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RESEARCH PAPER

siRNA Knock-Down of RANK Signaling to Control Osteoclast-Mediated Bone Resorption

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ABSTRACT

Purpose To demonstrate the ability of small interfering (si)RNA targeting the cell receptor, RANK, to control osteoclast function in cultures of both primary and secondary osteoclasts and their precursor cells.

Methods siRNA targeting RANK was transfected into both RAW264.7 and primary bone marrow cell cultures. RANK knock-down by siRNA and functional inhibition were assessed in both mature osteoclast and their precursor cell cultures. RANK mRNA message and protein expression after the transfections were analyzed by PCR and Western blot, respectively. Off-target effects were assessed. The inhibition of osteoclast formation was evaluated using tartrate-resistant acid phosphatase (TRAP) assay, and subsequent bone resorption was determined by resorption pit assay.

Results Both osteoclasts and osteoclast precursors can be targeted by siRNA in serum-containing media. Delivery of siRNA targeting RANK to both RAW 264.7 and primary bone marrow cell cultures produces short term repression of RANK expression without off-targeting effects, and significantly inhibits both osteoclast formation and bone resorption. Moreover, data support successful RANK knock-down by siRNA specifically in mature osteoclast cultures.

Conclusions RANK is demonstrated to be an attractive target for siRNA control of osteoclast activity, with utility for development of new therapeutics for low bone mass pathologies or osteoporosis.

KEY WORDS bone resorption · macrophage · osteoclasts · RANK · small interfering RNA

INTRODUCTION

With mean life expectancy increasing worldwide, degenerative skeletal diseases become more significant. Age-related hormonal changes are correlated with enhanced osteoclast activity observed in this patient population. Reduced bone density is highly associated clinically with the risk of fragility fractures (1–3), decreasing the eligibility for orthopedic implants, significantly impairing many patients' quality of life. The osteoclast is the major cell type responsible for bone resorption. Together with the bone-forming osteoblast, the osteoclast regulates the homeostasis of skeletal mass and continual turnover (4). Increased osteoclast function induces excessive osteoclast-mediated bone resorption, leading to bone loss-associated diseases, including Paget's disease (5), osteoporosis (6), hypercalcemia (7) and metastatic bone disease (8).

As large multi-nucleated cells, osteoclasts originate from mononuclear precursors of the monocyte-macrophage cell lineage (9). Osteoclastogenesis, cell maintenance and activation involve complex pathways with intricate relationships between multiple signaling molecules. Macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear

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factor κB (RANK), and RANK ligand (RANKL) are known to be key molecules initiating osteoclast formation. Interaction between M-CSF and its receptor, c-Fms, generates signals for osteoclast precursor cell survival and proliferation (10). By contrast, osteoclastogenesis is modulated by positive interactions between RANK and RANKL and negative interactions between RANKL and osteoprotegerin (OPG). RANK is a transmembrane signaling receptor expressed on haematopoietic precursor cells and osteoclasts (11). Interaction of RANKL is required for osteoclast formation, activation, and calcium homeostasis (11,12). It has also been reported that interaction of RANK and RANKL increases survival of the mature osteoclast *in vitro* and *in vivo* (13). RANK signals through the key adapter molecule, TNF receptor-associated factor (TRAF) 6, and RANK cytoplasmic domains, to regulate formation and activation through osteoclast-specific gene expression (11). Mice lacking RANK, TRAF 6, or RANKL are deficient in osteoclasts and lack osteoclastogenesis (12,14–16). OPG, a soluble protein of the TNF receptor family, secreted by osteoblasts, competitively binds RANKL (11,17), consequently acting as a decoy receptor to block osteoclastogenesis and suppress osteoclast survival (18,19). Thus, positive regulator RANKL and negative regulator OPG are normally coordinated to modulate bone degradation and formation homeostasis by competitive interactions with RANK. RANK is therefore a central factor in this bone metabolic regulatory pathway.

RNA interference (RNAi) (20) is a relatively recent development with increasing utility as a sequence-specific post-transcriptional gene silencing tool (21). Because systemic siRNA targeting has proven very challenging, local or topical siRNA therapeutics have been most actively investigated. Successful delivery approaches include ocular, respiratory, CNS, skin and vaginal sites, where local siRNA delivery accesses desired cell target populations directly (22–26). To date, siRNA targeting of RANK responsible for osteoclast formation and function has not been reported. The purpose of this study is to assess the utility of RNA interference (RNAi) methods to target RANK in regulating osteoclast formation and function *in vitro*. Reduction of RANK expression using siRNA specific to RANK is expected to suppress bone resorption by osteoclasts, ultimately increasing bone density and potentially preventing bone mass loss. In order to determine the efficacy of this RANK-targeting siRNA, both RAW and primary cells were evaluated for RANK message and protein expression after siRNA delivery. Functional assessments included inhibition of osteoclast formation by TRAP assay, and functional suppression of osteoclasts by bone resorption pit assay. Results show that osteoclast formation and osteoclast-mediated bone resorption can be significantly suppressed using siRNAs targeting RANK.

MATERIALS AND METHODS

Immortalized Murine Monocyte-Macrophage Cell Line Culture

A subclonal line of murine monocytic pre-osteoclastic RAW264.7 cells, purchased from the American Type Culture Collection (ATCC), was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone®, UT) and 1% penicillin-streptomycin (Gibco), defined for all cell cultures as "complete media." To induce osteoclast formation *in vitro*, RAW cells (passage number controlled to less than 10) were cultured on 24-well plates in complete media at the density of 4×10^4 cells per well supplemented with 100 ng/ml of RANKL. Complete media with RANKL was changed every other day.

Primary Murine Cell Harvest and Differentiation

C57BL/6 male mice (6–8 weeks old, Jackson labs) were maintained in a specific pathogen-free facility at the University of Utah. All procedures were performed as approved by the Institutional Animal Care and Use Committee of University of Utah. Bone marrow cells (BMC) were harvested from murine tibias and femurs of C57BL/6 male mice and differentiated into osteoclast precursors using previously described methods (27–29). Briefly, BMC were cultured in α-MEM (Gibco) containing 10% FBS and 1% penicillin-streptomycin overnight at a density of 1×10^6 cells/ml. Non-adherent cells were harvested the next day and immediately seeded into 24-well tissue culture plates in complete media with 30 ng/ml M-CSF (R&D Systems) at a density of 1×10^6 cells per well. After 2 days of culture, attached cells were used as osteoclast precursors. To generate osteoclasts, precursor cells were incubated in 200 ng/ml RANKL and 30 ng/ml M-CSF (R&D Systems) in complete media, refreshed every other day. RANKL working concentrations to reliably generate osteoclasts from both RAW264.7 and primary cell cultures in serum media were experimentally determined.

sRANKL Expression

A glutathione-S-transferase (GST)-tagged sRANKL construct was generated by cloning the murine sRANKL SalI/NotI fragment, coding 470–951 nucleotides, into the plasmid pGEX-4 T-1 (a generous gift of Dr. M. F. Manolson, University of Toronto). The expressed protein was harvested as previously described (29–31). GST-tagged sRANKL was purified by Glutathione Sepharose 4B affinity resin (Amersham Pharmacia Biotech), dialyzed against phosphate-

buffered saline (PBS, Gibco) and concentrated using Amicon Ultra centrifuge tubes (Millipore). Protein concentration was determined by BCA protein assay kit (Pierce).

siRNA Transfection of Cells

An siGENOME SMARTpool (Dharmacon) containing four different siRNA sequences, all designed to target murine RANK, as well as four pure individual siRNAs (**1**, sense 5′–GAGCAGAACUGACUCUAUGUU- 3′, antisense 5′-CAUAGAGUCAGUUCUGCUCUU-3′; **2**, sense 5′-GCGCAGACUUCACUCCAUAUU-3′, antisense 5′-UAUGGAGUGAAGUCUGCGCUU-3′; **3**, sense 5′-CC AAGGAGGCCCAGGCUUAUU-3′, antisense 5′-UAAGC CUGGGCCUCCUUGGUU-3′; **4**, sense 5′-CAAGAAGU GUGUGAAGGUAUU-3′, antisense 5′-UACCUUCACA CACUUCUUGUU-3′), and a non-targeting control siRNA (sense: 5′-UAGCGACUAAACACAU CAAUU-3′, antisense: 5′-UUAUCGCUGAUUUGUGUAGUU-3′), were purchased from Dharmacon. DharmaFECT 4 (DF4, Dharmacon) was used as the cationic lipid cell transfection reagent. RAW cells were seeded at a density of 4×10^4 cells per well in 24-well plates in DMEM at 37°C with 5% CO_2 overnight. Transfection with siRNA/DF4 complexes was then carried out in complete media. Primary BMC were seeded at 1×10^6 cells per well in 24-well plates in complete media containing 30 ng/ml M-CSF for 2 days. Subsequently, siRNA transfection was immediately performed. Transfection reagent DF4 and siRNA were prepared according to manufacturer's instructions (Dharmacon). Final dosing concentrations of all siRNAs provided to each well were 125 nM in a total volume of 1.0 microliter DF4. Cell uptake of siRNA complexes was performed by incubating cells with siRNA complexes in complete media at 37°C with 5% CO_2. In osteoclast formation and pit formation assays, cells were transfected by siRNA complexes in complete media with 100 ng/ml RANKL (RAW cells) or 30 ng/ml M-CSF and 200 ng/ml RANKL (BMC). Non-specific knock-down of DF4 was assessed by using non-targeting siRNA dosed under identical conditions. Multiple cell transfections were carried out identically each day over the successive first 3 days, or on alternating days, as specified in each figure. In the case of transfection of osteoclast cultures, RAW cells and primary BMC were seeded in 24-well plates and treated as mentioned above to generate osteoclasts. Mature osteoclasts were purified essentially as described elsewhere (28, 32) by gently washing with PBS without Ca^{2+} and Mg^{2+} (Gibco). By tapping the plate, most mononuclear cells were detached, while multinucleated osteoclasts remained on the plate. Osteoclasts were transfected by incubating with siRNA complexes prepared as above in complete media containing 100 ng/ml RANKL (RAW cells) or 200 ng/ml RANKL and 30 ng/ml M-CSF (BMC).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated 48 h after siRNA transfection using an RNeasy Mini Kit (Qiagen). Up to 4 micrograms of RNA were used to make cDNA with the SuperScript III 1st strand RT kit for PCR (Invitrogen). PCR primers were designed for RANK (5′-AGATGTGGTCTGCAGCTC TTCCAT-3′, 5′-ACACACTTCTTGCTGACTGGAG GT-3′) and cyclophilin B (housekeeping control, 5′- AGC GCTTCCCAGATGAGAACTTCA-3′, 5′-GCAATGG CAAAGGGTTTCTCCACT-3′) using Primerquest software purchased from Integrated DNA Technologies (IDT). PCR was performed with iTaq DNA polymerase (Bio-Rad), 1.5 mM magnesium chloride, 200 μM each of dNTPs, 500 nM of each primer, and 2 μL of the cDNA. Reactions were performed using the following protocol: 95°C melt, 60°C anneal and 72°C extension in the iCycler Thermal cycler (Bio-Rad). PCR products were analyzed on ethidium bromide-stained TBE-based 2% agarose gels run at 100 V for 30 min and visualized with UV light.

Real-Time Quantitative PCR (*q*PCR) Analysis

cDNA was prepared as described above. Primers for RANK (5′-TAGGACGTCAGGCCAAAGGACAAA- 3′, 5′-AGGGCCTACTGCCTAAGTGTGTTT-3′, Probe: 56-FAM/TGAAGGTGCCAGGGAAATTCAAG AAAGA/36-TAMSp) and cyclophilin B (5′-TCCGGCAA GATCGAAGTGGAGAAA-3′, 5′-AACCTT GTGA CTGGCTACCTTCGT-3′, Probe: 56-FAM/TCAT CCCTCTAAGCAGCTGTCTGTGT/36-TAMSp) were designed using Primerquest software and purchased from IDT. Experiments were performed using 7900HT Sequence Detection System and data were analyzed using SDS RQ manager (Applied Biosystems).

RANK Western Immunoblot Assay

Cells were lysed in RIPA buffer (Sigma) supplied with 1× protease inhibitor cocktail (Pierce) and 1 mM phenylmethylsulfonyl fluoride (Sigma) (32). Insoluble material was removed by centrifuging at 15,000 rpm at 4°C for 5 min after 20 min on ice. Protein concentration was measured with a BCA protein assay kit (Pierce). Heat-denatured samples were separated on 4–12% SDS-polyacrylamide gels (Invitrogen) and blotted on PVDF filters (Bio-Rad). After blocking with 5% (w/v) dry milk in 0.5% Tween 20 in TBS (TBST), the filter was incubated overnight in primary antibody against RANK (BAF692; R&D systems) in 5%

BSA/TBST with constant shaking. After three washes with TBST, the membrane was incubated with streptravidin-HRP (RPN 1231; Amersham Pharmacia Biotech Ltd.). The housekeeping control was detected with antibody against cyclophilin B (PA1-027; Affinity BioReagents) and HRP-conjugated donkey anti-rabbit antibody (SA1-200, Affinity BioReagents). Secondary antibodies were detected with chemiluminescence reagent (Santa Cruz Biotechnology), and band images were captured using a Molecular Imager Gel Doc XR System (Bio-Rad).

Tartrate-Resistant Acid Phosphatase (TRAP) Assay

Cells were stained for TRAP using a leukocyte acid phosphatase kit (Sigma) according to the instructions. Osteoclasts staining positive with at least three nuclei were counted as TRAP-positive cells.

Bone Resorption Pit Assay

Bovine bone was sawed into 0.2–0.3 mm thick slices (a gift from Dr. S. Miller, University of Utah) and washed as described (29) before placing small pieces into 24-well plates. A total of 4×10^4 RAW cells were plated and cultured on bone slices in DMEM for 12 h, and then cells were transfected with siRNA in complete media with 100 ng/ml RANKL (Day 1). BMC were plated on bone slices at a density of 1×10^6 per well in 30 ng/ml M-CSF complete media for 2 days. Thereafter, siRNA transfection was performed in 200 ng/ml RANKL and 30 ng/ml M-CSF-containing complete media (Day 1). The media was changed every other day. To observe osteoclast-generated bone resorption, slices were stained using a previously described method (29). Pit numbers per frame were counted from random fields under microscopic observation.

Cell Imaging

Live adherent cells, TRAP-stained images and pit images were photographed using a Nikon Eclipse TE 2000-U microscope, with Photometrics Coolsnap ES camera (gray scale, Roper Scientific) or QImagine RETIGA EXi color 12-bit camera (color, Canda), using Metamorph™ software (Molecular Devices) or QCapture™ software (QImaging). An average of 15 frames per well were taken randomly for TRAP assay analysis, and 10 frames were acquired randomly from each bone slice for pit formation analysis (except for triple siRNA transfection sequences in primary BMC, where 5 frames were acquired).

Statistical Analysis

Analysis of variance (ANOVA) followed by two-tailed student's *t*-test was used for statistical analysis. All experiments were repeated three times. Error bars represent the standard error of the mean. Results were considered statistically significant if $p<0.05$.

RESULTS

Optimization of siRNA Transfection in Cultures of RAW Cells

DharmaFECT 4 (DF4) was chosen as a siRNA transfection reagent since it was appropriate for mouse and rat cell lines based on the manufacturer's instructions. Transfection conditions in serum-containing media were optimized using a commercial murine-targeted siRNA pool (SMARTpool). Each siRNA sequence was then tested individually for their effectiveness to knock down RANK expression in cells in complete media. While *q*PCR results for sample siRNA-1 and -2 RANK knock-down were statistically indistinguishable, siRNA-2 was selected for further study since it produced the greatest overall knock-down effect (Fig. 1a, $p=0.009$). Non-specific knock-down of RANK by DF4 evaluated using non-targeting siRNA and DF4 demonstrates little effect by PCR analysis, shown in Fig. 1b. Similarly, treatment of mature osteoclasts with RANK siRNA-2 also significantly suppressed RANK expression in cultures compared with untreated osteoclasts (Fig. 1c). Additionally, in the presence of RANKL, small osteoclasts continued to fuse into larger ones in control cultures (Fig. 1d), while in RANK siRNA-treated cultures (Fig. 1e), the speed of cell fusion was suppressed after treatment, and the osteoclast size was smaller than those in controls.

Effects of Multiple Serial siRNA Transfections on RANK Expression in RAW Cells

RANK expression knock-down by multiple serial RANK and non-targeting siRNA transfections of RAW cells were tested using *q*PCR analysis (Fig. 1f). Serial transfections were performed every other day. mRNA was extracted on Day 3 (single siRNA transfection on Day 1), Day 5 (double siRNA transfections on Day 1 and 3), Day 7 (three siRNA transfections on Day 1, 3, and 5) and Day 9 (four siRNA transfections on Day 1, 3, 5, and 7), respectively. Compared to control (no treatment) groups, RANK siRNA-2 significantly suppressed RANK expression in all four situations ($p<0.001$). There was no significant difference between the control groups and the non-targeting siRNA groups on

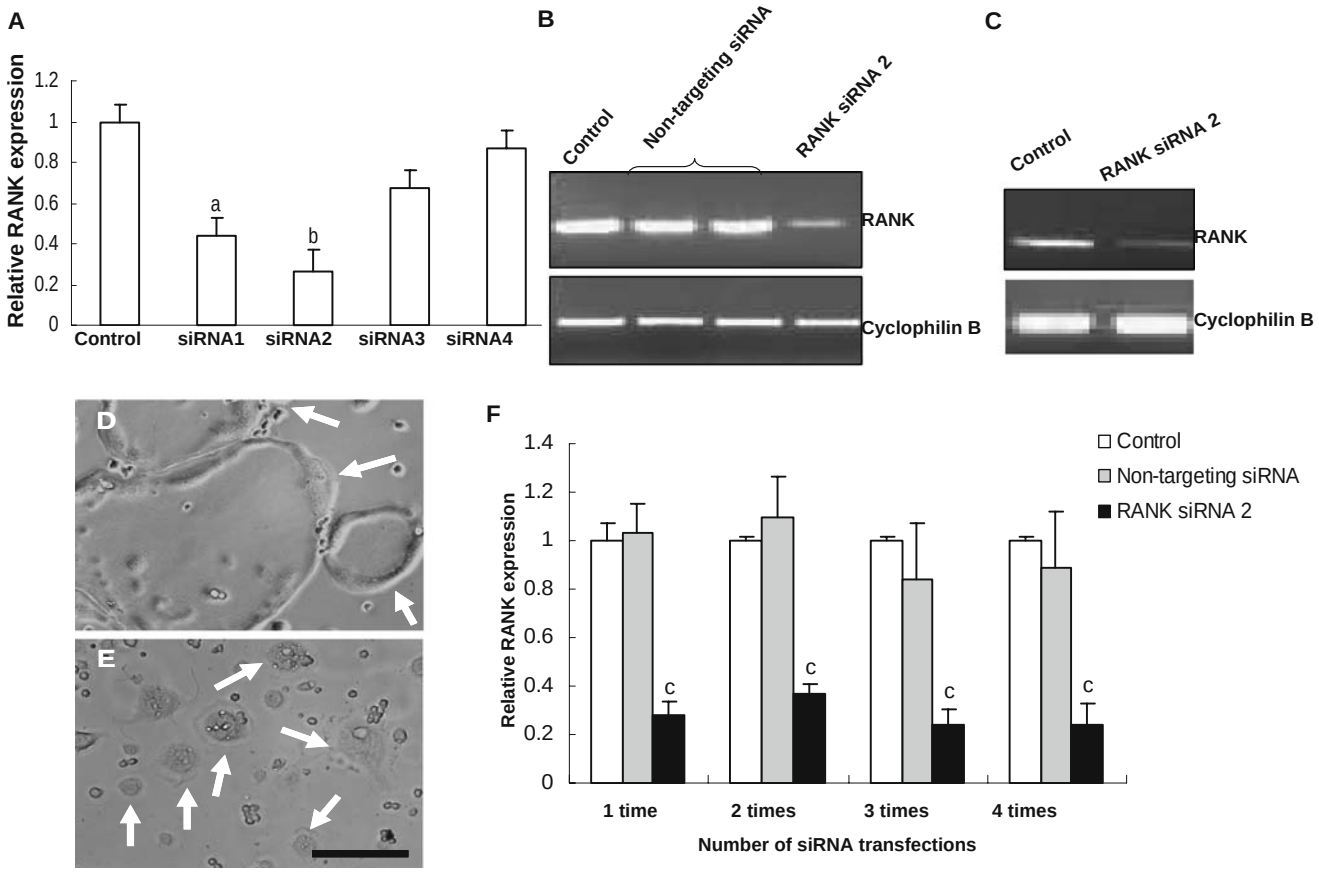

Fig. 1 RANK mRNA expression knock-down in cultured RAW cells. RNA was harvested 48 h post-transfection. (**A**) Four RANK siRNAs were assayed by *q*PCR analysis for the greatest knock-down effects. ($^{a}p < 0.05$, $^{b}p < 0.01$, each compared with controls without treatment). (**B**) DF4 was tested for non-specific knock-down of RANK using non-targeting siRNA. (**C**) RANK siRNA-2 was used to knock down the RANK gene expression in RAW-derived mature osteoclasts. Representative images of (**E**) RAW-derived osteoclasts (*arrows*) after a 48-h knock-down by RANK siRNA, compared with (**D**) controls with no treatment. (10X magnification) (**F**) RANK mRNA expression knock-down in RAW cell culture after multiple serial transfections determined by performing *q*PCR. ($^{c}p < 0.001$, RANK targeting groups *vs.* controls with no treatment).

RANK expression for one ($p=0.2$), two ($p=0.49$), three ($p=0.23$), or four ($p=0.51$) serial transfections, indicating that DF4 had no significant effect on expression of RANK in the absence of specific siRNA. In addition, compared with non-targeting siRNA transfections, the RANK siRNA-2 groups showed significant reduction of RANK expression for all four dosing situations: one ($p=0.00163$), two ($p=0.0007$), three ($p=0.0056$), and four ($p=0.0049$) serial siRNA transfections ($p<0.01$).

Effects of RANK siRNA on Protein Expression in RAW Cells

RANK protein expression was detected by Western blotting after single and multiple transfections, respectively. For a single transfection, RANK protein was at most suppressed 3 days post-transfection and then began to recover (Fig. 2a). Multiple transfections were performed in two ways. First, RAW cells were transfected successively daily in the first three culture days (Day 1 to Day 3). Protein was harvested on Day 5, 6, and 7. RANK knock-down was maintained until Day 6 and then started to return from Day 7 (Fig. 2b). Second, RAW cells were transfected three times serially but on every other day (Day 1, 3, and 5), and protein was harvested from Day 7 to Day 9. Fig. 2c clearly shows that protein knock-down effects could be prolonged until Day 9 with serial transfections.

Effects of RANK siRNA on Osteoclast Formation and Pit Resorption in RAW Cells

RAW cells were transfected with RANK siRNA or non-targeting siRNA in complete media with 100 ng/ml RANKL. For each siRNA, cells were dosed either once (Day 1), or three times serially on alternate days (Day 1, 3, and 5). To evaluate osteoclast formation, TRAP assay was performed on Day 7. Results are summarized in Fig. 3f. Both controls with no siRNA exposure (Fig. 3a)

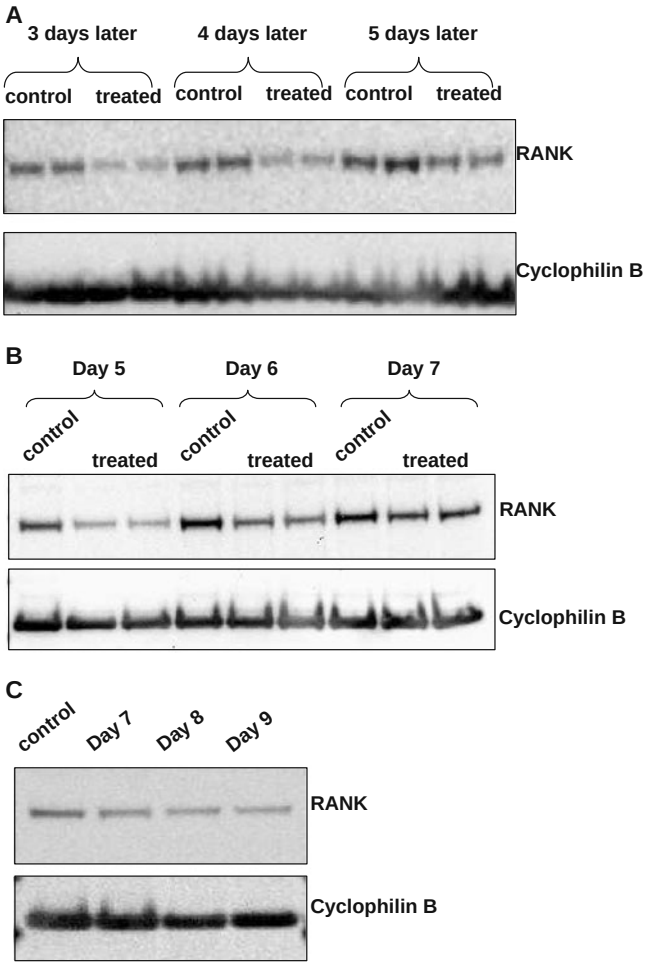

Fig. 2 Western blot analysis of RANK protein knock-down in RAW cell cultures. Western blotting analyzed 30 μg of cell lysate per sample. (**A**) Protein was harvested at 3, 4, and 5 days after single RANK siRNA-2 transfection. (**B**) RANK protein expression after three successive RANK siRNA-2 transfections. Protein was harvested on Day 5, 6, and 7. (**C**) RANK protein expression after three serial RANK siRNA-2 transfections on Day 1, 3, and 5. Protein was harvested on Day 7, 8, and 9.

and cells treated with non-targeting siRNA (single transfection, Fig. 3b; three transfections, Fig. 3c) showed strongly TRAP-positive multi-nucleate giant cells. Compared to controls, both single (Fig. 3d, $p=0.007$) and three-dose transfections with RANK siRNA (Fig. 3e, $p=0.00015$) showed significant reductions in numbers of TRAP-positive multi-nucleate cells in culture. There was no significant difference in the number of osteoclasts between controls and the single non-targeting siRNA transfection groups ($p=0.17$). Although three serial non-targeting transfections exert some influence on osteoclast formation by the transfection reagent ($p=0.042$), comparisons between three serial transfections of non-targeting *versus* RANK siRNA show that osteoclast numbers were significantly reduced in the RANK siRNA-treated groups ($p<0.001$). The same trend exists for comparisons between single transfections of non-targeting *versus* RANK targeting siRNAs ($p=0.017$). Three-dose RANK siRNA transfection groups showed reduced osteoclast numbers compared with single-dose groups, but not significantly different ($p=0.13$). Resorption pit assay was performed 6 days post-single transfection. Formation of resorption pits on bone slices was markedly curtailed in the siRNA-treated groups (Fig. 3g, $p=0.02$) compared to controls (no treatment) that displayed many pits formed by osteoclasts.

Effects of siRNA on RANK mRNA and Protein Expression in Primary BMC Cultures

To ensure that RANK siRNA-2 retained the most powerful knock-down effects, differences in siRNA knock-down for the 4 different RANK siRNAs were tested on primary BMC cultures in serum media. RT-PCR results clearly demonstrated that RANK siRNA-2 was the most efficient at down-regulating BMC RANK mRNA expression (Fig. 4a). Therefore, RANK siRNA-2 was used for subsequent transfections in primary BMC. Consistently, RANK protein production was reduced both by single siRNA transfection (Fig. 4b) and three serial transfections on alternate days (Fig. 4c). Furthermore, RANK siRNA transfection was also able to suppress RANK message expression in primary BMC-induced mature osteoclasts. PCR products showed significantly reduced RANK mRNA expression in osteoclast cultures after single transfection (Fig. 4d), as well as reduced RANK protein expression by Western blot assay (Fig. 4e). Similarly to RAW cell cultures, BMC-induced osteoclasts had reduced rates of cell fusion after transfection (Fig. 4g) compared to large osteoclasts formed in control cultures (Fig. 4f).

Effects of RANK siRNA on Osteoclast Formation and Activity in Primary BMC Cultures

TRAP assays were performed on Day 5 cell cultures for single siRNA transfections. Compared with controls (Fig. 5b), significant reductions in numbers of TRAP-positive multi-nucleate giant cells post-transfection (Fig. 5c, $p=0.001$) were observed. Differences in numbers of TRAP-positive cells are summarized in Fig. 5a. Primary osteoclast precursor cells were cultured on bovine bone slices to assess effects of siRNA on osteoclast function. Resorption pit assay was performed on Day 5 for single transfections. Controls without siRNA treatment produced abundant resorption pits on bone slices as detected by microscopy (4X magnification). RANK siRNA treatment inhibited osteoclastic bone resorption with significant reductions in resorption pit numbers (Fig. 5d, $p=0.04$).

DISCUSSION

We provide evidence that siRNA delivered to cells in serum-containing media can successfully and specifically inhibit RANK expression both in osteoclast precursors and mature osteoclasts from RAW secondary and primary BMC. This results in suppression of cell-based bone resorption mechanisms by reducing the number and activity of osteoclasts in cultures.

RANK plays an essential role in regulating osteoclastogenesis (33). Activation of RANK by its ligand, RANKL, is required for the formation and activation of osteoclasts (12,15). Similar to RANK, OPG can also bind RANKL to act as a competitive inhibitor by blocking RANK interaction (17,34,35). Since the elucidation of the RANK/RANKL/OPG signaling pathways, RANKL and OPG have been actively investigated as therapeutic targets. Several OPG-based approaches to regulate bone mass and reduce bone resorption have been reported in animal models (17,18,36) and in a human trial (37). However, reliable delivery of a large protein therapeutic poses several challenges, including limited stability, relevant dosing and bioavailability (short half-life), possible host immune responses to recombinant products, patient compliance (parenteral requirements) and

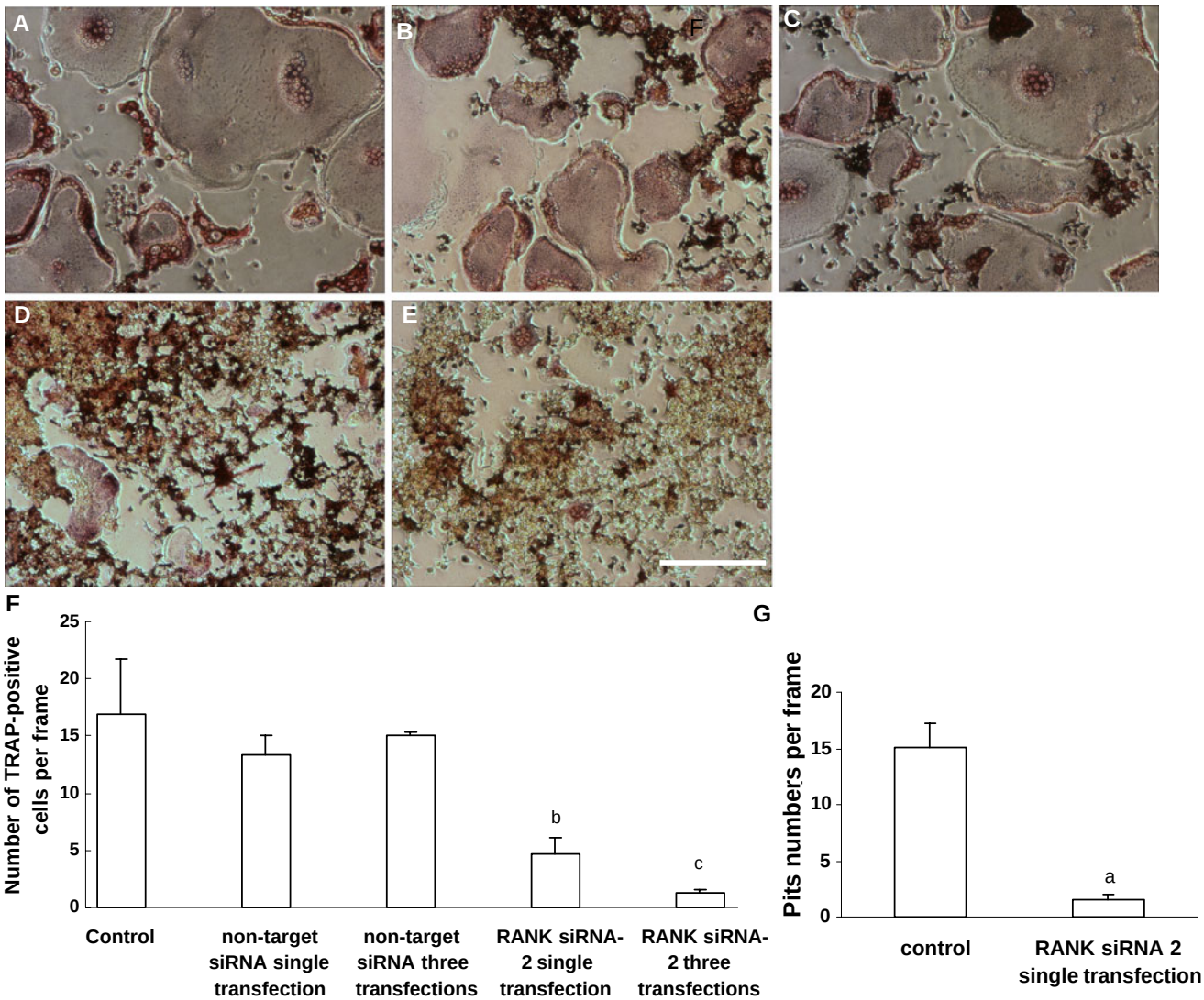

Fig. 3 Effects of RANK siRNA and non-targeting siRNA on osteoclast formation determined by TRAP assay in RAW cell cultures. Cells were TRAP-stained and counted 7 days post-transfection. Cultures were performed in the presence of RANKL (**A**) with no siRNA treatment for positive controls, (**B**) transfected with non-targeting siRNA once on Day 1, (**C**) transfected with non-targeting siRNA three times serially every other day, (**D**) transfected with RANK siRNA once on Day 1, and (**E**) three times serially every other day. (**F**) Comparison of the number of TRAP-positive multi-nucleate cells formed after siRNA dosing. ($^{b}p < 0.01$, $^{c}p < 0.001$, RANK siRNA-2 single and three transfection groups *vs.* controls). (scale bar = 250 μm) Inhibition of osteoclast-mediate bone resorption by RANK siRNA was evaluated by pit formation assay. (**G**) Difference of resorption pit numbers per frame (10X magnification) between RANK siRNA untreated and treated RAW cells ($^{a}p < 0.05$).

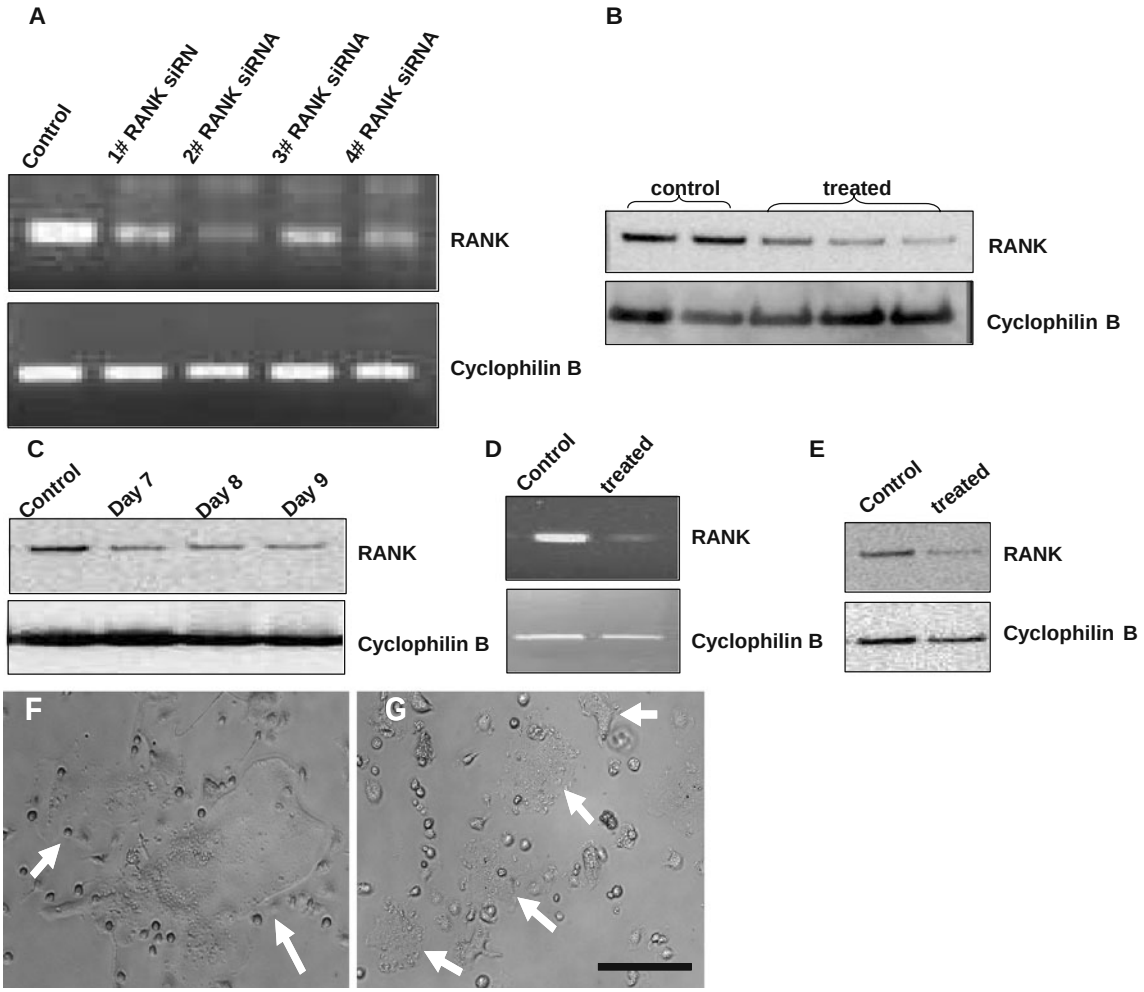

Fig. 4 RANK knock-down by RANK siRNA dosing to primary BMC and BMC-derived osteoclasts in serum-based culture. RNA was harvested 48 hours post-transfection. Protein was harvested 3 days post-transfection, except from the mature osteoclasts, harvested at 48 h post-transfection. Western blotting analyzed 30 μg of cell lysate per sample. (**A**) Four RANK siRNAs were analyzed by RT-PCR for the greatest knock-down effect. (**B**) Lysates analyzed by Western blot for RANK protein expression suppression by a single dose of RANK siRNA. (**C**) RANK protein expression after three serial siRNA transfections on every other day. Protein was harvested on Day 7, 8, and 9. Inhibition of target RANK gene expression in primary cellinduced osteoclasts at the mRNA and protein levels as shown by (**D**) PCR, and (**E**) Western blot. Representative images of primary BMC-derived osteoclasts (arrows) after 48-h knock-down by (**G**) RANK siRNA compared with (**F**) controls. (10X magnification).

complex formulating issues for sustained protein release (38–40). A fully human monoclonal antibody targeting RANKL, denosumab, is currently investigated as a subcutaneous injection in late-stage clinical development for bone metabolic therapies (41–43). Possible safety issues of this anti-RANKL antibody include cross-reactions with OPG or RANK-activated endogenous antibodies. Therefore, other therapeutic options are necessary. RNAi is an alternative approach to RANK and RANKL control in this same context. In this approach, siRNA molecules silence gene expression in a sequence-specific manner by causing degradation of corresponding endogenous mRNA (20,44,45). In fact, complete absence of either RANKL or RANK (as shown in RANKL and RANK knock-out mice) eliminates RANKL-RANK signaling between osteoblasts and immature osteoclasts (12,15). This depletes functioning mature osteoclasts, removes intrinsic bone deposition control mechanisms and eventually causes osteopetrosis in these knock-out mutants. Therefore, due to these concerns as well as other side effects (i.e., targeting undesired cell populations expressing RANK and general non-specific targeting), site- and temporally selective, as well as reversible control over RANK-RANKL activity, rather than its complete, irreversible abolition, is considered more appealing for developing new clinical approaches to osteoporosis therapies. In this study, siRNA targeting RANK was transfected into serum-based secondary macrophage cultures, primary monocyte-macrophage cultures, and culture-generated osteoclasts to observe RANK mRNA knock-down and suppression of protein expression. While RNAi has noted issues with mammalian cell delivery efficiency and specific targeting (46), one advantage of siRNA in the context of RANK is

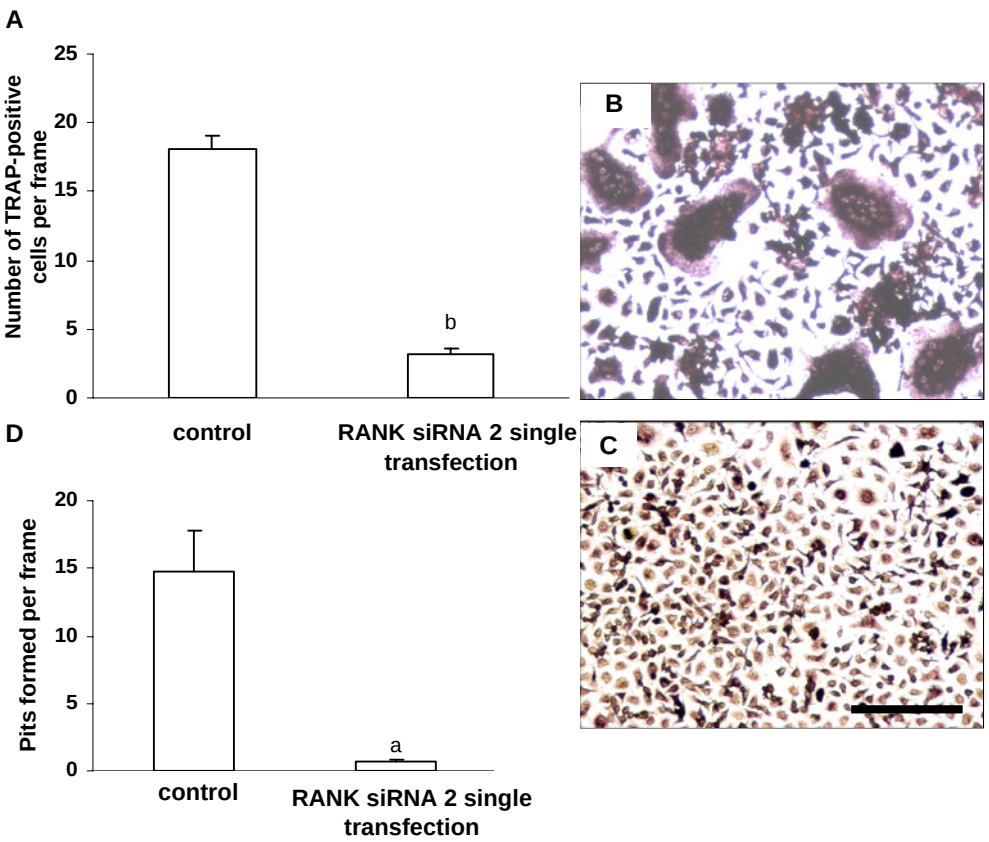

Fig. 5 Inhibition of primary cell-induced osteoclast formation and activity by RANK siRNA. (**A**) Inhibition of osteoclast formation by a single RANK siRNA dose (Day 1). TRAP assay was performed on Day 5 ($^{b}p < 0.01$). Resorption pit assay was performed on same day. Representative image of TRAP-stained cells in (**B**) control groups with no treatment, and (**C**) treated groups with single siRNA dose. (**D**) Comparison of average pit number for cell-treated bovine bone slices per frame (4X magnification) between controls (no treatment) and single dose of RANK siRNA-2 ($^{a}p < 0.05$). (scale bar = 250 μm).

the transient gene knock-down experienced (5~6 days, depending on the target, cell type, and frequency of target protein expression typical to that cell) with relatively small amounts of siRNA dosed. This provides an alternative control feature for RANK suppression in cells compared to mutants completely lacking RANK or protein-based systemic antagonism approaches. Additionally, clinical osteoporosis bone augmentation approaches (47,48) permit local placement of siRNA-releasing carriers directly into osteoporotic sites by injection, permitting renewable, local siRNA delivery to bone RANK at these sites, and avoiding systemic delivery issues.

As osteoclasts central to both bone metabolic control and RANK presentation derive from fusions of haematopoietically sourced circulating cells and tissue-resident differentiated macrophages, assessing and comparing the utility of siRNA knock-down effects in both secondary and primary macrophage cultures was considered an important milestone. Immortalized commercial RAW264.7 monocyte-macrophages are commonly cultured as macrophage surrogates, despite the general lack of reporting of phenotypic indicators or fidelity to primary macrophages and their relatively high rates of contamination from commercial sources (49). Some limitations with the accuracy of RAW cell comparisons to primary macrophages in model assays have already been noted (27,50). Aggressive endocytosis and proliferation rates in RAWs, characteristic of tumor-derived cell lines, as well as higher frequency and intensity of both gene up-regulation and protein expression, may all alter the intensity and duration of siRNA effects (51). Nevertheless, as RAW264.7 cells have been frequently used for *in vitro* generation of osteoclasts (32,52–54), this study compared RAW cells to primary BMC for effects of siRNA on osteoclast behavior.

RANK siRNA-2 provides the highest knock-down of the RANK mRNA for both RAW and primary BMC cultures. A dose of 125 nM RANK siRNA and the commercial cationic lipid transfection reagent, DF4, demonstrated successful inhibition of the target RANK gene expression at the mRNA level 48 h after transfection in serum-based culture conditions. In addition, evidence indicates that RANK message knock-down is not caused by the transfection reagent, as there was no significant reduction of RANK mRNA expression in cells transfected with non-targeting siRNA/DF4 complexes. Moreover, the accumulating effects of multiple serial transfections were evaluated in RAW cells, showing sustained knock-down effects in serum-containing culture media. A likely explanation for the similar RANK knock-down effects observed with the three and four transfection cycles could be the increasing cell numbers by the third and fourth transfection cycles diluting effects of constant siRNA dosing.

Protein expression analysis of both cell cultures further confirmed RANK knock-down using siRNA. In RAW cell

cultures, with single transfections, RANK protein levels were suppressed after 3 days in culture. Subsequently, RANK protein production began to recover, due possibly to rapid cell proliferation and dilution of siRNA within cells. Multiple transfections (i.e., 3 serial doses) were performed, successively in the first 3 days or serially every other day throughout the culture period. In the latter case, RANK protein expression can be suppressed until Day 9. The same prolonged protein suppression was observed in BMC as well, suggesting that changing the frequency of multiple siRNA transfections can extend protein knock-down. Therefore, serial siRNA transfections performed every other day was considered more efficective and hence used for multiple transfections in subsequent assays.

Since RANK also resides on mature osteoclasts and positively regulates osteoclast activation and survival, it was also important to target RANK on mature osteoclasts. We found that mature osteoclasts induced from both RAW and primary BMC cultures can be successfully transfected by siRNA, producing significant reductions in both RANK message and protein compared with untreated controls. In support of this, we found that in both secondary and primary cultures, after osteoclasts were transfected by RANK siRNA, further fusion was suppressed. This corresponds to a previous report showing that RANKL stimulation is essential for cell fusion of osteoclasts (55). Large osteoclasts have much greater bone resorbing capability than small osteoclasts under same conditions (32,56,57); therefore, the bone resorbing activity of osteoclasts is suppressed after RANK siRNA transfection at least in part by controlling osteoclast fusion and size.

We further assessed RANK siRNA effects on osteoclast formation. In RAW cell cultures, osteoclast numbers were reduced significantly in both single and three serial RANK-siRNA-treated groups. No significant changes were observed in the number of osteoclasts in single non-targeting siRNA-treated cultures compared with controls, consistent with minimal apoptosis and cell viability influences from transfection. Three serial transfections of non-targeting siRNA showed significant differences when compared to controls, though the average numbers were similar. A likely explanation may be a negative role played by the transfection reagent in multiple dosing. However, comparisons between three serial transfections of non-targeting siRNA and RANK siRNA showed significant differences in osteoclast number, which confirms the inhibition effects on osteoclast differentiation by specific siRNA transfection. Though the average osteoclast number in three serially RANK siRNA-transfected cell cultures was lower than that for single-dose groups, there was no significant difference within the seven culture days, indicating that osteoclast formation is sensitive to RANK expression level and a single transfection at culture initiation is sufficient to inhibit both osteoclast formation and activity for a cycle of osteoclast formation in RAW cells. Hence, single doses of siRNA were applied for evaluation of effects on osteoclast bone-resorption activity. Similar results were obtained in primary BMC cultures; single dose of RANK siRNA led to a significant reduction in numbers of TRAP-positive cells. Furthermore, we have established that RANK siRNA is a potent inhibitor of osteoclast-mediated bone resorption, resulting in significant reduction of bone resorption pit number. Bone resorption activity was largely inhibited by RANK siRNA in both cultures. We attribute this in part to fewer osteoclasts formed, demonstrated by TRAP assay results, consistent with RANK mRNA and protein knock-down specific to siRNA introduction in monocyte-macrophage precursor and mature osteoclast serum-based cultures. All experiments with primary cells duplicated the results and conclusions from RAW cell cultures, confirming the general knock-down effect of RANK siRNA in these cell types in serum-based culture.

In summary, we conclude that siRNA can be successfully used to specifically inhibit RANK expression both in RAW and primary macrophage cell cultures, and also in osteoclast precursors and mature osteoclasts in serum-based transfections. Since RANK is an essential receptor in the membranes of both osteoclast precursors and osteoclasts and plays a key role in osteoclast formation and function, control of RANK has important fundamental and translational implications. The experimental results follow theoretical predictions: formation of osteoclasts is strongly suppressed after siRNA treatment, and the bone resorption pit numbers are largely reduced as well. In addition, our analysis demonstrates that a single dose of RANK siRNA is sufficient to inhibit both osteoclast formation and activity for a cycle of osteoclast formation. These data suggest that siRNA against RANK could be a powerful tool for inhibiting osteoclast-mediated bone resorption and improving bone mass maintenance. Extensions of this concept to treatment of osteoporosis or low bone mass using siRNA administration could be interesting if reasonable RANK control at desired locations could be achieved.

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