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MicroRNA-17/20a inhibits glucocorticoid-induced osteoclast differentiation and function through targeting RANKL expression in osteoblast cells

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ABSTRACT

Glucocorticoids act on the osteoblasts to up-regulate the expression of RANKL, which is very important in the etiology of glucocorticoid-induced osteoclast differentiation and bone resorption. The mechanisms of this process are still not completely understood. Recent studies have shown that glucocorticoids mediate osteoblast function by decreasing the expression of microRNA-17–92a cluster. Coincidentally, we found that the microRNA-17/20a (microRNA-17, microRNA-20a) seed sequences were also complementary to a sequence conserved in the 3′-untranslated region of RANKL mRNA. Therefore, we hypothesized that glucocorticoids might promote osteoblast-derived RANKL expression by down-regulating microRNA-17/20a, which favors differentiation and function of the osteoclasts. In the present study, Western blot analysis showed that microRNA-17/20a markedly lowered the levels of RANKL protein and attenuated dexamethasone-induced RANKL expression in the osteoblasts. The post-transcriptional repression of RANKL by microRNA-17/20a was further confirmed by the luciferase reporter assay. Furthermore, we found that dexamethasone-induced osteoclast differentiation and function were significantly attenuated in co-culture with osteoblast over-expressed microRNA-17/20a and osteoclast progenitors. These results showed that microRNA-17/20a may play a significant role in glucocorticoid-induced osteoclast differentiation and function by targeting the RANKL expression in osteoblast cells.

Introduction

Glucocorticoids (GCs) are frequently prescribed for the treatment of inflammatory and immune disorders, such as rheumatoid arthritis (RA), asthma, Crohn's disease as well as in transplantation [1–3]. Although GCs are effective anti-inflammatory agents, prolonged GC administration often results in several adverse effects, one of the most severe of which is GC-induced osteoporosis (GIO) [4]. GIO is considered to be the most common cause of secondary osteoporosis and remains a challenging issue for physicians. The consequences of GIO are bone loss and fragility fractures, which occur in 30–50% of the patients receiving long-term GC treatment [5]. In fact, patients suffering from GIO experience vertebral fractures at a higher rate than those with primary osteoporosis [6]. Despite the frequency and severity of GIO, its pathogenesis still remains obscure.

It is well known that bone remodeling is a dynamic metabolic balance, which is maintained by tethering of the activities of osteoclasts and osteoblasts [7]. Osteoblasts, derived from pleiotropic mesenchymal stem cells, are essential for bone formation. Osteoclasts, derived from hematopoietic stem cells, are the sole bone-resorbing cells. Osteoclasts undergo differentiation and fusion resulting in large multinucleated cells in the presence of receptor activator of nuclear factor κB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF), both of them produced by stromal cells/osteoblasts. Furthermore, an in vivo study also demonstrated that RANKL produced by the osteoblasts contributes toward the development of osteoclasts [8]. Previous studies have shown that GIO could be caused by disrupting bone metabolic balance. The bone metabolic balance was disrupted due to action on osteoclasts to increase bone resorption in the initial phase of GC exposure and, further by acting on osteoblasts to reduce bone formation in the

Abbreviations: GCs, Glucocorticoids; RA, Rheumatoid arthritis; GIO, GC-induced osteoporosis; RANKL, Receptor activator of nuclear factor B ligand; M-CSF, Macrophage-colony stimulating factor; OPG, Osteoprotegerin; miRNAs, MicroRNAs; 3′-UTR, 3′-untranslational region; α-MEM, Alpha-minimal essential media; DMEM, Dulbecco's Modified Eagle Medium; Dex, Dexamethasone; AMOs, 2′-O-methyl antisense inhibitory oligonucleotides; NC, Negative control; RANK, Receptor activator of nuclear factor κB; TRAP, Tartrate-resistant acid phosphatase.

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later phase [9,10]. Previous investigations have also demonstrated that the induction of osteoblast apoptosis and inhibition of osteoblast proliferation and differentiation were involved in reduction of bone formation by GCs. Moreover, the enhanced osteoclastogenesis in GIO could be either due to the prolongation of the osteoclasts life span or because of action on the stromal cells/osteoblasts to up-regulate the RANKL expression and down-regulate the expression of its decoy receptor osteoprotegerin (OPG) [11,12]. Although large number of studies was conducted to explore the effect of GCs on osteoblasts and osteoclasts, the precise molecular events underlying the regulatory effect of GCs on these cells need to be further elucidated.

MicroRNAs (miRNAs) are single-stranded RNAs with 19–25 nucleotides in length that regulate several pathways including the development timing, organogenesis, cell apoptosis, proliferation and differentiation. The miRNAs bind to the 3′-untranslational region (3′-UTR) of target mRNAs and either block the translation or initiate the transcript degradation [13,14]. The miRNAs may also increase translation of selected mRNAs in a cell cycle-dependent manner [15]. Recent studies have demonstrated the importance of miRNAs in the control of osteoblast and osteoclast differentiation and function [16–19]. Furthermore, it has been confirmed that microRNA-29a could protect against GC-induced inhibition of bone mass homeostasis and improve osteoblast differentiation and mineral acquisition through regulation of the Wnt and Dkk-1 signaling pathways [20]. Notably, our previous study demonstrated that microRNA-17–92a cluster mediated the GC-induced osteoblast apoptosis through targeting Bim [21]. Coincidentally, computational and bioinformatics-based approach predicted that microRNA-17/20a (microRNA-17, microRNA-20a) seed sequences were also complementary to a sequence conserved in the 3′-UTR of RANKL mRNA. Therefore, we hypothesized that microRNA-17/20a might play a significant role in the up-regulation of osteoblast-derived RANKL expression by GCs, which favors differentiation and function of osteoclasts.

In this study, we identified that RANKL is a target gene of microRNA-17/20a. Furthermore, GCs could increase RANKL expression by down-regulation of microRNA-17/20a in osteoblasts, which indirectly enhances osteoclastogenesis and bone resorption. Therefore, miRNAs and miRNA-mediated gene silencing may contribute to GC-induced osteoclast differentiation and function by targeting RANKL expression in osteoblast cells.

Materials and methods

Cell and tissue culture

Osteoblastic cells were obtained from neonatal murine calvaria using the methods previously described [22]. MC3T3-E1 cell line was supplied by Shanghai Institute of Orthopaedics and Traumatology. Both cell types were cultured with alpha-minimal essential media (α-MEM) (Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin. HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM). The cultures were supplemented with 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin. Parietal bones from 7-day-old C57BL/6 mice were dissected and cut into four pieces. The bones were incubated for 72 h in α-MEM containing 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin.

Co-culture

The osteoblast cells were cultured with differentiation medium (α-MEM containing 10% FCS, 50 ug/ml ascorbic acid, 4 mmol/L beta-glycerophosphate) (Sigma-Aldrich, St. Louis, USA) for 24 h. The cells were then seeded at a density of 2.5×10^4 per well in 24-well plates in α-MEM medium. Osteoblasts cultured in 24-well plates were divided into different groups. Osteoblasts in the control group were cultured in normal culture medium. Dexamethasone (Dex) (Sigma-Aldrich, St. Louis, USA) group were treated with 100 nM Dex for 72 h. MicroRNA group were transfected with microRNA-17/20a or/and inhibitors for 48 h. MicroRNA together with Dex group cells were pretreated with 100 nM Dex for 24 h followed by transfection with microRNA-17/20a or/and inhibitors for 48 h under 100 nM Dex.

Two-month-old C57BL/6 mice were euthanized and bone marrow cells from each femur and tibia were flushed and seeded. Twenty-four hours later, the non-adherent cells were harvested and mononuclear cells were isolated by Ficoll density centrifugation (GE Healthcare, Uppsala, Sweden). Purified mononuclear cells were seeded at a density of 2.5×10^4 per well mixed with osteoblasts of different treatment groups. The co-cultured cells then were grown in α-MEM supplemented with 10 nM 1,2,5-dihydroxyvitamin D (Sigma-Aldrich, St. Louis, USA) and 1 μM Prostaglandin E2 (Sigma-Aldrich, St. Louis, USA) [23].

In vivo treatment of mice

All procedures involving animals were approved by the Shanghai Jiao Tong University School of Medicine Animal Study Committee and were carried out in accordance with the guide for the humane use and care of laboratory animals. Seven-day-old neonatal C57BL/6 mice were used for this study. According to the report from Gronowicz et al. [24], stock solutions of 1 mg/ml Dex were prepared in ethanol. Dosing solutions were prepared by diluting the stock solution with normal saline. After weight measurement, mice were given daily sc injections of Dex (1.0 mg/kg BW). At 72 h, mice were weighed and euthanized. The entire calvarium was removed for real-time PCR and Western blot analysis.

Western blot analysis

The protein samples were extracted from osteoblasts, with the procedures essentially the same as described in detail elsewhere [25,26]. Protein samples (~50 μg) were fractionated by SDS-PAGE (7.5–10% polyacrylamide gels). Separated proteins were blot transferred onto a nitrocellulose membrane. After blocking with 0.1% Tween 20 and 5% non-fat dry milk in Tris-buffered saline at room temperature for 1 h, the membrane was incubated overnight at 4 °C in one of the following primary antibodies: RANKL (Peprotech, Rocky Hill, NJ) (1:400) and β-actin (Santa Cruz, CA, USA) (1:1000) as an internal control. The membrane was incubated with horseradish peroxidase-conjugated secondary antibody (1:2000) for 1 h and detected using the Enhanced Chemiluminescence (ECL) Western blot System (Amersham Biosciences).

Synthesis of miRNAs and sequences of microRNA-17/20a inhibitors

MicroRNA-17 and microRNA-20a were synthesized by Integrated DNA Technologies (IDT). The sequences of microRNA-17 and microRNA-20a inhibitors (AMOs; 2′-O-methyl antisense inhibitory oligonucleotides) used are as follows: anti-microRNA-17: 5′-CUACCUGCA CUGUAAGCACUUUG-3′, anti- microRNA-20a: 5′-CUACCUGCAC UAUAAGCACUUUA-3′. Negative control (NC) used is as follows: sense: 5′-UUCUCCGAACGUGUCACGUTT-3′, antisense: 5′-ACGUGACACGUUC GGAGAATT-3′. DNA fragments of the 3′-UTRs of RANKL mRNA containing the putative microRNA-17 and microRNA-20a binding sequence were synthesized by Invitrogen. These fragments were then respectively cloned into the multiple cloning sites downstream the luciferase gene (HindIII and SpeI sites) in the pMIR-REPORT™ luciferase miRNA expression reporter vector (Ambion Inc., Austin, USA), as described elsewhere [26].

Transfection of miRNAs and luciferase assay

After 24 h of starvation in serum-free medium, HEK293 cells (1×10^5 per well) were transfected with 1 μg microRNA-17/20a or 1 μg PGL3-target DNA (firefly luciferase vector) and 0.1 μg PRL-TK (TK-driven Renilla luciferase expression vector), with Lipofectamine

2000 (Invitrogen, Carlsbad, CA), as referring to the manufacturer's instructions. Luciferase activities were measured 48 h after transfection with a dual luciferase reporter assay kit (Promega Corporation, Madison, USA) on a luminometer (Lumat LB9507).

Quantification of miRNA levels

The mirVanaTM qRT-PCR miRNA Detection Kit (Ambion Inc, Austin, USA) was used in conjunction with real-time PCR with SYBR Green I for quantification of microRNA-17 and microRNA-20a transcript, as detailed elsewhere [25,26].

TRAP staining

Tartrate-resistant acid phosphatase (TRAP) staining was used as a marker for mature osteoclasts. Cells were fixed and stained for TRAP activity using a Leukocyte acid phosphatase kit (Sigma-Aldrich, St. Louis, USA). Pre-osteoclasts and mature multinucleated osteoclasts (more than three nuclei) appeared as dark red cells, the number of which were counted by light microscopy, the experiment was performed in duplicate on three independent occasions. Ten fields were counted for each group.

Pit formation assay

Bone resorption activity was assessed by pit formation assay performed according to the method reported by Feng et al. [27] with slight modification. Purified mononuclear cells and osteoblasts were co-cultured on bovine cortical bone slices in 24-well plates. After 6 days the slices were placed for 10 min in 1 M NH_4OH and were sonicated to remove the cells. The cell-free slices were stained in 1% toluidine blue and 1% sodium borate for 1 min. The experiment was repeated three times. The resorption pits appeared dark blue and were viewed by light microscopy. The percentage of pit area to a "random field of view" was counted.

Statistics

Where indicated, experimental data are reported as mean ± SD of triplicate independent samples. Statistical analysis was performed

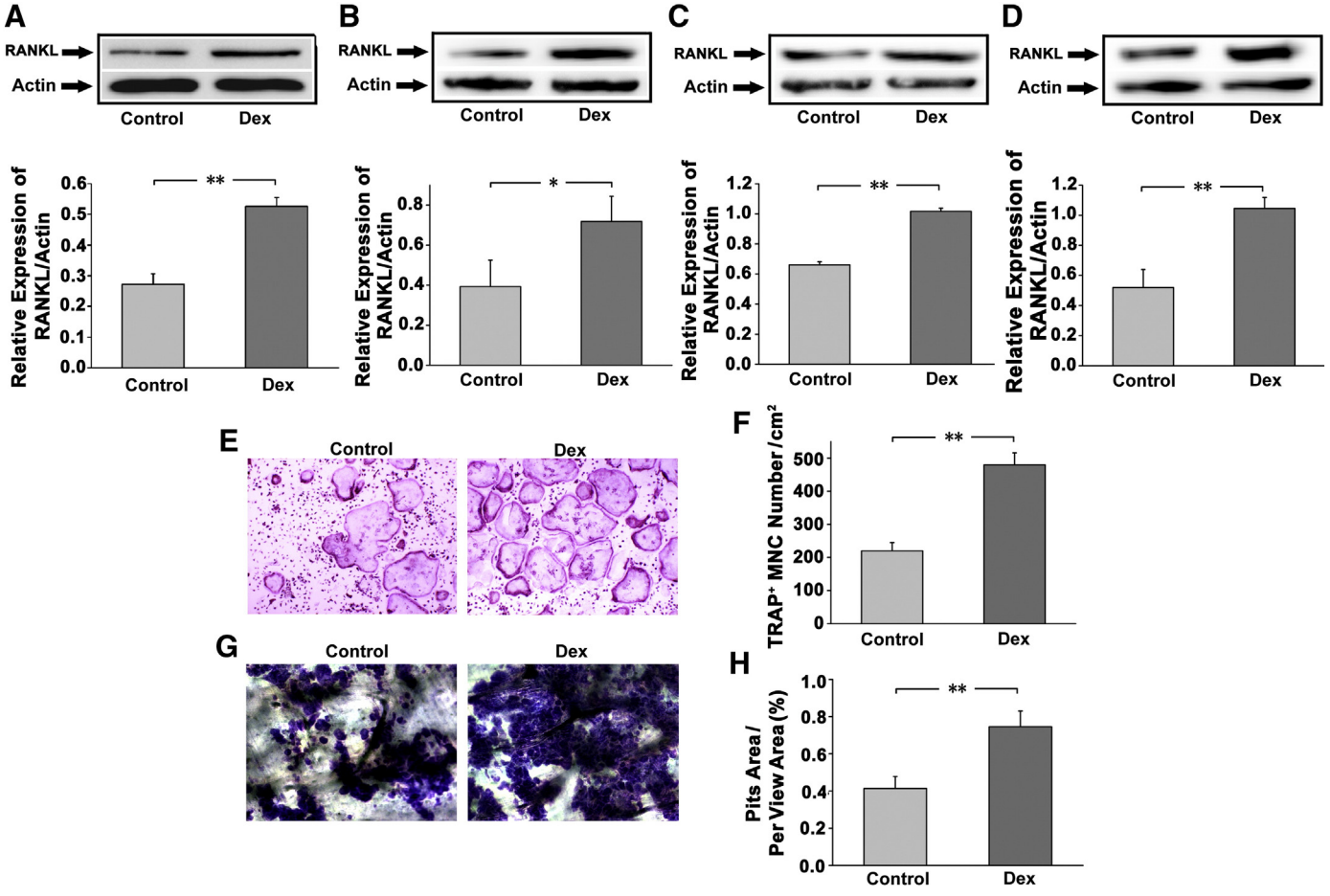

Fig. 1. Dex stimulates osteoblast-derived RANKL expression, leading to an increase in osteoclastogenesis and bone resorption. A: The level of RANKL protein in the calvarias from mice treated with Dex for 3 days was analyzed by Western blot. $n = 3$, $^{**}P < 0.01$. B: The level of RANKL protein in mouse calvarias treated with 100 nM Dex for 72 h in vitro was analyzed by Western blot. $n = 3$, $^{*}P < 0.05$. C: The level of RANKL protein in MC3T3-E1 cells treated with 100 nM Dex for 72 h was analyzed by Western blot. $n = 3$, $^{**}P < 0.01$. D: The level of RANKL protein in osteoblasts from mouse calvariae treated with 100 nM Dex for 72 h was analyzed by Western blot. $n = 3$, $^{**}P < 0.01$. E. TRAP staining in co-culture of osteoclast progenitors and osteoblasts treated with Dex for 72 h. F: Summarized data showed that the number of $TRAP^{+}$ multinuclear osteoclasts was significantly increased in co-culture of osteoclast progenitors and osteoblasts treated with Dex for 72 h. $n = 3$, $^{**}P < 0.01$. G: Resorption pit formation in co-culture of osteoclast progenitors and osteoblasts treated with Dex for 72 h. H: Summarized data showed that a significantly increased pit formation was observed in co-culture of osteoclast progenitors with Dex-induced osteoblasts compared with that in the control group. $n = 3$, $^{**}P < 0.01$. Dex indicates dexamethasone.

with one-way ANOVA followed by Dunnett's test where appropriate. Differences were considered to be statistically significant at $P \leq 0.05$.

Results

Dex stimulates osteoblast-derived RANKL expression, leading to an increase in osteoclastogenesis and bone resorption

The regulation of RANKL expression by Dex in osteoblasts was studied in both in vivo and in vitro models. Initially, Western blot analysis of RANKL expression was performed in the calvarias sample from the mice treated with Dex for 3 days. We observed that Dex caused a significant increase of RANKL protein expression (Fig. 1A). In agreement with this result, treatment of mouse calvariae with 100 nM Dex for 72 h in vitro resulted in an increased expression of RANKL (Fig. 1B). In addition, Dex also stimulated the expression of RANKL in MC3T3-E1 cells and mouse calvarial osteoblasts (Figs. 1C, D).

To examine the effect of Dex-induced osteoblast-derived RANKL expression on osteoclasteogenesis, we assessed the osteoclast differentiation, in the co-culture of osteoblasts with osteoclasts, using TRAP staining. We observed that the number of $TRAP^+$ multinuclear osteoclasts was significantly increased in the co-culture of osteoclast progenitors and osteoblasts pre-treated with Dex for 72 h (Figs. 1E, F). Furthermore, to examine the potential function of Dex-induced osteoblast-derived RANKL expression in bone resorption, we then assessed resorption pit formation by performing co-culture of osteoclast progenitors with osteoblasts pre-treated with Dex, on bovine cortical bone slices. As shown in (Figs. 1G, H), we found a significantly increased pit formation in the co-culture of osteoclast progenitors with osteoblasts pre-treated with Dex compared with those in the control group. Taken together, these results strongly indicate that the up-regulation of RANKL expression by Dex in osteoblasts is an essential regulator of osteoclast differentiation and function.

Involvement of microRNA-17/20a in Dex-induced osteoblast-derived RANKL expression

We have previously demonstrated that microRNA-17–92a could mediate GC-induced osteoblast apoptosis through targeting Bim. Coincidentally, computational and bioinformatics-based approach including *miRanda*, *TargetScan*, and *PicTar* predicted that microRNA-17/20a seed sequences were also complementary to a sequence conserved in the 3′-UTR of RANKL mRNA (Figs. 2A, B). Thus, we hypothesized that microRNA-17/20a might be involved in the process of Dex-induced osteoblast-derived RANKL expression. First, we examined the effect of Dex on the expression of microRNA-17/20a in vivo. We observed that Dex caused a significant repression of microRNA-17/20a expression in the calvarias from mice treated with Dex for 3 days (Fig. 2C). Furthermore, treatment of mouse calvariae in vitro with 100 nM Dex for 72 h resulted in decreased expression of microRNA-17/20a (Fig. 2D). In addition, Dex also repressed microRNA-17/20a expression in the osteoblast cells line, MC3T3-E1 and mouse calvarial osteoblasts (Figs. 2E, F).

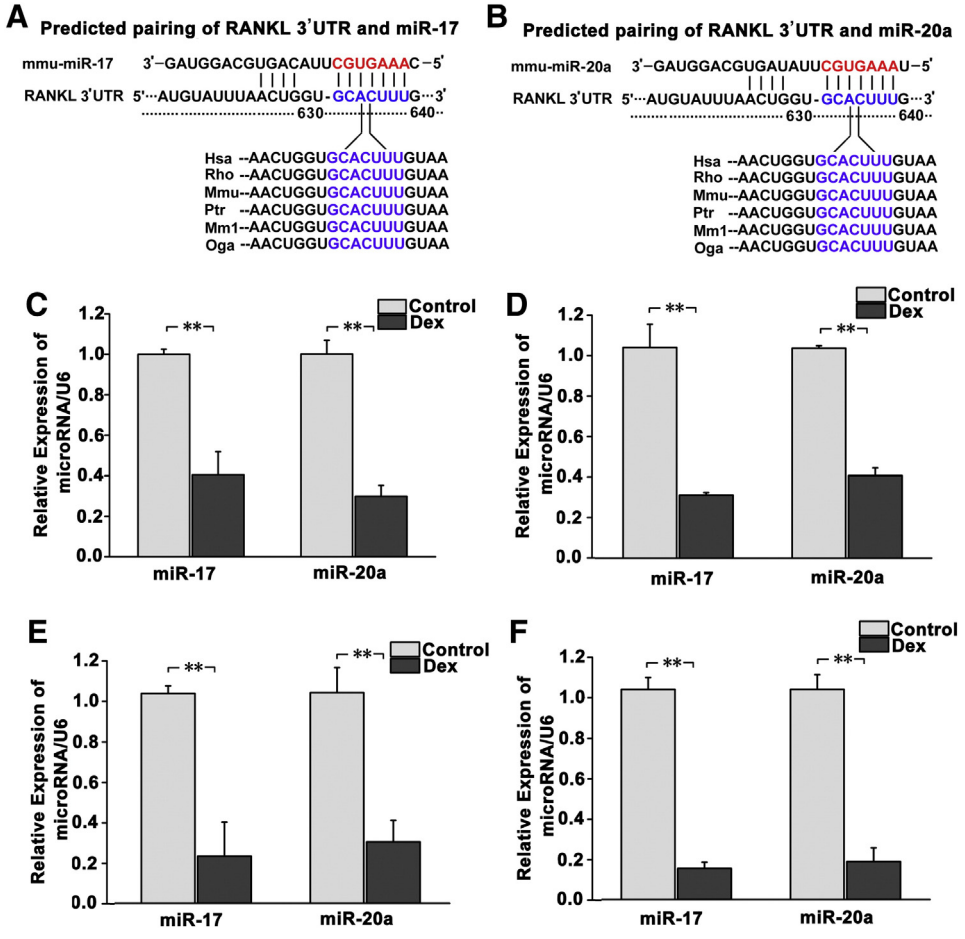

Fig. 2. Downregulation of microRNA-17/20a expression by Dex in osteoblasts. A and B: The sequences showing the unique sites of miRNA::mRNA complementarity between microRNA-17/20a and RANKL. C: Real-time PCR analysis of microRNA-17, microRNA-20a expression in the calvarias from mice treated with Dex for 3 days. $n = 3$, $^{**}P < 0.01$. D: Real-time PCR analysis of microRNA-17, microRNA-20a expression in mouse calvariae treated with 100 nM Dex for 72 h in vitro. $n = 3$, $^{**}P < 0.01$. E: Real-time PCR analysis of microRNA-17, microRNA-20a expression in MC3T3-E1 cells treated with 100 nM Dex for 72 h in vitro. $n = 3$, $^{**}P < 0.01$. F: Real-time PCR analysis of microRNA-17, microRNA-20a expression in osteoblasts from mouse calvariae treated with 100 nM Dex for 72 h in vitro. $n = 3$, $^{**}P < 0.01$. Dex indicates dexamethasone; miR indicates microRNA; 3′UTR, 3′- untranslational region.

To verify the interactions between microRNA-17/20a and RANKL, we placed the 3′-UTRs of RANKL into the 3′-UTR of a luciferase reporter plasmid to construct the chimeric vectors. Transfection of HEK293 cells by the chimeric constructs with microRNA-17/20a resulted in a smaller luciferase activity relative to transfection of the chimeric plasmid alone, suggesting that RANKL was the target gene of microRNA-17/20a (Fig. 3A). To further validate that the RANKL were indeed repressed post-transcriptionally by microRNA-17/20a, we measured the effect of microRNA-17/20a on RANKL protein expression. Western blot analysis showed that microRNA-17/20a markedly lowered the levels of RANKL proteins in MC3T3-E1 cells and mouse calvariae osteoblasts. To further verify the effect of microRNA-17/20a, we performed reciprocal experiments wherein we transfected osteoblasts with AMOs against microRNA-17/20a (AMO-17/20a). Co-application of microRNA-17/20a and AMO-17/20a almost completely abolished the effect of microRNA-17/20a. Moreover, application of the AMO-17/20a alone increased the levels of RANKL in MC3T3-E1 cells and mouse calvariae osteoblasts, indicating that there is a basal level of microRNA-17/20a activity in osteoblasts (Figs. 3B, C).

We consequently investigated whether microRNA-17/20a were involved in Dex-induced RANKL expression in osteoblasts. To this end, we observed the effect of Dex on RANKL expression in osteoblasts transfected with microRNA-17/20a or/and AMO-17/20a. Western blot analysis showed that microRNA-17/20a markedly lowered Dex-induced RANKL expression in MC3T3-E1 cells and mouse calvariae osteoblasts. Co-application of microRNA-17/20a and AMO-17/20a almost completely abolished the effect of microRNA-17/20a. Moreover, application of the AMO-17/20a alone increased the levels of RANKL in MC3T3-E1 cells and mouse calvariae osteoblasts treated with Dex, indicating that microRNA-17/20a are involved in Dex-induced RANKL expression in osteoblasts (Figs. 3D, E).

MicroRNA-17/20a inhibit Dex-induced osteoclastogenesis and bone resorption through targeting RANKL expression in osteoblast cells

We examined whether microRNA-17/20a targeting RANKL expression in osteoblast cells was involved in regulation of osteoclast differentiation and function. Our results showed that the number of $TRAP^{+}$ multinuclear osteoclasts was significantly decreased from co-culture of osteoclast progenitors with osteoblasts pre-transfected with microRNA-17/20a for 48 h. The depletion of microRNA-17/20a with AMO-17/20a in the osteoblasts resulted in an increased osteoclast differentiation in the osteoblast/osteoclast co-culture system. In contrast, AMO-17/20a facilitated osteoblast-induced osteoclastogenesis (Figs. 4A, B). Furthermore, to examine the potential function of microRNA-17/20a in regulation of osteoclastic bone resorption by osteoblasts, we then

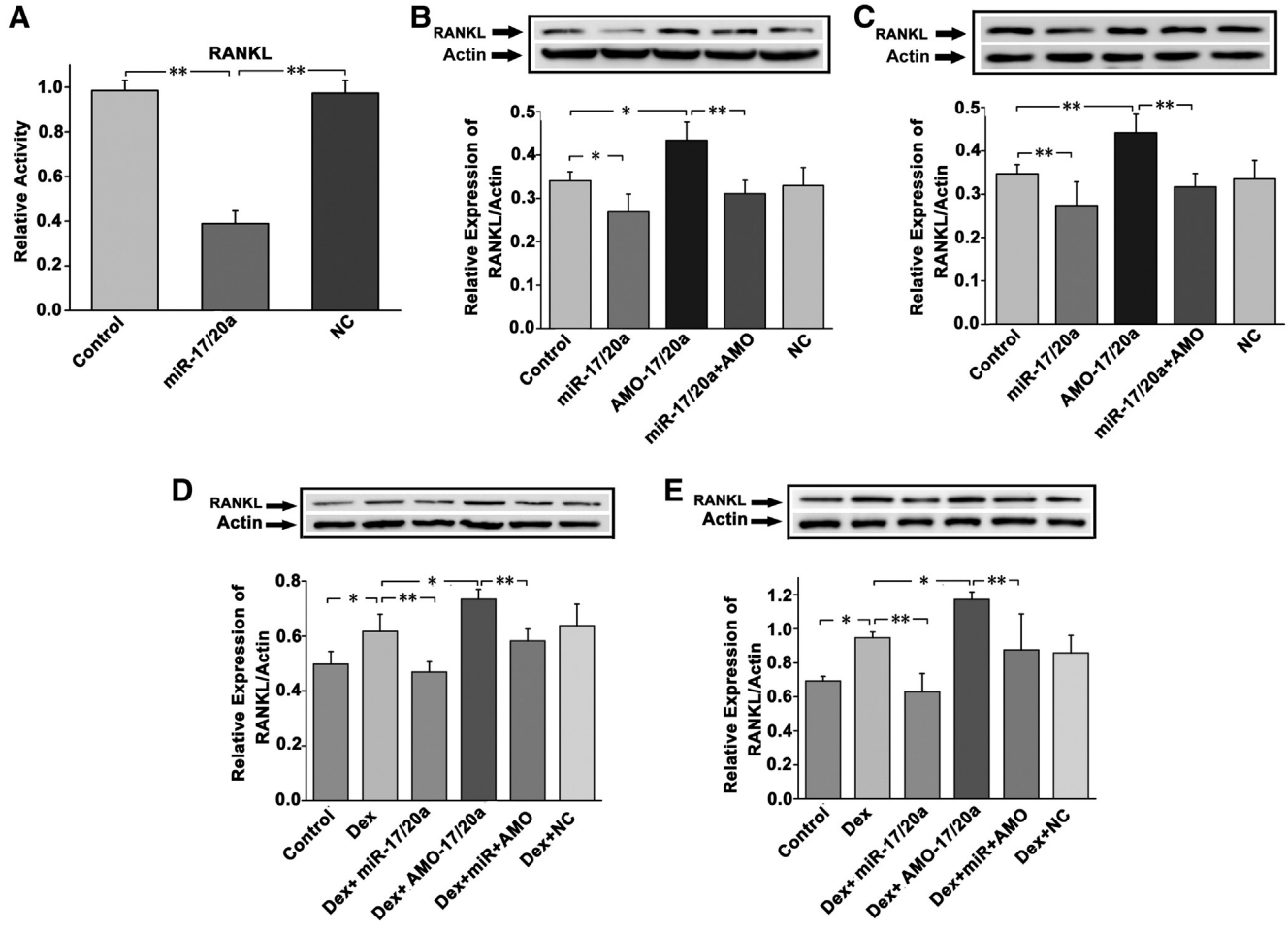

Fig. 3. The effect of microRNA-17/20a on RANKL in osteoblasts. A: Verification of RANKL as cognate targets of microRNA-17/20a. Data on luciferase reporter activities show the interaction between microRNA-17/20a and RANKL 3′-UTRs. $n = 3$, $^{**}P < 0.01$. B: Western blot analysis of RANKL expression in MC3T3-E1 cells transfected with microRNA-17/20a mimics or/and blocker. $n = 3$, $^{**}P < 0.01$, $^{*}P < 0.05$. C: Western blot analysis of RANKL expression in osteoblasts transfected with microRNA-17/20a mimics or/and blocker. $n = 3$, $^{**}P < 0.01$. D: The effect of Dex on RANKL expression in MC3T3-E1 cells transfected with microRNA-17/20a mimics or/and blocker was observed by Western blot. $n = 3$, $^{**}P < 0.01$, $^{*}P < 0.05$. E: The effect of Dex on RANKL expression in osteoblasts transfected with microRNA-17/20a mimics or/and blocker was observed by Western blot. $n = 3$, $^{**}P < 0.01$, $^{*}P < 0.05$. Dex indicates dexamethasone; miR indicates microRNA; NC, negative control; AMO, 2′-O-methyl antisense inhibitory oligonucleotides.

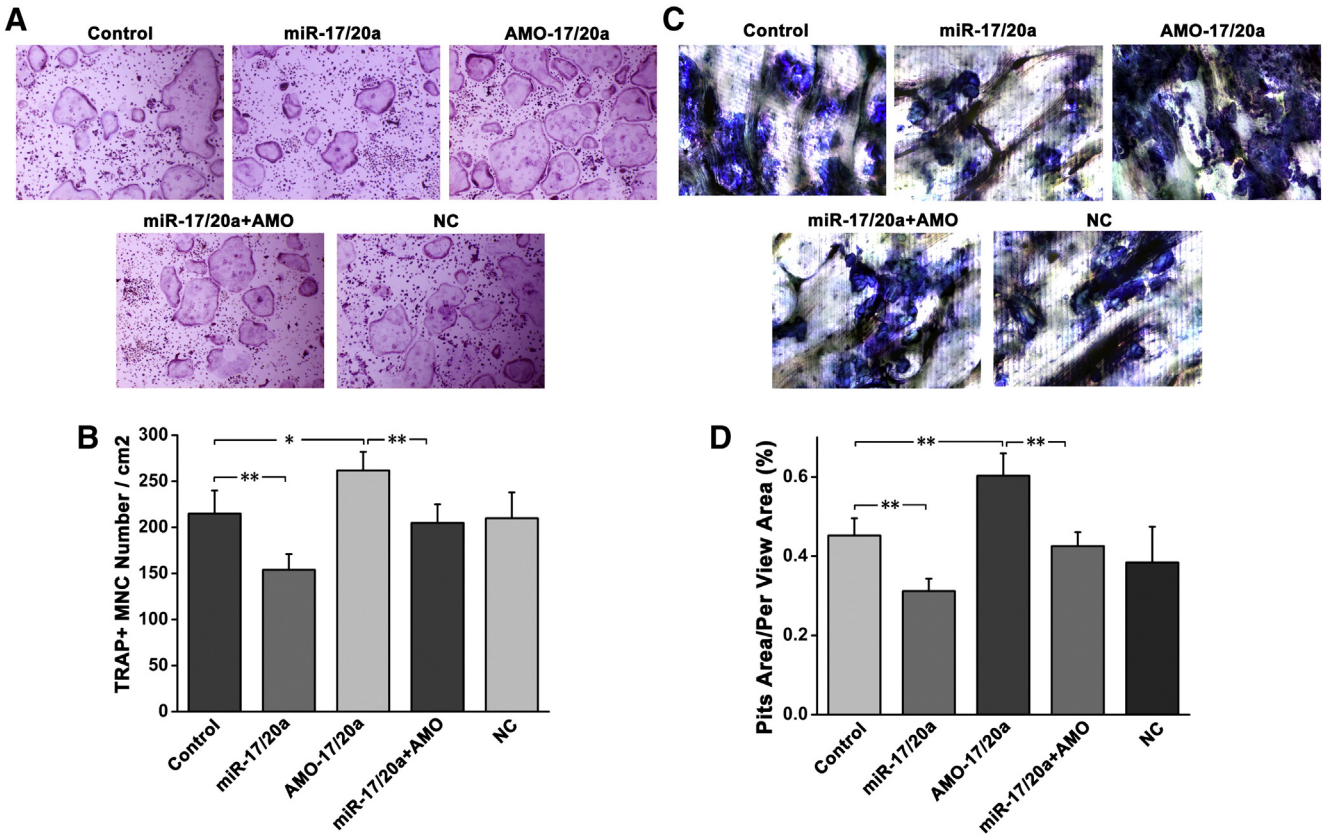

Fig. 4. MicroRNA-17/20a targeting RANKL expression in osteoblast cells is involved in the regulation of osteoclast differentiation and function. A: TRAP staining in co-culture of osteoclast progenitors and osteoblasts transfected with microRNA-17/20a mimics and blocker. B: Summarized data showed that over-expression of microRNA-17/20a significantly decreased osteoblast-induced osteoclast differentiation. However, microRNA-17/20a depletion significantly increased the ability of osteoblast-induced osteoclastogenesis. $n = 3$, $^{**}P < 0.01$, $^{*}P < 0.05$. C: Resorption pit formation in co-culture of osteoclast progenitors and osteoblasts transfected with microRNA-17/20a mimics or/and blocker. D: Summarized data showed that over-expression of microRNA-17/20a significantly decreased osteoblast-induced osteoclast bone resorption. However, microRNA-17/20a depletion significantly increased the ability of osteoblast-induced osteoclast bone resorption. $n = 3$, $^{**}P < 0.01$. miR indicates microRNA; NC, negative control; AMO, 2′-O-methyl antisense inhibitory oligonucleotides.

assessed resorption pit formation by co-culture of osteoclast progenitors with osteoblasts over-expressing microRNA-17/20a, on bovine cortical bone slices. As shown in Figs. 4C, D, we found a significantly decreased pit formation in co-culture of osteoclast progenitors with osteoblasts over-expressing microRNA-17/20a compared with that in control group. However, AMO-17/20a facilitated osteoblast-induced osteoclastic bone resorption. These results strongly suggest that microRNA-17/20a is an essential regulator of osteoblast-induced osteoclast differentiation and function.

To further confirm that Dex-induced osteoclast differentiation and function were mediated by microRNA-17/20a targeting RANKL expression in osteoblast cells, we observed the number of $TRAP^{+}$ multinuclear osteoclasts and pit formation by co-culture of osteoclast progenitors with osteoblasts pre-transfected with microRNA-17/20a or/and AMO-17/20a under Dex. We observed that microRNA-17/20a significantly decreased Dex-induced osteoclastogenesis and pit formation in the co-culture of osteoclast progenitors with osteoblasts. However, AMO-17/20a facilitated Dex-induced osteoclast differentiation and bone resorption in the co-culture of osteoclast progenitors with osteoblasts (Figs. 5A–D). These results suggest that microRNA-17/20a is an essential regulator of Dex-induced osteoclast differentiation and function in osteoclast/osteoblast co-culture system.

Comparison of microRNA-17/20a expression levels under various conditions

Successful delivery of microRNA-17/20a, AMO-17/20a and negative control (NC) to the cells were further verified by comparing the microRNA-17/20a levels 48 h after transfection of the constructs in cultured MC3T3-E1 cells and mouse calvariae osteoblasts. Transfection resulted in approximately nine to eleven-fold increases in microRNA-17/20a levels (Figs. 6A, B). It is worth noting that the microRNA-17/20a levels were dynamic after transfection. Our data were collected at a specific time which was 48 h after transfection because the plateau level was reached then. These results proved the feasibility of all experiments.

Discussion

Increased concentrations of GCs can cause the development of Cushing's syndrome, with severe osteoporosis. Previous studies have revealed that GCs up-regulate the expression of RANKL and M-CSF by directly acting on the osteoblasts, which is essential for osteoclastogenesis [28]. This indirect action of GCs via osteoblast cells is one of the mechanisms for GC-induced osteoclastogenesis. However, the cellular and molecular mechanisms of GC-induced expression of RANKL in osteoblasts remain elusive. Here we demonstrated that GCs can increase RANKL expression through down-regulating microRNA-17/20a in osteoblasts, which enhances osteoclastogenesis and bone resorption.

The inhibition of osteoblastogenesis and the apoptosis of osteoblasts and osteocytes induced by GCs are considered to be the primary cause for bone loss [29,30]. However, the involvement of osteoclasts in the early phase of great bone loss has not been satisfactorily elucidated and is still controversial, as inhibitory and stimulatory effects of GCs on osteoclastic differentiation and bone-resorbing activity have been reported. Dempster et al. demonstrated that GCs caused a dose-dependent decrease in the amount of bone resorbed, which was accompanied by a parallel decrease in osteoclast number [31]. Kim et al. observed that GCs inhibited the proliferation of osteoclasts from bone

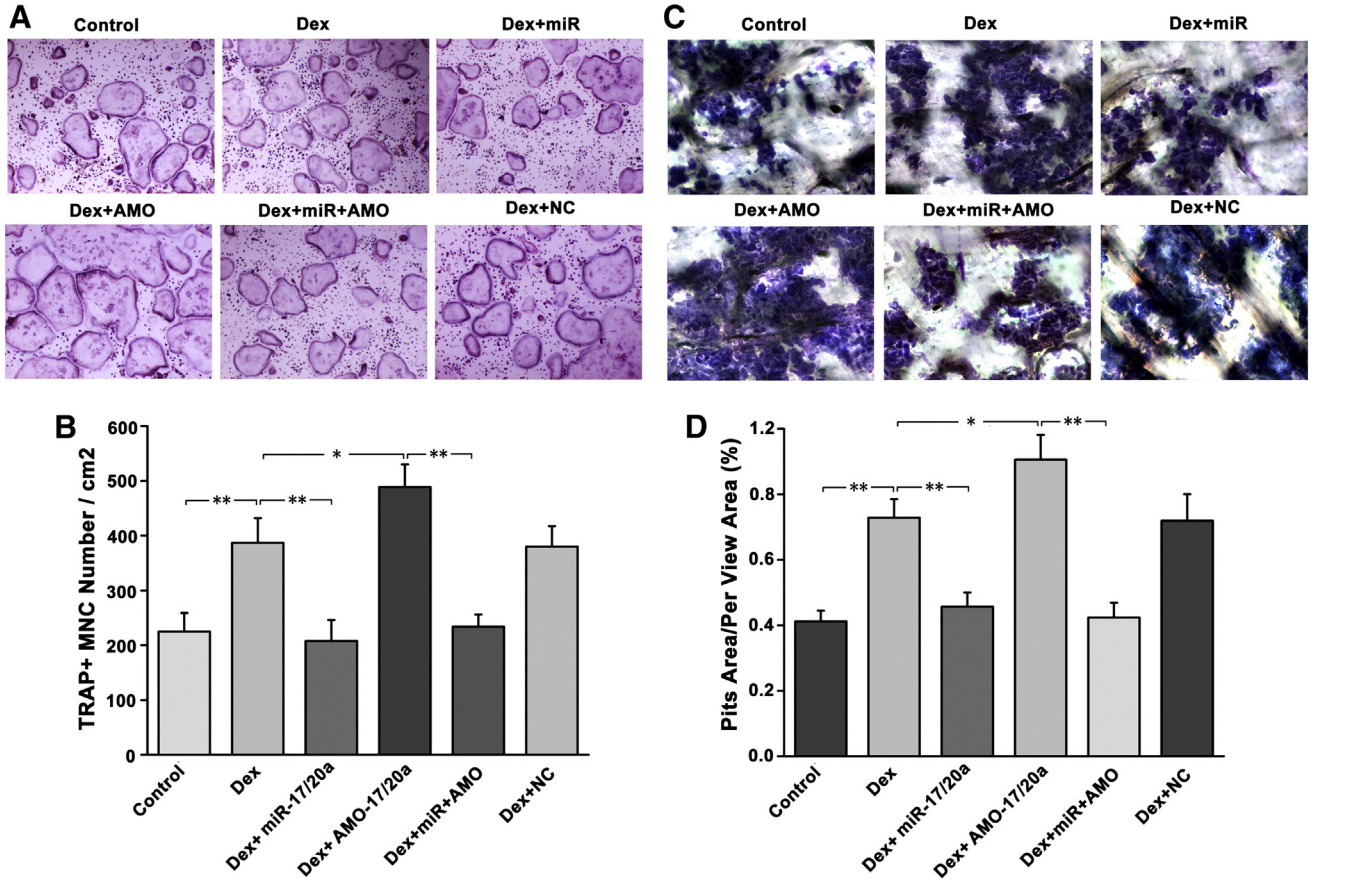

Fig. 5. The effect of microRNA-17/20a on osteoclastogenesis and bone resorption in co-culture of osteoclast progenitors with osteoblasts treated with Dex. A: TRAP staining in co-culture of osteoclast progenitors and osteoblasts transfected with microRNA-17/20a mimics or/and blocker under Dex treatment. B: Summarized data showed that over-expression of microRNA-17/20a significantly decreased in co-culture of osteoclast progenitors with osteoblasts treated with Dex. However, microRNA-17/20a depletion significantly increased the ability of osteoclastogenesis in co-culture of osteoclast progenitors with osteoblasts treated with Dex. $n = 3$, $^{**}P < 0.01$, $^{*}P < 0.05$. C: Resorption pit formation in co-culture of osteoclast progenitors and osteoblasts transfected with microRNA-17/20a mimics or/and blocker under Dex. D: Summarized data showed that over-expression of microRNA-17/20a significantly decreased osteoclast bone resorption in co-culture of osteoclast progenitors with osteoblasts treated with Dex. However, microRNA-17/20a depletion significantly increased the ability of osteoclast bone resorption in co-culture of osteoclast progenitors with osteoblasts treated with Dex. $n = 3$, $^{**}P < 0.01$, $^{*}P < 0.05$. Dex indicates dexamethasone; miR indicates microRNA; NC, negative control; AMO, 2′-O-methyl antisense inhibitory oligonucleotides.

marrow macrophages in a dose-dependent manner in vitro [32]. In addition, higher GC doses had no effect on osteoclast maturation but inhibited the ability of osteoclasts to reorganize their cytoskeleton. However, some studies also showed that Dex can stimulate osteoclast-like cells formation. Kaji et al. demonstrated that Dex stimulated osteoclast-like cells formation in the stromal cells-containing mouse bone cell cultures in a concentration-dependent manner [33]. Takuma et al. confirmed that Dex at low concentrations enhanced, but at high concentrations depressed, RANKL-induced osteoclast formation synergistically with TGF-β by acting at the early stage of osteoclast differentiation when bone marrow-derived osteoclast progenitors are primed toward osteoclasts [28]. Based on above results, we speculate that Dex at different concentrations and under varying culturing systems may affect osteoclastogenesis and bone resorption. In our study, we observed that osteoblasts treated with Dex could enhance osteoclastogenesis and bone resorption. Our results were consistent with those in previous studies. Many in vitro studies using cultures of bone marrow cells, in which osteoclast differentiation-supporting stromal/osteoblastic cells were present, showed the enhancement of osteoclast formation by GCs in the presence of osteotropic factors such as 1,2,5-dihydroxyvitamin D3 [30,34]. In our study, however, Dex-induced osteoclastogenesis and bone resorption through osteoblasts was examined through direct observation.

Several microRNAs have been found to be involved in GC-induced bone loss. More recently, Wang et al. demonstrated that microRNA-29a signaling protected against GC-induced disturbance of Wnt and Dkk-1 actions and improved osteoblasts differentiation and mineral acquisition [20]. Our previous studies indicate that the downregulation of microRNA-17 ~ 92a expression by Dex led to the Bim targeting and inhibition of osteoblast apoptosis [21]. In the current study, we confirmed that Dex acts on osteoblasts to up-regulate the expression of RANKL by decreasing microRNA-17/20a, which indirectly affects osteoclast differentiation and bone resorption. Although, these studies demonstrated that microRNAs might play a key role in the regulation of bone metabolism by Dex, there have been no reports to observe the effect of microRNAs on Dex directly mediated osteoclastogenesis and bone resorption. Future studies are expected to identify the role of microRNAs in the direct regulation of osteoclastogenesis and bone resorption by Dex. Furthermore, in our study, we did not explore how Dex regulated microRNA-17/20a expression in osteoblasts. Further studies are also required to uncover the progress of Dex regulation on microRNA-17/20a in osteoblasts.

In mammals, RANKL is expressed by osteoblasts and stromal cells and is known as the major osteoclast differentiation factor. It promotes osteoclastogenesis and maintains bone homeostasis through binding to receptor activator of nuclear factor κB (RANK), which is modulated by osteoprotegerin (OPG) [35]. Many studies recently demonstrated that microRNAs are involved in RANKL-induced osteoclastogenesis. Mizoguchi et al. showed that microRNA-31 is identified as one of the highly up-regulated miRNAs during osteoclast development under RANKL stimulation. Inhibition of microRNA-31 by specific antagomirs suppressed the RANKL-induced formation of osteoclast and bone resorption [36]. Cheng

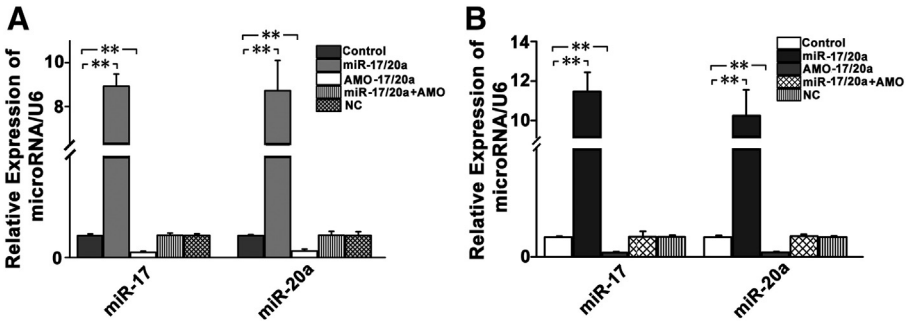

Fig. 6. Comparison of microRNA-17/20a expression levels under various conditions. A: MicroRNA-17/20a levels in MC3T3-E1 cells with transfection of sequence determined by real-time PCR. $n = 3$, $^{**}P < 0.01$. B: MicroRNA-17/20a levels in osteoblasts with transfection of sequence determined by real-time PCR. $n = 3$, $^{**}P < 0.01$. miR indicates microRNA; NC, negative control; AMO, 2′-O-methyl antisense inhibitory oligonucleotides.

et al. found that microRNA-148a was dramatically up-regulated during osteoclastic differentiation of circulating CD14 + peripheral blood mononuclear cells induced by RANKL [37]. Furthermore, microRNA-378, microRNA-223 and microRNA-21 may play an important role in RANKL-induced osteoclastogenesis [38]. However, to our knowledge, our study is the first to present the microRNAs targeting RANKL in osteoclastogenesis.

Two types of osteoblast cells, MC3T3-E1 cells and mouse calvarial osteoblasts, were used in this study. However, only osteoblasts from mouse calvarial were used in co-culture with osteoclast progenitors. In our study, we found that treatment of MC3T3-E1 cells with Dex greatly up-regulated the expression of RANKL protein, as determined by Western Blot analysis. However, treatment with Dex in MC3T3-E1 cells did not significantly stimulate osteoclastogenesis and bone resorption. We speculated that although the expression of RANKL protein in MC3T3-E1 cells treated with Dex was greatly up-regulated, it was not enough to significantly induce osteoclastogenesis and bone resorption. Similar results were also observed in previous reports. Touru et al. found that the expression of RANKL in MC3T3-E1 cells was very trivial and was detected only in the cells treated with Dex [39].

Conclusions

Our data provide new evidence that microRNA-17/20a play a dominant role in GC-mediated osteoblasts to induce osteoclastogenesis. The inhibitory effect of microRNA-17/20a on osteoclastogenesis and bone resorption can be attributable to the blocking of RANKL expression. This study is an effort to establish a molecular mechanism of GC-induced bone loss, and to provide insights into the potential contribution of miRNA in the regulation of osteoclast differentiation and bone resorption by GCs.

Author contributions

L.G. and C.G.S. were involved in the conception and hypothesis delineation; L.G. and C.G.S. designed the experiments, conducted the luciferase and quantitative real-time PCR experiments, and wrote the article; J.Q., H.B.Z. and H.K. performed TRAP staining and Pit formation assay; M.J., modified the manuscript; Q.Z. and P.H. performed a part of the luciferase, Western blot analysis; N.D.Q., Q.M.Y. and L.F.D. designed and conducted the animal studies.

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