Effects of Farnesyl Pyrophosphate Accumulation on Calvarial Osteoblast Differentiation

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Statins, drugs commonly used to lower serum cholesterol, have been shown to stimulate osteoblast differentiation and bone formation. Statins inhibit 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A reductase (HMGCR), the first step of the isoprenoid biosynthetic pathway, leading to the depletion of the isoprenoids farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). The effects of statins on bone have previously been attributed to the depletion of GGPP, because the addition of exogenous GGPP prevented statin-stimulated osteoblast differentiation in vitro. However, in a recent report, we demonstrated that the specific depletion of GGPP did not stimulate but, in fact, inhibited osteoblast differentiation. This led us to hypothesize that isoprenoids upstream of GGPP play a role in the regulation of osteoblast differentiation. We demonstrate here that the expression of HMGCR and FPP synthase decreased during primary calvarial osteoblast differentiation, correlating with decreased FPP and GGPP levels during differentiation. Zaragozic acid (ZGA) inhibits the isoprenoid biosynthetic pathway enzyme squalene synthase, leading to an accumulation of the squalene synthase substrate FPP. ZGA treatment of calvarial osteoblasts led to a significant increase in intracellular FPP and resulted in inhibition of osteoblast differentiation as measured by osteoblastic gene expression, alkaline phosphatase activity, and matrix mineralization. Simultaneous HMGCR inhibition prevented the accumulation of FPP and restored osteoblast differentiation. In contrast, specifically inhibiting GGPPS to lower the ZGA-induced increase in GGPP did not restore osteoblast differentiation. The specificity of HMGCR inhibition to restore osteoblast differentiation of ZGA-treated cultures through the reduction in isoprenoid accumulation was confirmed with the addition of exogenous mevalonate. Similar to ZGA treatment, exogenous FPP inhibited the mineralization of primary calvarial osteoblasts. Interestingly, the effects of FPP accumulation on osteoblasts were found to be independent of protein farnesylation. Our findings are the first to demonstrate that the accumulation of FPP impairs osteoblast differentiation and suggests that the depletion of this isoprenoid may be necessary for normal and statin-induced bone formation. (Endocrinology 152: 3113–3122, 2011)

Osteoporosis is a condition characterized by low bone mineral density that increases bone fragility and risk for fractures. Worldwide, there are approximately 9 million osteoporotic fractures each year, with over half of these occurring in Europe and the Americas. These fractures are a large cause of morbidity and mortality in the elderly and also represent a large economic burden (1–3). Most current treatments for osteoporosis impair bone resorption. Although this strategy is effective at preventing further mineral loss, anabolic agents are needed to restore bone in patients who have suffered substantial bone loss (4, 5). Mundy et al. (6) demonstrated that statins, drugs commonly used to lower serum cholesterol, stimulate bone formation through the induction of bone morpho-

Abbreviations: ALP, Alkaline phosphatase; BMP-2, bone morphogenetic protein-2; DGBP, digeranyl bisphosphonate; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; FPPS, FPP synthase; FTI, farnesyl transferase inhibitor; GGPP, geranylgeranyl pyrophosphate; GGPPS, GGPP synthase; GPP, geranyl pyrophosphate; HMG, 3-hydroxy-3-methylglutaryl; HMGCR, HMG-coenzyme A reductase; IPP, isopentenyl pyrophosphate; αMEM, α modified essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; N-BP, nitrogenous bisphosphonate; OCN, osteocalcin; qPCR, quantitative PCR; SOS, squalene synthase; ZGA, Zaragozic acid.
genetic protein-2 (BMP-2) expression in osteoblasts. These results have been supported by several in vitro (7–14) and in vivo (15, 16) studies. Additionally, higher bone mineral densities have been positively correlated with the administration of lipophilic statins to patients for hypercholesterolemia (17–20).

Statins inhibit 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A reductase (HMGCR), the first enzyme in the isoprenoid biosynthetic pathway (Fig. 1) (21, 22). HMGCR catalyzes the conversion of HMG-coenzyme A to mevalonate and is the rate-limiting step of cholesterol biosynthesis (23). Mevalonate undergoes two phosphorylation steps followed by decarboxylation, resulting in the production of isopentenyl pyrophosphate (IPP). IPP, a five-carbon moiety, is the precursor for isoprenoid metabolites, which are made up of multiples of the IPP isoprene building block (24). IPP isomerizes to dimethylallyl pyrophosphate (DMAPP), and together IPP and DMAPP are converted to the 10-carbon geranyl pyrophosphate (GGPP) by farnesyl pyrophosphate (FPP) synthase (FPPS). FPPS also catalyzes the production of the 15-carbon FPP by the addition of IPP to GPP. FPP is the branch point of the isoprenoid biosynthetic pathway. Squalene synthase (SQS) condenses two molecules of FPP to form squalene, the precursor for sterol synthesis. Alternatively, geranylgeranyl pyrophosphate (GGPP) synthase (GGPPS) catalyzes the production of the 20-carbon GGPP by the addition of IPP to FPP. FPP and GGPP are used in protein prenylation and are recognized by farnesyl transferase and geranylgeranyl transferase I and II, respectively (24–26).

Through inhibition of HMGCR, statins deplete all of these isoprenoids and impair protein prenylation. Published data that the addition of exogenous GGPP prevented the effects of statins on osteoblast differentiation in vitro suggested that statin-stimulated osteoblast differentiation is dependent on the depletion of GGPP and diminution of protein geranylgeranylation (10, 13). This led us to previously hypothesize that specific inhibition of GGPPS would similarly stimulate osteoblast differentiation. In contrast, we found that the specific inhibition of GGPPS reduced osteoblastic differentiation; the effects were not prevented by the addition of exogenous GGPP. Interestingly, we noted that the GGPPS substrate FPP decreased during MC3T3-E1 preosteoblast differentiation. Inhibition of GGPPS caused an increase in intracellular FPP and prevented the differentiation-associated decrease of this isoprenoid (27).

In this study, we sought to determine whether isoprenoids upstream of GGPP, specifically FPP, negatively regulate osteoblast differentiation, potentially playing a role in normal and statin-stimulated bone formation.

Materials and Methods

Primary cell isolation and culture

The animal protocol used for isolation of primary cells was approved by the Institutional Animal Care and Use Committee at the University of Iowa. Primary rat osteoblast cells were obtained by three sequential enzyme digestions of calvariae from 2-d-old neonatal Sprague Dawley rats (Harlan Laboratories, Inc., Indianapolis, IN). Digestions were performed with 0.05% collagenase type I (Sigma, St. Louis, MO) and 1% trypsin (Invitrogen, Carlsbad, CA) in serum-free α modified essential medium (αMEM) (Invitrogen) at 37°C with shaking. The first two digestions (10 and 20 min, respectively) were discarded. The last digestion (60 min) was collected, and cells were centrifuged and resuspended in α-MEM containing 10% fetal bovine serum and 1× penicillin-streptomycin (Invitrogen). Cells were grown in a humidified atmosphere with 5% CO₂ at 37°C in 10-cm plates. Upon confluence, cells were subcultured for experiments at a density of 1 × 10⁴ cells/cm².

All experiments were carried out in osteoblast differentiation medium. This consisted of α-MEM with 10 mM β-glycerophosphate (Sigma) and 50 μg/ml L-ascorbic acid (Fisher Scientific, Waltham, MA). Compounds used for experimental treatments included Zaragozic acid (ZGA) (Sigma), lovastatin (Sigma), mevalonate (Sigma), farnesyl transferase inhibitor (FTI)-277 (Sigma), or digeranyl bisphosphonate (DGBP) (David Wiemer, University of Iowa). Treatments were replaced every 3–4 d until the termination of the experiment. ZGA, FTI-277, and DGBP were dissolved in water. Lovastatin and mevalonic acid lactone were subjected to lactone hydrolysis, followed by dilution with water or RPMI medium, respectively.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay

Cells were plated in 24-well plates. Upon confluence, cells were treated for 72 h. MTT (Calbiochem, San Diego, CA) was added to cells, and the reaction was incubated at 37°C with 5% CO₂ for 3 h. The reaction was terminated with MTT stop solution.
lution (HCl, Triton X-100, and isopropyl alcohol). Plates were shaken overnight at 37 C. Absorbance was measured at 540 nm with reference wavelength at 650 nm.

### Real-time quantitative PCR (qPCR)

Primary calvarial osteoblasts were plated in six-well plates. Treatment was applied when cells reached confluency. Total RNA was isolated from each well using Qiagen kits (QIAGEN, Valencia, CA). During the isolation, a deoxyribonuclease step was performed (QIAGEN). One microgram of RNA was reverse transcribed into cDNA using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Real-time qPCR was performed with Sybr Green Master Mix (Applied Biosystems, Foster City, CA) on an ABI SDS 7900 HT (Applied Biosystems). The real-time protocol consisted of 2 min at 50 C, 10 min at 95 C, followed by 40 cycles of 95 C (15 sec) and 60 C (1 min). Dissociation curves were obtained after the real-time qPCR to ensure the proper amplification of target cDNA. Primers were obtained from Integrated DNA Technologies (Iowa City, IA) and eluted in Tris-EDTA buffer (Ambion, Austin, TX). Sequences and amplicon lengths are found in the Table 1.

### Mineralization assay

Primary calvarial osteoblasts were plated in 24-well plates. Treatment began when cells reached confluency. After 14 d of culture, cells were fixed for 1 h in ice-cold 70% ethanol. Cells were then washed thoroughly with deionized water; mineralization was detected with 40 mM Alizarin red (pH 4.2) (Sigma) for 15 min. After staining, cells were washed thoroughly with deionized water to remove nonspecific Alizarin red. Images were captured using a Canon EOS Rebel XS (Canon, Lake Success, NY). Mineralization was quantified as described previously (28). Briefly, Alizarin red was eluted with 10% acetic acid. Plates were then washed thoroughly with deionized water; mineralization was compared with an Alizarin red standard curve; values were expressed as total micromoles Alizarin red per well.

### Alkaline phosphatase (ALP) activity assay

Calvarial osteoblasts were plated in 12-well plates. One week after the onset of treatment, cells were washed twice with PBS (Invitrogen). Cells were lysed with 0.2% Triton X-100 (Sigma) and subjected to two freeze thaw cycles. Cells were transferred to 1.5-ml tubes and centrifuged. The supernatants were used in the ALP assay. Five-milligram ALP substrate tablets (p-nitrophenyl phosphate) (Sigma) were dissolved in alkaline buffer (40 mg/10 ml; Sigma). Cell lystate or control lysis buffer was transferred to a 96-well plate, and substrate solution was added to each well. The assay was carried out at 37 C for 10 min. Absorbance was read at 405 nm on a Thermomax Microplate reader (Molecular Devices, Foster City, CA) and eluted in Tris-EDTA buffer (Ambion, Austin, TX). Sequences and amplicon lengths are found in the Table 1.

### FPP/GGPP quantitation

Calvarial osteoblasts were plated in 10-cm plates. Upon confluency, cells were treated for 24 h. FPP and GGPP levels were determined as previously reported by reverse phase HPLC (29). Briefly, cells were washed twice with PBS (Invitrogen), and isoprenoid prenylphosphates were extracted from cells and used as substrates for incorporation into fluorescent GCVLS or GCVLL peptides by farnesyltransferase or geranylgeranyl transferase I. The prenylated fluorescent peptides were separated by reverse phase HPLC and quantified by fluorescence detection. Total FPP and GGPP levels were normalized to total protein content as measured by bicinchoninic assay. Values are expressed as ALP units per milligram protein per minute.

### Table 1. Rat primers for real-time qPCR analysis

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F, Forward; R, reverse.
Statistical analysis

Data are expressed as mean ± SE of the mean. All experiments were repeated at least twice with similar results. Differences between two groups were compared using unpaired Student’s t tests. Comparisons between multiple groups were made by one-way ANOVA followed by the post hoc Bonferroni t test. Statistical significance was defined by P values less than 0.05.

Results

Isoprenoid biosynthetic pathway during osteoblast differentiation

To determine intracellular levels of the isoprenoids FPP and GGPP during osteoblast differentiation, calvarial osteoblasts were treated with differentiation medium for 0, 4, 7, or 10 d. At each end point, intracellular isoprenoids were extracted and quantified by HPLC as described in Materials and Methods. Both FPP and GGPP decreased during calvarial osteoblast differentiation (Fig. 2A).

Expression of isoprenoid pathway enzymes was also assessed. Calvarial osteoblasts were treated with differentiation medium for 0, 2, 4, or 7 d. At each end point, cells were analyzed for expression of the isoprenoid pathway enzymes HMGCR, FPPS, and GGPPS. Expression of HMGCR and FPPS decreased during osteoblast differentiation (Fig. 2B). In contrast, GGPPS levels increased modestly at d 7. Differentiation was assessed by expression of the mature osteoblast marker osteocalcin (OCN) (Fig. 2C).

ZGA leads to an accumulation of FPP

To determine the effect of the SQS inhibitor, ZGA (Fig. 1), on intracellular FPP and GGPP levels in osteoblasts, calvarial osteoblasts were treated with ZGA (1–10 μM) for 24 h. FPP and GGPP were both increased by treatment with ZGA (Fig. 3). GGPP increased maximally with 1 μM ZGA. In contrast, FPP, which was increased significantly with 1 μM ZGA, increased to a greater degree with higher doses of ZGA (5–10 μM). Control levels of FPP and GGPP in primary calvarial osteoblasts are 3.8 ± 0.3 and 1.5 ± 0.3 pmol/mg protein, respectively.

ZGA inhibits osteoblast mineralization and osteoblast gene expression

To examine whether ZGA inhibits osteoblast matrix mineralization, calvarial osteoblasts were treated with 1–10 μM ZGA for 14 d followed by Alizarin red staining of calcium deposition. As shown in Fig. 4, A and B, ZGA significantly impaired matrix mineralization, with 5 and 10 μM ZGA resulting in nearly total prevention of mineralization.

The effect of ZGA on osteoblast viability was assessed by MTT assays. Cells were treated with 0.5–25 μM ZGA for 72 h. As shown in Fig. 4C, maximal inhibition of MTT reduction was approximately 40%, and this occurred with concentrations greater than or equal to 5 μM ZGA. MTT is reduced by a variety of cellular enzymes, as well as by the mitochondrial transport chain, and is commonly used as a measurement of cellular proliferation. This suggests that ZGA may inhibit osteoblast proliferation. However, decreased cellular MTT reduction may also reflect toxicity. Therefore, apoptosis was assessed by Western blot analysis of poly(ADP-ribose) polymerase and caspase-3 cleavage. Primary calvarial cells were treated with 1–50 μM ZGA for 72 h. No cleavage of these proteins.

FIG. 2. The isoprenoid biosynthetic pathway is down-regulated during differentiation of calvarial osteoblasts. A, Intracellular FPP and GGPP decrease during osteoblast differentiation. Data are expressed as picomoles per milligram protein, percent d 0 (mean ± SEM), n = 2. B, Expression of the isoprenoid pathway enzymes HMGCR and FPPS decreases during osteoblast differentiation. mRNA levels were quantified by real-time qPCR and normalized to the housekeeping gene GAPDH. Data are expressed as relative units (mean ± SEM), n = 3. C, OCN expression increased on d 2–7 showing differentiation of calvarial osteoblasts. Expression was normalized to the housekeeping gene GAPDH, and data are expressed as relative units (mean ± SEM), n = 3.
expression of Col1a1 gene, the transcription factor for type I collagen, was significantly reduced compared with ZGA alone. ZGA also increased GGPP levels (Fig. 3). One micromolar DGBP, which specifically targets GGPPS (30, 31), the enzyme downstream of FPPS (Fig. 1), led to a slight accumulation of FPP. Cotreatment of 5 μM ZGA with 1 μM DGBP led to a slight decrease in matrix mineralization and ALP activity, with no effect on osteoblast viability (Fig. 5D). In contrast to lovastatin, 1 μM DGBP did not prevent the inhibition of osteoblast viability, ALP activity, or matrix mineralization by ZGA treatment of calvarial osteoblasts (Fig. 6, C–E). Higher concentrations of DGBP (5 μM) in combination with ZGA led to a reduction of GGPP below control levels (Fig. 6A). However, similarly to the 1 μM DGBP cotreatment, this concentration did not restore osteoblast mineralization (Fig. 6B). The effect of 5 μM DGBP alone on mineralization is not shown due to the toxicity of this treatment over the 2-wk time course in the absence of exogenous GGPP or isoprenoid accumulating agents.

Exogenous FPP inhibits osteoblast matrix mineralization

To determine whether the effects of ZGA on osteoblast differentiation are due specifically to the accumulation of FPP, primary calvarial osteoblasts were treated with increasing concentrations of ZGA (1–10 μM). FPP and GGPP are expressed as picomoles per milligram protein, fold vehicle (mean ± SEM). a, P < 0.05 vs. vehicle FPP; b, P < 0.05 vs. vehicle GGPP; c, P < 0.05 vs. 1 μM ZGA FPP, n = 2.

Inhibition of osteoblast differentiation and mineralization by ZGA is prevented by inhibition of HMGCR but not GGPPS

To assess whether the effect of ZGA to inhibit osteoblast differentiation and matrix mineralization is due to the accumulation of isoprenoids, osteoblasts were treated with lovastatin in the presence or absence of ZGA. As described earlier, statins inhibit HMGCR, resulting in depletion of mevalonate and its metabolites, including FPP and GGPP. Figure 5A demonstrates that 1 μM lovastatin alone led to a reduction in intracellular FPP and GGPP. Five micromolar ZGA, as shown earlier, led to a significant increase in intracellular FPP levels. Cotreatment of 1 μM lovastatin with 5 μM ZGA led to a significant increase in FPP compared with control. However, the FPP accumulation was significantly reduced compared with ZGA alone. ZGA also increased GGPP levels (Fig. 3). One micromolar DGBP, which specifically targets GGPPS (30, 31), the enzyme downstream of FPPS (Fig. 1), led to a slight accumulation of FPP. Cotreatment of 5 μM ZGA and 1 μM DGBP slightly reduced the intracellular increase in GGPP (Fig. 5B). As expected, there was no significant reduction in FPP accumulation compared with ZGA alone (Fig. 5A).

Figure 5, B–E, shows that 1 μM lovastatin alone had only minimal effects on the matrix mineralization and ALP activity of calvarial osteoblasts, with no effect on osteoblast viability. Similar to previous experiments, 5 μM ZGA reduced osteoblast viability, ALP activity, and matrix mineralization of primary osteoblasts. Cotreatment of 5 μM ZGA with 1 μM lovastatin resulted in a significant restoration of matrix mineralization, osteoblast viability, and ALP activity compared with ZGA alone. This suggests that the effect of ZGA to inhibit osteoblast proliferation, differentiation, and matrix mineralization is due to the accumulation of isoprenoids.

To confirm that the inhibition of osteoblast differentiation and matrix mineralization by ZGA was not due to the slight increase in GGPP, DGBP cotreatments were used. Alone, 1 μM DGBP led to a slight decrease in matrix mineralization (Fig. 5C) and ALP activity (Fig. 5E), with no effect on osteoblast viability (Fig. 5D). In contrast to lovastatin, 1 μM DGBP did not prevent the inhibition of osteoblast viability, ALP activity, or matrix mineralization by ZGA treatment of calvarial osteoblasts (Fig. 6, C–E). Higher concentrations of DGBP (5 μM) in combination with ZGA led to a reduction of GGPP below control levels (Fig. 6A). However, similarly to the 1 μM DGBP cotreatment, this concentration did not restore osteoblast mineralization (Fig. 6B). The effect of 5 μM DGBP alone on mineralization is not shown due to the toxicity of this treatment over the 2-wk time course in the absence of exogenous GGPP or isoprenoid accumulating agents.

Restoration of osteoblast differentiation by lovastatin cotreatment is due to prevention of isoprenoid accumulation

To determine whether the restoration of osteoblastic differentiation and matrix mineralization by lovastatin cotreatments was through prevention of isoprenoid accumulation, mevalonate add-back experiments were per-
formed. Addition of 5 mM mevalonate alone had no effect on matrix mineralization as measured by Alizarin red staining (Fig. 7A). Five micromolar ZGA inhibited mineralization, and this was restored by cotreatment with 5 μM lovastatin. Addition of 5 mM mevalonate to the ZGA and lovastatin cotreatment significantly decreased mineralization compared with ZGA and lovastatin alone. These data suggest that the restoration of osteoblast differentiation by lovastatin is due to the prevention of isoprenoid accumulation and not an off target effect of lovastatin.

The effect of FPP accumulation to inhibit osteoblast differentiation is not due to increased protein farnesylation

To test whether the negative effects of FPP accumulation on osteoblast differentiation are due to increased protein farnesylation, cells were treated with a FTI in the presence or absence of ZGA. FTI-277 was used at concentrations of 0.1, 1.0, and 10.0 μM alone or in combination with 5 μM ZGA. As shown in Fig. 7B, FTI-277 had no significant effect on osteoblast mineralization at any concentration tested. In combination with ZGA, FTI-277 did not have any significant effect on ZGA-induced inhibition of osteoblast differentiation. This suggests that the accumulation of FPP is acting independently of farnesylation to inhibit osteoblast differentiation.

Discussion

These results demonstrate that isoprenoids upstream of GGPP, specifically FPP, have negative effects on osteoblast differentiation. This was evidenced by ZGA treatment, which increased FPP and GGPP isoprenoid levels, leading to the inhibition of osteoblast differentiation. The inhibitory effect of ZGA on osteoblasts could be prevented by inhibition of HMGCR and the reduced accumulation of FPP but not by specific inhibition of GGPPS. Additionally, exogenous FPP inhibited the matrix mineralization of primary calvarial osteoblasts.

These results have interesting implications for the mechanism of statin-stimulated osteoblast differentiation. As described earlier, several in vitro (7–14) and in vivo (15, 16) studies have demonstrated positive effects of statins on osteoblast differentiation and bone formation. These effects have been attributed to the depletion of GGPP, because the addition of exogenous GGPP prevented statin-stimulated osteoblast differentiation in vitro (10, 13). Consistent with this paradigm, several studies have found that nitrogenous bisphosphonates (N-BP), which inhibit FPPS (32–34) and similarly lead to GGPP depletion (Fig. 1), have been shown to stimulate osteoblast differentiation (35–41). It is important to note, however, that the effects of N-BP on osteoblasts are unclear, because some studies have shown effects of N-BP to inhibit matrix mineralization and trigger osteoblast apoptosis (42–45).

Our studies show that the isoprenoids FPP and GGPP, as well as the expression of HMGCR and FPPS, decrease during osteoblast differentiation. In contrast to a previous study by Yoshida et al. (46), expression of GGPPS did not decrease with differentiation. This discrepancy may be due to their use of MC3T3-E1 preosteoblasts vs. the primary calvarial osteoblasts used in this study. The results presented here suggest that depletion of more than just GGPP
plays a role in osteoblast differentiation. Consistent with this, Takase et al. (47) demonstrated that the anabolic protein parathyroid hormone led to a reduction in mevalonate kinase expression. Importantly, the results presented in this study and that of Yoshida et al. (46) agree that the activity of the isoprenoid pathway decreases during osteoblast differentiation.

ZGA treatment of primary calvarial osteoblasts led to an increase in intracellular FPP and GGPP levels, as well as decreased osteoblast differentiation and matrix mineralization. Because of previously published data demonstrating the negative effects of geranylgeraniol (46) and GGPP (27) on MC3T3-E1 preosteoblast differentiation, it was important to determine whether the effect of ZGA on osteoblast differentiation was due to the increase in intracellular GGPP. It was found that the negative effects of ZGA on osteoblast differentiation were due to accumulation of upstream isoprenoid metabolites, such as FPP, not GGPP. Several lines of evidence support this conclusion. GGPP increased maximally at a concentration of 1 μM ZGA. However, inhibition of osteoblastic gene expression, ALP activity, as well as maximal inhibition of matrix mineralization, did not occur with this concentration. Secondly, addition of DGBP, which prevented the increase in intracellular GGPP in ZGA-treated cultures, did not prevent the inhibition of osteoblast viability, ALP activity, and matrix mineralization. In contrast, addition of lovastatin, which inhibits HMGCR, leading to decreased FPP accumulation (and likely other upstream isoprenoids, which were not measured in this study), restored osteoblast viability, ALP activity, and matrix mineralization. These experiments suggest that ZGA inhibits osteoblast differentiation and matrix mineralization through the accumulation of isoprenoid metabolites upstream of GGPP.

Interestingly, addition of exogenous FPP decreased the matrix mineralization of primary calvarial osteoblasts in our study, supporting a role for FPP accumulation specifically in regulating osteoblast differentiation. It appeared that exogenous FPP inhibited matrix mineralization to a lesser degree than ZGA. It is likely that we cannot attain the intracellular concentration achieved with ZGA. However, inhibition of osteoblast viability, ALP activity, and matrix mineralization by ZGA in the presence or absence of an inhibitor to HMGCR (Lov 1 μM) or GGPP (DGBP 1 μM). A. The accumulation of FPP by ZGA was prevented by inhibition of HMGCR but not inhibition of GGPPs. Values are expressed as picomoles per milligram protein, fold vehicle (mean ± SEM). a, P < 0.05 compared with vehicle; b, P < 0.05 compared with ZGA-treated cells, n = 2. B. The increase in GGPP by ZGA was reduced by inhibition of GGPPs. Data are expressed as picomoles per milligram protein, fold vehicle (mean ± SEM), n = 2. C. Inhibition of HMGCR prevented the inhibition of mineralization by ZGA, whereas inhibition of GGPPs did not. Osteoblast mineralization was assessed by Alizarin red staining followed by elution and quantification of dye. Values are expressed as micrograms of Alizarin red per well (mean ± SEM). a, P < 0.05 compared with vehicle control; b, P < 0.05 compared with ZGA-treated cells, n = 3. D. Cell proliferation was assessed by MTT assay. Inhibition of HMGCR prevented the inhibition of proliferation by ZGA, whereas inhibition of GGPPs did not. E. ALP activity was assessed. Inhibition of HMGCR prevented the inhibition of ALP activity by ZGA, whereas inhibition of GGPPs did not. Values were normalized to protein content. Data are expressed as percent vehicle, ALP units per milligram protein per minute (mean ± SEM). a, P < 0.05 compared with vehicle control; b, P < 0.05 compared with ZGA-treated cells, n = 3. Lov, Lovastatin.
modulating the activities of the farnesoid X receptor (FXR) (49) and liver X receptor (LXR) receptors (50), respectively. Das et al. (48) showed that FPP binds and activates several nuclear receptors, including the thyroid hormone receptor, estrogen receptor, and glucocorticoid receptor. In a more recent report, Vukelic et al. (51) demonstrated that modulation of FPP levels altered wound healing through modulation of glucocorticoid receptor activities. The potential role of FPP activating nuclear hormone receptors remains a potential mechanism of ZGA-mediated inhibition of osteoblast differentiation and matrix mineralization. In addition to roles activating nuclear hormone receptors, it has been reported that FPP stimulates TRPV3 (52) and G-protein coupled receptor 92 (53) and antagonizes the lysophosphatidic acid 3 receptor (54). In support of a nonpyrenylation role for FPP in inhibiting osteoblast differentiation, the effects of ZGA on osteoblast mineralization were not prevented by inhibition of farnesylation.
In summary, we demonstrate for the first time here that expression of the isoprenoid pathway enzymes HMGCR and FPPS are down-regulated during osteoblast differentiation, and the accumulation of isoprenoids upstream of GGPP, specifically FPP, impairs osteoblast differentiation. The individual effects of FPP and other upstream isoprenoids will be the focus of future investigations. Finally, our results suggest a role for depletion of FPP in normal and statin-stimulated osteoblast differentiation.

Acknowledgments

We thank Dr. Tong in the Hohl lab for assistance in measuring FPP and GGPP levels and Dr. David Wiemer at the University of Iowa for supplying DGBP.

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This work was supported by the Roy J. Carver Charitable Trust as a Research Program of Excellence and the Roland W. Holden Family Program for Experimental Cancer Therapeutics.

Disclosure Summary: M.M.W. has nothing to disclose. R.J.H. is an inventor of digeranyl bisphosphonate (DGBP) and has a financial interest in Terpenoid Therapeutics, Inc., which has an exclusive license to develop DGBP.

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