Genetics and Molecular Research

MicroRNA-338-3p inhibits glucocorticoid-induced osteoclast formation through RANKL targeting

X.H. Zhang[1]*, G.L. Geng[2]*, B. Su[1], C.P. Liang[1], F. Wang[1] and J.C. Bao[3]

[1]Department of Physical Therapy,
Wuxi Tongren International Rehabilitation Hospital, Wuxi, Jiangsu, China
[2]School of Nursing, Nantong University, Nantong, Jiangsu, China
[3]Department of Rehabilitation,
Wuxi Tongren International Rehabilitation Hospital, Wuxi, Jiangsu, China

*These authors contributed equally to this study.
Corresponding author: B. Su
E-mail: larferi@126.com

Genet. Mol. Res. 15 (3): gmr.15037674
Received September 18, 2015
Accepted June 16, 2016
Published August 26, 2016
DOI http://dx.doi.org/10.4238/gmr.15037674

ABSTRACT. The differentiation deficiencies of osteoclast precursors (pre-OCs) may contribute to osteoporosis. Research on osteoporosis has recently focused on microRNAs (miRNAs) that play crucial roles in pre-OC differentiation. In the current study, we aimed to analyze the expression and function of the glucocorticoid (GC)-associated miRNA-338-3p (miR-338-3p) in osteoclast formation. We found that dexamethasone induced osteoclast differentiation and inhibited miR-338-3p expression. Overexpression of an miR-338-3p mimic in osteoclast precursor cells attenuated GC-induced osteoclast formation and bone resorption, whereas inhibition of miR-338-3p reversed these effects. The expression of the nuclear factor κB ligand RANKL, a

potential target gene of miR-338-3p, was inversely correlated with miR-338-3p expression in pre-OCs. Furthermore, we demonstrated that RANKL was directly regulated by miR-338-3p and re-introduction of RANKL reversed the inhibitory effects of miR-338-3p on osteoclast formation and bone resorption. Taken together, these findings demonstrate that miR-338-3p may play a significant role in GC-induced osteoclast differentiation and function by targeting RANKL in osteoclasts.

Key words: MicroRNA-338-3p; Glucocorticoid; Osteoclast formation; RANKL

INTRODUCTION

Glucocorticoids (GCs) are frequently prescribed anti-inflammatory and immunosuppressive agents (Schäcke et al., 2002). Long-term GC administration often results in several adverse effects, one of which is GC-induced osteoporosis (Capozzi et al., 2013). GC-induced osteoporosis is considered the most common cause of secondary osteoporosis, and occurs rapidly at a rate of 6-12% within the first year of therapy (Albaum et al., 2014). Despite the frequency and severity of GC-induced osteoporosis, there is little known about the management of this disease, and thus the precise molecular events underlying GC-induced osteoporosis need to be further elucidated.

Bone homeostasis is characterized by an intimate balance between bone-forming osteoblasts and bone-resorbing osteoclasts (Weinstein and Manolagas, 2000). In osteoporosis, the balance between osteoclast and osteoblast activities is disrupted. The dysregulation of bone resorbing osteoclasts significantly contributes to aging and drug-induced osteoporosis (Makras et al., 2015). MicroRNAs (miRNAs) are a class of endogenous, non-coding, single-stranded RNAs 19-24 nucleotides in length, which block translation or initiate transcript degradation via binding to the 3'-untranslated region (3'-UTR) of target mRNAs (Zamore and Haley, 2005). miRNAs have been implicated in various pathophysiological events including embryogenesis, organogenesis, differentiation, metabolism, proliferation, and apoptosis (Couzin, 2007). Recent research on osteoclasts has focused on miRNAs as they have been shown to play crucial roles in their differentiation and function, indicating that miRNAs are novel regulatory factors of osteoclastogenesis (Tang et al., 2014). Therefore, miRNAs may represent new therapeutic targets for the pharmacological control of bone diseases.

The aim of the current study was to investigate the association of miR-338-3p expression with osteoclast formation and bone resorption. Additionally, we investigated target genes of miR-338-3p, which may meditate osteoclast formation and bone resorption. Lastly, we assessed whether miR-338-3p was involved in GC-induced osteoclast formation and bone resorption.

MATERIAL AND METHODS

Cell culture

Murine osteoclast precursors (pre-OCs) were prepared from bone marrow cells as previously described (Youn et al., 2013). Briefly, femurs were aseptically removed from

female C57BL/6J mice aged 6-8 weeks, and bone marrow cells were flushed out with a sterile 21-gauge syringe. The cells were grown in α-minimum essential medium (α-MEM, Gibco; Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) with M-CSF (30 ng/mL) in a cell culture incubator at 37°C and 5% CO_2 for 3 days. The adherent cells were used as pre-OCs. For osteoclast differentiation of pre-OCs, cells were cultured with M-CSF (30 ng/mL; Sigma-Aldrich, St. Louis, MO, USA) and dexamethasone (Dex; Sigma-Aldrich) for 4 days.

RNA oligonucleotide and cell transfection

The miR-338-3p mimic or inhibitor and control miRNA mimic or inhibitor were purchased from Genepharma (Shanghai, China). When cell confluency reached 70%, the cells were transfected with the miRNA mimic/inhibitor (50 nM) using the Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. All of the assays were performed 2 days after transfection.

RNA extraction and miRNA expression assay

Total RNA was isolated using the miRNeasy Kit (Qiagen, Courtabœuf, France) according to manufacturer instructions. Total RNA (10 ng) was used for reverse transcription using the miScript Reverse Transcription Kit (Qiagen). The expression of miR-338-3p was quantified using the miScript Primer Assay and the miScript Universal Primer (Qiagen). Quantitative polymerase chain reaction (qPCR) was performed using the QuantiTect SYBR Green PCR Kit (Qiagen) on the Applied Biosystems 7500 Real-Time PCR System (ABI 7500, Life Technologies). The relative quantification of miR-338-3p was calculated using the $2^{-\Delta\Delta Ct}$ method. The data were normalized using the U6 small nuclear RNA as an internal control, and measured relative to a calibrator sample as the external control.

Tartrate-resistant acid phosphatase (TRAP) staining

TRAP staining was used as a marker for mature osteoclasts, and was performed using the Acid Phosphatase kit (Sigma-Aldrich) according to the manufacturer protocol. Briefly, cells were fixed by immersing them in fixative solution at room temperature for 30 s. Then, the cells were rinsed thoroughly in deionized water followed by incubation with TRAP-staining fluid at 37°C protected from light for 50 min. After removal of the TRAP solution, the cells were washed 3 times with distilled water. The TRAP-positive multinuclear cells were imaged and recorded using an inverted microscope (BX51; Olympus Corp., Tokyo, Japan).

Pit formation assay

Bone resorption activity was assessed by pit formation assay as previously described (Feng et al., 2009). Purified osteoblasts were co-cultured with bovine bone slices on 24-well plates. After 14 days, the slices were placed in 1 M NH_4OH for 10 min to remove adherent cells and then stained with 0.1% toluidine blue and 1% sodium borate for 1 min. The resorption pits appeared dark blue and were viewed by light microscopy. Pit area versus total bone area of each slice was measured using the Image Pro Plus software, version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Western blot assays

Cultured cells were lysed in extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and protease inhibitors). Protein fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then probed with primary antibodies against RANKL (Peprotech, Rocky Hill, NJ, USA) and GAPDH (Cell Signaling Technology, Boston, MA, USA), which was used as a loading control. The bands were visualized with the ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA).

Luciferase assays

The segment of the 3'-UTR of RANKL predicted to interact with miR-338-3p or a mutated sequence containing the potential binding region were synthesized by PCR and inserted via the *Bgl*II and *Hin*dIII restriction sites downstream of the luciferase open reading frame into the pMiR-Report reporter vector (Ambion Inc., Austin, TX, USA). The correct clones were confirmed by sequencing. Pre-OCs were seeded onto a 24-well plate (1 x 10^4 cells per well), and were co-transfected with a luciferase reporter construct (200 ng) and miRNA mimic/inhibitor (50 nM) using Lipofectamine 2000 according to manufacturer instructions. Luciferase activity was measured 2 days after transfection using the Dual-Luciferase Assay System (Promega, Madison, WI, USA). Luminescent signals were quantified by a luminometer (Glomax, Promega), and each value from the firefly luciferase construct was normalized to those of Renilla luciferase in the assay.

Statistical analysis

Results are reported as means ± SD, and all statistical analyses were performed using the SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA). The statistical significance between groups were analyzed using the Student *t*-test or one-way analysis of variance (ANOVA). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Dex stimulates osteoclast formation and inhibits miR-338-3p expression

To examine the effect of Dex on osteoclast formation, we assessed osteoclast differentiation in co-cultures with different concentrations of Dex for 4 days using TRAP staining. As shown in Figure 1A and B, the number of $TRAP^+$ multinucleated osteoclasts was significantly increased after Dex treatment, and this effect increased with increased Dex concentration. Furthermore, we observed that Dex caused a significant decrease in miR-338-3p expression in a dose-dependent manner (Figure 1C). These data suggest that Dex is an essential regulator of osteoclast differentiation and function.

miR-338-3p is essential for Dex-induced osteoclast formation

In order to elucidate the role of miR-338-3p in osteoclast differentiation, we treated

pre-OCs with miR-338-3p mimic or miR-338-3p inhibitor during the course of osteoclast differentiation. The results showed that cellular miR-338-3p levels were substantially up-regulated by miR-338-3p mimic treatment and markedly down-regulated by miR-338-3p inhibitor treatment (Figure 2A). Functionally, the number of $TRAP^+$ multinucleated osteoclasts per well was substantially reduced in the miR-338-3p mimic treatment group and markedly increased in the miR-338-3p inhibitor treatment group compared to that in the negative control group (Figure 2B and C). Additionally, the miR-338-3p mimic markedly blocked resorption pit formation, whereas the miR-338-3p inhibitor significantly increased pit formation (Figure 2B and D). Taken together, these results suggest that miR-338-3p could regulate Dex-induced osteoclast formation and bone resorption.

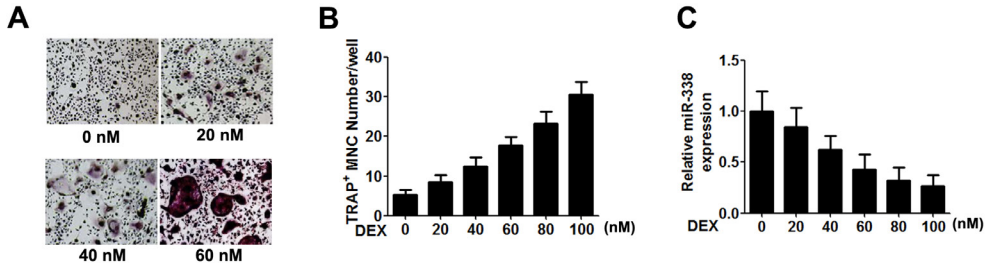

Figure 1. Dexamethasone (Dex) induces osteoclast formation and suppresses miR-338-3p expression. Pre-OCs were cultured for 4 days in the presence of different concentrations of Dex (0, 20, 40, 60, 80, 100 nM). **A.** Cultured cells fixed and stained for TRAP. **B.** Numbers of $TRAP^+$ MNCs counted. **C.** Mature miR-338-3p expression determined by qPCR. Data are reported as means ± SD. *P < 0.05 *vs* control group, N = 5.

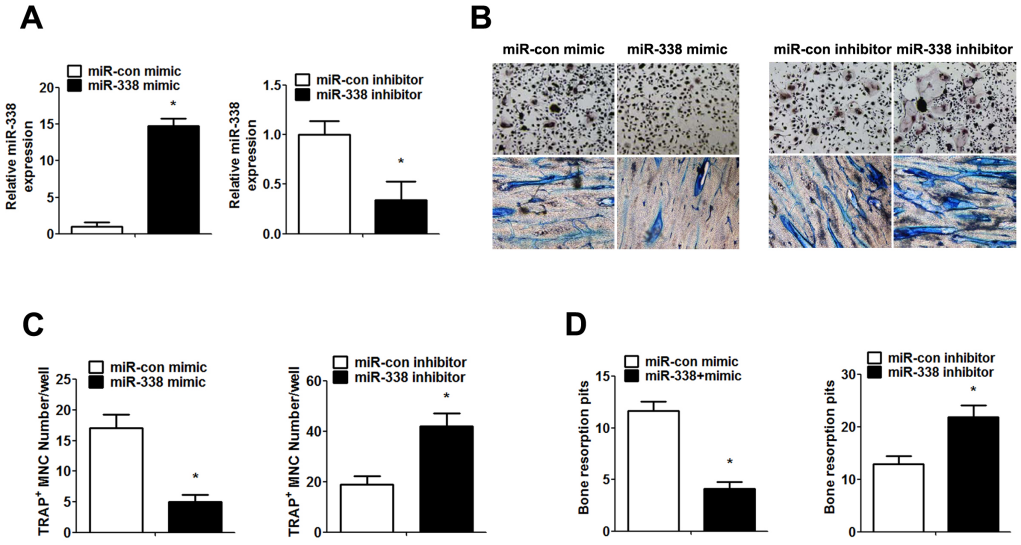

Figure 2. miR-338-3p inhibits Dex-induced osteoclast formation. Pre-OCs were cultured for 2 days in the presence of Dex (60 nM). Pre-OCs were transfected with the control miRNA (miR-con) or miR-338-3p mimic/inhibitor for 2 days. **A.** Expression level of miR-338-3p detected by qPCR. **B.** Representative TRAP staining and bone resorption pit images. **C.** Numbers of $TRAP^+$ MNCs and bone resorption pits counted. Data are reported as means ± SD. *P < 0.05 *vs* control group, N = 5.

miR-338-3p directly targets RANKL in osteoclasts

To identify candidate targets of miR-338-3p, we used bioinformatic tools (TargetScan and miRBase) for miRNA target prediction, by which the RANKL gene was identified as a potential target. Western blot analysis demonstrated that the introduction of miR-338-3p into pre-OCs down-regulated RANKL expression, whereas miR-338-3p inhibitor strongly induced the expression of RANKL in osteoclasts (Figure 3A). To determine whether miR-338-3p directly targets RANKL by binding to the 3'-UTR of RANKL in osteoclasts, we cloned wild-type and mutant RANKL 3'-UTR segments containing the binding site of miR-338-3p into a luciferase reporter gene system (Figure 3B). The results showed that overexpression of miR-338-3p led to significantly reduced luciferase activity for wild-type RANKL, whereas knockdown of miR-338-3p increased luciferase activity for wild-type RANKL. In contrast, the activity of the luciferase reporter gene linked to the 3'-UTR of mutant RANKL did not change in the presence of miR-338-3p mimic/inhibitor (Figure 3C and D).

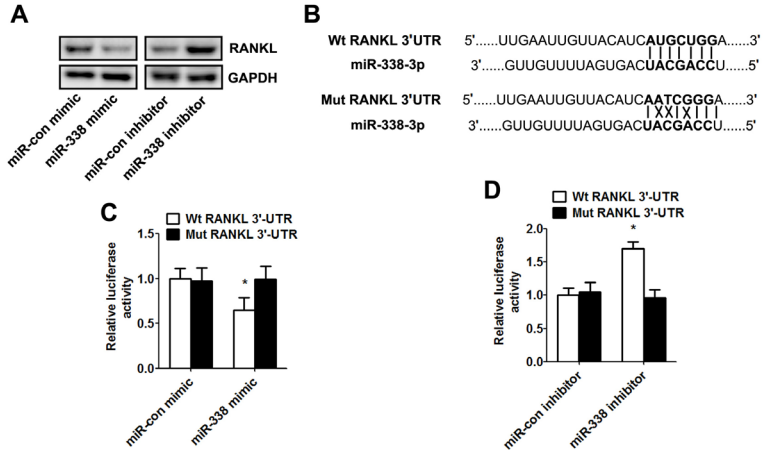

Figure 3. miR-338-3p negatively regulates RANKL expression in osteoclasts. Pre-OCs were transfected with control (miR-con) mimics or miR-338-3p for 2 days. **A.** RANKL protein level detected by western blot in osteoclasts. **B.** Alignment between RANKL and miR-338-3p, as predicted by bioinformatic analysis of sequences. The predicted binding sites between miR-338-3p and the 3'-UTR sequence of RANKL is shown (solid lines indicate matching base pairs and crosses represent non-matching base pairs). **C.** and **D.** Pre-OCs co-transfected with the luciferase reporter plasmid carrying the 3'-UTR sequences of wild-type RANKL or mutant RANKL and miR-338-3p mimic/inhibitor. The data are reported as means ± SD. *P < 0.05 *vs* control group, N = 5.

Re-introduction of RANKL averts the inhibitory effect of miR-338-3p on osteoclast formation and function

To investigate whether RANKL is a functional target of miR-338-3p in Dex-induced osteoclast differentiation, pre-OCs transfected with the miR-338-3p mimic were co-cultured with exogenous RANKL. Western blot analysis demonstrated that there was increased expression of RANKL protein after treatment (Figure 4A). Functionally, restoration of RANKL expression strongly blocked miR-338-3p-mediated down-regulation of osteoclast formation and bone resorption (Figure 4B-D). These results indicate that miR-338-3p regulates osteoclast formation and function through RANKL.

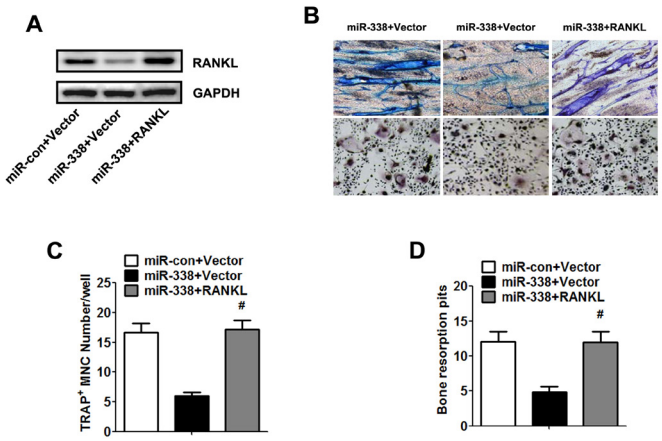

Figure 4. Inhibitory effect of miR-338-3p is rescued by RANKL. Pre-OCs were cultured for 2 days in the presence of Dex (60 nM). Pre-OCs were transfected with the control miRNA (miR-con) or miR-338-3p mimic/inhibitor for 2 days. Transfected cells were further cultured with RANKL (100 ng/mL). **A.** Expression of RANKL protein analyzed by western blot. **B.** Representative TRAP staining and bone resorption pit images. **C.** Numbers of $TRAP^+$ MNCs counted. **D.** Numbers of resorption pits quantified. The data are reported as means ± SD. $^{\#}P < 0.05$ *vs* miR-338-3p+Vector group, N = 5.

DISCUSSION

The identification and validation of novel biomarkers for osteoporosis is a high priority not only for identifying patients at high risk for fracture and monitoring the efficacy of anti-resorptive therapies, but also for defining novel therapeutic strategies (Makras et al., 2015). Despite great advances in the understanding of mechanisms in bone metabolism over the past few years, major limitations still exist in managing osteoporosis. Long-term GC therapy, the leading cause of secondary osteoporosis, leads to a rapid reduction in bone formation through inhibition of osteoblast differentiation and increased osteoclast activity (Tory et al., 2015). Since large numbers of clinical practice guidelines have been used to treat osteoporosis, very little is known about GC-induced osteoporosis management. Therefore, the precise molecular events underlying the regulatory effects of GCs on osteoblasts and osteoclasts need to be further elucidated.

Here, we demonstrated that GCs indeed boosted osteoclast formation and miR-338-3p expression. Overexpression of miR-338-3p inhibited GC-induced osteoclast formation and bone resorption, while inhibition of miR-338-3p expression reversed these effects. Previous studies showed that miR-338-3p expression is significantly down-regulated during the osteoblastic differentiation of bone marrow stromal cells, and that overexpression of miR-338-3p could inhibit osteoblast differentiation, indicating that miR-338-3p plays an important role during osteoblast differentiation and serves as a potential modulator of osteoporosis via its effect on osteoblasts (Liu et al., 2014; Guo et al., 2015). Our study first demonstrated that miR-338-3p acts as a regulator of osteoclast formation and exerts its biological function in bone resorption.

Additionally, we revealed that RANKL is a direct target gene for miR-338-3p, and we found that miR-338-3p negatively regulated RANKL expression by directly targeting the 3'-UTR of RANKL mRNA in osteoclasts. RANKL, a key cytokine for osteoclast differentiation, promotes osteoclast formation and maintains bone homeostasis through binding to the receptor

activator of nuclear factor κB (RANK) (Danks and Takayanagi, 2013). Furthermore, RANKL induces cell-cell fusion of $TRAP^+$ mononuclear pre-OCs to become multinuclear mature osteoclasts at the late stages of osteoporosis (Kim et al., 2015). A recent study confirmed that Dex acts on osteoblasts to up-regulate the expression of RANKL, which indirectly affects osteoclast differentiation and bone resorption (Shi et al., 2014). Furthermore, accumulating evidence demonstrates that miRNAs are involved in RANKL-induced osteoporosis (Tang et al., 2014). In the current study, we demonstrated that the inhibitory effect of miR-338-3p on osteoclast differentiation and bone resorption could be reversed by exogenous RANKL treatment, suggesting that miR-338-3p inhibited GC-induced osteoporosis by targeting RANKL expression.

Our data provide new evidence that miR-338-3p plays a dominant role in GC-mediated osteoclast formation to induce osteoporosis. The inhibitory effect of miR-338-3p on osteoclast differentiation and bone resorption can be attributable to the blocking of RANKL expression. In conclusion, we provide strong evidence to establish a molecular mechanism for GC-induced bone loss and insights into new therapeutic approaches for osteoporosis.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the Hospital Administration Center Foundation of Wuxi (#YGZXL1320).

REFERENCES

Albaum JM, Youn S, Levesque LE, Gershon AS, et al. (2014). Osteoporosis management among chronic glucocorticoid users: a systematic review. *J. Popul. Ther. Clin. Pharmacol.* 21: e486-e504.

Capozzi A, Casa SD, Altieri B and Pontecorvi A (2013). Chronic low-dose glucocorticoid inhalatory therapy as a cause of bone loss in a young man: case report. *Clin. Cases Miner. Bone Metab.* 10: 199-202.

Couzin J (2007). Genetics. Erasing microRNAs reveals their powerful punch. *Science* 316: 530. http://dx.doi.org/10.1126/science.316.5824.530

Danks L and Takayanagi H (2013). Immunology and bone. *J. Biochem.* 154: 29-39. http://dx.doi.org/10.1093/jb/mvt049

Feng S, Deng L, Chen W, Shao J, et al. (2009). Atp6v1c1 is an essential component of the osteoclast proton pump and in F-actin ring formation in osteoclasts. *Biochem. J.* 417: 195-203. http://dx.doi.org/10.1042/BJ20081073

Guo DW, Han YX, Cong L, Liang D, et al. (2015). Resveratrol prevents osteoporosis in ovariectomized rats by regulating microRNA-338-3p. *Mol. Med. Rep.* 12: 2098-2106.

Kim K, Kim JH, Kim I, Lee J, et al. (2015). MicroRNA-26a regulates RANKL-induced osteoclast formation. *Mol. Cells* 38: 75-80.

Liu H, Sun Q, Wan C, Li L, et al. (2014). MicroRNA-338-3p regulates osteogenic differentiation of mouse bone marrow stromal stem cells by targeting Runx2 and Fgfr2. *J. Cell. Physiol.* 229: 1494-1502. http://dx.doi.org/10.1002/jcp.24591

Makras P, Delaroudis S and Anastasilakis AD (2015). Novel therapies for osteoporosis. *Metabolism* 64: 1199-1214. http://dx.doi.org/10.1016/j.metabol.2015.07.011

Schäcke H, Döcke WD and Asadullah K (2002). Mechanisms involved in the side effects of glucocorticoids. *Pharmacol. Ther.* 96: 23-43. http://dx.doi.org/10.1016/S0163-7258(02)00297-8

Shi C, Qi J, Huang P, Jiang M, et al. (2014). MicroRNA-17/20a inhibits glucocorticoid-induced osteoclast differentiation and function through targeting RANKL expression in osteoblast cells. *Bone* 68: 67-75. http://dx.doi.org/10.1016/j.bone.2014.08.004

Tang P, Xiong Q, Ge W and Zhang L (2014). The role of microRNAs in osteoclasts and osteoporosis. *RNA Biol.* 11: 1355-1363. http://dx.doi.org/10.1080/15476286.2014.996462

Tory HO, Solomon DH and Desai SP (2015). Analysis of quality improvement efforts in preventing glucocorticoid-induced osteoporosis. *Semin. Arthritis Rheum.* 44: 483-488. http://dx.doi.org/10.1016/j.semarthrit.2014.09.011

Weinstein RS and Manolagas SC (2000). Apoptosis and osteoporosis. *Am. J. Med.* 108: 153-164. http://dx.doi.org/10.1016/S0002-9343(99)00420-9

Youn BU, Kim K, Kim JH, Lee J, et al. (2013). SLAT negatively regulates RANKL-induced osteoclast differentiation. *Mol. Cells* 36: 252-257. http://dx.doi.org/10.1007/s10059-013-0159-x

Zamore PD and Haley B (2005). Ribo-gnome: the big world of small RNAs. *Science* 309: 1519-1524. http://dx.doi.org/10.1126/science.1111444