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Small Interfering RNA Knocks Down the Molecular Target of Alendronate, Farnesyl Pyrophosphate Synthase, in Osteoclast and Osteoblast Cultures

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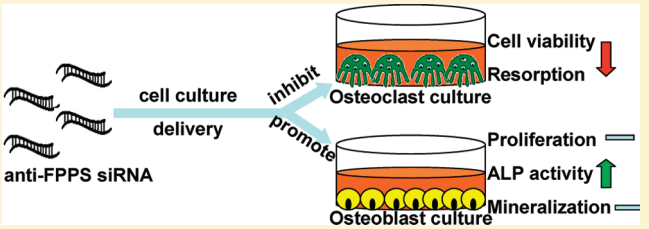
ABSTRACT: Farnesyl pyrophosphate synthase (FPPS), an enzyme in the mevalonate pathway, is the inhibition target of alendronate, a potent FDA-approved nitrogen-containing bisphosphonate (N-BP) drug, at the molecular level. Alendronate not only inhibits osteoclasts but also has been reported to positively affect osteoblasts. This study assesses the knockdown effects of siRNA targeting FPPS compared with alendronate in both osteoclast and osteoblast cultures. Primary murine bone marrow cell-induced osteoclasts and the preosteoblast MC3T3-E1 cell line were used to assess effects of anti-FPPS siRNA compared with alendronate. Results show that both FPPS mRNA message and protein knockdown in serum-based culture is correlated with reduced osteoclast viability. FPPS siRNA is more potent than 10 μM alendronate, but less potent than 50 μM alendronate on reducing osteoclast viability. Despite FPPS knockdown, no significant changes were observed in osteoblast proliferation. FPPS knockdown promotes osteoblast differentiation significantly but not cell mineral deposition. However, compared with 50 μM alendronate dosing, FPPS siRNA does not exhibit cytotoxic effects on osteoblasts while producing significant effects on ostoblast differentiation. Both siRNA and alendronate at tested concentrations do not have significant effects on cultured osteoblast mineralization. Overall, results indicate that siRNA against FPPS could be useful for selectively inhibiting osteoclast-mediated bone resorption and improving bone mass maintenance by influencing both osteoclasts and osteoblasts in distinct ways.



KEYWORDS: farnesyl pyrophosphate synthase, siRNA, osteoclasts, osteoblasts, alendronate, bone metabolism, mineralization

INTRODUCTION

Osteoporosis, a metabolic bone disease and leading cause of osteoporotic fragility fractures in both men and women, is rapidly becoming a global healthcare crisis as average life expectancy increases worldwide. It is defined as a disorder of calcium and phosphate metabolism characterized by low bone mass and microarchitectural deterioration.[1] With decades of clinical experience, bisphosphonates are the most used pharmacological approach to treat osteoporosis currently,[2] due to their significant inhibition of osteoclast-mediated bone resorption. Nitrogen-containing bisphosphonates (N-BPs) are more potent than their non-nitrogen-containing bisphosphonate analogues in suppressing osteoclast activity. However, since "avascular osteonecrosis" in patients receiving pamidronate (N-BP) therapy was first described by Marx in 2003,[3] their general side effects, including gastrointestinal irritation, bone/joint pain and jaw osteonecrosis,[3−5] and their long half-life[6] have clouded their therapeutic efficacy. Furthermore, due to their severe suppression of bone turnover,[7,8] long-term bisphosphonate therapy can increase the risk of fractures, such as atypical fracture as a potential complication which was first reported in 2005.[9,10] Therefore, designing an improved therapeutic that retains N-BPs' inhibition of bone resorption with significant reductions in its side effects will be highly significant.

The major intracellular target of alendronate, one of the most potent N-BPs, is farnesyl pyrophosphate (FPP) synthase (FPPS), a key enzyme in the mevalonate pathway.[11−15] The mevalonate pathway is ubiquitous in mammalian cells, producing essential lipids including cholesterol and isoprenoids that are critical for post-translational prenylation of proteins regulating cell apoptosis, such as Ras and Rho.[16,17] FPPS catalyzes the synthesis of the C_{15} metabolite farnesyl pyrophosphate (FPP) through the sequential condensation of isopentenyl pyrophosphate (IPP), starting with dimethylallyl pyrophosphate (DMAPP) and then with the resultant geranyl pyrophosphate (GPP). FPP is also used as the substrate to produce the C_{20} isoprenoid geranylgeranyl

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pyrophosphate (GGPP). Both FPP and GGPP are required for post-translational prenylation of small GTPases. FPP is therefore an essential isoprenoid intermediate in the mevalonate pathway required for the post-translational prenylation of essential GTPase signaling proteins.[18] Crystallography studies reveal that, as potent inhibitors of osteoclastic activity, alendronate competitively binds with FPPS in the GPP/DMAPP binding site and the FPPS/alendronate complex can be further stabilized by binding with IPP.[13,15] Downregulation of post-translational prenylation of GTP-binding proteins results in perturbed cell activity and the induction of osteoclast apoptosis.[19] However, interestingly, increasing evidence suggests that these bisphosphonates also have an anabolic effect on osteoblasts. Specifically, N-BPs were shown to induce human osteoblast differentiation and mineralization in culture by inhibiting the mevalonate pathway.[20] Therefore, a therapeutic that avoids known side effects for N-BPs but reliably suppresses FPPS to inhibit the mevalonate pathway in both osteoclasts and osteoblasts could increase bone formation in the control of osteoporosis.

RNA interference (RNAi) is a potent therapeutic gene silencing tool to transiently knock down gene-specific mRNA expression levels by exploiting a natural intracellular cytoplasmic mRNA regulatory phenomenon in mammalian species.[21−23] Gene silencing using short interfering RNAs (siRNAs) has many potential therapeutic applications.[24] We therefore examined the potential of administering small interfering RNA (siRNA) to suppress N-BP's molecular target, FPPS, in both osteoclasts and osteoblasts in vitro and compared its effects with the clinically familiar N-BP, alendronate. Osteoclasts were obtained from murine primary bone marrow monocytes.[25,26] The preosteoblastic cell line MC3T3-E1 was used since it has been widely exploited as a culture model for osteoblast differentiation.[27] Expression levels of FPPS message and protein are monitored after siRNA transfections. Osteoclast viability and osteoblast proliferation, differentiation and mineral deposition post-transfections in serum culture were evaluated.

MATERIALS AND METHODS

Cell Cultures. Primary Murine Cell Harvest and Differentiation. C57BL/6 male mice (6−8 weeks old, Jackson Laboratories) 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 (BMCs) were harvested from murine tibias and femurs of C57BL/6 male mice and differentiated into osteoclast precursors using previously described methods.[25,26,28] Briefly, BMCs were cultured in α-MEM (Gibco) containing 10% heat-inactivated fetal bovine serum (FBS, Hyclone, UT) and 1% penicillin−streptomycin (Gibco) overnight at a density of 1×10^6 cells/mL, defined for all cell cultures as "complete media". Nonadherent 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, adherent cells were used as osteoclast precursors. To generate osteoclasts, these 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 primary cell cultures in serum media were experimentally determined. Mature osteoclasts, distinguished by multinucleated, large cell bodies were purified essentially as described elsewhere by gently washing with PBS without Ca^{2+} and Mg^{2+} (Gibco).[26,29] It usually took 5 days of incubation, for at least 80% of the plate remains covered with osteoclasts. By tapping the plate, most mononuclear cells were detached and removed, while multinucleated osteoclasts remained on the plate.

Osteoblast Culture. The immature osteoblast-like cell line MC3T3-E1 (subclone 4, ATCC, USA) cells were cultured at 4×10^4 cells/well in 24-well plates. To induce differentiation, cells were maintained in complete media with 50 μg/mL ascorbic acid (AA, Sigma) and 10 mM β-glycerophosphate (Sigma).[30] Medium was refreshed every other day.

sRANKL Expression. A glutathione-*S*-transferase (GST-tagged sRANKL construct was generated by cloning the murine sRANKL *Sal*I/NotI fragment, coding 470−951 nucleotides, into the plasmid pGEX-4T-1 (a generous gift of Dr. M. F. Manolson, University of Toronto). The expressed protein was harvested as previously described.[25,31,32] 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 a BCA protein assay kit (Pierce).

siRNA Transfection of Osteoclasts and Osteoblasts. Four pure individual ON-TARGETplus siRNAs all designed to target murine FPPS (target sequences: siRNA1, 5′-GUC AAG UAC AAG ACG GCUU-3′; siRNA2, 5′-GAA AAG AGG UAC AAA UCGA-3′; siRNA3, 5′-AGA AAG UGA CCC CGG AAUU-3′; siRNA4, 5′-CCU AGA GUA CAA UGC CUUA-3′) and a nontargeting control siRNA (sense, 5′-UAG CGA CUA AAC ACA UCA AUU-3′; antisense, 5′-UUA UCG CUG AUU UGU GUA GUU-3′) were purchased from Dharmacon (USA). DharmaFECT 4 (DF4, Dharmacon) was used as the cationic lipid cell transfection reagent in complete media. Mature osteoclasts were prepared as described above, residual monocytes were removed from culture, and siRNA transfection was immediately performed. Transfection reagent DF4 and siRNA were prepared according to the manufacturer's instructions (Dharmacon). Final dosing concentrations of all siRNAs provided to each well were 125 nM in a total volume of 1.0 μL of DF4. Cell uptake of siRNA complexes was performed by incubating cells with siRNA complexes in complete media with 30 ng/mL M-CSF and 200 ng/mL RANKL at 37 °C with 5% CO_2. Nonspecific knockdown from DF4 transfection was assessed by using nontargeting siRNA dosed under identical conditions. Multiple repeated siRNA cell transfections were performed on alternating days as specified in each figure legend.

MC3T3-E1 cells were cultured at 4×10^4 cells/well in 24-well plates in complete media overnight. Transfections were performed early the next day. Final dosing concentrations of all siRNAs provided to each well were identical to osteoclasts. Cell uptake of siRNA complexes was performed by incubating the cells with siRNA complexes in complete media with 50 μg/mL AA and 10 mM β-glycerophosphate at 37 °C with 5% CO_2.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Total cellular RNA was isolated from each well at different time points after siRNA transfection using an RNeasy Mini Kit (Qiagen). Up to 4 μg of RNA was used to make cDNA with the SuperScript III first strand RT kit for PCR (Invitrogen). PCR primers were designed for FPPS (5′-TGC TGG TAT CAG AAG CCA GGC ATA-3′, 5′-TGC TGG TAT CAG AAG CCA GGC ATA-3′) and cyclophilin B (housekeeping control, 5′- AGC

GCT TCC CAG ATG AGA ACT TCA-3′, 5′-GCA ATG GCA AAG GGT TTC TCC ACT-3′) using Primerquest software and purchased from Integrated DNA Technologies (IDT). PCR was performed by routine methods described previously.[33]

Western Immunoblot Assay. The assay was performed as described previously.[33] Briefly, cells were lysed and protein concentration was measured using 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). The filter was incubated overnight in primary antibody human antimouse FPPS (HCA012, AbD SeroTec) in 5% BSA/TBST. Then the membrane was incubated with goat anti-human IgG (0500−0099, AbD SeroTec). 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).

Cell Viability. The relative number of viable osteoclasts or osteoblasts in each well was determined using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, WI, USA). To terminate culture, medium was replaced with 300 μL/well of fresh complete medium with the addition of 60 μL of Cell Titer 96 Aqueous One Solution, including three wells containing only medium for background subtraction. Cells were then incubated at 37 °C with 5% CO_2 for 2 h, and optical absorbance at 490 nm was then determined using a plate reader (TECAN GENIOS Plus).

Alkaline Phosphatase Activity. To evaluate the differentiation of MC3T3-E1 cells, alkaline phosphatase (ALP) activity was determined. MC3T3-E1 cells were seeded at 4×10^4 cells/well in 24-well plate and transfected with siRNA or incubated with complete medium containing various concentrations of alendronate (gift from Prof. J. Kopeček, University of Utah). ALP activity was determined using a QuantiChrom Alkaline Phosphatase Assay Kit (BioAssay Systems, CA). Alkaline phosphatase activity values were normalized to the relative number of viable cells as determined directly using the above-mentioned cell proliferation assay.

Cell Mineralization. The degree of mineralization of cultured MC3T3-E1 cells was determined using Alizarin red staining,[20] sensitive to the identification of cell calcification in vitro. Briefly, medium was aspirated and the cells were rinsed twice with PBS. Then cells were fixed with ice-cold 70% (v/v) ethanol for 1 h. Ethanol was removed, and the cells were then stained with 2% Alizarin Red S (Sigma) in deionized water (pH 4.2) for half an hour at room temperature. The staining solution was removed, and the cells were rinsed five times with deionized water. After removal of the water, cells were incubated in PBS for 15 min at room temperature on a shaker. After PBS was removed, the cells were rinsed with PBS again. Dye destaining was performed by incubating the cells with 10% (w/v) cetylpyridinium chloride in 10 mM sodium phosphate (pH 7) washing buffer at room temperature. The washing buffer was then transferred to a 96-well plate, and the optical absorbance at 562 nm was measured using a plate reader (TECAN GENIOS Plus). The concentration of Alizarin Red S in the wash buffer was determined according to Alizarin Red S standards. Mineralization values were normalized to the relative number of viable cells determined by using Cell Titer 96 Aqueous One Solution Cell Proliferation Assay as described above. Mineralization produced in culture after triplicate serial siRNA transfections was measured the day following the last transfection.

Statistical Analysis. Cell viability, ALP and mineralization results are reported as mean ± standard deviation relative to control. Significance is determined using the two-tailed Student's *t*-test. All experiments were repeated three times. Results are considered statistically significant if $p < 0.05$.

RESULTS

Successful FPPS Message and Protein Knockdown in Both Osteoclast and Osteoblast Cultures. Since this study's focus was not on assessing transfection efficiency but rather on assessing post-transfection knockdown of target, the established commercial cationic cell transfection surfactant, DharmaFECT 4 (DF4), was used for siRNA transfection based on its frequency of successful use and manufacturer's instructions claiming it "appropriate" for mouse and rat cell lines. Importantly, siRNA transfections were all performed in serum-containing media to avoid serum-free medium-induced cell stress. Each siRNA sequence was tested individually for effectiveness to knock down FPPS expression in osteoclasts in complete medium. siRNA-2 produced the greatest overall knockdown effect from the commercial library and, therefore, was used for all further siRNA transfections of both osteoclast and osteoblast cultures. Nonspecific knockdown of FPPS target by DF4, evaluated using nontargeting siRNA and DF4 in both cultures, is not significant by PCR analysis (Figure 1). Significant downregulation of FPPS gene and protein expression was observed in osteoclast cultures (Figure 1A,C). Since all assays on osteoblasts were performed 5 days post-transfection, gene and protein expression levels were monitored prior to this on days 2 to 5. Treatment of osteoblasts with FPPS siRNA-2 also significantly suppressed FPPS message expression in cultures until day 4, when cell assays were performed, compared to untreated and nontargeting siRNA-transfected osteoblasts (Figure 1B). Significantly, sustained downregulation of FPPS protein expression was also observed 5 days post-transfection (Figure 1D).

Comparison of FPPS siRNA and Alendronate on Osteoclast Cytotoxicity. Osteoclast viability assays were performed 3 days after siRNA transfections in serum-based cultures. Osteoclast numbers were significantly reduced in FPPS siRNA-treated groups compared with control groups (70.3% of the control, $^*p = 0.03$, Figure 2A). Compared with nontargeting siRNA which showed 98.4% viability of the untreated control, FPPS siRNA significantly suppressed viability ($^*p = 0.015$), indicating that reduced cell viability results from specific FPPS gene knockdown, but not from nonspecific effects of siRNA or transfection reagent dosing. Osteoclasts were treated with alendronate at two different concentrations (10 μM and 50 μM). After three day culture (the same duration as siRNA transfection), cell viability was reduced to 95.5% ($^*p = 0.025$) in the 10 μM alendronate-treated group and to 40.3% ($^{**}p = 0.009$) in the 50 μM alendronate-treated group. Both cell viability decreases are significant compared with control (Figure 2B).

Comparison of FPPS siRNA and Alendronate Treatments on MC3T3-E1 Preosteoblast Cell Proliferation. FPPS siRNA and alendronate effects on MC3T3-E1 preosteoblast cell proliferation were compared five days post-treatment. As shown in Figure 3A, FPPS siRNA had little effect on cell proliferation (95.9% of the control) compared with nontreated control groups, as well as nontargeting siRNA-treated groups (103.7% of the

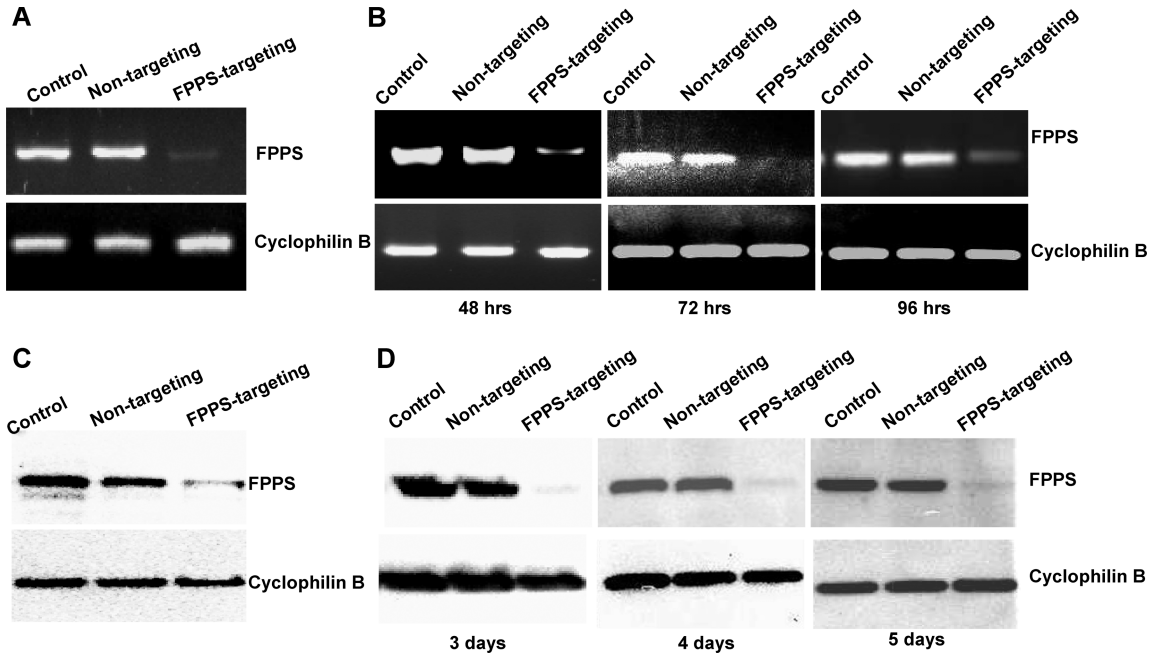

Figure 1. Successful FPPS message and protein knockdown in both osteoclast and osteoblast cultures. FPPS message knockdown by FPPS siRNA in (A) osteoclast culture 48 h post-single transfection and (B) osteoblast culture 48 to 96 h post-single transfection compared with untreated cells and cells transfected with nontargeting siRNA. Inhibition of target FPPS gene expression at the mRNA level is shown by PCR. FPPS protein knockdown by FPPS siRNA in (C) osteoclast 3 days post-single transfection and (D) osteoblast cultures 3 to 5 days post-single transfection compared with untreated cells and cells transfected with nontargeting siRNA. Protein expression was evaluated by Western blotting.

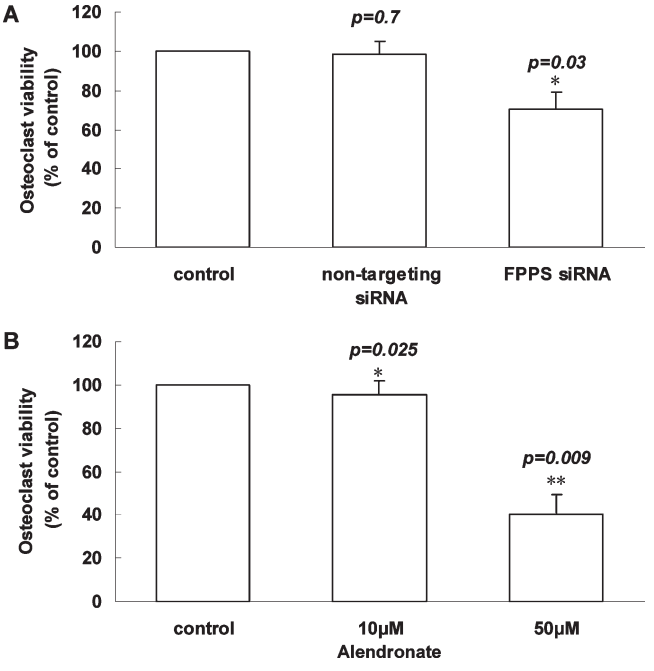

Figure 2. Both FPPS siRNA and alendronate reduce osteoclast cell viability. Cytotoxicity was determined using the Cell Titer 96 Aqueous One Solution Cell Proliferation assay. (A) Cultured osteoclast viability comparing nontargeting siRNA and FPPS siRNA three days post-transfection. (B) Osteoclast viability compared for 10 μM and 50 μM alendronate three days postdosing to cultures (p values shown are obtained by comparing each group with controls, $n = 3$).

control). Furthermore, there is no significant difference between FPPS siRNA and nontargeting siRNA groups ($p = 0.55$). For alendronate, three different concentrations (10 μM, 50 μM and 100 μM) were initially used. The 100 μM alendronate dose showed significant evidence of cell death (>90%), a likely result of bisphosphonate toxicity, and this concentration was not continued. Compared with nontreated controls, there is no significant inhibition of cell proliferation at 10 μM (Figure 3B, $p = 0.908$), but when alendronate's concentration increased to 50 μM, cell viability was reduced to 54% compared to control (Figure 3B, $^{***}p < 0.001$). There is also a significant difference in cell viability between the 50 μM and 10 μM groups ($^{***}p < 0.001$).

Comparison of FPPS siRNA and Alendronate Treatments on MC3T3-E1 Preosteoblast Cell Differentiation and Mineralization. MC3T3-E1 preosteoblast cell differentiation was detected by measuring ALP activity. After five-day culture, the effects of nontargeting siRNA, FPPS siRNA, and alendronate (10 μM and 50 μM) on MC3T3 cell differentiation were compared. As shown in Figure 4A, nontargeting siRNA had no effect on osteoblastic differentiation compared with control (99.5%, $p = 0.977$). However, FPPS siRNA treatment increased cell ALP activity and showed a significant 21% increase compared to nontreated control groups ($^{*}p = 0.049$). When alendronate concentration was 10 μM, cell ALP activity was reduced to 85.9% compared to control, but was not significant ($p = 0.12$). However, 50 μM alendronate treatment significantly increased cell ALP activity to 164% (Figure 4B, $^{*}p = 0.017$).

Full comparison of these effects on cell mineralization among nontargeting siRNA, FPPS siRNA and alendronate (10 μM and 50 μM) is summarized in Figure 5. Nontargeting siRNA reduced MC3T3 cell mineralization to 81% of control ($p = 0.081$). FPPS siRNA of single dose had no significant effect on cell mineralization (99.5%, $p = 0.758$) after 5-day culture compared with control (p value between nontargeting siRNA and FPPS siRNA groups is 0.078). There is no significant influence of 10 μM alendronate on

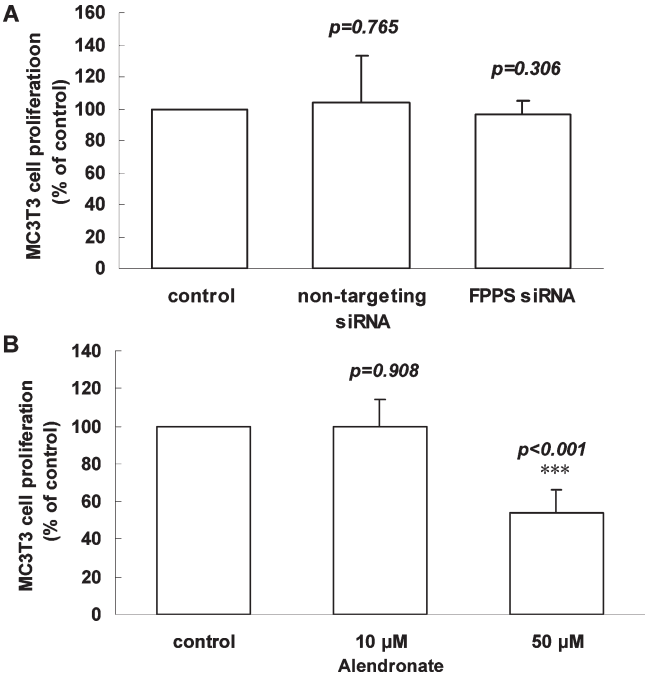

Figure 3. FPPS siRNA is not as potent as alendronate (50 μM) on reducing cultured MC3T3-E1 preosteoblast cell proliferation. Cell proliferation was determined using the Cell Titer 96 Aqueous One Solution Cell Proliferation assay. (A) Osteoblast viability comparing nontargeting siRNA and FPPS siRNA five days post-single transfection in serum-based cultures. (B) The comparison of osteoblast viability compared for 10 μM and 50 μM alendronate five days postdosing to cultures. (p values shown are obtained by comparing each group with controls, n = 3).

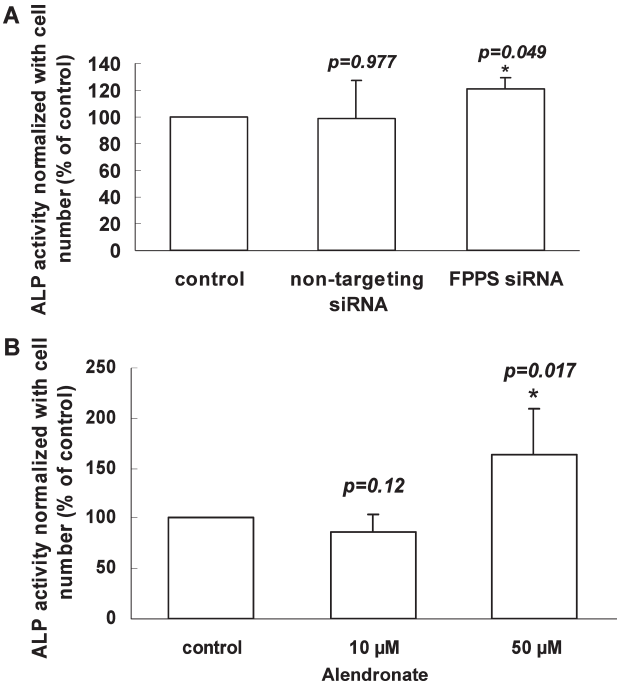

Figure 4. Both FPPS siRNA and alendronate (50 μM) significantly increase ALP activity during MC3T3-E1 preosteoblast differentation. Cell differentiation was monitored by measuring ALP activity. (A) Osteoblast ALP activity compared for nontargeting siRNA and FPPS siRNA five days post-single transfection in serum-containing cultures. (B) Osteoblast ALP activity compared for 10 μM and 50 μM alendronate five days postdosing to cultures (p values shown are obtained by comparing each group to controls, n = 3).

cell mineralization (95.3%, p = 0.318). However, 50 μM alendronate increased cell mineralization to 155.5% compared to controls (p = 0.058). Cell mineralization under triplicate FPPS siRNA transfections on every other day showed 9.98% increase compared with control (not significantly different). Triplicate nontargeting siRNA transfection showed only 0.51% increase compared with control (Figure 5C). In addition, both nontargeting and FPPS siRNA (single and triple dosing) have no observed influence on osteoblast proliferation (Figure 5D).

■ DISCUSSION

Bisphosphonates are potent inhibitors of bone resorption and widely used in clinic to treat postmenopausal osteoporosis. However, more attention has been drawn recently to their side effects, such as bisphosphonate-associated osteonecrosis and their long half-life that complicates fracture healing.[7] Inhibition of FPPS in the mevalonate pathway has been implicated as the mechanism of action for select N-BPs at the molecular level in both osteoclasts and osteoblasts.[11−13,20,34] Osteoclasts and osteoblasts are closely correlated with each other; therefore, in our study use of siRNA to knock down FPPS expression in both osteoclasts and osteoblasts was examined and compared with N-BP alendronate. Alendronate is one of the most potent FDA-approved N-BPs,[35] commonly prescribed for the treatment and prevention of osteoporosis. Actual dosing ranges of alendronate to which bone cells are exposed under pharmacological conditions are unknown. Many studies have shown that bisphosphonate affects cells in a dose-dependent manner in cultures.[20,36,37] We therefore employed three alendronate concentrations (10 μM, 50 μM and 100 μM) in cell cultures. Alendronate at 100 μM concentration significantly reduced cell viability in both cell types, consistent with the observation of Im et al.[38] who showed inhibited cellular proliferation when alendronate was used at 100 μM. We chose alendronate working concentration within the range of 10−100 μM since significant effects on cells have been observed in other studies.[20,36,37,39]

FPPS siRNA-2 provides the highest knockdown of FPPS mRNA in primary BMC cultures in our preliminary studies (data not shown) and was used for all experiments. A dose of 125 nM siRNA and the commercial cationic lipid transfection reagent, DF4, demonstrated successful inhibition of both the target FPPS gene and protein expression 48 h and 72 h, respectively, after transfection in serum-based culture for osteoclasts. In osteoblast cultures, successfully sustained gene and protein knockdown were obtained up to 5 days post-transfections. In addition, evidence indicates that FPPS message knockdown is not caused by the transfection reagent, as there was no significant reduction of FPPS mRNA or protein expression in cells transfected with nontargeting siRNA/DF4 complexes.

Classically, the majority of reports regarding bisphosphonates have been focused on their effects on reducing osteoclast bone resorbing activities.[40−45] A variety of modes of drug action on osteoclasts have been explored. The present study focused on cytotoxic depletion of osteoclasts since both in vitro and in vivo studies provide evidence that bisphosphonates reduce osteoclast numbers and activity.[43] FPPS siRNA showed significant inhibition on growth of cultured murine bone marrow cell-induced osteoclasts. This suppression is stronger than that from 10 μM

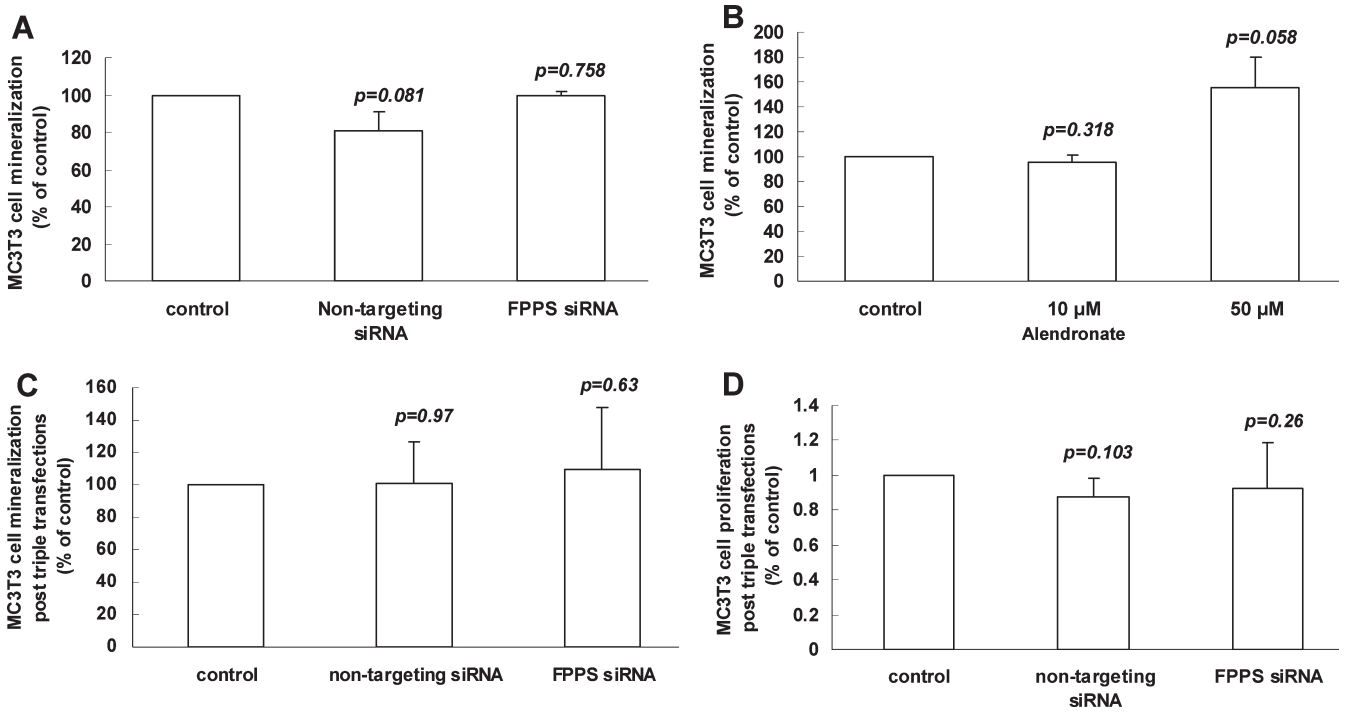

Figure 5. Neither FPPS siRNA nor alendronate significantly affect MC3T3-E1 preosteoblast cell mineralization. Mineralization of MC3T3-E1 cell cultures was determined using alizarin red staining. (A) Osteoblast mineral deposition compared for nontargeting siRNA and FPPS siRNA five days post-single transfection and (C) post-triple transfections in serum-based cultures. (B) Osteoblast mineral deposition compared for 10 μM and 50 μM alendronate five days postdosing to cultures. (D) Osteoblast proliferation after triplicate nontargeting and FPPS siRNA transfections (p values shown are obtained by comparing each group to controls, $n = 3$).

alendronate, but not as potent as that from alendronate at 50 μM. Cell viability can be reduced to 40% by alendronate at 50 μM compared with the nontreated control. FPPS siRNA effects on cell viability are less than expected based on the substantial mRNA and protein knockdown results, exhibiting about 30% reduction compared with control. A possible explanation for this could be intrinsic disproportionate relationships between cell viability and FPPS enzyme activity. Unlike other functional cell proteins, e.g. cell receptors where decreased protein expression leads to proportionate decreases in functions, enzymatic turnover activity can remain significant despite relative knockdown, distinguishing functional differences between knockdown and knockout. Inhibition of the mevalonate pathway has been linked to bisphosphonate-induced apoptosis in macrophages and human myeloma cells in vitro.[46,47] These reports suggest that reduced osteoclast viability results from FPPS siRNA-induced cell apoptosis by preventing protein prenylation in osteoclasts.

Except for the effect of bisphosphonate on osteoclast apoptosis, it was also reported to be able to inhibit osteoclast formation in long-term human marrow culture alone[40] or by acting on osteoblastic cells.[48] In addition, mevalonate pathway is essential for producing DMAPP and IPP which involve in the biosynthesis of molecules for cell memebrane maintenance. Taken together, it would be interesting to further investigate the effects of FPPS siRNA on osteoclastogenesis and osteoclast podosome assembly and actin ring formation which play a critical role of osteoclastic bone resorption.

Increasing evidence showing that N-BPs directly affect osteoblast proliferation, differentiation and mineralization[37,49,50] in ways distinct from those of osteoclasts prompted study of the role of FPPS siRNA on the mevalonate pathway in MC3T3-E1 preosteoblast cells cultures. Changes in MC3T3-E1 cell viability in response to FPPS siRNA addition to cultures was compared to single alendronate treatments. As expected, nontargeting siRNA does not significantly influence cultured cell proliferation compared with controls without any treatment. However, FPPS siRNA addition does not significantly change cell proliferation compared with both control and nontargeting siRNA. This suggests that FPPS knockdown is not sufficient to influence MC3T3-E1 viability within 5 days. No difference is observed on cell viability between cells without any treatment and those treated with 10 μM alendronate. However, when alendronate concentration increased to 50 μM, cell viability was inhibited dramatically. During culture, significant numbers of dead cells were not evident, suggesting that the decreased cell viability results from suppression of cell proliferation, but not cytotoxicity. This observation is not surprising since similar alendronate inhibition of cell proliferation is also reported with human fetal osteoblast cells,[37] osteoclasts,[43,51] and primary osteoblasts.[50,52] Though some opposing effects have been reported,[38,53,54] this may be due to variations in experiments, including cell types, culture duration, bisphosphonate analogue chemistries and concentrations applied.

While FPPS siRNA did not influence osteoblast viability significantly, it increases total alkaline phosphatase activity significantly compared with controls lacking any treatment. These observations indicate that FPPS knockdown enhances osteoblast differentiation without affecting cell proliferation. Stronger induction of alkaline phosphatase activity was obtained by treating these cells with 50 μM alendronate. These findings concur with those of a previous study suggesting that bisphosphonates enhance osteoblast progression from the proliferation stage to the matrix

maturation stage.[37] However, culture mineralization was increased in MC3T3 cells treated with 50 μM alendronate by 55.5%, nearly significant compared with control (p = 0.058) and correlates with previous reports[20] showing increased human fetal osteoblast cell mineralization by pamidronate and zoledronate. Thus, N-BP alendronate augments cultured cell mineralization within 5 days post-treatment. In contrast, for osteoblasts treated with siRNA, there is no significant difference in mineralization between the control and nontargeting siRNA or FPPS siRNA after single transfections. To investigate the possibility for disproportionate functional knockdown of cell enzyme (ALP) activity, triplicate siRNA transfections dosed every other day were performed to minimize FPPS expression during the subsequent cell assay period. While triplicate dosing of nontargeting siRNA produces no observable effects on cell mineralization, multiple FPPS transfections generates about 10% increase of cell mineralization compared with control. This observed increase may suggest some small cell mineralization enhancement from FPPS knockdown. However, FPPS knockdown did not enhance cell mineralization compared with 50 μM alendronate. Although alendronate at 50 μM can promote cell mineralization by 56%, this effect is obscured by its severe suppression of cell proliferation. Cell mineral deposition by 50 μM alendronate in term of percentage of cell proliferation (mineralization % $\times$ proliferation %) is 84% compared to the controls, while mineralization from FPPS single transfection is 95% and by triple transfections is 101.7% of the controls. Overall, the effect of FPPS knockdown on cell mineralization is not significant. Based on the siRNA results, the direct effect of FPPS changes on cell mineralization is not clear. Osteoclasts and osteoblasts closely communicate and interact with each other in vivo to regulate bone remodeling. Bisphosphonates can act indirectly on osteoclasts through their action on osteoblast lineage cells.[39,55−58] Therefore, further study is required to determine whether FPPS siRNA effects on osteoblasts seen in this study indirectly influence osteoclasts.

In summary, single-dose FPPS siRNA, unlike results obtained from osteoclast cultures, did not show any significant cytotoxicity in MC3T3-E1 preosteoblasts. Compared with 50 μM alendronate which exhibits potent inhibition of cell viability, FPPS siRNA has much milder effects on cell proliferation. Both FPPS siRNA and 50 μM alendronate can promote cell differentiation significantly, but FPPS siRNA increases ALP activity without significantly inhibiting cell proliferation. Within 5 days post-treatment, FPPS siRNA did not evidence an influence on cell mineral deposition.

CONCLUSION

Significantly, to the best of our knowledge, this is the first report to knock down the molecular target of clinically common nitrogen-containing bisphosphonates using siRNA, and comparing these effects to alendronate, both on osteoclasts and osteoblasts in similar cultures. Results from both the culture of primary murine bone marrow cell-induced osteoclasts and MC3T3-E1 preosteoblast cells showed that FPPS siRNA suppresses osteoclast viability significantly, but with less potency than alendronate at 50 μM. There is no observed effect of FPPS siRNA on osteoblast proliferation. However, osteoblast differentiation can be significantly increased by FPPS siRNA. Both FPPS siRNA and alendronate show no significant effects on osteoblast mineral deposition. In summary, siRNA targeting FPPS has a beneficial effect at the cellular level of inhibiting excessive bone resorption and increasing overall bone mass maintenance by its effects on both osteoblasts and osteoclasts and suggest that it has clinical potential.

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ABBREVIATIONS USED

AA, ascorbic acid; FPP, farnesyl pyrophosphate; FPPS, farnesyl pyrophosphate synthase; M-CSF, macrophage-colony stimulating factor; RANK, receptor activator of nuclear factor κB; RANKL, RANK ligand; N-BPs, nitrogen-containing bisphosphonates; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; GST, glutathione-*S*-transferase; RNAi, RNA interference; siRNA, small interfering RNA; BMC, bone marrow cell; DF, DharmaFECT; ALP, alkaline phosphatase; PBS, phosphate buffered saline; α-MEM, α-minimal essential medium; FBS, fetal bovine serum; PCR, polymerase chain reaction.

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