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RESEARCH PAPER

Protein isoprenylation regulates osteogenic differentiation of mesenchymal stem cells: effect of alendronate, and farnesyl and geranylgeranyl transferase inhibitors

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BACKGROUND AND PURPOSE

Protein isoprenylation is an important step in the intracellular signalling pathway conducting cell growth and differentiation. In bone, protein isoprenylation is required for osteoclast differentiation and activation. However, its role in osteoblast differentiation and function remains unknown. In this study, we assessed the role of protein isoprenylation in osteoblastogenesis in a model of mesenchymal stem cells (MSC) differentiation.

EXPERIMENTAL APPROACH

We tested the effect of an inhibitor of farnesylation [farnesyl transferase inhibitor-277 (FTI-277)] and one of geranylgeranylation [geranylgeranyltransferase inhibitor-298 (GGTI-298)] on osteoblast differentiating MSC. In addition, we tested the effect of alendronate on protein isoprenylation in this model either alone or in combination with other inhibitors of isoprenylation.

KEY RESULTS

Initially, we found that levels of unfarnesylated proteins (prelamin A and HDJ-2) increased after treatment with FTI-277 concomitantly affecting osteoblastogenesis and increasing nuclear morphological changes without affecting cell survival. Furthermore, inhibition of geranylgeranylation by GGTI-298 alone increased osteoblastogenesis. This effect was enhanced by the combination of GGTI-298 and alendronate in the osteogenic media.

CONCLUSIONS AND IMPLICATIONS

Our data indicate that both farnesylation and geranylgeranylation play a role in osteoblastogenesis. In addition, a new mechanism of action for alendronate on protein isoprenylation in osteogenic differentiating MSC *in vitro* was found. In conclusion, protein isoprenylation is an important component of the osteoblast differentiation process that could constitute a new therapeutic target for osteoporosis in the future.

Abbreviations

ALP, alkaline phosphatase; EDTA, ethylenediamine tetra-acetic acid; EGTA, ethylene glycol tetraacetic acid; FCS, fetal calf serum; FTI, farnesyl transferase inhibitor; GGPP, geranylgeranyl-pyrophosphate; MSC, mesenchymal stem cells; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium; OCN, osteocalcin; OIM, osteoblastogenesis induction media; pNPP, p-nitrophenyl phosphate; RAP-1, ras-like protein 1; RUNX2, runt-related transcription factor 2; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling

Introduction

Protein isoprenylation is an important step in the intracellular signalling pathway conducting cell growth and differentiation. Isoprenylated proteins have key roles in membrane attachment and protein functionality, have been shown to have a central role in some cancers and are likely to also be involved in the pathogenesis and progression of atherosclerosis and Alzheimer's disease (McTaggart, 2006). Isoprenylation occurs by two different mechanisms: farnesylation and geranylgeranylation (Rusiñol and Sinensky, 2006). These two mechanisms involve the activity of two different enzymes: the Caa*X* prenyltransferase protein farnesyltransferase (FTase) and protein geranylgeranyltransferase type I (GGTase-I), which add either a 15-carbon farnesyl group or a 20-carbon geranylgeranyl group, respectively, to the cysteine found within the Caa*X* motif (Rusiñol and Sinensky, 2006). This addition will induce the activation of multiple proteins required for cell differentiation and function (Sebti, 2005).

In order to exert their function, some proteins involved in cell differentiation require either farnesylation or geranylgeranylation. For instance, lamin A/C is a protein of the nuclear envelope that requires farnesylation in order to participate in osteoblastogenesis (Rivas *et al.*, 2009) and HDJ-2 is a chaperone protein that activates a multiple cell trafficking process after farnesylation (McTaggart, 2006). In contrast, other proteins typically require geranylgeranylation. The characteristic example is the ras-like protein 1 (RAP-1), which after geranylgeranylation activates endocytosis and cell differentiation (Coxon *et al.*, 2005; Taniguchi *et al.*, 2008).

In bone, protein isoprenylation has been found to be essential for osteoclastogenesis and bone resorption (Coxon *et al.*, 2000; Russell, 2006). This process is inhibited in osteoclasts by nitrogen-containing bisphosphonates (Coxon *et al.*, 2000; Russell, 2006), which are the most common treatment for osteoporosis. This effect is induced mostly through the inhibition of geranylgeranylation rather than farnesylation (Coxon *et al.*, 2000). Indeed, osteoclast precursors treated with alendronate, a nitrogen-containing bisphosphonate, exhibited diminished differentiation, disruption in their cytoskeleton, inhibition of bone resorption and higher level of apoptosis, all associated with inhibition of geranylgeranylation (Russell, 2006). This effect is not exclusive to bisphosphonates, since treatment of bone marrow cells during osteoclastogenesis with the specific inhibitor of protein geranylgeranylation GGTase inhibitor-298 (GGTI-298) resulted in similar changes to those induced by alendronate (Coxon *et al.*, 2000). In general, inhibition of geranylgeranylation by bisphosphonates has an anti-fracture effect through the inhibition of bone resorption and thus results in an increase in bone mass (Coxon *et al.*, 2000).

In contrast to the well-known role of protein isoprenylation in osteoclasts, its role in osteoblast function and differentiation remains unknown. It would be expected that, as in other cell lines (McTaggart, 2006), protein isoprenylation would play an important role during osteoblast differentiation of mesenchymal stem cells (MSC). In addition, recent reports of a direct effect of alendronate on MSC differentiation (Duque and Rivas, 2007; Fu *et al.*, 2008) suggest that this effect may be exerted through the regulation of protein isoprenylation during osteoblast differentiation. In this study, we hypothesized that protein isoprenylation plays an important role in osteogenic differentiation of MSC and that the osteogenic effect of alendronate on differentiating MSC could be exerted through the regulation of this process. In the present study, we treated osteogenic differentiating MSC with FTase inhibitor-277 (FTI-277) and GGTI-298 alone or in combination with a known osteogenic dose of alendronate (Duque and Rivas, 2007). In addition, we investigated the effect of inhibiting either farnesylation or geranylgeranylation on MSC differentiation and survival. Our data showed that inhibition of either farnesylation or geranylgeranylation affected osteoblast differentiation and function in a negative and positive manner respectively. Furthermore, addition of alendronate to the osteogenic media enhanced the effect of protein isoprenylation inhibitors on osteoblast differentiating MSC. Taken together, our data suggest that protein isoprenylation is an important component of the osteoblast differentiation process that could constitute a new therapeutic target for osteoporosis in the future.

Methods

Reagents

FTI-277 and GGTI-298 were purchased from Sigma Chemical (St. Louis, MO, USA), alendronate was provided by Merck Pharmaceuticals (Whitehouse Station, NJ, USA). Alendronate was dissolved in phosphate-buffered saline (PBS) and the pH was adjusted to 7.4 with 1 M NaOH and then filter-sterilized by using a 0.2 μm filter. FTI-277 and GGTI-298 were dissolved in dimethyl sulphoxide and then filter-sterilized using a 0.2 μm filter. Other reagents were from Sigma Chemical Co. unless stated otherwise.

Osteogenic differentiation of MSC in vitro

Human MSC were purchased from Lonza (Walkersville, MD, USA). These cells were obtained from healthy 24-year-old male donors (n = 3). Cells at passages three and four from time of marrow harvest were used in these experiments. The induction of osteogenic differentiation of MSC was as previously described (Duque and Rivas, 2007). Briefly, cells were plated at a density of 5×10^5 cells per well in 100 cm^2 dishes containing MSC growth medium (BioWhittaker, Walkersville, MD, USA) with 10% fetal calf serum (FCS) and incubated at 37°C for 24 h. After the cells reached 60% confluence, media was replaced with either MSC growth medium or osteoblastogenesis induction medium (OIM) prepared with MSC growth medium, 10% FCS, dexamethasone (200 μM) β glycerol phosphate (1 M) and ascorbic acid (100 mM). Media was changed every 3 days. Osteogenesis was assessed at week 3 using alkaline phosphatase (ALP) staining and activity and mineralization was assessed at week 3 using Alizarin red staining.

Cells treatment

Human MSC were plated in 6-well plates (3×10^3 cells per well). After confluence, MSC growth media was replaced with OIM containing FTI-277 (5 or 10 μM) alone or in combination with alendronate (10^{-7} to 10^{-9} M) and GGTI-298 (2.5, 5, 7.5 and 10 μM) alone or in combination with alendronate

(10^{-7} to 10^{-9} M). Media was replaced every 3 days. In all experiments, untreated differentiating MSC were used as control.

Western blot analysis

Mesenchymal stem cells were treated as previously described and then lysed at week 2 in 20 mM Tris–HCl, pH 7.5, 200 mM DTT, 200 mM KCl, 0.5 mL glycerol and protease inhibitor tablets (Roche Diagnostics Canada, Laval, QC, Canada), freeze-thawed three times in a dry ice-ethanol bath and centrifuged at 13 000× *g* for 15 min to remove insoluble material. Before electrophoresis, cell lysates were equalized as determined with a commercial kit (Bio-Rad, Hercules, CA, USA) and then dissolved in SDS electrophoresis buffer (Bio-Rad). Thirty micrograms of protein per well were separated on SDS-polyacrylamide gels and subsequently electrotransfered to polyvinylidene difluoride membranes. After blocking with PBS containing 0.1% Tween 20 and 10% non-fat dry milk, membranes were incubated overnight at 4°C using an antibody directed against runt-related transcription factor 2 (Runx2) (1:100; Oncogene, Cambridge, MA, USA), osteocalcin (OCN) (1:200), chaperone protein HDJ-2 (1:400), prelamin A/C (recognizing farnesylated and unfarnesylated proteins) (1:400), and unprenylated (sc-1842) and total RAP-1 (1:400) (sc-65), all from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The bound antibodies were detected with the corresponding secondary antibodies (1:10 000) conjugated with horseradish peroxidase. Blots were developed by enhanced chemiluminescence using Perkin-Elmer reagents (Perkin-Elmer, Boston, MA, USA). The Western blot bands were quantified using the Image Processing Tool Kit v.5 Plus (Fovea Pro 3.0, Asheville, NC, USA).

Identification of nuclear morphological changes by bisbenzimide (Hoechst 33342) staining

Cells were plated in two chamber slides (Nalge Nunc, Rochester, NY, USA) at a density of 5000 per cm^2, induced to differentiate and treated as previously described. After 2 weeks of differentiation and treatment cells were fixed using 70% ethanol for 20 min. After thorough washing in PBS, cells were stained for nuclear blue fluorescence using blue-fluorescent bisbenzimide trihydrate dye (Hoechst 33342, Invitrogen, Burlington, ON, Canada) nuclei were then observed via UV light using an Olympus IX-70 microscope (Olympus, London, UK). Cells with nuclear morphological changes were considered those with blebbing, irregular shape, nodulation and vacuolization (Capell *et al.*, 2005). The number of cells showing nuclear morphological changes in a minimum of 100 cells in ten fields per well was quantified by three different observers, which were blind to the treatment conditions of the samples. This experiment was repeated three times.

Identification of the effect of FTI-277 and GGTI-298 with and without alendronate on osteoblast differentiation and activity

Human MSC were plated in 4 cm^2 dishes in a density of 4×10^4 cells per dish. At 60% confluence, media was replaced with OIM containing either FTI-277 (5 and 10 μM) or GGTI-298 (2.5–10 μM) with and without alendronate (10^{-8} M). At week 1, media was aspirated and cells were stained for ALP using RR-blue+ staining. For this staining, cells were washed first with 10 mL PBS and then fixed with citrate-buffered acetone for 30 s. After new washing, cells were stained with a solution consisting of diazonium salt (Fast Blue RR Salt capsule) and 2 mL naphthol AS-MX phosphate alkaline solution and then incubated at 18–26°C for 30 min. ALP activity was quantified using the SigmaFAST p-nitrophenyl phosphate (pNPP). Briefly, after incubation with ALP conjugate, cells were washed and 200 μl of pNPP substrate solution added to each well. Plates were incubated in the dark for 30 min at room temperature and then read at 405 nm by spectophotometry (FLUOstar, BMG Labtech, Durham, NC, USA).

At late osteogenic differentiation (week 3), calcium deposition was also quantified using Alizarin red staining. Briefly, after Alizarin red staining, matrix mineralization was quantified by extracting the Alizarin red staining with 100 mM cetypyridinium chloride at room temperature for 3 h. The absorbance of the extracted Alizarin red was measured at 570 nm. Experiments were performed in duplicated wells and were repeated three times.

Determination of cell viability and apoptosis

To test whether treatment with inhibitors of isoprenylation has any effect on cell survival, differentiating MSC were seeded in 96-well plates. At 60% confluence, media was replaced with OIM containing either FTI-277 (5 and 10 μM) or GGTI-298 (2.5–10 μM) with and without alendronate (10^{-8} M). At timed intervals (week 1, 2 and 3 of differentiation), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS)-Formazan cell viability assays (Promega, Madison, WI, USA) were performed and corrected for cell number as previously described (Rivas *et al.*, 2007). Briefly, a stock solution of MTS was dissolved in PBS at a concentration of 5 $mg \cdot mL^{-1}$ and was added in a 1:10 ratio (MTS/DMEM) to each well, incubated at 37°C for 2 h, and the optical density determined at a wavelength of 490 nm on a microplate reader model 3550 (FLUOstar, BMG Labtech). The percentage of survival was defined as [(experimental absorbance – blank absorbance) / (control absorbance – blank absorbance)] × 100, where the control absorbance is the optical density obtained for 1×10^4 cells per well (number of cells plated at the start of the experiment), and blank absorbance is the optical density determined in wells containing medium and MTS alone. This experiment was replicated three times.

Furthermore, for quantification of apoptosis, terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) reaction was performed using the Apoptag fluorescein direct *in situ* apoptosis detection kit (Chemicon, Temecula, CA, USA) as previously described (Duque *et al.*, 2004). Briefly, 6×10^5 MSC were seeded in two-well glass chamber slides (Nalge Nunc) and induced to differentiate in osteoblastogenesis induction medium for 48 h at which point they were treated with either FTI-277 (5 and 10 μM) or GGTI-298 (2.5–10 μM) with and without alendronate (10^{-8} M). After 72 h of treatment, cells were fixed in 4% paraformaldehyde for 10 min, washed in 10 mM Tris–HCl, pH 8.0, and preincubated for 10 min at room temperature in the reaction

buffer for terminal deoxynucleotidyl transferase reaction (200 mM potassium cacodylate, 0.22 mg·mL^{-1} BSA and 25 mM Tris–HCl, pH 6.6). The preincubation buffer was then removed, and a reaction mixture containing 500 U·mL^{-1} terminal deoxynucleotidyl transferase, 25 mM $CoCl_2$ and 40 μM biotinylated dUTP was added for 60 min at 37°C. The reaction was terminated by the addition of 300 mM NaCl and 30 mM sodium citrate for 25 min at room temperature and for 60 min at room temperature in the dark. Propidium iodide was added to cell suspensions at a concentration of 5 μg·mL^{-1}. Slides were mounted and observed through fluorescence microscopy. The proportion of apoptotic cells was quantified in ten fields per well by three different observers, which were blind to the treatment conditions. This experiment was repeated three times. Jurkat cells induced to apoptosis by serum deprivation were used as a positive control.

Furthermore, occurrence and mechanism of apoptosis were assessed by analysis of caspase-3/7 activity. MSC (6×10^5) were seeded in 6-well plates and left in culture in MSC growth medium until 60% confluence at which point they were treated with OIM containing either FTI-277 (5 and 10 μM) or GGTI-298 (2.5–10 μM) with and without alendronate (10^{-8} M). After 72 h of treatment, cells were lysed in ice-cold buffer (20 mM Tris, pH 7.9, 1 mM EDTA, 1 mM EGTA, 0.5 mM DTT, 400 mM NaCl and 0.5 mL glycerol) containing protease inhibitor tablets (Roche Diagnostics Canada), freeze-thawed 3 times in a dry ice-ethanol bath and centrifuged at 13 000× *g* for 15 min to remove insoluble material. Protein concentrations were determined as previously described. Caspase-3/7 activity was measured by using Caspase Glo-3/7 assay systems (Promega). Samples (100 μL) were gently mixed with Caspase-Glo substrate (100 μL) and the luminescence of each sample was measured by using Luciferase assay system (Promega).

Statistical analysis

All results are expressed as mean ± SEM of three replicate determinations, and statistical comparisons are based on two-way analysis of variance (ANOVA) or Student's *t*-test. A probability value of $P < 0.05$ was considered significant.

Results

Role of protein farnesylation in osteogenic differentiation of MSC

We assessed the effect of FTI-277 on HDJ-2 and prelamin A. These two proteins, when unprenylated, are suitable markers of effective inhibition of farnesylation (Kelland *et al.*, 2001). As shown in Figure 1, after 2 weeks of treatment FTI-277 inhibited both HDJ-2 and prelamin A farnesylation as demonstrated by the appearance of a second band, indicating higher levels of unfarnesylated protein. This effect appears to be dose-dependent as suggested by the intensity of the band (Figure 1B and C). Lamin C was not affected since it does not require farnesylation to be activated (Kelland *et al.*, 2001).

Furthermore, we treated osteogenic differentiating MSC with a dose of alendronate that has been shown to stimulate osteoblastogenesis *in vitro* (Duque and Rivas, 2007), which also corresponds to the therapeutic dose of alendronate *in vivo* (Stepensky *et al.*, 2002). We observed that at week 2 of differentiation, alendronate reverted the inhibitory effect of FTI-277 on HDJ-2 and prelamin A farnesylation (Figure 1A–C).

Inhibition of protein farnesylation decreases osteoblast differentiation and activity

We tested the effect that inhibition of protein farnesylation could have on osteoblast differentiation and mineralization by looking at ALP and Alizarin red staining respectively. As in our previous report (Duque and Rivas, 2007), we found that cells treated with alendronate showed higher levels of ALP (twofold) and mineralization (1.6-fold) as compared with untreated cells ($P < 0.01$)(Figure 2A–C). Treatment with FTI-277 significantly affected osteoblast differentiation (approx. –50 ± 10%) and function (approx. –60 ± 15%, $P < 0.01$) with a higher inhibitory effect seen at a dose of 10 μM (Figure 2A–C). Furthermore, addition of alendronate to FTI-277-treated cells showed a recovery in osteoblast differentiation and mineralization (Figure 2A–C). Finally, the levels of expression of the transcription factors for osteoblastogenesis Runx2 and OCN were decreased by a high dose of FTI-277 (10 μM) (Figure 2D and E). This effect was also reverted by treatment with alendronate in a dose-dependent manner (Figure 2D and E).

Alendronate reverses nuclear morphological changes induced by inhibition of protein farnesylation

Figure 3 shows the quantification of nuclear morphological changes in osteogenic differentiating MSC exposed to alendronate, FTI-277 or both. Cells treated with FTI-277 alone showed significantly higher percentage of nuclear morphological changes (9 ± 1%) than either untreated (2.8 ± 0.8%) or alendronate-treated (1.4 ± 0.6%) cells (Figure 3B) ($P < 0.001$). Finally, the combination of FTI-277 and alendronate induced a significant decrease in nuclear morphological changes back to the levels seen in cells grown in osteogenic media (3.8 ± 0.7) ($P < 0.001$).

Cell survival after treatment with alendronate and/or FTI-277

To test whether treatment with either FTI-277 with or without alendronate affects cell survival or induced apoptosis in differentiating osteoblasts, we used the MTS-Formazan assay to test survival and TUNEL and caspase-3/7 assays for apoptosis detection. There were no differences in either cell survival (Figure 3C) or percentage of either TUNEL positive cells (Figure 3D) or caspase-3/7 activity (Figure 3E) between treated and untreated cells. In this study, cell proliferation was not measured since MSC do not actively proliferate after committing to differentiate into the osteoblast lineage (Pittenger *et al.*, 1999).

Role of protein geranylgeranylation in osteoblast differentiation of MSC

We tested the effect that inhibition of geranylgeranylation could have on osteoblast differentiation and mineralization. Treatment with GGTI-298 at a low dose (2.5 μM) had a

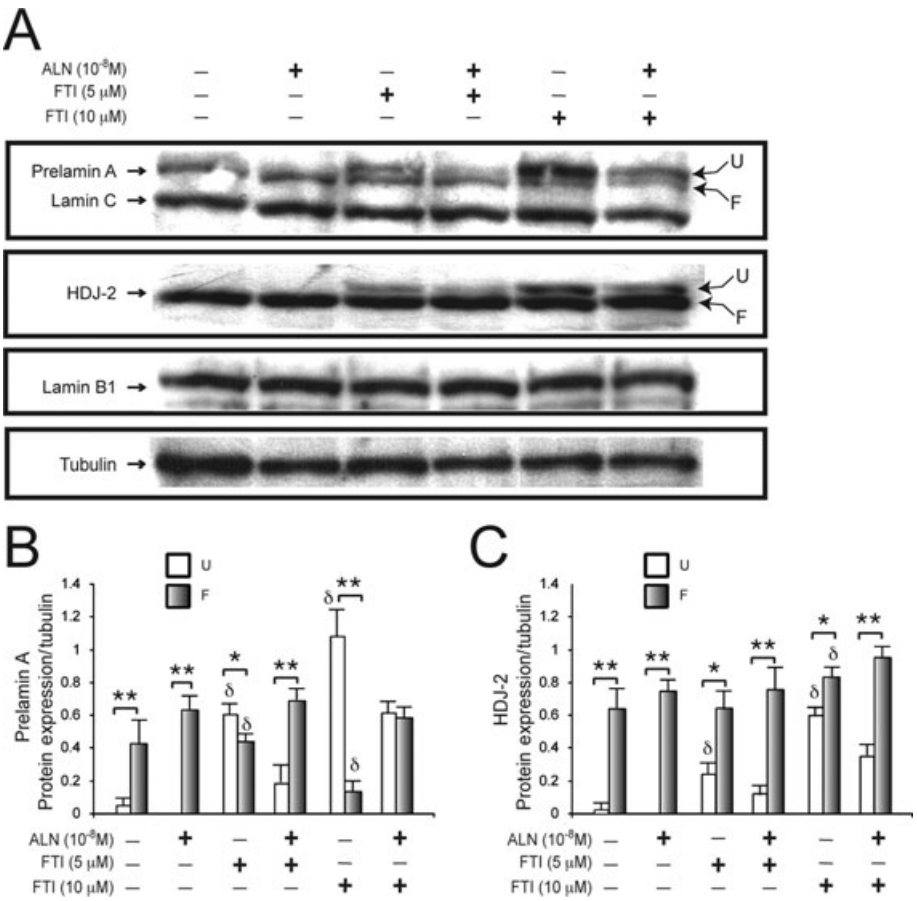

Figure 1

Effect of farnesyl transferase inhibitor-277 (FTI-277) with (+) and without (–) alendronate (ALN) on the processing of farnesylated proteins in osteogenic differentiating mesenchymal stem cells (MSC). Human MSC were plated and induced to differentiate as described in *Methods*. Human MSC were treated with osteogenic media containing vehicle, ALN alone (10^{-8} M) and FTI-277 (5 and 10 μM) alone or in combination with ALN. Media was replaced every 3 days for 2 weeks. The cells were then lysed and the lysates immunoblotted with antibodies against farnesylated (F) and unfarnesylated (U) prelamin A and HDJ-2. (A) Treatment with FTI-277 significantly increased the levels of unfarnesylated prelamin A in a dose-dependent manner. This effect was reverted by addition of ALN to the media. (B and C) The relative intensity to these proteins is presented in the histogram as a ratio of β-tubulin expression. Results are representative of three separate experiments. $^{*}P < 0.01$, $^{**}P < 0.001$, significant difference between unfarnesylated and farnesylated protein. $^{\delta}P < 0.01$ represents a significant difference between FTI-277-treated cells with versus without ALN.

stimulating effect on osteoblast differentiation and mineralization only in combination with alendronate (10^{-8} M) (Figure 4A–C). In contrast, GGTI-298 at a dose of 5 μM significantly increased osteoblast differentiation and function (Figure 4A–C), independently of the presence of alendronate (10^{-8} M) in the media. In contrast, a higher dose of GGTI-298 (7.5 μM) induced higher levels of ALP at week 1 but was unable to increase mineralization at week 3 (Figure 4A and B). The combination of GGTI-298 (7.5 μM) and alendronate (10^{-8} M) increased osteoblast differentiation and function similar to the levels found after treatment with alendronate alone (Figure 4A–C). Finally, a higher dose of GGTI-298 (10 μM) induced 85% cell mortality at week 1 and affected MSC differentiation. Higher and lower doses of alendronate (10^{-7} and 10^{-9}) were unable to either favour osteoblastogenesis or affect the cells' response to increasing doses of GGTI-298 (data not shown).

Indeed, conditions showing the higher osteogenic effect of GGTI-298 alone or in combination with alendronate were those showing the higher levels of ungeranylgeranylated RAP-1 (Figure 4D and E). In contrast, the levels of farnesylated and unfarnesylated prelamin A/C were not affected by the presence of GGTI-298 in the media. Furthermore, those conditions in which inhibition of geranylgeranylation induced higher osteogenesis closely correlated with higher levels of Runx2 and OCN expression (Figure 4D and E), with the higher level of change found in cells treated with the most efficient osteogenic dose of GGTI-298 (5 μM), independently of the presence of alendronate in the media. Finally, a similar proportion of nuclear morphological changes and cell survival at week 2 of differentiation was found between GGTI-277-treated and untreated cells at a dose of 2.5, 5 and 7.5 μM alone (data not shown) or in combination with alendronate (~93 ± 3% of cell survival in all groups) (Figure 4F).

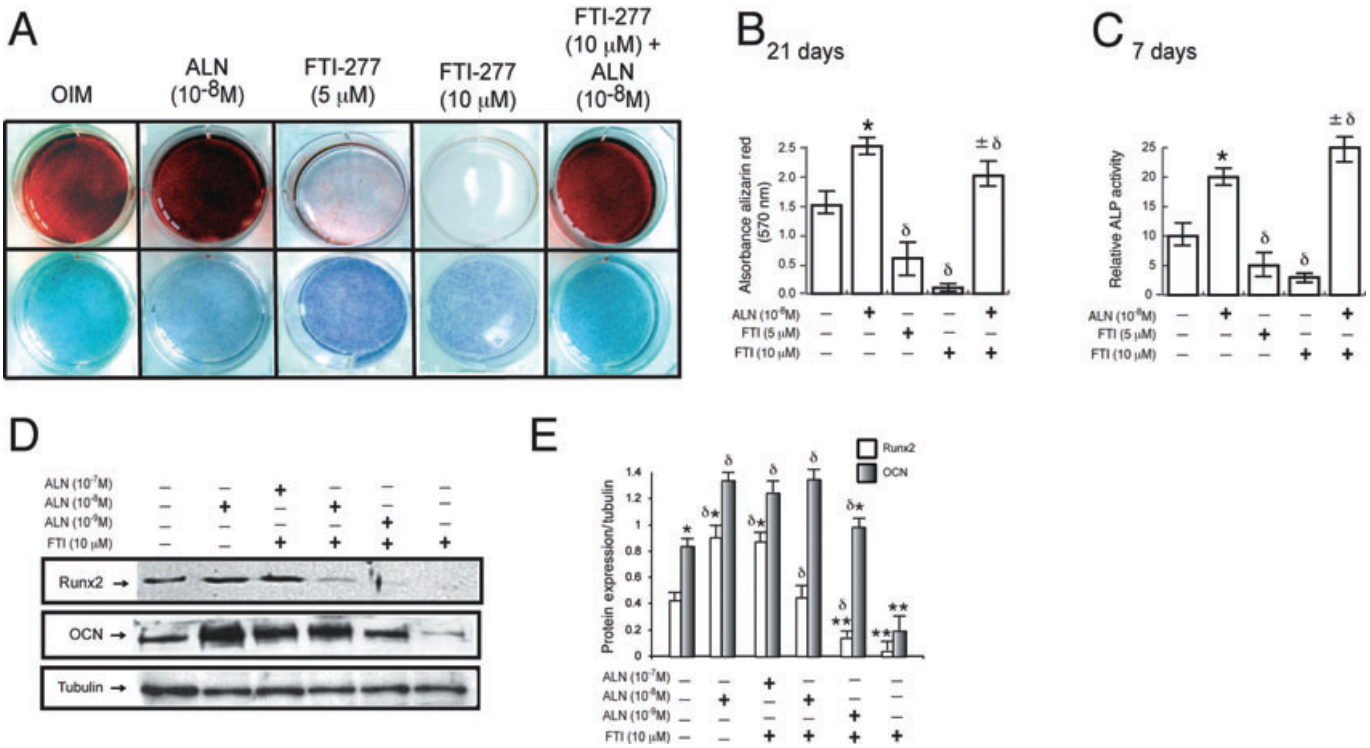

Figure 2

Inhibition of protein farnesylation affects osteoblastic differentiation and activity. (A) Osteogenic differentiation was determined by Alizarin red (upper panels) and alkaline phosphatise (ALP) (lower panels) staining indicating mineralization and differentiation respectively. Both mineralization and differentiation were significantly affected by treatment with farnesyl transferase inhibitor-277 (FTI-277) in a dose-dependent manner. This effect was reverted by addition of alendronate (ALN) (10^{-8} M) to the media. (B and C) Absorbance of the extracted Alizarin red S staining (B) and alkaline phosphatase activity (C) were measured at 570 and 405 nm respectively. At week 3, Alizarin red staining quantification shows a significantly higher mineralization in the ALN-treated cells as compared with untreated cells. Treatment with FTI-277 significantly affected mineralization. This effect was reverted by ALN. A similar effect on alkaline phosphatase activity was found at week 1 of differentiation. Six wells were analysed per experimental condition. Results are representative of three separate experiments. $^{*}P < 0.01$ represents a significant difference between treatment conditions. $^{\delta}P < 0.01$ represents a significant difference between FTI-277-treated and vehicle-treated cells. $^{\pm}P < 0.01$ represents a significant difference between FTI-277 with (+) ALN-treated and ALN-treated cells. (D and E) Changes in the expression of bone-specific proteins at week 2 of differentiation detected by Western blot. Addition of ALN (10^{-8} M) to the media increased the expression of both runt-related transcription factor 2 (Runx2) and osteocalcin (OCN). Addition of FTI-277 decreased protein expression. This effect was reverted by addition of ALN to the media in a dose-dependent manner. Membranes were stripped and immunoblotted for tubulin levels to demonstrate equal loading of proteins. Results are representative of three separate experiments. $^{*}P < 0.01$, $^{**}P < 0.001$ represent a significant difference between ALN- and vehicle-treated conditions; $^{\delta}P < 0.01$ represents a significant difference between FTI-277-treated and ALN with and without FTI-277 cells.

In contrast, cells treated with GGTI-298 at a dose of 10 μM showed 100% mortality at week 1 of differentiation (data not shown).

Discussion

The post-translational modification of proteins by the addition of isoprenoids has been recognized as a key physiological process for facilitating cellular protein–protein interactions, membrane-associated protein trafficking and cell differentiation (Sebti, 2005; McTaggart, 2006). In the musculoskeletal system, protein isoprenylation plays an essential role in the differentiation and activity of osteoclasts (Coxon *et al.*, 2000; Vigouroux *et al.*, 2001) as well as synovial fibroblasts (Abeles *et al.*, 2007). However, its role in the process of osteoblast differentiation and activation remains unknown. In the present study, we found that protein isoprenylation also plays a role in osteoblastic differentiation of MSC. In addition, we found that the role of protein isoprenylation is markedly different in osteoblast as compared with osteoclast differentiation. Our results indicate that protein farnesylation is required for osteoblast differentiation of MSC. In contrast, inhibition of protein geranylgeranylation facilitates osteoblastogenesis. Furthermore, considering a recent reported role of alendronate in osteoblastogenesis (Duque and Rivas, 2007; Fu *et al.*, 2008) and the well-known effect of this compound on protein isoprenylation (Plotkin *et al.*, 1999; Russell, 2006), we tested the effect of alendronate on protein isoprenylation in osteogenic differentiating MSC. Our results show that in osteogenic differentiating MSC alendronate potentiated the effect of GGTI-298 on geranylgeranylation as well as reverted the effect of FTI-277 on protein farnesylation, both in favour of osteoblastogenesis.

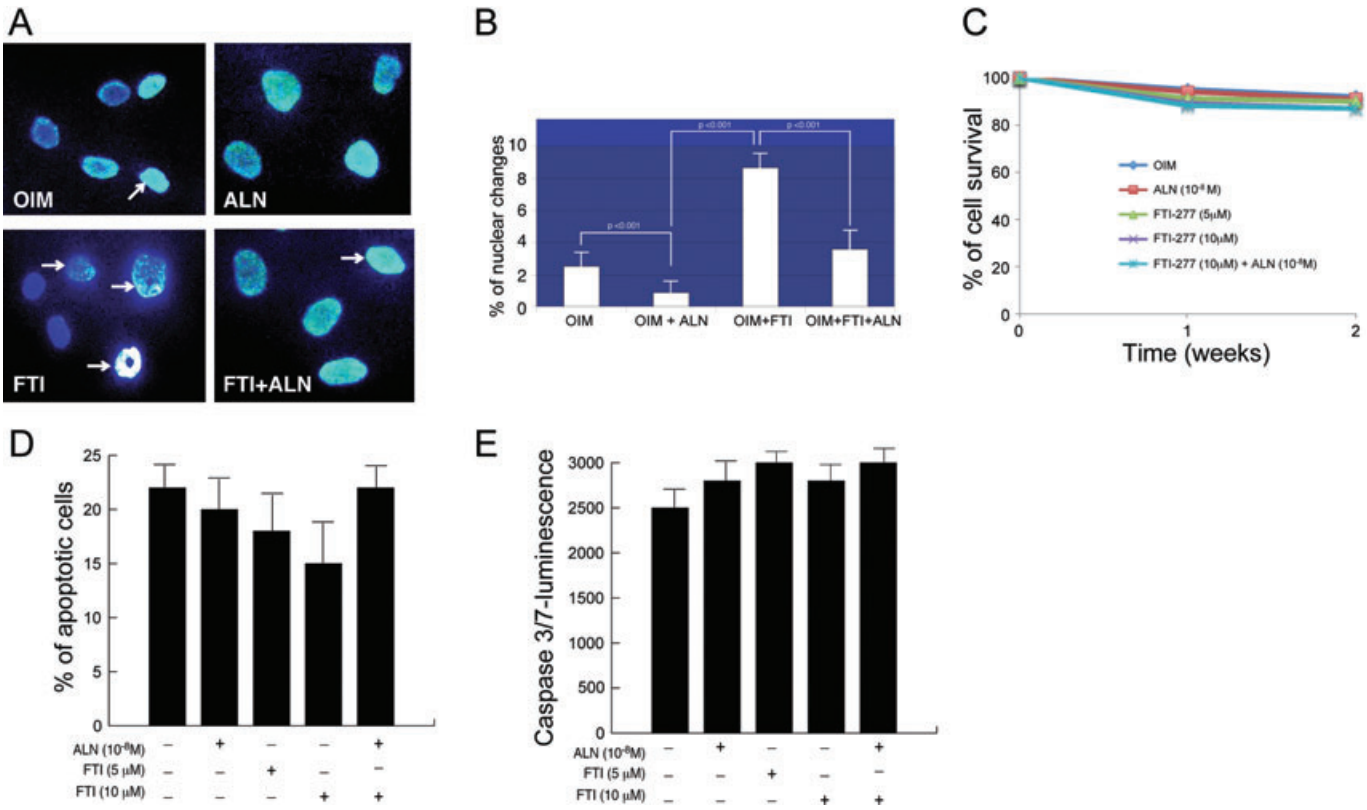

Figure 3

Inhibition of protein farnesylation and nuclear morphological changes in osteogenic differentiating mesenchymal stem cells (MSC). Cells were plated and induced to differentiate as previously described. At week 2 of differentiation, cells were fixed and stained using bisbenzimide trihydrate dye (blue Hoechst). One hundred nuclei per field were classified as either having changes or remaining normal as described in *Methods*. Three independent observers without knowledge of the treatments did these classifications. (A) The changes in nuclear morphology suggestive of nuclear blebbing (white arrows) after treatment with farnesyl transferase inhibitor-277 (FTI-277) (5 μM), alendronate (ALN) (10^{-8} M) or FTI with (+) ALN. Cells treated with FTI-277 show a significantly higher proportion of nuclear morphological changes as compared to untreated cells. (B) In contrast, the proportion of nuclear morphological changes was significantly reduced after addition of ALN both in the presence or absence of FTI. Morphologically, cells treated with ALN showed bigger and better defined nuclei than FTI-277-treated cells. Photomicrographs were taken at ×100 magnification. (B) The quantification of cells positive for nuclear morphological changes obtained from three replicates of the experiments; three independent data sets were averaged. (C) MSC were induced to differentiate in 96-well plates. At 60% confluence, media was replaced with osteoblastogenesis induction media (OIM) containing FTI-277 (5 and 10 μM) with and without ALN (10^{-8} M). MTS-Formazan cell viability assay was performed. Cell survival was not affected by the presence of FTI-277, ALN or both. (D) Quantification of differentiating cells showing nuclear fragmentation by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay after treatment with FTI-277 (5 and 10 μM), ALN or both showed no difference as compared with vehicle-treated cells. (E) Caspase-3/7 activity was assayed using the Caspase-Glo luminescence assay and data represent the mean values (SD) of triplicate cultures. Treatment with FTI-277 (5 and 10 μM), ALN or both showed no difference as compared with vehicle-treated cells.

Protein isoprenylation by either farnesylation or geranylgeranylation is known to be required for cell differentiation (McTaggart, 2006). This is the case of synovial fibroblasts, in which farnesylation is required for their differentiation and function (Abeles *et al.*, 2007). In contrast, recent evidence indicates that osteoclastogenesis is enhanced by inhibition of lamin A/C, a protein that requires farnesylation (Rauner *et al.*, 2009) whereas protein geranylgeranylation is required for osteoclast differentiation and function (Coxon *et al.*, 2000).

In the case of stem cells, few studies have looked at the role of protein isoprenylation in stem cell differentiation. Lee *et al.* (2007) have reported that inhibition of geranylgeranylation in mouse embryonic stem cells affects cell renewal and proliferation. In contrast, using an osteoblastic cell line, Yoshida *et al.* (2006) have reported that inhibition of isoprenylation of geranylgeranyl-pyrophosphate (GGPP) is critical for the cell-to-cell contact-induced differentiation. Finally, we have recently reported that pharmacological inhibition of FTase decreases adipocyte differentiation of MSC *in vitro* (Rivas *et al.*, 2007).

A common approach to assess the role of protein isoprenylation in cell biology is the use of pharmacological inhibition of both, farnesylation and geranylgeranylation (Coxon *et al.*, 2000; Abeles *et al.*, 2007; Igarashi *et al.*, 2007). The most commonly used inhibitors of farnesylation and geranylgeranylation are FTI-277 and GGTI-298 respectively (Adjei *et al.*, 2000; Coxon *et al.*, 2000; Abeles *et al.*, 2007; Igarashi *et al.*,

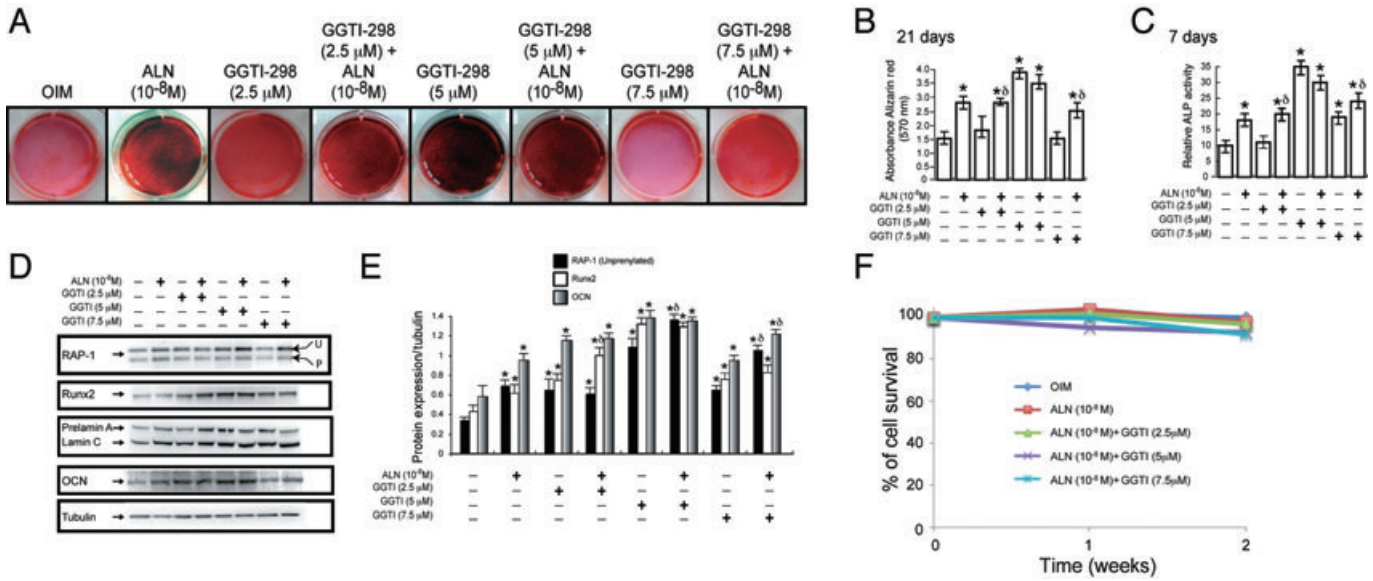

Figure 4

Inhibition of geranylgeranylation has a positive effect on osteoblastic differentiation and activity. (A) Treatment of osteogenic differentiating mesenchymal stem cells (MSC) with geranylgeranyltransferase inhibitor-298 (GGTI-298) at a dose of 5 μM increased osteoblastogenesis and stimulated mineralization. This effect was not affected by the presence of alendronate (ALN) in the media. (B and C) The absorbance of the extracted Alizarin red S staining (B) and alkaline phosphatase activity (C) were measured at 570 and 405 nm respectively. At week 3, Alizarin red staining quantification shows a significantly higher mineralization and alkaline phosphatase activity in the GGTI-277 (5 μM)-treated cells as compared with untreated cells [osteoblastogenesis induction media (OIM)]. This effect was not changed by the presence of ALN in the media. Six wells were analysed per experimental condition. Results are representative of three separate experiments. $^{*}P < 0.01$ represents a significant difference between either ALN- or GGTI-298-treated cells versus untreated cells. $^{\delta}P < 0.01$ represents a significant difference between GGTI-298-treated and GGTI-298 with (+) ALN-treated cells. (D and E) Changes in the expression of geranylgeranylated [ras-like protein 1 (RAP-1)], farnesylated (lamin A/C) and bone-specific proteins [runt-related transcription factor 2 (Runx2) and osteocalcin (OCN)] after treatment with GGTI-298. (D) Cells treated with osteogenic media alone showed baseline levels of ungeranylgeranylated RAP-1 (U) and farnesylated lamin A/C. (E) Treatment with both ALN and GGTI-298 significantly increased ungeranylgeranylated RAP-1 expression. In addition, levels of Runx2 expression were increased after treatment with both ALN and GGTI-298. Finally, OCN expression was significantly increased by inhibition of protein geranylgeranylation. Membranes were stripped and immunoblotted for tubulin levels to demonstrate equal loading of proteins. The figure shows averages of expression after three replicates. Results are representative of three separate experiments. $^{*}P < 0.01$ represents a significant difference between treated and untreated conditions. $^{\delta}P < 0.01$ represents a significant difference between GGTI-298-treated and GGTI-298 with (+) and without (–) ALN-treated cells. (F) MSC were induced to differentiate in 96-well plates. At 60% confluence, media was replaced with OIM containing GGTI-298 (2.5–7.5 μM) with and without ALN (10^{-8} M). MTS-Formazan cell viability assay was performed. Cell survival was not affected by the presence of GGTI-298, ALN or both.

2007). Other studies have used bisphosphonates due to their known inhibitory effect on protein isoprenylation in osteoclasts (Mönkkönen *et al.*, 2006; Russell, 2006). For the purposes of this study, and as no previous studies have looked at the role of protein isoprenylation in osteogenic differentiating MSC, we decided to use a similar methodological approach to the one previously used in other cell lines (Coxon *et al.*, 2000; Mönkkönen *et al.*, 2006; Abeles *et al.*, 2007). Initially, we looked at the effect that inhibitors of protein isoprenylation have on osteoblast differentiation. Subsequently, we assessed the effect of alendronate in this model.

Inhibition of farnesylation by FTI-277 arrested osteoblastogenesis and decreased the expression of the osteogenic transcription factors Runx2 and OCN in a dose-dependent manner, without affecting cell survival or inducing apoptosis. This effect is somewhat similar to the effect of FTI-277 on differentiating adipocytes (Rivas *et al.*, 2007) and suggests that different levels of protein farnesylation are required for the successful differentiation of MSC into either osteoblasts or adipocytes.

Furthermore, addition of alendronate to FTI-277-treated cells prevented the nuclear morphological changes usually induced by inhibition of farnesylation (Young *et al.*, 2005) and reverted the inhibitory effect of FTI-277 on osteoblastogenesis and osteogenic factors. This effect could be relevant when considered together with recent evidence showing that protein farnesylation in general, and farnesylated lamin A/C in particular (Akter *et al.*, 2008), could be required for successful differentiation of stem cells into osteoblasts (Duque and Rivas, 2006; Duque, 2007; Scaffidi and Misteli, 2008). In fact, mice lacking the metallopeptidase that converts prelamin A to lamin A show bone changes compatible with osteoporosis and lack of bone formation (Bergo *et al.*, 2002; Young *et al.*, 2005; Rivas *et al.*, 2009) and treatment with zoledronic acid, another bisphosphonate, would correct their bone phenotype (Varela *et al.*, 2008). In summary, our data suggest that farnesylated proteins are required for osteoblastogenesis.

Interestingly, this finding contrasts with the recent evidence reporting that inhibition of farnesylated lamin A/C activity stimulates osteoclastogenesis (Rauner *et al.*, 2009). Taken together, this evidence suggests that induction of protein farnesylation, particularly lamin A/C, in differentiating bone cells could constitute a new therapeutic approach to promote bone formation and inhibit osteoclastic activity. In addition, the anti-fracture effect of alendronate on bone cells could be partially explained by this mechanism.

A particularly interesting finding of this study was that inhibition of protein geranylgeranylation has a stimulant effect on osteoblast differentiation and function. This effect is contrary to the well-known effect of inhibition of geranylgeranylation on osteoclasts, which includes inhibition of protein prenylation, disruption of cytoskeleton organization and cellular trafficking and induction of apoptosis (Russell, 2006). In contrast, and in agreement with another differentiation model (Li *et al.*, 2010), treatment of differentiating MSC with GGTI-298 has a direct effect on the expression of osteogenic proteins suggesting that, contrary to stimulators of farnesylation, inhibitors of geranylgeranylation could constitute a new approach to the regulation of bone turnover through the stimulation of osteoblastogenesis while decreasing osteoclast differentiation and function.

However, the effect of GGTI-298 seems to be exerted within a narrow therapeutic range. Whereas a dose of 2.5 μM showed a minimal effect, a dose of 10 μM induced cell death, which was not prevented by the addition of alendronate to the media. Nevertheless, GGTI-298 seems to have an optimal osteogenic dose (5 μM), which correlates with the higher levels of expression of unprenylated RAP-1, induces the higher levels of Runx2 and OCN, and is independent of the presence of alendronate in the media. Taken together, this evidence suggests that, as in a previous report (Yoshida *et al.*, 2006), inhibition of geranylgeranylation has an osteogenic effect on differentiating MSC. However, further *in vivo* studies looking at the appropriate dosing of protein isoprenylation inhibitors to induce osteoblastogenesis and bone formation are still required.

Our results may not be in contradiction to those of Orriss *et al.* (2009) and Idris *et al.* (2008). These two groups reported that nitrogen-containing bisphosphonates inhibit protein prenylation nodule formation in osteoblast cultures. However, their results cannot be compared with ours as in their studies the investigators used mature calvarian osteoblasts, whereas in the present study we have used a well-established model of MSC treated at an early stage of their osteogenic differentiation and thus showing a positive response to bisphosphonates.

Finally, the role of alendronate in this model requires specific analysis. The dose of alendronate (10^{-8} M) was selected not only based on its osteogenic effect on MSC *in vitro* (Duque and Rivas, 2007), where concentration curves were tested in this model, but also because this dose closely correlates with the pharmacokinetics of alendronate in soft tissues after either intermittent or continuous administration (Stepensky *et al.*, 2002). In contrast to its effect on osteoclasts, in our model, alendronate showed a minimal inhibitory effect on geranylgeranylation, which was much lower than the effect of GGTI-298. The presence of alendronate in the differentiation media was unable to potentiate osteogenesis in cells treated with GGTI-298 at its optimal osteogenic dose (5 μM), and was also unable to rescue cells from apoptosis induced by higher doses of GGTI-298 (10 μM). This is not a surprising finding as the combination of alendronate with other osteogenic compounds such as vitamin D and parathyroid hormone has been shown to have an antagonistic effect (Duque and Rivas, 2007; Issack *et al.*, 2007).

In summary, the results of this study demonstrate that protein isoprenylation plays an important role in osteoblastic differentiation of MSC. Both induction of farnesylation and inhibition of geranylation play a role in bone formation. This evidence provides a new understanding for the role of protein isoprenylation in bone formation and suggests the regulation of isoprenylation as a new therapeutic approach for osteoporosis.

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Conflict of interest

G. D. has received research grants from Procter and Gamble, USA, Novartis Pharmaceuticals, Australia and Merck, USA.

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