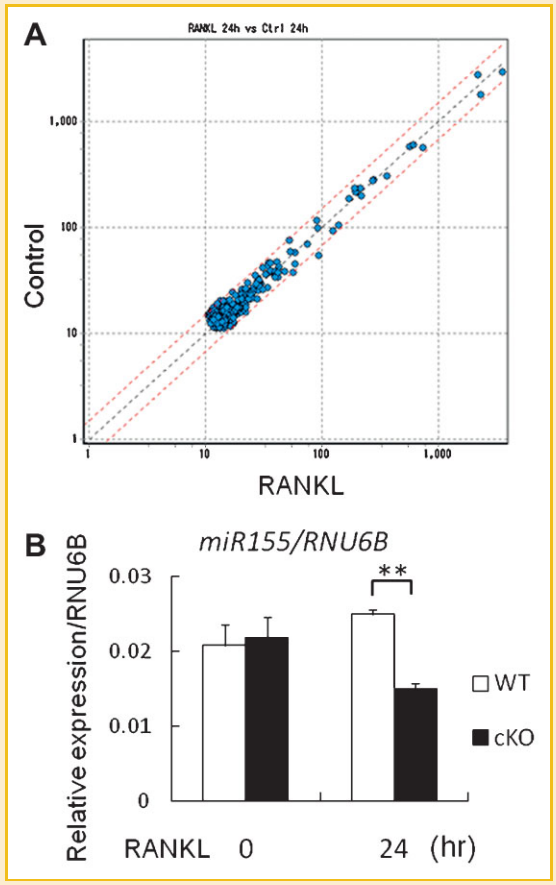


Fig. 6. Osteoclast-specific Dicer deficiency reduces the levels of miR-155. A: MicroRNA array analysis was performed on BMMs subjected to in vitro osteoclastogenesis. The bone marrow cells were cultured in the presence of 10 ng/ml M-CSF alone or combination of 10 ng/ml M-CSF and 100 ng/ml RANKL for 24 h. The profile of miRNA expression was visualized by scatter plot of normalized signal intensities. Horizontal axis is for cells treated with M-CSF and RANKL. Vertical axis is for cells treated only with M-CSF. B: Osteoclast-specific Dicer deficiency does not alter the basal levels of miR-155 in bone marrow derived macrophages treated with RANKL. However, it reduces miRNA levels after treatment with RANKL. Values were normalized against control (RNU6B). Wild-type (WT); mice with Dicer deficiency in osteoclasts (cKO). Asterisk indicates that the difference is statistically significant ($P < 0.01$).

imply that Dicer deficiency in osteoclasts suppressed the activity of osteoblasts in vivo via coupling event.

### DICER DEFICIENCY IN OSTEOCLASTS SUPPRESSES EXPRESSION LEVELS OF OSTEOBLASTIC PHENOTYPE-RELATED GENE

To further examine the influence of Dicer deficiency in osteoclasts on osteoblasts, mRNA expression of the genes encoding osteoblast phenotype-related proteins was examined. RT-PCR analysis on bone revealed that Dicer deficiency in osteoclasts suppressed the mRNA expression levels of type I collagen (*Col1a1*), the major product of osteoblasts (Fig. 7E). Dicer deficiency in osteoclasts also reduced the mRNA expression levels of osteocalcin (*Bglap1*) (Fig. 7F), which is a specific product of osteoblasts. We further examined the osteoblast regulatory transcription factor, which is a specific product of osteoblasts. Dicer deficiency in osteoclasts suppressed the levels of runt related transcription factor 2 (*Runx2*) mRNA (Fig. 7G), indicating that Dicer deficiency in osteoclasts affects osteoblast phenotype expression in vivo. In contrast, Rankl/opg ratio was not significantly decreased in cKO mice (data not shown).

### DICER DEFICIENCY IN OSTEOBLASTS DOES NOT ALTER BONE MASS

We further examine Dicer function in osteoblasts by specifically deleting Dicer in osteoblasts using 2.3 kb col I promoter-cre mice. 3D-μCT analysis was conducted. Dicer deficiency in osteoblasts did not alter the levels of the trabecular bone mass (Fig. 8A), trabecular thickness (Fig. 7B), and trabecular number (Fig. 7C) compared to WT mice. Thus, Dicer regulates bone metabolism mainly via its action in regulation of bone resorption.

## DISCUSSION

We showed that Dicer is involved in regulation of osteoclastogenesis and osteoclastic control of bone metabolism. Dicer deficiency in osteoclasts suppressed the levels of osteoclast number and osteoclast surface in vivo and also reduced osteoclastic development in culture indicating endogenous regulation by Dicer of the cells in osteoclastic lineage. Therefore, Dicer in osteoclasts is a critical regulator that acts cell autonomously to modulate bone resorption. In fact, bone mass was increased in Dicer deficient mice indicating the importance of Dicer in control of bone mass and metabolism.

Osteoclastic differentiation is regulated by multiple steps of events including the levels of progenitor cell population derived from hematopoietic stem cells, microenvironmental signals (M-CSF and RANKL), transcription factors such (TRAF6, NFkB, c-Fos, NFATc1), ITAM receptors, calcium oscillation, and calcineurin [Takayanagi, 2007]. Recent reports also revealed the presence of estrogen receptors in osteoclasts [Nakamura et al., 2007]. Identification of the involvement of Dicer in the regulation of osteoclastic activity adds a new layer of regulatory system, that is, microRNAs, to the control of the osteoclasts.

Decreased osteoclast number associated with increased bone mass in osteoclast-specific dicer-deficient mice suggests that expression of certain miRNA-target gene would be altered by Dicer deficiency in osteoclasts. As Dicer produces miRNAs that inactivate their target gene(s) via reduction of mRNA or protein levels, the putative function of the target genes would be to suppress osteoclastogenesis. As RANKL treatment induces differentiation of osteoclasts from BMMs, miRNA levels were examined using miRNA array systems before and after RANKL treatment of BMMs, to identify the candidate miRNAs required for regulation of osteoclastogenesis. In this setting, no major changes were detected in the levels of miRNA expression. We cannot rule out the possibility that our analysis would not be sensitive enough to detect the differences, which might have been confounded by heterogeneity of cell types in BMMs cultures. In fact, osteoclast progenitors are not predominant populations as shown in flow-cytometry. Nevertheless, our results indicated that miRNA expression during osteoclastogenesis is relatively stable, although their functional importance was suggested by in vivo and in vitro observations using Ctsk-driven

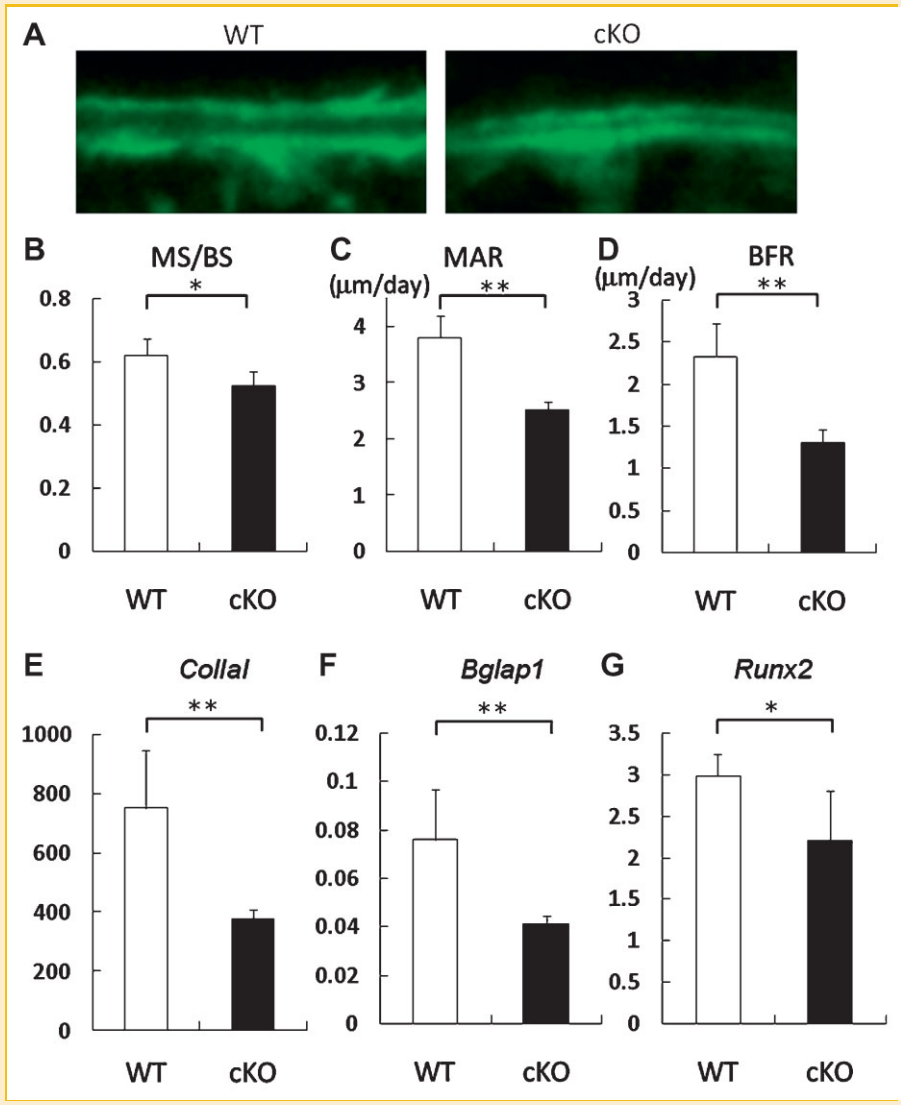


Fig. 7. Dicer deficiency in osteoclasts decreases osteoblastic activities in vivo. Calcein double labeling of the bone (A). Dynamic histomorphometric parameters. MS/BS (B); MAR (A,C); BFR (D). Expression levels of mRNAs encoding *Collal* (E), *Osteocalcin* (F), and *Runx2* (G) were examined based on real-time RT-PCR. Data were normalized against *Gapdh* expression levels. Wild-type (WT); mice with Dicer deficiency in osteoclasts (cKO). Asterisks indicate that the difference is statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$).

conditional knock-out of dicer gene in mice. In cKO mice, miRNA expression would be declined after cells begin to express *Ctsk* and *Cre*. This may up-regulate expression of target genes that impair osteoclastogenesis. Individual effect of each target gene may be small. However, accumulation of defects in multiple miRNAs would result in dysregulation of osteoclastogenesis.

We observed that Dicer deficiency suppresses expression of miR-155 in BMMs upon the treatment with RANKL. miR-155 expression is induced in macrophages and is enhanced by inflammatory stimuli [O'Connell et al., 2007]. We observed that RANKL treatment interacts with Dicer deficiency to suppress the expression of miR-155. Therefore, miR-155 target genes, for example, SHIP that suppresses osteoclasts, may be up-regulated by the reduction of miR-155 to lead to the suppression of osteoclasts and increase in bone mass observed in this study.

Osteoclast-specific dicer deficiency also decreased bone formation activity in vivo. In contrast, the levels of in vitro mineralized nodule formation were similar regardless of the genotypes (data not shown). Decrease in in vivo function of osteoblasts would suggest a coupling of bone resorption and bone formation in vivo. Although identity of coupling factors is not fully clear, we observed that one of the possible molecules, *Efnb2*, was downregulated in cKO mice. Investigation of regulatory signals for coupling between osteoclasts and osteoblasts would clarify the effect of dicer deficiency in the cKO mice observed in our study.

Conditional KO mice showed smaller body size with shorter bone length than WT mice. Shorter bone length has been reported in several examples of mutant mice deficient in genes required for osteoclastogenesis, such as Atp6i [Li et al., 1999], Clcn7 [Kornak et al., 2001], TRAF6 [Lomaga et al., 1999], and TRAP [Hollberg et al.,

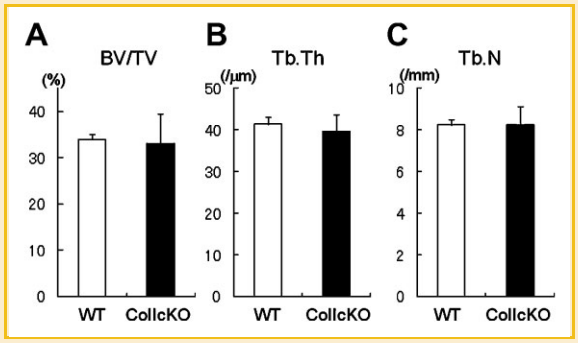


Fig. 8. Dicer deficiency in osteoblasts does not affect bone volume. Dicer floxed mice were crossed with 2.3 kb type I collagen promoter Cre mice. Dicer deficiency in osteoblasts did not change the levels of bone volume/tissue volume (BV/TV) (A), trabecular thickness (Tb.Th) (B) and trabecular number (Tb.N) (C). Wild-type (WT); mice with Dicer deficiency in osteoblasts (CollcKO).

2002]. This similarity may imply that developmental impairment of osteoclastogenesis would generally affect long-bone growth by as yet unidentified mechanisms. During skeletal development, dicer and miRNAs are reported to play critical roles in the regulation of chondrocyte proliferation and differentiation [Kobayashi et al., 2008]. Our results suggest that dicer and miRNAs may also play their roles in osteoclasts during endochondral bone development and longitudinal bone growth.

Recently, CD11b promoter-driven cre system was used to suppress Dicer and the miR-223 was implicated in osteoclast regulation [Sugatani and Hruska, 2007, 2009]. These mice showed mild osteopetrosis due to decreased bone resorption but bone formation was not altered in contrast to our observation that cathepsin K-Cre-induced Dicer cKO showed high bone mass with decrease in both osteoclasts and osteoblasts. The difference between their mice and ours was possibly due to diversity in the Cre systems. Furthermore, they reported that positive feedback loop of Pu.1, miR-223, NFI-A, and M-CSFR might regulate the osteoclast differentiation. Cathepsin-K Cre system does not influence macrophage but expresses cre in osteoclasts. Their hypothesis may not be applied to explain the bone phenotype of our cathepsin K-Cre-driven Dicer cKO mice. Multiple events may be involved in the regulatory network of miRNA target actions including both suppression and activation of osteoclastogenesis.

In conclusion, Dicer deficiency in osteoclasts suppresses osteoclast development and trans-suppresses osteoblasts. Thus, Dicer is involved at least in part in the regulation of osteoclasts to control themselves to contribute to the maintenance of adult bone mass.

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