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Journal of Cellular Biochemistry 114:1760–1771 (2013)

Journal of Cellular Biochemistry

Rapamycin Inhibits BMP-7-Induced Osteogenic and Lipogenic Marker Expressions in Fetal Rat Calvarial Cells

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ABSTRACT

Bone morphogenetic proteins (BMPs) promote osteoblast differentiation and bone formation in vitro and in vivo. BMPs canonically signal through Smad transcription factors, but BMPs may activate signaling pathways traditionally stimulated by growth factor tyrosine kinase receptors. Of these, the mTOR pathway has received considerable attention because BMPs activate P70S6K, a downstream effector of mTOR, suggesting that BMP-induced osteogenesis is mediated by mTOR activation. However, contradictory effects of the mTOR inhibitor rapamycin (RAPA) on bone formation have been reported. Since bone formation is thought to be inversely related to lipid accumulation and mTOR is also important for lipid synthesis, we postulated that BMP-7 may stimulate lipogenic enzyme expression in a RAPA-sensitive mechanism. To test this hypothesis, we determined the effects of RAPA on BMP-7-stimulated expression of osteogenic and lipogenic markers in cultured fetal rat calvarial cells. Our study showed that BMP-7 promoted the expression of osteogenic and lipogenic markers. The effect of BMP-7 on osteogenic markers was greater in magnitude than on lipogenic markers and was temporally more sustained. RAPA inhibited basal and BMP-7-stimulated osteogenic and lipogenic marker expression and bone nodule mineralization. The acetyl CoA carboxylase inhibitor TOFA stimulated the expression of osteoblast differentiation markers, whereas palmitate suppressed their expression. We speculate that the BMP-7-stimulated adipogenesis is part of the normal anabolic response to BMPs, but that inappropriate activation of the lipid biosynthetic pathway by mTOR could have deleterious effects on bone formation and could explain paradoxical effects of RAPA to promote bone formation. J. Cell. Biochem. 114: 1760–1771, 2013.

KEY WORDS: BMP-7; OSTEOBLAST DIFFERENTIATION; ADIPOGENIC DIFFERENTIATION; MTOR; RAPAMYCIN; FRC

Bone morphogenetic proteins (BMPs) induce new bone formation by promoting the commitment of osteoblast precursors to the osteogenic lineage, and the concerted proliferation and differentiation of these precursors into mineralizing osteoblasts [Friedlaender et al., 2001; Chen et al., 2004; Kim and Choe, 2011]. BMPs bind to and activate receptors with intrinsic serine/threonine kinase activity, leading to the phosphorylation and nuclear localization of Smad transcription factors [Massague, 2000; ten Dijke et al., 2003]. However, increasing evidence suggests that BMP-induced osteoblast differentiation requires pathways that typically are associated with signaling through growth factor receptor tyrosine kinases [Derynck and Zhang, 2003; Zhang, 2009]. Inhibitor and molecular genetic studies suggest that the PI3K/Akt pathway is required for BMP activation of the osteoblastic differentiation program [Mukherjee and Rotwein, 2009]. Nevertheless, downstream pathways that mediate PI3K/Akt stimulated osteogenesis are largely uncharacterized. One candidate pathway is the mTOR signaling system [Zoncu et al., 2011]. This system consists of two distinct complexes, mTORC1, which contains the rapamycin sensitive subunit raptor and mTORC2, which contains the rapamycin (RAPA) insensitive rictor subunit. Akt activates mTORC1, which phosphorylates two downstream effectors: P70S6 kinase and eIF4E binding protein (eIF4E-BP), thereby increasing general and specific protein synthesis. In contrast, mTORC2 may activate Akt but is blocked by P70S6 kinase [Averous and Proud, 2006]. Evidence that mTOR regulates osteogenesis comes primarily from studies showing

The authors declare no competing interests. None of the authors have a competing financial interest in any companies providing reagents for this study.

Grant sponsor: NIH (NIA); Grant numbers: AG 037746, AG 040612.

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Manuscript Received: 12 October 2012; Manuscript Accepted: 7 February 2013

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 26 February 2013

DOI 10.1002/jcb.24519 • © 2013 Wiley Periodicals, Inc.

that mTOR inhibitors such as RAPA alter the osteoblastic differentiation program in vitro and/or bone formation in vivo [Isomoto et al., 2007; Singha et al., 2008; Niziolek et al., 2009; Martin et al., 2010]. However, conflicting results have been obtained, with reports of both stimulatory [Lee et al., 2010; Martin et al., 2010] and inhibitory [Isomoto et al., 2007; Singha et al., 2008; Niziolek et al., 2009] effects of RAPA on bone formation. Pleiotropic effects of mTOR could explain these discordant reports. Specifically, mTOR signaling has been shown to increase fatty acid biosynthesis, decrease fatty acid oxidation and increase triglyceride accumulation in a number of cell types and has been proposed to be responsible for lipotoxicity in metabolic tissues [Chakrabarti et al., 2010; Laplante and Sabatini, 2010; Li et al., 2010].

Previous studies have shown that BMP-7 increases both adipogenic and osteogenic differentiation of osteoblastic precursors in vitro [Asahina et al., 1996; Chen et al., 2001] and that RAPA inhibits BMP-7-stimulated osteoblast differentiation [Shoba and Lee, 2003]. In the present study, we have tested the hypothesis that BMP-7 stimulates both osteogenesis and adipogenesis in primary cultures of fetal rat calvarial (FRC) cells via a RAPA-sensitive pathway. Our study showed that BMP-7 promoted the expression of osteogenic and lipogenic markers and that RAPA inhibited basal and BMP-7-stimulated osteogenic and lipogenic marker expression and bone nodule mineralization. Moreover, we showed that exposure of FRC cultures to the fatty acid palmitate inhibited expression of osteogenic markers. The inhibition was partly prevented by TOFA, an inhibitor of the rate limiting enzyme acetyl CoA carboxylase in the lipogenic pathway. Our data demonstrate that mTOR mediates osteogenesis and lipogenesis in differentiating osteoblasts and provide experimental evidence that fatty acid accumulation may have an adverse effect on osteogenesis.

MATERIALS AND METHODS

MATERIALS

Alpha minimum essential medium (αMEM), Hanks' balanced salt solution, penicillin–streptomycin stock, and trypsin/EDTA were all purchased from Gibco/Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Gemini Bio-Products (Woodland, CA). Recombinant human BMP-7 was provided by Stryker Biotech (Hopkinton, MA). Primary and HRP-tagged secondary antibodies used in the present study were purchased from Santa Cruz (Santa Cruz, CA) or Cell Signaling Technologies (Danvers, MA). Rapamycin was obtained from Cell Signaling Technologies. Palmitate and TOFA were purchased from Sigma (St. Louis, MO) and Cayman Chemicals (Ann Arbor, MI), respectively.

FETAL RAT CALVARIAE (FRC) CELL CULTURE

Rats were handled and killed according to the protocol approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio, TX, USA. Primary osteoblastic cells were prepared from calvaria of 19-day-old fetal rats as described previously [McCarthy et al., 1988; Aronow et al., 1990; Yeh et al., 1996] and were cultured in αMEM plus 10% FBS, 100 U/ml penicillin, and 100 mg/ml of streptomycin sulfate, at 37°C with 5% CO_2. For experimentation, confluent FRC cells were incubated in serum-free αMEM in the presence or absence of BMP-7 (200 ng/ml) as described in figure legends. The BMP-7 concentration used for the present study was based on published data showing maximum enhancement of alkaline phosphatase (AP) activity under these experimental conditions [Yeh et al., 1997]. For experiments with RAPA, FRC cultures were treated with RAPA (10 and 100 nM) for various times as indicated in the figure legends. For experiments with palmitate (250 μM) and TOFA (10^{-6} M), FRC cultures were treated for 48 h and terminated.

WESTERN BLOT ANALYSIS

Cellular proteins were analyzed by Western blot analysis as described [Yeh et al., 2010]. Briefly, cells were lysed in ice-cold buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 1% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 20 mM β-glycerol phosphate, and phosphatase inhibitor cocktails 1 and 2 [Sigma P2850 and P5726]). After 30 min on ice, lysates were centrifuged at 12,000 rpm for 15 min at 4°C. Equal amounts of protein from each sample were loaded onto a 4–20% Novex Tris–Glycine gel (Invitrogen). After electrophoresis, proteins were transferred onto Immobilon-P membranes (Millipore, Bedford, MA). Membranes were soaked in Tris-buffered saline containing 5% milk and 0.1% Tween-20 (TBST), incubated with a primary antibody overnight, followed by a secondary antibody—horse radish peroxide conjugate. Membranes were washed in TBST and treated with chemiluminescence reagents (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL). Proteins were detected by exposure of membrane to X-ray films (Phenix, Hayward, CA), which were subsequently scanned with an Epson Expression 1680 scanner (Long Beach, CA). The intensity of the protein signals was analyzed by NIH Image J software.

ALKALINE PHOSPHATASE ACTIVITY ASSAY

AP activity was measured as described previously [Yeh et al., 1997]. Briefly, FRC cells were rinsed with phosphate-buffered saline (PBS) and lysed in 0.1% Triton X-100 in PBS for 1 h at 4°C with gentle agitation. Total cellular AP activity was measured with a commercial assay kit (Sigma Chemical Co., St Louis, MO). Absorbance of the reaction mixture was measured at 405 nm using a microplate reader (Thermo Labsystems, Chantilly, VA). Protein was measured according to the method of Bradford [1976] using bovine serum albumin as a standard. AP activity was expressed as nanomoles of *p*-nitrophenol liberated per microgram of total cellular protein.

FORMATION OF MINERALIZED BONE NODULES AND ALIZARIN RED S STAINING

Near confluent FRC cells in 12-well plates grown in αMEM containing 5% FBS, β-glycerol phosphate (5 mM) and ascorbic acid (100 μg/ml) were treated with solvent vehicle or BMP-7 (200 ng/ml) in the absence or presence of RAPA as indicated in the figure legend. Media were refreshed every 3 days. Images of cell cultures were captured with an Olympus CK2 inverted microscope equipped with a charge-coupled device (CCD) camera. At the end of the experiment, cultures were stained with alizarin red S (AR-S) for mineralized bone nodules according to the method of Stanford et al. [1995]. To

quantify the mineralized nodule, the AR-S stained culture was extracted with 5% sodium dodecyl sulfate in 0.5 N HCl and the absorbance at 405 nm of the extracted dye was measured [Yeh et al., 2010].

RNA EXTRACTION AND REAL-TIME RT-PCR

mRNA expression in FRC cells was analyzed by qRT-PCR as described previously [Yeh et al., 2010]. Briefly, total RNA was extracted using RNA-STAT-60 reagent/chloroform, precipitated with isopropanol and collected by centrifugation, washed with 75% ethanol, and dissolved in nuclease-free water. RNA (2.5 μg) was used to synthesize cDNAs using the high-capacity Reverse Transcription kits (Applied Biosystems, Foster City, CA) in the Eppendorf Mastercycler (Westbury, NY). Real-time PCR was performed on the ABI7500 Fast Real-Time PCR System using the universal condition (1 cycle at 50°C for 2 min; 1 cycle at 95°C for 10 min; 40 cycles at 95°C for 15 s and 60°C for 1 min) with the TaqMan Universal PCR Master Mix. Taqman gene expression probes for osterix (Osx, Rn02769744_s1), Osteocalcin (OCN, Rn00566386_g1), Bone Sialoprotein (BSP2, Rn00561414_m1), Runx2 (Rn01512298_m1), acetyl-CoA carboxylase alpha (ACC1, Rn00573474_m1), ATP citrate lyase (ACL, Rn00566411_m1), fatty acid synthase (FAS, Rn00569117_m1), and internal controls β-2-microglobulin (B2M, Rn00560865_m1) were purchased from Applied Biosystems. Target gene expression was normalized to that of B2M; duplicates were determined using the $\Delta\Delta C_T$ method.

STATISTICAL ANALYSIS

Data are presented as mean ± SD. All data were analyzed using Student's *t*-test. $P < 0.05$ is considered statistically significant.

RESULTS

BMP-7 STIMULATES OSTEOBLAST CELL DIFFERENTIATION IN FRC CULTURES

Rodent calvarial cells have been previously shown to have the potential for osteoblastogenesis and adipogenesis [Aubin, 1998; Bellows and Heersche, 2001; Hasegawa et al., 2008; Steenhuis et al., 2009; Yoshiko et al., 2010]. Moreover, we and others have shown that BMP-7 stimulates the expression of characteristic osteogenic markers in several cell systems, including the FRC system [Asahina et al., 1996; Yeh et al., 1996; Chen et al., 2001; Kang et al., 2009]. Specifically, in long-term cultures of FRC cultures, we have shown that BMP-7 stimulates Smad5 phosphoryation, mRNA expression of the osteoblast specific transcription factor Runx2 [Yeh et al., 2006], AP, OCN, bone sialoprotein (BSP), and type I collagen (TIC) [Yeh et al., 2000]. For the purpose of directly comparing the magnitude and timing of the effects of BMP-7 on lipogenic and osteogenic marker expression in FRC cultures, we repeated measurements of osteogenic markers in the current study. BMP-7 stimulated mRNA expression of Osx, an osteoblast specific transcription factor, in a time-dependent manner (Fig. 1A). In control cultures, no significant change in Osx mRNA levels were observed as a function of time. The extent of stimulation by BMP-7 on Days 1, 2, 3, and 4 was approximately 15-, 18-, 26-, and 36-fold, respectively, compared to the Day 1 control. BMP-7 also stimulated mRNA expression of OCN, an osteoblast differentiation marker, in a time-dependent manner (Fig. 1B). The extent of stimulation by BMP-7 on Days 1, 2, 3, and 4 was about 2-, 5-, 8-, and 21-fold, respectively, compared to the Day 1 control. No significant changes in OCN mRNA levels in control cultures were detected over the 4 day period. Figure 1C shows that BMP-7 did not stimulate AP activity on Day 1 post treatment. However, BMP-7 stimulated AP activity on Days 2, 3, and 4 by approximately 4-, 9-, and 14-fold, respectively, compared to the Day 1 control. A slight but insignificant increase in AP activity was observed in control cultures over the 4 day period.

Figure 2 shows the effect of BMP-7 on formation of mineralized bone nodules as detected by AR-S staining. No bone nodules were detectable in control cultures up to 17 days of culture. By comparison, BMP-7 stimulated bone nodule formation in a time-dependent manner (Fig. 2). Bone nodules were detectable beginning on Day 4 and the nodules grew larger on Day 8. Mineralized bone nodules were detectable on about Day 10 post BMP-7 treatment and more mineralization was observed on Day 12. To quantify the extent of mineralized bone nodule formation, cultures on Day 9, 12, 15, and 17 were stained with AR-S and the intensity of AR-S stain was quantified spectrophotometrically. Based on AR-S staining, BMP-7 stimulated mineralized bone nodule formation by 9- to 10-fold, compared to the same day control (Fig. 2). Taken together, the present data demonstrates that BMP-7 stimulates expression of osteoblast cell differentiation markers in FRC cultures as a function of time. More importantly, these data on the expression of osteogenic markers provides a spatial and temporal frame work for our subsequent studies on effects of BMP-7 on lipogenic marker expression in FRC cultures.

EFFECTS OF BMP-7 ON EXPRESSION OF LIPOGENIC MARKERS IN FRC CULTURES

The effects of BMP-7 on the mRNA levels of acetyl CoA carboxylase (ACC), ATP citrate lyase (ACL), and fatty acid synthase (FAS) in FRC cultures were determined by qRT-PCR (Fig. 1). These biochemical markers were selected because the products of these mRNAs catalyze critical steps in the de novo fatty acid biosynthetic pathway. ACC mRNA levels did not change significantly in control cultures over the 4 day culture period (Fig. 1D). By comparison, BMP-7 stimulated ACC mRNA expression by about twofold, compared to the same day control throughout the time period of the experiment. The ACL mRNA levels in control cultures decreased gradually over the 4 day period so that by Day 4 it was about 50% compared to the Day 1 control (Fig. 1E). However, BMP-7 stimulated ACL mRNA expression by about twofold ($P < 0.01$), compared to the same day control throughout the 4 day culture period. FAS mRNA levels in control cultures decreased gradually over the period of the experiment, such that their Day 4 value was about 70% of that on Day 1 (Fig. 1F). By comparison, BMP-7 stimulated FAS mRNA levels by about twofold ($P < 0.01$) throughout the 4 day period, compared to the vehicle-treated same day controls.

Effects of BMP-7 on the protein levels of ACC and FAS were also determined by Western blot analysis (Fig. 3A). The basal ACC protein level increased slightly, but statistically insignificantly, as a function of culture time. BMP-7 stimulated ACC protein levels by

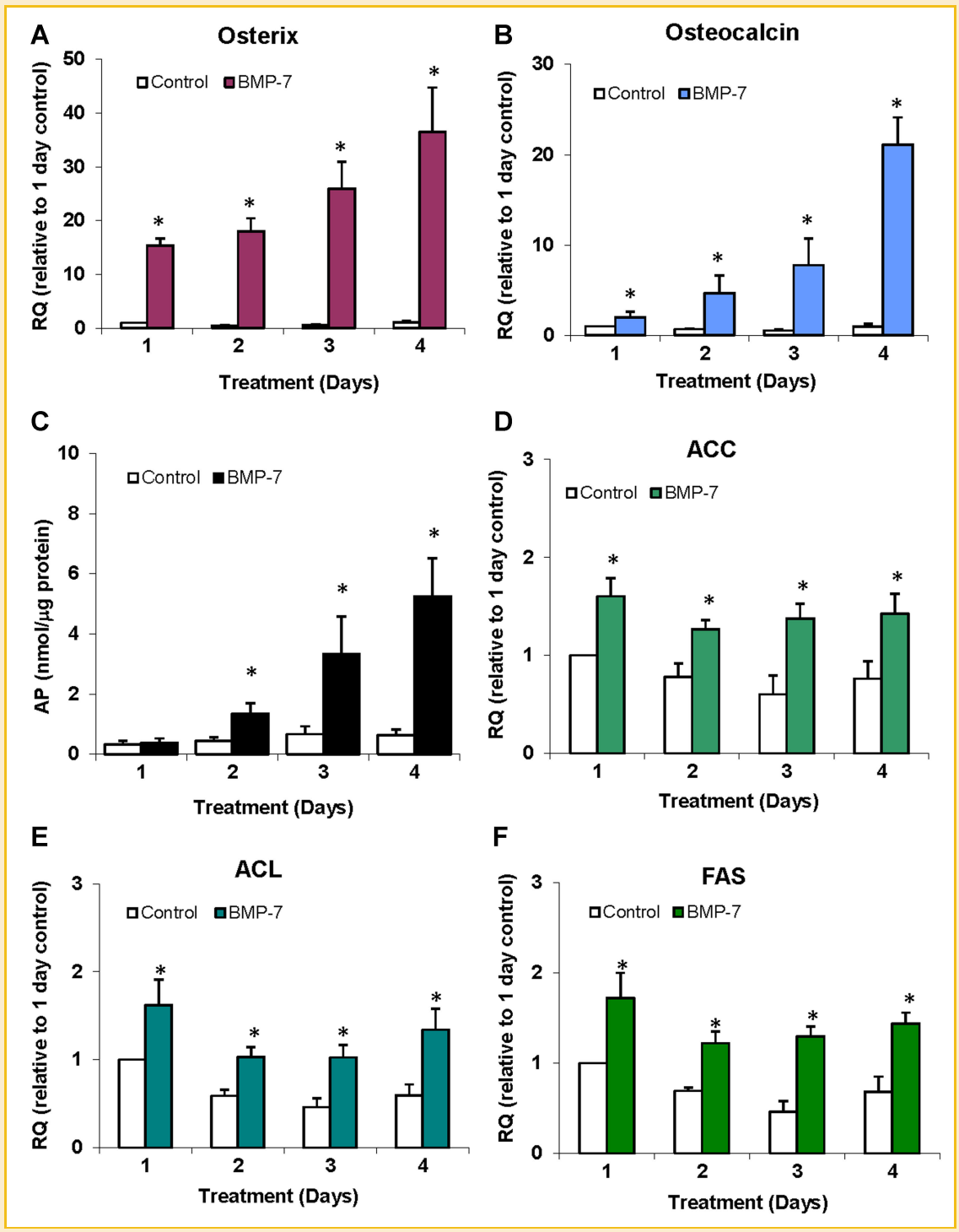

Fig. 1. Effects of BMP-7 on mRNA expression of osteogenic and lipogenic differentiation markers in FRC cell cultures. (A) Osterix, (B) osteocalcin, (C) AP activity, (D) acetyl CoA carboxylase (ACC), (E) ATP citrate lyase (ACL), and (F) fatty acid synthase (FAS). Confluent FRC cultures were incubated in serum-free αMEM-containing solvent or BMP-7 (200 ng/ml). (A,B,D–F) At the indicated days, cells in six-well plates were harvested, total RNA were isolated, and mRNA levels were measured by qRT-PCR as described in Materials and Methods. Values are the means ± SD of three to five independent experiments of three different FRC preparations and are normalized to the same day control. * indicates a $P < 0.05$ and is considered statistically significant. (C) At the indicated days, cells in 48-well plates were lysed with 0.05% Triton X-100/PBS as described in Materials and Methods Section. AP activity was expressed as nanomoles of *p*-nitrophenol per microgram of protein. Values are the means ± SD of five independent experiments (six replicates/condition) of three different FRC preparations and are normalized to Day 1 control. * indicates a $P < 0.05$ and is considered statistically significant.

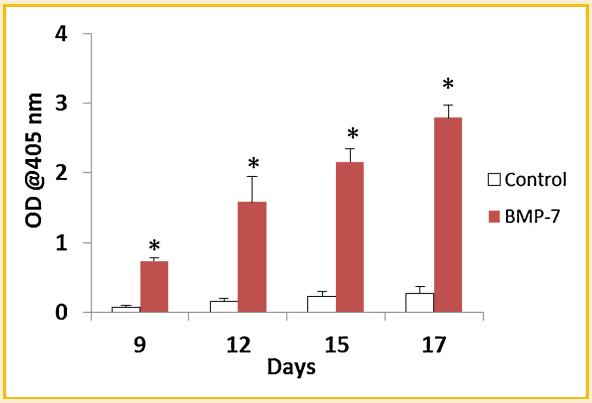

Fig. 2. Effects of BMP-7 on bone nodules formation in FRC cell cultures as a function of time. Confluent FRC cells in 12-well plates were treated and grown in αMEM containing solvent or BMP-7 (200 ng/ml). Media were changed every 3 days. FRC cultures from Days 9, 12, 15, and 17 were stained with AR-S and the extent of staining was quantified spectrophotometrically. Values are the means ± SD of three independent experiments of three different FRC preparations. * indicates a $P < 0.05$ and is considered statistically significant.

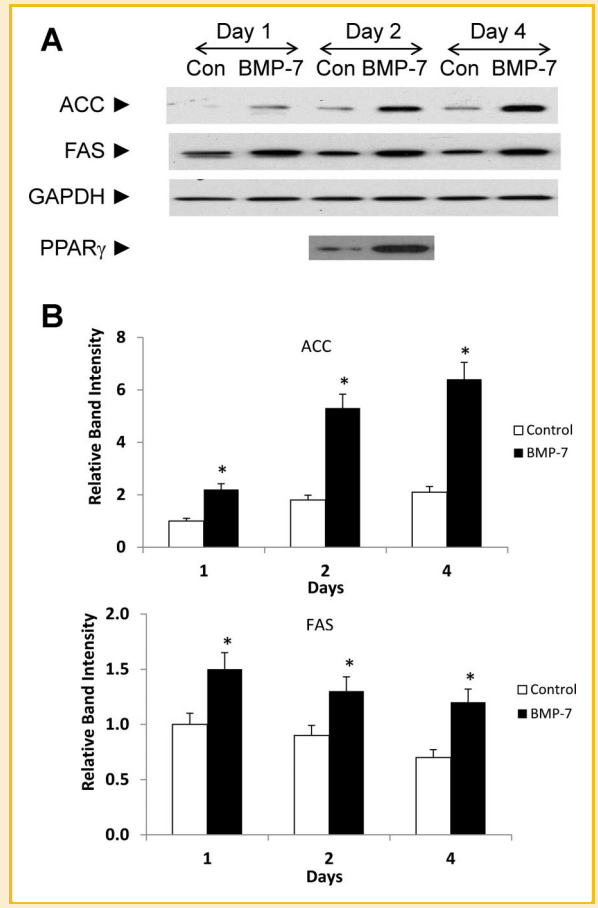

Fig. 3. Western blot analysis of acetyl CoA carboxylase (ACC), fatty acid synthase (FAS), and PPARγ protein expression in FRC cell cultures. A: Representative blots are shown of three to five independent experiments of three different FRC preparations. B: Western blots were scanned and the band intensity was measured. Values were normalized to Day 1 control as 1. * indicates a $P < 0.05$ and is considered statistically significant.

twofold ($P < .0.05$) on Day 1 and about threefold ($P < 0.01$) on Days 2 and 4, compared to the same day vehicle-treated controls (Fig. 3B). FAS protein levels decreased as a function of time in vehicle-treated cultures such that by Day 4 it was about 60% compared to the Day 1 control. By comparison, BMP-7 stimulated FAS protein levels by 1.5-, 1.5-, and twofold ($P < 0.05$) on Days 1, 2, and 4, compared to the same day control (Fig. 3B). In addition, Western blot analysis shows that BMP-7 stimulates the nuclear proteins peroxisome proliferator-activated receptor gamma (PPARγ) that is known to play a central role in adipocyte differentiation (Fig. 3A). GAPDH levels were used as controls and were not altered by BMP-7. Taken together, the present data demonstrates that BMP-7 stimulates expression of adipogenic markers in FRC cultures as a function of time.

ROLE OF MTOR IN OSTEOBLAST CELL DIFFERENTIATION AND LIPOGENESIS IN FRC

To assess the role of mTOR in osteoblastogenesis and lipogenesis in FRC cells, we first determined the basal levels of total mTOR and phospho (p)-mTOR and total P70S6K and phospho (p)-P70S6K, a downstream effector of mTOR, by Western blot analysis (Fig. 4A). The effects of BMP-7 on their levels were also measured. The basal level of total mTOR and phosphor (p)-mTOR was low on Day 1 but increased significantly on Days 2 and 4, compared to Day 1 control. However, the ratio of phosphor-mTOR/mTOR remained unchanged throughout the 4 days in the control cultures. Whereas BMP-7 did not stimulate total mTOR level, compared to the same day control, BMP-7 stimulated phosphorylation of mTOR on Days 1 and 4 by approximately three- and twofold ($P < 0.01$), compared to the same day control (Fig. 4B). In control cultures, the basal level of total P70S6K did not change significantly over the 4 day culture period. BMP-7 did not change total P70S6K, compared to the same day control, but BMP-7 significantly increased (p)-P70S6K levels by 1.5-fold ($P < 0.01$) on Days 1 and 4, compared to the same day control (Fig. 4B).

Next, we measured the effect of RAPA on mTOR and p70S6K in control and BMP-7-treated FRC cultures by Western blot analysis (Fig. 5A). RAPA (10 and 100 nM) did not change the level of total mTOR in control and BMP-7-treated cultures. However, RAPA reduced phosphorylated mTOR in control cultures by approximately 50% (Fig. 5B); 10 nM and 100 nM of RAPA were equally effective. RAPA (10 and 100 nM) also reduced the BMP-7-stimulated mTOR phosphorylation to the control level (Fig. 5B). RAPA did not change either the level of total P70S6K proteins in control or BMP-7-treated cultures (Fig. 5A). By comparison, BMP-7 stimulated the level of phosphorylated P70S6K by threefold ($P < 0.05$). RAPA (10 and 100 nM) completely blocked the BMP-7-stimulated phosphorylation (Fig. 5A).

We then examine effects of RAPA on the expression of several osteogenic differentiation markers, including AP activity, the osteoblast specific transcription factor Osx, OCN, and bone sialoprotein (BSP) in FRC cultures. As shown in Figure 6, RAPA

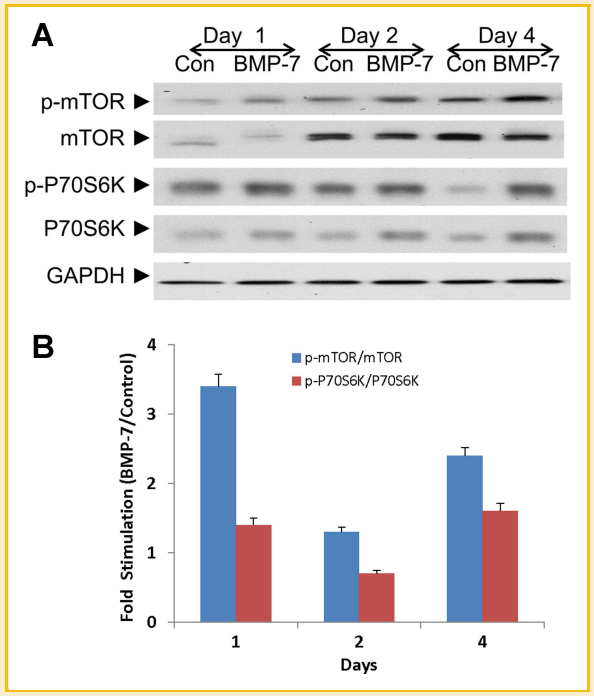

Fig. 4. Western blot analysis on effects of BMP-7 on phosphorylation of mTOR and P70S6K in FRC cell cultures. A: Representative blots are shown of three to five independent experiments of three different FRC preparations. B: Western blots were scanned and band intensity was measured. The ratios of p-mTOR/mTOR and p-P70S6K/P70S6K were first calculated from the band intensity. The ratios of p-mTOR/mTOR in BMP-7-treated cultures to p-mTOR/mTOR in control cultures are presented. Similarly, the ratios of p-P70S6K/P70S6K in BMP-treated cultures to p-P70S6K/P70S6K in control cultures are presented.

(10 and 100 nM) did not inhibit AP activity or mRNA expression of Osx, OCN, and BSP in control cultures, but it inhibited FAS mRNA expression by about twofold, compared to the control cultures not treated with RAPA. By contrast, RAPA inhibited the BMP-7 stimulated AP activity by about fourfold (Fig. 6A). Whereas RAPA alone did not change basal osterix mRNA expression, RAPA (10 or 100 nM) reduced BMP-7-stimulated osterix mRNA expression by approximately 50%, compared to cultures treated with BMP-7 (Fig. 6B). RAPA alone did not change basal osteocalcin mRNA expression, but RAPA (10 nM) reduced osteocalcin mRNA expression by about 70% (<0.05) compared to cultures treated with BMP-7, but by about 50% (<0.05) at 100 nM (Fig. 6C). The difference between 10 and 100 RAPA was insignificant. RAPA alone reduced basal BSP mRNA expression slightly but insignificantly. By comparison, RAPA reduced BSP mRNA expression in BMP-7-stimulated BSP mRNA expression by 50% ($P < 0.05$) and 90% ($P < 0.01$) at 10 and 100 nM, respectively (Fig. 6D).

We also examined effects of RAPA on expression of mRNA level of fatty acid synthase (FAS), a key enzyme in lipid synthesis. RAPA, at both concentrations tested, slightly reduced ($P < 0.1$) basal FAS mRNA expression. BMP-7 alone stimulated FAS mRNA expression by about twofold ($P < 0.05$) compared to the vehicle-treated control. RAPA (10 and 100 nM) inhibited the BMP-7-stimulated FAS mRNA expression by about twofold ($P < 0.05$; Fig. 6E).

Effects of RAPA on BMP-7 stimulated mineralized bone nodule in FRC cultures were examined as a function of time. Figure 7A shows a panel of representative photographs of cultures treated with RAPA in the presence or absence of BMP-7. No nodule formation was observed in control- or RAPA-treated cultures (top panels). However, RAPA significantly inhibited the BMP-7-stimulated mineralized bone nodule formation in FRC cultures (bottom panels). The results were quantified by treating these cultures with AR-S followed by spectrophotometric measurement of the AR-S staining intensity (Fig. 7B). BMP-7 stimulated nodule formation in a time-dependent manner such that nodule formation was stimulated by three-, five-, and fivefold on Days 9, 15, and 17, respectively, compared to the same day untreated controls. In the presence of RAPA, nodule formation was inhibited by 60%, 70%, and 70%, compared to the same day BMP-7-treated sample, on Day 9, 15, and 17, respectively.

EFFECT OF PALMITATE AND TOFA

To further examine the potential relationship between osteoblastogenesis and lipid metabolism in bone, FRC cultures were treated with palmitate (250 μM), which has been shown previously to inhibit osteogenesis [Elbaz et al., 2010], the acetyl CoA carboxylase inhibitor TOFA (10^{-6} M), and the combination of palmitate + TOFA. The mRNA expression of Runx2 and OCN in FRC cultures were determined by qRT-PCR. As shown in Figure 8, palmitate treatment suppressed the mRNA expression of the osteogenic specific transcription factor Runx2 and OCN in FRC cultures to 33% and 40% of the vehicle-treated control, respectively. TOFA stimulated both Runx2 and OCN mRNA expression by 2.5- and threefold, respectively, compared to the vehicle-treated control (Fig. 8). The combination of palmitate and TOFA stimulated Runx2 mRNA expression only by 1.2-fold ($P < 0.1$), compared to the vehicle-treated control but by about fourfold ($P < 0.01$), compared to the palmitate-treated value. In contrast, the combination of palmitate and TOFA stimulated OCN mRNA expression by about twofold ($P < 0.05$), compared to the vehicle-treated control, but was not to the same level as by TOFA alone. Taken together, the present data shows that palmitate suppressed expression of osteoblast markers in FRC cultures, whereas inhibition of ACC, the rate-limiting enzyme in the lipogenic pathway, by TOFA, stimulated expression of osteoblast differentiation markers. At the concentrations of TOFA and palmitate used in these experiments, TOFA was able to partially abate the inhibitory effect of palmitate on osteoblast differentiation.

DISCUSSION

Previously published studies on effects of BMPs on osteogenesis and adipogenesis have yielded conflicting results. Several studies using bone marrow cultures revealed that BMPs, including BMP-7, were capable of stimulating both osteogenesis and adipogenesis [Chen et al., 2001; Kang et al., 2009; Mikami et al., 2011]. Others indicated that BMP-2 stimulated osteogenesis but inhibited adipogenesis in

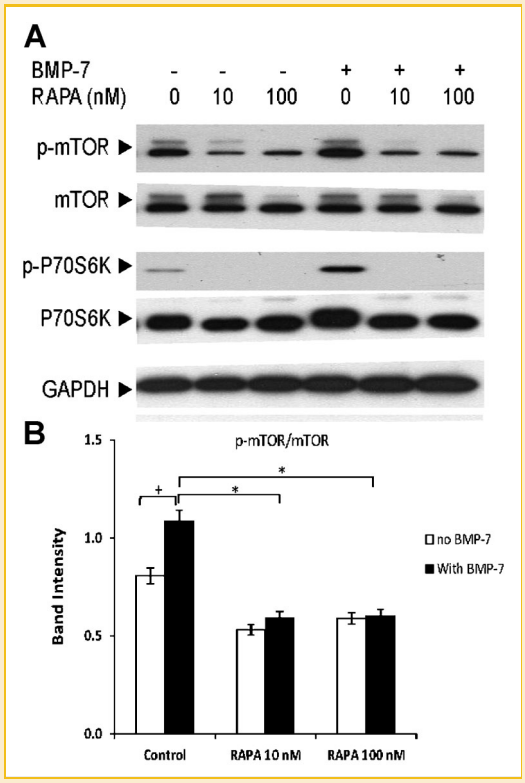

Fig. 5. Effects of rapamycin on total and phosphorylated mTOR and P70S6K in control and BMP-7-treated FRC cell cultures. A: Representative blots are shown of three to five independent experiments of three different FRC preparations. B: Ratios of p-mTOR/mTOR are presented. + indicates a $P < 0.05$, compared to vehicle-treated control. * indicates a $P < 0.01$, compared to the BMP-7-treated sample. $P < 0.01$ and $P < 0.05$ are considered statistically significant.

immortalized human marrow cells [Gori et al., 1999]. The present study was designed to determine the effects of BMP-7 on the expression of lipogenic differentiation markers in FRC cells, which are more differentiated along the osteogenic pathway than are bone marrow cells. Previous studies have shown that FRC cells possess the potential for osteoblastogenesis and adipogenesis [Aubin, 1998; Bellows and Heersche, 2001; Hasegawa et al., 2008; Steenhuis et al., 2009; Yoshiko et al., 2010]. Our current results not only confirm that BMP-7 stimulates expression of osteogenic differentiation markers in FRC cultures, but also demonstrate that it is capable of stimulating the expression of lipogenic markers.

BMPs exert potent effects on osteoblast differentiation in vitro and bone formation in vitro and in vivo. Although BMPs, including BMP-7, canonically signal by activating a serine/threonine kinase receptor complex, which then phosphorylates the SMAD transcription factors, the precise mechanisms by which BMP-7 promotes osteoblastic differentiation via other signaling pathways are not as well established. The results of our study strongly suggest that BMP-7 promotes osteoblast differentiation of FRC cells by activating the mTOR pathway. The mTOR inhibitor RAPA inhibited both basal and BMP-7-stimulated osteoblast differentiation as assessed by expression of biochemical markers including AP, osterix, osteocalcin, and bone sialoprotein. Our current results confirm and extend our previously published work [Shoba and Lee, 2003] showing that BMP-7 activated P70S6K phosphorylation, which is a downstream target of mTOR, and that RAPA inhibited BMP-7-induced AP activity as well as mRNA expression of several osteoblast differentiation markers.

Published studies, utilizing mesenchymal stem cells, embryonic stem cells, MC3T3, osteosarcoma and C2C12 cell lines, have revealed stimulatory and inhibitory effects of RAPA on osteoblast differentiation. For example, in ROS osteosarcoma cells, RAPA inhibited proliferation but promoted osteogenic differentiation [Ogawa et al., 1998]. Also, in C2C12 cells rapamycin promoted osteogenic differentiation [Vinals et al., 2002]. In contrast, rapamycin was shown to inhibit the osteogenic differentiation of the MC3T3 osteoblast cell line and primary mouse bone marrow cells [Singha et al., 2008]. These effects were attributed to inhibition of proliferation. Moreover, expression of P70S6 kinase promoted Runx2 expression in COS cells [Singha et al., 2008]. More recently, rapamycin was reported to stimulate osteogenic differentiation of human embryonic stem cells in association with activation of endogenous BMP/Smad signaling [Kang et al., 2009; Lee et al., 2010; Martin et al., 2010]. A similar enhancement of osteoblastic differentiation by a newer generation of mTOR inhibitor was observed in human and mouse mesenchymal stem cells, SaOS osteosarcoma cells, and mouse calvarial cultures [Martin et al., 2010]. Moreover, RNA silencing of mTOR in SaOS cells enhanced their osteoblastic differentiation [Martin et al., 2010]. Similar to what was observed in human embryonic fibroblasts, the mTOR inhibitor was associated with up-regulation of BMP/Smad signaling [Kishimoto et al., 2010; Lee et al., 2010]. BMP-2 induces the phosphorylation of mTOR in lung cancer cell lines [Langenfeld et al., 2005]. Recently, Xian et al. [2012] reported that IGF1 stimulated osteoblast differentiation in bone marrow cultures and phosphorylation of mTOR in wild-type mice, and the bone mass and mineralization in IGF1r flox/flox mice were decreased. These authors further suggested that bone mass is maintained by activation of mTOR in mesenchymal stem cells.

Although the reasons for the discrepant results are not readily apparent, one possible explanation for the enhanced osteogenic differentiation seen when mTOR is inhibited in osteoblastic progenitors is that mTOR promotes lipid accrual in a variety of cell types, by stimulating lipid synthesis and inhibiting lipid oxidation [Chakrabarti et al., 2010]. Aberrant activation of mTOR has been postulated to result in lipotoxicity, and bone marrow lipogenesis has been implicated in osteoporosis. Indeed lipid accrual and adipogenic differentiation are thought to inhibit osteogenic differentiation [Kishimoto et al., 2010]. However, our results and those of others also suggest that in whole cell populations, osteogenesis and adipogenesis are not necessarily mutually exclusive. We found that BMP-7 not only increased expression of osteogenic markers but also increased expression of lipid synthesizing enzymes.

Our results show that BMP-7 stimulates PPARγ expression. Consistent with our findings are those of Kang et al. [2009] who, using the C3H10T½ mesenchymal stem cell line, reported that several BMPs including BMP-7 promoted both adipogenic and osteogenic differentiation. Moreover, overexpression of PPARγ-2

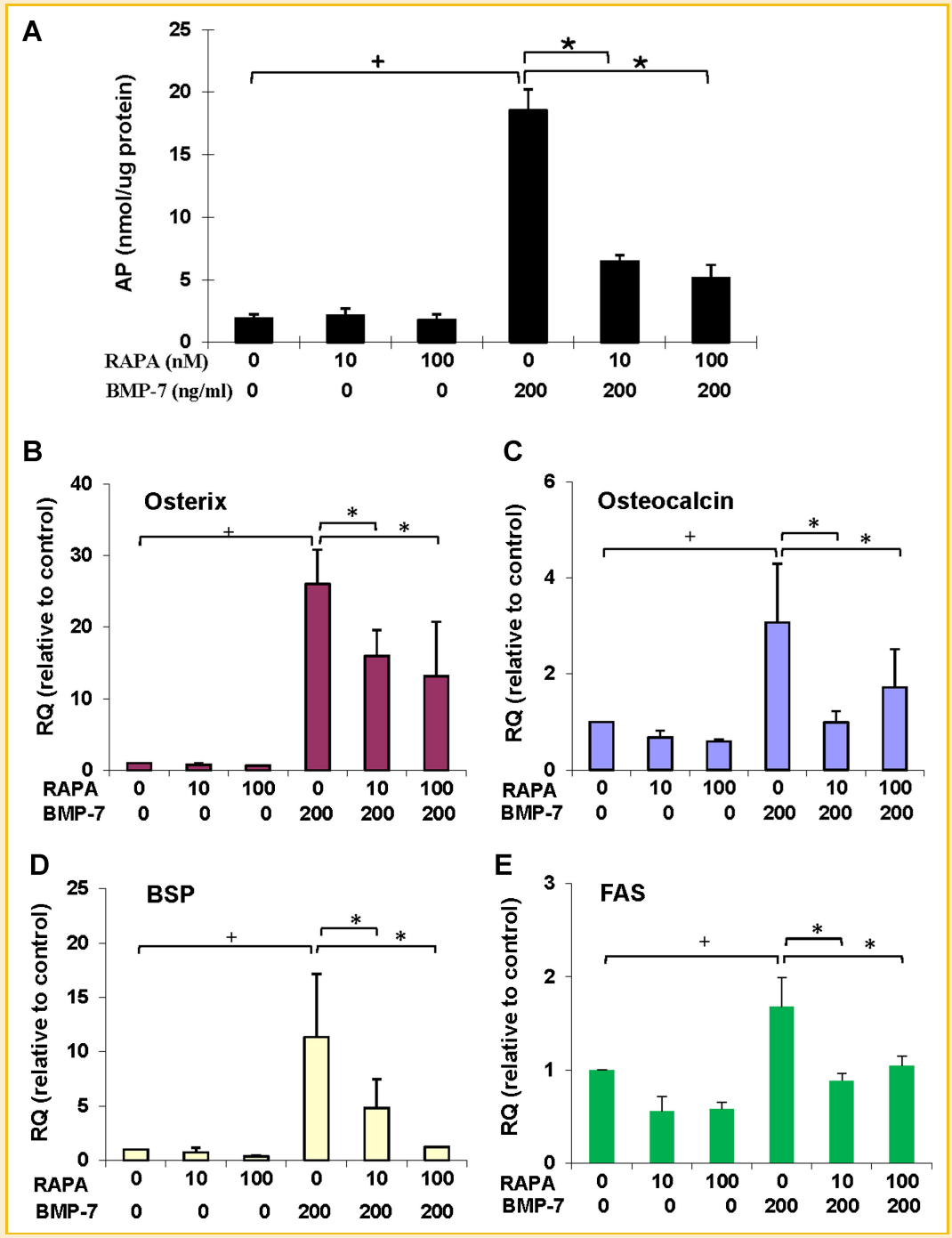

Fig. 6. Effects of rapamycin on (A) AP activity, and mRNA expression of (B) Osterix, (C) OCN, (D) BSP, and (E) FAS in control and BMP-7-treated FRC cell cultures. Values are the means ± SD of three to five independent experiments of three different FRC preparations and are normalized to vehicle-treated control. + indicates a $P < 0.05$, compared to vehicle-treated control. * indicates a $P < 0.05$, compared to the BMP-7-treated sample. $P < 0.05$ is considered statistically significant.

promoted osteogenic and adipogenic differentiation, whereas cells deficient in PPARγ exhibited decreased BMP-induced adipogenic and osteogenic differentiation [Hasegawa et al., 2008]. However, when individual cells were analyzed within the whole cell population, it was found that the same cells that expressed osteogenic makers did not express adipogenic markers and vise versa. Mikami et al. [2011] using a cell line derived from newborn rat calvariae reported that BMP-2 inhibited adipogenic markers while stimulating osteogenic markers at both early and late stages of differentiation. However, Yoshiko et al. [2010] showed that

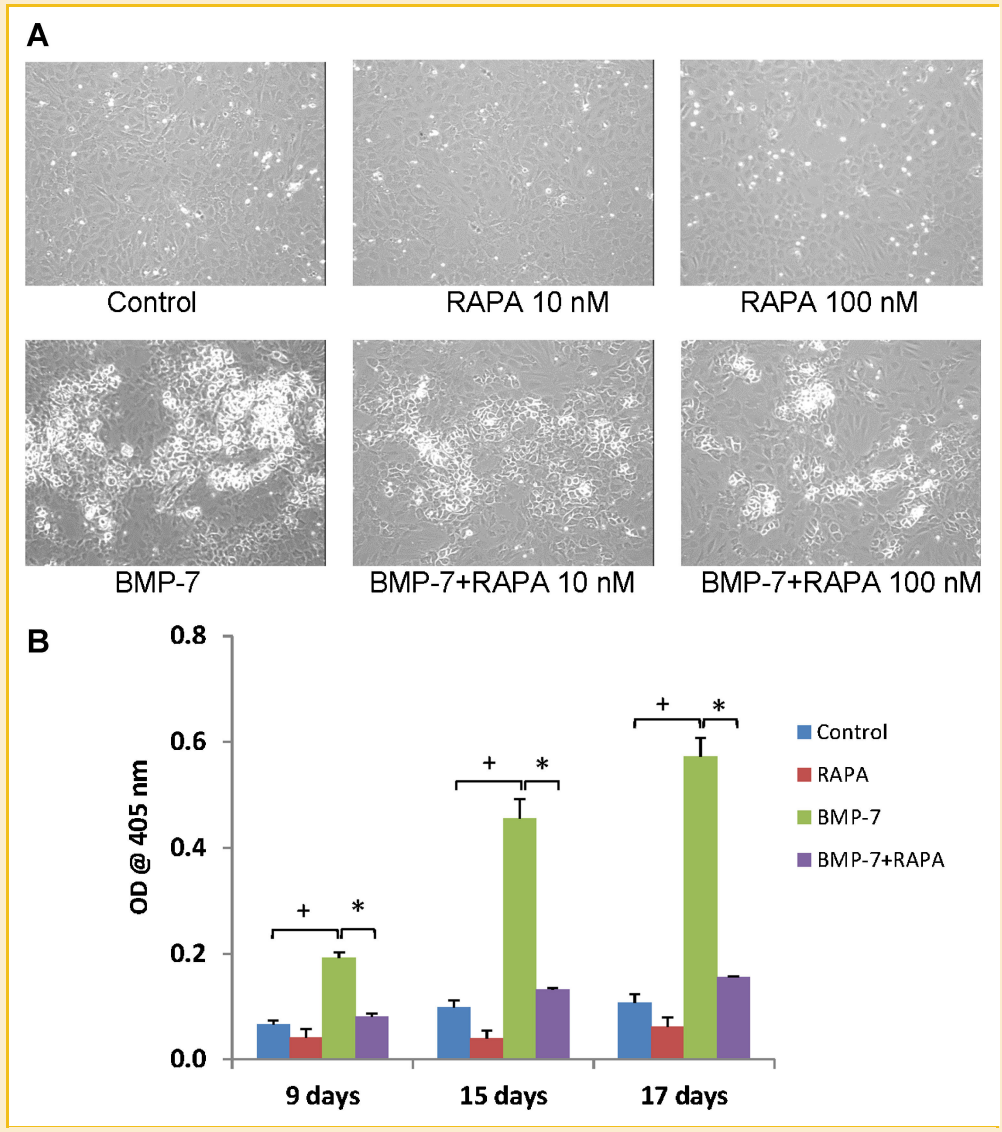

Fig. 7. Formation of mineralized bone nodules in FRC cell cultures in the presence or absence of RAPA and BMP-7. Confluent FRC cells grown in αMEM in 12-well plates were treated as indicated. Media were changed every 3 days. A: On Day 9, nodules were clearly discernable in BMP-7-treated cells under the phase contrast microscope. B: Quantitative results of AR-S staining of FRC cell cultures treated with solvent (as control), RAPA alone (10 nM), BMP-7 alone (200 ng/ml), and BMP-7 (200 ng/ml) plus RAPA (10 nM) on Days 9, 15, and 17. Values are the means ± SD of three independent experiments of three different FRC preparations and are normalized as indicated. + and * indicate a $P < 0.05$, compared to the same day control and the BMP-7-treated sample, respectively. $P < 0.05$ value is considered statistically significant.

activation of PPARγ promoted both osteogenic and adipogenic differentiation in a subset of calvarial pre-osteoblasts. Hasegawa et al. [2008] demonstrated that, whereas PPARγ activation consistently induced adipogenic markers while inhibiting osteogenic marks in bone marrow stromal cells, a subset of calvarial cells could be induced to exhibit both adipogenic and osteogenic phenotypes. Thus, it is possible that different cells have different capacities to differentiate down osteogenic and adipogenic phenotypes, and that rat calvarial cells may exhibit a greater degree of bi-potentiality in this respect than do bone marrow cells. Indeed, our kinetics show that over time the expression of lipogenic enzymes after addition of BMP-7 to calvarial cultures declines while osteogenic marker expression increases, although lipogenic enzymes expression is always higher in BMP-7 treated cells than in the control cells. This could also reflect responses of different cells within the population.

Our results do not elucidate the precise mechanisms by which mTOR activates the osteogenic and lipogenic phenotypes. Known downstream effectors of mTOR include p70S6 kinase and eIF4E-BP (see review [Zoncu et al., 2011]). Although studies in which P70S6K was overexpressed in COS cells showed that Runx2 was induced in the P70S6K overexpressing cells [Singha et al., 2008], it is not yet known how P70S6K could induce the osteogenic program. Moreover, although RNAi silencing of P70S6K enhances bone formation [Martin et al., 2010], it is not clear that P70S6K is the effector of mTOR-mediated adipogenesis. For example, in rat liver, a

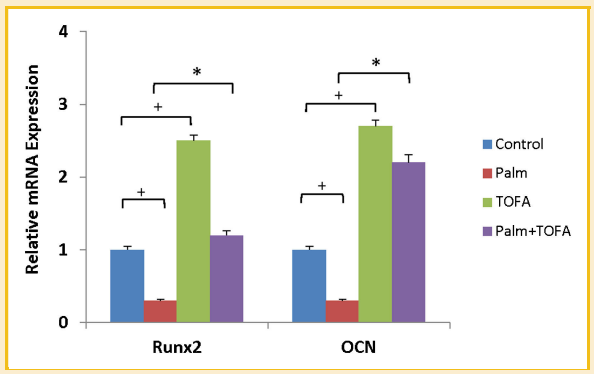

Fig. 8. Effects of palmitate and TOFA on relative mRNA expression levels of Runx2 and osteocalcin (OCN) in FRC cell cultures. FRC cells were cultured as described in Materials and Methods Section until confluent, followed by addition of vehicle control (DMSO), palmitate (250 μM), TOFA (10^{-6} M), or the combination of palmitate (250 μM), and TOFA (10-6 M). Cultures were terminated 48 h after treatment. All data was calculated using the ΔΔCT method and compared to endogenous expression of β-2 microglobulin. + indicates a $P < 0.01$, compared to the vehicle control. * indicates a $P < 0.01$, compared to the palmitate only-treated cultures.

P70S6K inhibitor did not prevent mTOR from activating the lipogenic program [Li et al., 2010].

An important question is what is the physiological role of the BMP-7-induced lipogenesis in bone cells? This is directly related to the question of what is the metabolic fate of the de novo synthesized fatty acids? It is possible that the increase in lipogenic enzyme expression by BMP-7 is part of the anabolic response of osteoblasts to BMPs. The de novo synthesized fatty acids may be esterified with glycerol to produce triglycerides. The glycerol would be derived either from glycolysis or through the process of glyceroneogenesis, by which glycerol is synthesized from gluconeogenic substrates [Nye et al., 2008]. Alternatively or in addition, the de novo fatty acids could enter into other pathways.

Our results showed that exogenous palmitate inhibited osteoblast differentiation. One possible explanation is that if palmitate is oxidized, this may inhibit glucose uptake and cause insulin resistance in osteoblasts as it does in metabolically active tissues [Nye et al., 2008]. If glucose consumption is important in osteoblast action, loss of glucose metabolism could be deleterious. However, it is also possible that some of the exogenous palmitate is esterified to glycerol to form triglycerides. Our observation that TOFA increases basal osteoblast differentiation and prevents palmitate from inhibiting osteoblast differentiation is interesting, and suggests an alternative mechanism. TOFA is a pharmacological inhibitor of ACC, which catalyzes the carboxylation of acetyl CoA to malonyl CoA. Malonyl CoA is the precursor of long-chain fatty acids as a substrate of fatty acid synthase. In addition, malonyl CoA is a potent allosteric inhibitor of carnitine palmitoyl transferase-1 (CPT-1), which is the rate-limiting step in beta-oxidation of fatty acids. Thus inhibition of ACC not only will inhibit fatty acid biosynthesis, but also is predicted to stimulate fatty acid oxidation. It is possible that palmitate is poorly oxidized, leading to accumulation of fatty acyl CoA, diacylglycerols, and ceramide, which are believed to be toxic species in cells [Holland et al., 2007; Chibalin et al., 2008; Elbaz et al., 2010]. Accordingly, reduction of malonyl CoA using TOFA is expected to reduce the generation of these species from palmitate by causing greater beta-oxidation.

Although our studies were conducted in isolated cell cultures, it is also important to consider the consequences of our findings with respect to whole body energy metabolism. The present data showed that BMP-7 increased osteocalcin expression. Recently, bone-derived, undercarboxylated osteocalcin has been shown to promote insulin secretion and improve insulin sensitivity in metabolic tissues [Ducy, 2011; Karsenty and Oury, 2012; Schwetz et al., 2012]. Thus, BMP-7 signaling in bone could promote systemic insulin sensitivity and provide serum insulin which may then promote further osteocalcin decarboxylation. In turn, a positive feed forward mechanism may be established whereby bone derived osteocalcin provides the basis for supplying the optimal energy requirements for bone remodeling.

Overall, the present data shows that exposure of FRC cultures to excess lipid suppresses expression of osteoblast markers, whereas inhibition of the rate limiting enzyme in the lipogenic pathway stimulates expression of osteoblast differentiation markers. Although our results do not clearly support a conclusion that rapamycin could be used effectively to prevent age-related osteoporosis, it is possible that targeting individual cells within an osteoprogenitor cell population with an ACC inhibitor may indeed promote osteogenic differentiation and become a basis for practical treatment of age related bone loss. The current findings employing the primary culture system provide an important basis for experimentation to provide further insights to the potential role of mTOR in osteogenesis and adipogenesis in bone. The current observations also provide a foundation to address future questions, such as mTOR degradation and RAPA-insensitive effect, and the relationship between mTOR signaling and Smad-dependent signaling. Future studies should also focus on understanding precise molecular switch that activate the osteogenic and lipogenic programs in developing and regenerating bones. Moreover, future studies will be required to determine how BMPs and mTOR regulate flux through glucose and lipid metabolic pathways and whether BMP/mTOR signaling alters the carboxylation of osteocalcin to effect improvements in whole body insulin/glucose homeostasis.

ACKNOWLEDGMENTS

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

REFERENCES

Aronow MA, Gerstenfeld LC, Owen TA, Tassinari MS, Stein GS, Lian JB. 1990. Factors that promote progressive development of osteoblast phenotype in cultured fetal rat calvaria cells. J Cell Physiol 143:213–221.

Asahina I, Sampath TK, Hauschka PV. 1996. Human osteogenic protein-1 induces chondroblastic, osteoblastic, and/or adipocytic differentiation of clonal murine target cells. Exp Cell Res 222:38–47.

Aubin JE. 1998. Advances in the osteoblast lineage. Biochem Cell Biol 76:899–910.

Averous J, Proud CG. 2006. When translation meets transformation: The mTOR story. Oncogene 25:6423–6435.

Bellows CG, Heersche JN. 2001. The frequency of common progenitors for adipocytes and osteoblasts and of committed and restricted adipocyte and osteoblast progenitors in fetal rat calvaria cell populations. J Bone Miner Res 16:1983–1993.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Chakrabarti P, English T, Shi J, Smas CM, Kandror KV. 2010. Mammalian target of rapamycin complex 1 suppresses lipolyisis, stimulates lipogenesis, and promotes fat storage. Diabetes 59:775–781.

Chen TL, Shen WJ, Kraemer FB. 2001. Human BMP-7/OP-1 induces the growth and differentiation of adipocytes and osteoblasts in bone marrow stromal cell cultures. J Cell Biochem 82:187–199.

Chen D, Zhao M, Mundy GR. 2004. Bone morphogenetic proteins. Growth Factors 22:233–241.

Chibalin AV, Leng Y, Vieira E, Krook A, Bjornholm M, Long YC, Kotova O, Zhong Z, Sakane F, Steiler T, Nylen C, Wang J, Laakso M, Topham MK, Gilbert M, Wallberg-Henriksson H, Zierath JR. 2008. Downregulation of diacylglycerol kinase delta contributes to hyperglycemia-induced insulin resistance. Cell 132:375–386.

Derynck R, Zhang YE. 2003. Smad-dependent and Smad-independent pathways in TGF-b family signaling. Nature 425:577–584.

Ducy P. 2011. The role of osteocalcin in the endocrine cross-talk between bone remodeling and energy metabolism. Diabetologia 54:1291–1297.

Elbaz A, Wu X, Rivas D, Gimble JM, Duque G. 2010. Inhibition of fatty acid biosynthesis prevents adipocyte lipotoxicity on human osteoblasts in vitro. J Cell Mol Med 14:982–991.

Friedlaender GE, Perry CR, Cole JD, Cook SD, Cierny G, Muschler GF, Zych, GA, Calhoun JH, LaForte AJ, Yin S. 2001. Osteogenic Protein-1 (Bone Morphogenetic Protein-7) in the treatment of tibial nonunions. J Bone Joint Surg Am 83A(Suppl 1):S151–S158.

Gori F, Thomas T, Hicok KC, Spelsberg TC, Riggs BL. 1999. Differentiation of human marrow stromal precursor cells: Bone morphogenetic protein-2 increases OSF2/CBFA1, enhances osteoblast commitment, and inhibits late adipocyte maturation. J Bone Miner Res 14:1522–1535.

Hasegawa T, Oizumi K, Yoshiko Y, Tanne K, Maeda N, Aubin JE. 2008. The PPARgamma-selective ligand BRL-49653 differentially regulates the fate choices of rat calvaria versus rat bone marrow stromal cell populations. BMC Devel Biol 8:71.

Holland WL, Brozinick JT, Wang LP, Hawkins ED, Sargent KM, Liu Y, Narra K, Hoehn KL, Knotts TA, Siesky A, Nelson DH, Karathanasis SK, Fontenot GK, Birnbaum MJ, Summers, SA. 2007. Inhibition of ceramide synthesis ameliorates glucocorticoid-, saturated-fat-, and obesity-induced insulin resistance. Cell Metab 5:167–179.

Isomoto S, Hattori K, Ohgushi H, Nakajima H, Tanaka Y, Takakura Y. 2007. Rapamycin as an inhibitor of osteogenic differentiation in bone marrow-derived mesenchymal stem cells. J Orthop Sci 12:83–88.

Kang Q, Song WX, Luo Q, Tang N, Luo JY, Luo X, Chen J, Bi Y, He BC, Park JK, Jiang W, Tang Y, Huang J, Su Y, Zhu GH, He Y, Yin H, Hu Z, Wang Y, Chen L, Zuo GW, Pan XC, Shen J, Vokes T, Reid RR, Haydon RC, Luu HH, He TC. 2009. A comprehensive analysis of the dual roles of BMPs in regulating adipogenic and osteogenic differentiation of mesenchymal progenitor cells. Stem Cell Develop 18:545–559.

Karsenty G, Oury F. 2012. Biology without walls: The novel endocrinology of bone. Annu Rev Physiol 74:87–105.

Kim M, Choe S. 2011. BMPs and their clinical potentials. BMB Reports 44:619–634.

Kishimoto K, Kato A, Osada S, Nishizuka M, Imagawa M. 2010. Fad104, a positive regulator of adipogenesis, negatively regulates osteoblast differentiation. Biochem Biophys Res Commun 397:187–191.

Langenfeld EM, Kong Y, Langenfeld J. 2005. Bone morphogenetic protein-2–induced transformation involves the activation of mammalian target of rapamycin. Mol Cancer Res 3:679–684.

Laplante M, Sabatini DM. 2010. mTORC1 activates SREBP-1c and uncouples lipogenesis from gluconeogenesis. Proc Natl Acad Sci USA 107:3281–3282.

Lee KW, Yook JY, Son MY, Kim MJ, Koo DB, Han YM, Cho YS. 2010. Rapamycin promotes the osteoblastic differentiation of human embryonic stem cells by blocking the mTOR pathway and stimulating the BMP/Smad pathway. Stem Cell Develop 19:557–568.

Li S, Brown MS, Goldstein JL. 2010. Bifurcation of insulin signaling pathway in rat liver: mTORC1 required for stimulation of lipogenesis but not inhibition of gluconeogenesis. Proc Natl Acad Sci USA 107: 3441–3446.

Martin SK, Fitter S, Bong LF, Drew JJ, Gronthos S, Shepherd PR, Zannettino AC. 2010. NVP-BEZ235, a dual pan class I PI3 kinase and mTOR inhibitor, promotes osteogenic differentiation in human mesenchymal stromal cells. J Bone Miner Res 25:2126–2137.

Massague J. 2000. How cells read TGF-beta signals. Nature Rev Mol Cell Biol 1:169–178.

McCarthy TL, Centrella M, Canalis E. 1988. Further biochemical and molecular characterization of primary rat parietal bone cell cultures. J Bone Miner Res 3:401–408.

Mikami Y, Lee M, Irie S, Honda MJ. 2011. Dexamethasone modulates osteogenesis and adipogenesis with regulation of osterix expression in rat calvaria-derived cells. J Cell Physiol 226:739–748.

Mukherjee A, Rotwein P. 2009. Akt promotes BMP-2 mediated osteoblast differentiation and bone development. J Cell Sci 122:716–726.

Niziolek PJ, Murthy S, Ellis SN, Sukhija KB, Hornberger TA, Turner CH, Robling AG. 2009. Rapamycin impairs trabecular bone acquisition from high-dose but not low-dose intermittent Parathyroid Hormone treatment. J Cell Physiol 221:579–585.

Nye C, Kim J, Kalhan SC, Hanson RW. 2008. Reassessing triglyceride synthesis in adipose tissues. Trends Endo Metab 19:356–361.

Ogawa T, Tokuda M, Tomizawa K, Matsui H, Itano T, Konishi R, Nagahata S, Hatase O. 1998. Osteoblastic differentiation is enhanced by rapamycin in rat osteoblast-like osteosarcoma (ROS 17/2.8) cells. Biochem Biophys Res Commun 249:226–230.

Schwetz V, Pieber T, Obermayer-Pietsch B. 2012. The endocrine role of the skeleton: Background and clinical evidence. Eur J Endo 166:959–967.

Shoba LNN, Lee JC. 2003. Inhibition of phosphatidylinositol 3-kinase and p70S6 kinase blocks Osteogenic Protein-1 induction of alkaline phosphatase activity in fetal rat calvaria cells. J Cell Biochem 88:1247–1255.

Singha UK, Jiang Y, Yu S, Luo M, Lu Y, Zhang J, Xiao G. 2008. Rapamycin inhibits osteoblast proliferation and differentiation in MC3T3-E1 cells and primary mouse bone marrow stromal cells. J Cell Biochem 103:434–446.

Stanford CM, Jacobson PA, Eanes ED, Lembke LA, Midura RJ. 1995. Rapidly forming apatitic mineral in an osteoblastic cell line (UMR 106-01 BSP). J Biol Chem 270:9420–9428.

Steenhuis P, Carr KM, Pettway GJ, Ignelzi MA. 2009. Osteogenic and adipogenic cell fractions isolated from postnatal mouse calvaria. Cells Tissues Organs 190:150–157.

ten Dijke P, Fu J, Schaap P, Roelen BA. 2003. Signal transduction of bone morphogenetic proteins in osteoblast differentiation. J Bone Joint Surg Am 85A(Suppl 3):34–38.

Vinals F, Lopez-Rovira T, Rosa JL, Ventura F. 2002. Inhibition of PI3K/p70 S6K and p38 MAPK cascades increases osteoblastic differentiation induced by BMP-2. FEBS Lett 510:99–104.

Xian L, Wu X, Pang L, Lou M, Rosen CJ, Qiu T, Crane J, Frassica F, Zhang L, Rodriguez JP, Jia X, Yakar S, Xuan S, Efstratiadis A, Wan M, Cao X. 2012. Matrix IGF-1 maintains bone mass by activation of mTOR in mesenchymal stem cells. Nat Med 18:1095–1101.

Yeh LCC, Adamo ML, Kitten AM, Olson MS, Lee JC. 1996. Osteogenic protein-1-mediated insulin-like growth factor gene expression in primary cultures of rat osteoblastic cells. Endocrinology 137:1921–1931.

Yeh LCC, Adamo ML, Olson MS, Lee JC. 1997. Osteogenic protein-1 and insulin-like growth factor synergistically stimulate rat osteoblastic cell differentiation and proliferation. Endocrinology 138:4181–4190.

Yeh LCC, Unda R, Lee JC. 2000. Osteogenic Protein-1 differentially regulates the mRNA expression of bone morphogenetic proteins and their receptors in primary cultures of osteoblasts. J Cell Physiol 185: 87–97.

Yeh LCC, Zavala MC, Lee JC. 2006. C-type natriuretic peptide enhances osteogenic protein-1-induced osteoblastic cell differentiation via Smad5 phosphorylation. J Cell Biochem 97:494–500.

Yeh LCC, Ma X, Matheny RW, Jr., Adamo ML, Lee JC. 2010. Protein kinase D mediates the synergistic effects of BMP-7 and IGF-I on osteoblastic cell differentiation. Growth Factors 28:318–328.

Yoshiko Y, Oizumi K, Hasegawa T, Minamizaki T, Tanne K, Maeda N, Aubin JE. 2010. A subset of osteoblasts expressing high endogenous levels of PPARgamma switches fate to adipocytes in the rat calvaria cell culture model. PLoS ONE 5:e11782.

Zhang YE. 2009. Non-Smad pathways in TGF-β signaling. Cell Res 19: 128–139.

Zoncu R, Efeyan A, Sabatini DM. 2011. mTOR: From growth signal integration to cancer, diabetes and ageing. Nat Rev Mol Cell Biol 12: 21–35.