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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 knock-down 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 knock-down in serum-based culture is correlated with reduced osteoclast viability. FPPS siRNA is more potent than 10 μ M alendronate, but less potent than 50 μ M alendronate on reducing osteoclast viability. Despite FPPS knock-down, no significant changes were observed in osteoblast proliferation. FPPS knock-down promotes osteoblast differentiation significantly but not cell mineral deposition. However, compared with 50 μ M alendronate dosing, FPPS siRNA does not exhibit cytotoxic effects on osteoblasts while producing significant effects on osteoblast 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 micro-architectural deterioration.¹ With

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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 analogs 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-life⁶ 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 C₁₅ 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 C₂₀ isoprenoid geranylgeranyl 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} Down-regulation 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 week-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 (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”. 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, adherent cells were used as osteoclast precursors. To generate osteoclasts, these precursor cells were incubated in 200ng/ml RANKL and 30ng/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 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 μ g/ml ascorbic acid (AA, Sigma) and 10mM β -glycerophosphate (Sigma).³⁰ Media was refreshed every other day.

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-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 non-targeting 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 manufacturer's instructions (Dharmacon). Final

dosing concentrations of all siRNAs provided to each well were 125nM 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 with 30ng/ml M-CSF and 200ng/ml RANKL at 37°C with 5% CO₂. Non-specific knock-down from DF4 transfection was assessed by using non-targeting 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 50µg/ml AA and 10mM β-glycerophosphate at 37°C with 5% CO₂.

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 micrograms of RNA were used to make cDNA with the SuperScript III 1st 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 anti-mouse 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, media was replaced with 300µl/well of fresh complete media with the addition of 60µl of Cell Titer 96 Aqueous One Solution, including three wells containing only media for background subtraction. Cells were then incubated at 37°C with 5% CO₂ for 2 hours and optical absorbance at 490nm 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 media containing various concentrations of alendronate (gift from Prof. J. Kopecek, 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, media was aspirated and the cells were rinsed twice with PBS. Then cells were fixed with ice-cold 70% (v/v) ethanol for 1 hour. 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 removing the water, cells were incubated in PBS for 15 minutes 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 10mM 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

MTT, ALP and mineralization results are reported as mean $\pm$ 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 knock-down in both osteoclast and osteoblast cultures

Since this study's focus was not on assessing transfection efficiency but rather on assessing post-transfection knock-down 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 media-induced cell stress. Each siRNA sequence was tested individually for effectiveness to knock down FPPS expression in osteoclasts in complete media. siRNA-2 produced the greatest overall knock-down effect from the commercial library, and therefore, was used for all further siRNA transfections of both osteoclast and osteoblast cultures. Non-specific knock-down of FPPS target by DF4, evaluated using non-targeting siRNA and DF4 in both cultures, is not significant by PCR analysis (Figure 1). Significant down-regulation 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 non-targeting siRNA-transfected osteoblasts (Figure 1B). Significantly, sustained down-regulation 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 non-targeting 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 non-specific effects of siRNA or transfection reagent dosing. Osteoclasts were treated with alendronate at two different concentrations (10 μ M and 50 μ M). After three day-culture, (the same duration as siRNA transfection), cell viability was reduced to 95.5% ($*p=0.025$) in the 10 μ M alendronate-treated group and to 40.3% ($**p=0.009$) in the 50 μ M 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 non-treated control groups, as well as non-targeting siRNA-treated groups (103.7% of the control). Furthermore, there is no significant difference between FPPS siRNA and non-targeting siRNA groups ($p=0.55$). For alendronate, three different concentrations (10 μ M, 50 μ M and 100 μ M) were initially used. The 100 μ M alendronate dose showed significant evidence of cell death (>90%), a likely result of bisphosphonate toxicity, and this concentration was not continued. Compared with non-treated controls, there is no significant inhibition of cell proliferation at 10 μ M (Figure 3B, $p=0.908$), but when alendronate's concentration increased to 50 μ M, cell viability is reduced to 54% compared to control (Figure 3B, $***p<0.001$). There is also a significant difference in cell viability between the 50 μ M and 10 μ M 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 non-targeting siRNA, FPPS siRNA, and alendronate (10 μ M and 50 μ M) on MC3T3 cell differentiation were compared. As shown in Figure 4A, non-targeting 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 non-treated control groups ($*p=0.049$). When alendronate concentration was 10 μ M, cell ALP activity was reduced to 85.9% compared to control, but was not significant ($p=0.12$). However, 50 μ M alendronate treatment significantly increased cell ALP activity to 164% ($*p=0.017$).

Full comparison of these effects on cell mineralization among non-targeting siRNA, FPPS siRNA and alendronate (10 μ M and 50 μ M) is summarized in Figure 5. Non-targeting 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 non-targeting siRNA and FPPS siRNA groups is 0.078). There is no significant influence of 10 μ M alendronate on cell mineralization (95.3%, $p=0.318$). However, 50 μ M 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 non-targeting siRNA transfection showed only 0.51% increase compared with control (Figure 5C). In addition, both non-targeting 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 post-menopausal 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 μ M, 50 μ M and 100 μ M) in cell cultures. Alendronate at 100 μ M 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 μ M. We chose alendronate working concentration within the range of 10–100 μ M since significant effects on cells have been observed in other studies.^{20, 36, 37, 39}

FPPS siRNA-2 provides the highest knock-down of FPPS mRNA in primary BMC cultures in our preliminary studies (data not shown) and was used for all experiments. A dose of 125nM siRNA and the commercial cationic lipid transfection reagent, DF4, demonstrated successful inhibition of both the target FPPS gene and protein expression 48h and 72h, 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 knock-down is not caused by the transfection reagent, as there was no significant reduction of FPPS mRNA or protein expression in cells transfected with non-targeting 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 μ M alendronate, but not as potent as that from alendronate at 50 μ M. Cell viability can be reduced to 40% by alendronate at 50 μ M compared with the non-treated control. FPPS siRNA effects on cell viability are less than expected based on the substantial mRNA and protein knock-down 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 turn-over activity can remain significant despite relative knock-down, distinguishing functional differences between knock-down and knock-out. 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 alone⁴⁰ 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 membrane 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 pre-osteoblast cells cultures. Changes in MC3T3-E1 cell viability in response to FPPS siRNA addition to cultures was compared to single alendronate treatments. As expected, non-targeting 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 non-targeting 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 μ M alendronate. However, when alendronate concentration increases to 50 μ M, 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 knock-down enhances osteoblast differentiation without affecting cell proliferation. Stronger induction of alkaline phosphatase activity was obtained by treating these cells with 50 μ M 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 μ M alendronate by 55.5%, nearly significant compared with control ($p=0.058$) and correlates with previous reports²⁰ 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 non-targeting 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 non-targeting 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 μ M alendronate. Although alendronate at 50 μ M can promote cell mineralization by 56%, this effect is obscured by its severe suppression of cell proliferation. Cell mineral deposition by 50 μ M 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 50μM alendronate which exhibits potent inhibition of cell viability, FPPS siRNA has much milder effects on cell proliferation. Both FPPS siRNA and 50μM 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 50μM. 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

AA	ascorbic acid
FPP	farnesyl pyrophosphate
FPPS	farnesyl pyrophosphate synthase
M-CSF	macrophage-colony stimulating factor
RANK	receptor activator of nuclear factor B
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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Figure 1. Successful FPPS message and protein knock-down in both osteoclast and osteoblast cultures

FPPS message knock-down by FPPS siRNA in (A) osteoclast culture 48 hrs post-transfection and (B) osteoblast culture 48 to 96 hrs post-single transfection compared with untreated cells and cells transfected with non-targeting siRNA. Inhibition of target FPPS gene expression at the mRNA level is shown by PCR. FPPS protein knock-down by FPPS siRNA in (C) osteoclast 3 days post-single transfection and (D) osteoblast cultures 3 to 5 days post-transfection compared with untreated cells and cells transfected with non-targeting 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 non-targeting siRNA and FPPS siRNA three days post-transfection. (B) osteoclast viability compared for 10µM and 50µM alendronate three days post-dosing to cultures (p values shown are obtained by comparing each group with controls, n=3).

Figure 3. FPPS siRNA is not as potent as alendronate (50 μ M) on reducing cultured MC3T3-E1 pre-osteoblast cell proliferation

Cell proliferation was determined using the Cell Titer 96 Aqueous One Solution Cell Proliferation assay. (A) osteoblast viability comparing non-targeting siRNA and FPPS siRNA five days post-single transfection in serum-based cultures. (B) The comparison of osteoblast viability compared for 10 μ M and 50 μ M alendronate five days post-dosing to cultures. (p values shown are obtained by comparing each group with controls, n=3).

Figure 4. Both FPPS siRNA and alendronate (50μM) significantly increase ALP activity during MC3T3-E1 pre-osteoblast differentiation

Cell differentiation was monitored by measuring ALP activity. (A) osteoblast ALP activity compared for non-targeting siRNA and FPPS siRNA five days post-single transfection in serum-containing cultures. (B) osteoblast ALP activity compared for 10μM and 50μM alendronate five days post-dosing to cultures (p values shown are obtained by comparing each group to controls, n=3).

Figure 5. Neither FPPS siRNA nor alendronate significantly affect MC3T3-E1 pre-osteoblast cell mineralization

Mineralization of MC3T3-E1 cell cultures was determined using alizarin red staining. (A) osteoblast mineral deposition compared for non-targeting 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 μ M and 50 μ M alendronate five days post-dosing to cultures. (D) osteoblast proliferation after triplicate non-targeting and FPPS siRNA transfections (p values shown are obtained by comparing each group to controls, n=3).