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Rapamycin Inhibits Osteoblast Proliferation and Differentiation in MC3T3-E1 Cells and Primary Mouse Bone Marrow Stromal Cells

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Abstract While the roles of the mammalian target of rapamycin (mTOR) signaling in regulation of cell growth, proliferation, and survival have been well documented in various cell types, its actions in osteoblasts are poorly understood. In this study, we determined the effects of rapamycin, a specific inhibitor of mTOR, on osteoblast proliferation and differentiation using MC3T3-E1 preosteoblastic cells (MC-4) and primary mouse bone marrow stromal cells (BMSCs). Rapamycin significantly inhibited proliferation in both MC-4 cells and BMSCs at a concentration as low as 0.1 nM. Western blot analysis shows that rapamycin treatment markedly reduced levels of cyclin A and D1 protein in both cell types. In differentiating osteoblasts, rapamycin dramatically reduced osteoblast-specific osteocalcin (*Ocn*), bone sialoprotein (*Bsp*), and osterix (*Osx*) mRNA expression, ALP activity, and mineralization capacity. However, the drug treatment had no effect on osteoblast differentiation parameters when the cells were completely differentiated. Importantly, rapamycin markedly reduced levels of Runx2 protein in both proliferating and differentiating but not differentiated osteoblasts. Finally, overexpression of S6K in COS-7 cells significantly increased levels of Runx2 protein and Runx2 activity. Taken together, our studies demonstrate that mTOR signaling affects osteoblast functions by targeting osteoblast proliferation and the early stage of osteoblast differentiation. J. Cell. Biochem. 103: 434–446, 2008.

Key words: rapamycin; mammalian target of rapamycin; osteoblast; proliferation; differentiation; osteocalcin; Runx2

Rapamycin is an antiproliferative drug that has been clinically used as an immunosuppressant and a potential anti-cancer agent [Abraham and Wiederrecht, 1996; Faivre et al., 2006]. Rapamycin is a specific inhibitor of the mammalian target of rapamycin (mTOR), a highly conserved serine/threonine kinase. Rapamycin, in complex with immunophilin FKBP12 (FK506 binding protein), specifically binds to mTOR and interferes its function. mTOR is a central regulator of cell growth whose activity is involved in a wide spectrum of growth-related processes, including translation, transcription, autophagy, and actin organization [Faivre et al., 2006; Wullschleger et al., 2006]. mTOR elicits its functions mainly through controlling protein synthesis by two distinct mechanisms [Montagne et al., 1999]: (i) mTOR phosphorylates and inactivates 4E-BP1, a translation repressor that binds to and inhibits the translation initiation factor 4E (eIF-4E). Upon phosphorylation by mTOR, 4E-BP1 is inactivated and eIF-4E is released, thus resulting in an increased protein synthesis of 5′ capped mRNAs [Gingras et al., 2001], and (ii) mTOR phosphorylates and activates the ribosomal protein S6 kinase (S6K), which phosphorylates S6

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ribosomal protein, a component of the S40 ribosome subunit, thus facilitating protein translation. Activated p70S6K initiates the translation of a class of mRNAs containing a tract of polypyrimidine (TOP) in their 5′ untranslated region (UTR) [Jefferies et al., 1997]. In addition to translation regulation, mTOR/S6K signaling has been recently implicated in controlling apoptosis. It has been shown to phosphorylate pro-apoptotic protein Bad at Ser136 and prevents its interaction with Bcl-2, thus protecting cells from apoptosis [Freilinger et al., 2006].

mTOR activity is regulated mainly by the TSC-Rheb signaling pathway. The two tumor suppressors, TSC1 and TSC2, form a complex that displays a GTPase activating protein activity against Rheb, a Ras-like small GTPase. Deficiency in TSC function results in an enhanced Rheb activity, which in turn stimulates mTOR signaling. In animal models, the growth promoting effect of TSC (suppressor of mTOR) deficiency or Rheb (activator of mTOR) overexpression can be attenuated by inactivation of mTOR (or dTOR in Drosophila) [Gao et al., 2002]. mTOR elicits its pleiotropic function in the context of two distinct complexes termed mTOR complex 1 (mTORC1) and 2 (mTORC2), both of which contain several components that are conserved from yeast to human [Wullschleger et al., 2006]. Together with TSC, Rheb, and other components, mTOR controls cell size and proliferation and prevents apoptosis in response to nutrient availability, oxygen, and energy levels [Montagne et al., 1999; Zhang et al., 2000; Fingar et al., 2002; Inoki et al., 2003, 2006; Freilinger et al., 2006].

Through inhibition of mTOR and subsequent protein translation, rapamycin inhibits the transduction of the IL-2R signal and other cytokines relevant to the allograft rejection response. Because of these characteristics, rapamycin has been introduced into clinical practice as an immunosupressive agent. Furthermore, due to its antiproliferative and pro-apoptotic activities, rapamycin is studied for its anti-cancer activity in clinical trials [Faivre et al., 2006].

In human skeleton, bone mass is controlled by the numbers and the activities of osteoblasts, the bone-forming cells, and osteoclasts, the bone-resorbing cells. During bone formation, multipotential mesenchymal cells proliferate and differentiate into osteoblasts which synthesize and deposit a mineralizing extracellular matrix. Mature osteoblasts eventually become either osteocytes, bone lining cells or vanish because of apoptosis [Stein et al., 1996]. Runx2, the bone-specific product of the Cbfa1 gene, is a runt domain-containing transcription factor that is essential for osteoblast function and bone formation during embryogenesis and postnatal life [Komori et al., 1997; Mundlos et al., 1997]. Runx2 activates expression of osteoblast-specific genes, including those encoding osteocalcin, bone sialoprotein, osterix, and alkaline phosphatase [Banerjee et al., 1997; Ducy et al., 1997, 1999; Nakashima et al., 2002; Lian and Stein, 2003; Yang et al., 2003]. Its expression and activity are regulated by a number of factors including BMPs, FGF-2, PTH, TNF-α, and extracellular matrix signals, all of which play important roles in osteoblasts and bone in vivo and in vitro [Hurley et al., 1999; Montero et al., 2000; Gilbert et al., 2002; Zhang et al., 2002; Xiao et al., 2002a,b; Krishnan et al., 2003; Yang et al., 2003].

While the role of mTOR signaling in regulation of cell growth and proliferation have been extensively studied in many types of cells from *Drosophila* to human, surprisingly, there is very limited information available in the literatures regarding its independent actions in control of osteoblast proliferation, differentiation, and bone formation. In addition, as rapamycin being increasingly used as an immunosuppressant of organ-transplanted patients or anti-tumor agent, there is an urgent need to understand its effects on important tissues and organs such as bones under physiological and pathological conditions. This study examined the effects of rapamycin on osteoblast proliferation and differentiation in MC3T3-E1 subclone 4 (MC-4) cell line and primary mouse bone marrow stromal cells (BMSCs) in vitro.

MATERIALS AND METHODS

Reagents

Tissue culture media and fetal bovine serum were obtained from HyClone (Logan, UT). Other reagents were obtained from the following sources: Antibodies against phospho p70S6K and p70S6K from Cell Signaling (Beverly, MA), antibodies against Runx2, cyclin A, cyclin D1, cyclin D3, and horseradish peroxidase-conjugated goat anti-rabbit IgG from

Santa Cruz (Santa Cruz, CA), mouse monoclonal antibody against β-actin, alizarin red (AR-S), cetylpyridinium chloride (CPC), ascorbic acid (AA), and NaH_2PO_4 (inorganic phosphate or Pi) from Sigma (St. Louis, MO). Rapamycin was purchased from LC Laboratories (Woburn, MA). The drug was dissolved in DMSO. The final DMSO concentration in the culture media was less than 0.005%. All other chemicals were of analytical grade.

Cell Lines

The previously described MC3T3-E1 subclonal 4 (MC-4) cells with high osteoblast differentiation potential were used in this study [Xiao et al., 1997; Wang et al., 1999]. MC-4 cells express osteoblast phenotypic marker genes and mineralize only after growth in AA (ascorbic acid)-containing medium. MC-4 cells were maintained in AA-free α-MEM (Invitrogen, Carlsbad, CA), 10% fetal bovine serum, 1% penicillin/streptomycin and were not used beyond passage 15. COS-7 cells were maintained in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin.

Mouse Bone Marrow Stromal Cell Cultures (BMSC)

Isolation of mouse BMSCs was described previously [Xiao et al., 2002b]. Briefly, 6-week-old male C57BL/6 mice were sacrificed by cervical dislocation. Tibiae and femurs were isolated and the epiphyses were cut. Marrow was flushed with DMEM containing 20% FBS, 1% penicillin/streptomycin into a 60-mm dish and the cell suspension was aspirated up and down with a 20-gauge needle in order to break clumps of marrow. The cell suspension (marrow from 2 mice/flask) was then cultured in a T75 flask in the same media. After 10 days, cells reached confluency and were ready for experiments.

Cell Proliferation Assay

MTS assays were performed to assess the rate of cell proliferation according to manufacture's instruction (Promega, Madison, WI). Briefly, 1×10^4 cells/well were planted in 96-well plate in 100 μl proliferation medium (AA-free α-MEM medium with 10% FBS and 1% penicillin/streptomycin). Cells were incubated at 37°C for 24 h to allow attachment. The medium was replaced with fresh medium with or without rapamycin at indicated concentrations. After 72 h, 20 μl of CellTitre96AQ solution reagent was added into each well for 2 h. Finally, the absorbance was recorded at 490 nm using a 96-well plate reader. Alternately, cell proliferation was assessed by standard direct count with a hemocytometer (trypan blue staining).

Western Blot Analysis

Cells were washed with cold 1× PBS and lysed in 1× Passive Buffer (Promega) at RT for 20 min. Lysates were clarified by centrifugation (20 min, 13,000*g*, 4°C). Protein concentrations were determined by the method developed by Bio-Rad Laborotories, Inc. (Hercules, CA). Twenty five micrograms of total protein were fractionated on a 10% SDS–PAGE gel and transferred onto nitrocellulose membranes (Whatman, Schleicher & Schuell, Dassel, Germany). The membrane was blocked in 5% nonfat milk in Tris-buffered saline/Tween-20 (TBST) buffer, probed with antibodies against cyclin D1(1:1,000), cyclin D3(1:1,000), cyclin A(1:1,000), phospho-p70S6K(1:1,000), and total p70S6K(1:1,000) followed by incubation with anti-rabbit or anti-mouse antibodies conjugated with horseradish peroxidase (1:5,000); and visualized using an enhanced chemiluminescence kit (Pierce, Rockford, IL). Finally, blots were striped two times in buffer containing 65 mM Tris Cl (pH 6.8), 2% SDS, and 0.7% (v/v) β-mercaptoethanol at 65°C for 15 min and re-probed with β-actin antibody (Sigma) for normalization.

Real-Time RT-PCR

Total RNA was isolated using TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. Reverse transcription (RT) was performed using 2 μg of denatured RNA and 100 pmoles of random hexamers (Applied Biosystem, Foster, CA) in a total volume of 20 μl containing 12.5 U MultiScribe reverse transcriptase (Applied Biosystem) according to the manufacturer's instructions. PCR was performed on iCycler (BIO-RAD) using a SYBR® Green PCR Core Kit (Applied Biosystems). The DNA sequences of primers used for RT-PCR were: *Ocn*: 5′-TAG TGA ACA GAC TCC GGC GCT A-3′ (forward), 5′-TGT AGG CGG TCT TCA AGC CAT-3′ (reverse); *Bsp*: 5′-AAG AAG AGG AAG AGG AAG AAA ATG AGA ACG A -3′ (forward), 5′-GCT TCT TCT CCG TTG TCT CC-3′ (reverse); *Osx*: 5′-AGA GGT TCA CTC GCT CTG ACG A

(forward); 5′-TTG CTC AAG TGG TCG CTT CTG-3′ (reverse); β-*actin*: 5′-TCC TCC TGA GCG CAA GTA CTC T-3′ (forward), 5′-CGG ACT CAT CGT ACT CCT GCT T-3′ (reverse). For all primers the amplification was performed as follows: initial denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Melting curve analysis was used to confirm the specificity of the PCR products. Six samples were run for each primer set. The levels of mRNA were calculated by the ΔCT method [Wang et al., 2004]. *Ocn* and *Bsp* mRNAs were normalized to β-actin mRNA.

Mineralization Assay

Mineralization capacity was determined by Alizarin red (AR-S) staining according to a protocol kindly provided by Dr. Charles Sfeir of the University of Pittsburgh School of Dentistry. Briefly, cells were rinsed once with 1× PBS, fixed with cold 70% ethanol for 60 min, and rinsed 3 times with ddH_2O to remove ethanol. Cells were then stained with 40 mM AR-S (pH 4.2) (2 ml/35-mm dish) at RT for 15 min with rotation on a shaker. After that, cells were rinsed with ddH_2O five times to remove unbound AR-S and one time with 1× PBS to further reduce non-specific staining. Photographs were then taken. To quantify the AR-S stain, 10% cetylpyridinium chloride (CPC) was added to the dishes (2 ml/35-mm dish) at RT for 60 min with shaking. Aliquots of these AR-S extracts were taken, diluted in 10% CPC solution, and the concentration were determined by absorbance measurement at 562 nm on a 96-well multiple reader.

Alkaline Phosphatase (ALP) Assay

Briefly, cells were harvested in 1× Passive Buffer (Promega). Lysates were clarified by centrifugation (20 min, 13,000*g*, 4°C). Five microliters of cell extracts was added to each well (96-well plate) containing 150 μl p-nitrophenyl phosphate (pNPP) (Sigma) at 37°C for 10–60 min depending on the level of ALP activity in the extracts. ALP activity was determined by absorbance measurement at 405 nm on a 96-well plate reader. ALP activity was normalized to total protein.

DNA Constructs and Transfection

Wild-type or mutated p6OSE2-luc, pCMV5/β-gal, pCMV5/Runx2, and pCMV5/ATF4 expression vectors were described previously [Yang et al., 2004; Xiao et al., 2005]. pCMV5/S6K expression vector was kindly provided from Dr. Ken Inoki of the University of Michigan School of Medicine Ann Arbor. For protein expression study, COS-7 cells were plated on 35-mm dishes at a density of 5×10^4 cells/cm^2. After 24 h, cells were transfected with Lipofect-AMINE 2000 (InVitrogen) according to manufacturer's instructions. Each transfection contained 0.5 μg of pCMV5/Runx2 or pCMV5/ATF4 and 0.5 μg of pCMV5/β-gal or pCMV5/S6K expression vectors. After 36 h, whole cell extracts were used for Western blot analysis. For functional assay, COS-7 cells were transiently transfected with wild-type or mutated p6OSE2-luc (0.25 μg), pRL-SV40 (0.01 μg), and 0.5 μg of expression plasmids for β-gal, S6K, Runx2, or S6K plus Runx2. After 36 h, cells were harvested and assayed using the Dual Luciferase Assay Kit (Promega) on a Veritas™ Microplate Luminometer (Turner Biosystem, Inc., Sunnyvale, CA). Firefly luciferase activity was normalized to renilla luciferase activity for transfection efficiency.

Statistical Analysis

Results were expressed as means ± standard deviation (SD). Statistical differences between means for different groups were evaluated with Statview software (Abacus Concepts, Berkley, CA) using one-way analysis of variance (ANOVA). Students' *t*-test was used to test for differences between different groups. Differences with a $P < 0.05$ was considered as statistically significant. All experiments were repeated a minimum of three times with triplicate samples.

RESULTS

Rapamycin Inhibits Proliferation of MC3T3-E1 Subclone 4 (MC-4) Cells and Primary Mouse Bone Marrow Stromal Cells (BMSCs)

Rapamycin is known to inhibit cell proliferation in many types of cells. To determine the effect of rapamycin on osteoblast proliferation, we used MTS assay and direct cell count, both of which are widely used for measuring cell proliferation. MC-4 cells and BMSCs were seeded at low density (10^4 cells/well in 96-well plate) and treated with increasing concentrations of rapamycin or control vehicle in proliferation medium for 72 h. As shown in Figure 1A,B, rapamycin reduced proliferation

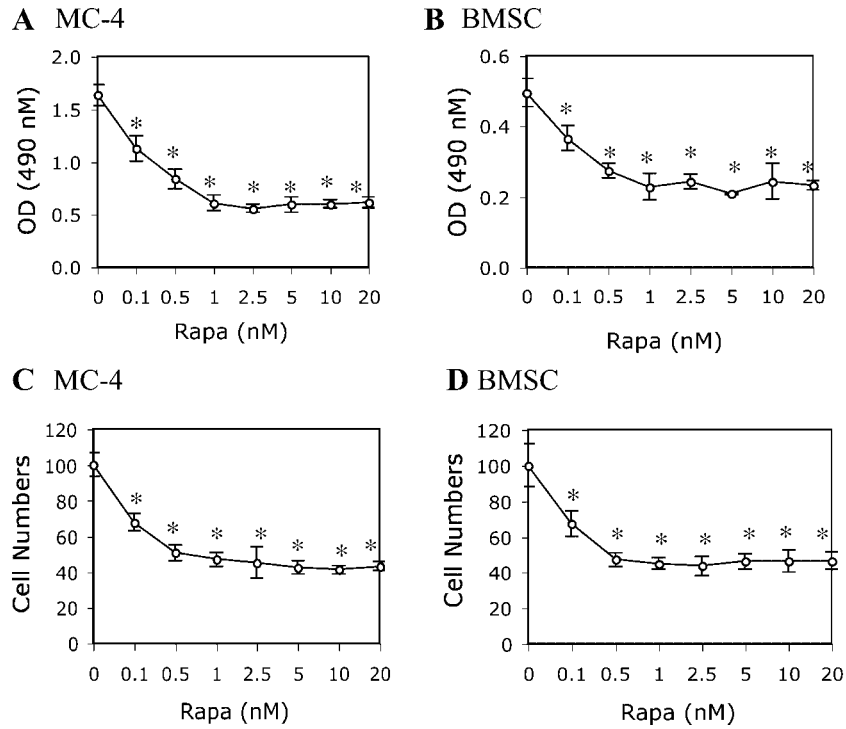

Fig. 1. Rapamycin inhibits cell proliferation. MC-4 cells (**A** and **C**), primary BMSCs (**B** and **D**) were seeded at 1×10^4 cells/well in 96-well plate ± rapamycin (0.1–20 nM) for 72 h. Cell proliferation rate was assessed by MTS assay (A and B) or direct count by trypan blue (C and D). Data are presented as the mean ± SD from triplicates. $^*P < 0.01$ compared to control group. Experiment was repeated three times and qualitatively identical results were obtained.

of MC-4 cells and BMSCs in a dose-dependent manner. These results were confirmed by direct cell count (Fig. 1C,D). Rapamycin (from 0.1 to 20 nM) was not cytotoxic to the cells under the described experimental conditions as demonstrated by trypan blue exclusion assay (data not shown).

Rapamycin Decreases the Levels of Cyclin A and D1 Proteins in Proliferating Osteoblasts

Cell-cycle progression from G1 to S phase is controlled by D-type cyclins. To explore the mechanism underlying the rapamycin-induced antiproliferative effect in osteoblasts, we determined the effect of rapamycin on the levels of these cyclin proteins. MC-4 cells were cultured in proliferation medium and treated with increasing concentration of rapamycin (from 0.01 to 10 nM) for 72 h. Cells were then lysed and the expression levels of cyclin A, D1, and D3 proteins were determined by Western blotting. As shown in Figure 2A, rapamycin significantly decreased the levels of cyclin A and D1 proteins in a dose-dependent manner. As shown in Figure 2B,C, rapamycin time-dependently reduced the levels of cyclin A and D1 proteins in both MC-4 cells and BMSCs. The levels of cyclin D3 protein were too low to be detected by Western blot analysis in both cell types (data not shown). Rapamycin rapidly blocked p70S6K phosphorylation without altering the levels of total p70S6K proteins in both cell types, thus demonstrating the specificity of the drug.

Rapamycin Inhibits Osteoblast-Specific Gene Expression and Mineralization in Differentiating Osteoblasts

MC-4 cells were used to evaluate effects of rapamycin on osteoblast differentiation because they express high levels of osteoblast phenotypic marker genes (i.e., *Ocn* and *Bsp*) and strongly mineralize after growth in AA/Pi-containing medium (differentiation medium) for 10 days [Xiao et al., 1997; Wang et al., 1999]. In these experiments, MC-4 cells were seeded at a density of 5×10^4 cells/cm^2 in 35-mm dishes and treated with and without rapamycin (10 nM) in differentiation medium for 10 days. Cells were then harvested for RNA isolation. The RNA levels of osteoblast-specific *Ocn* and

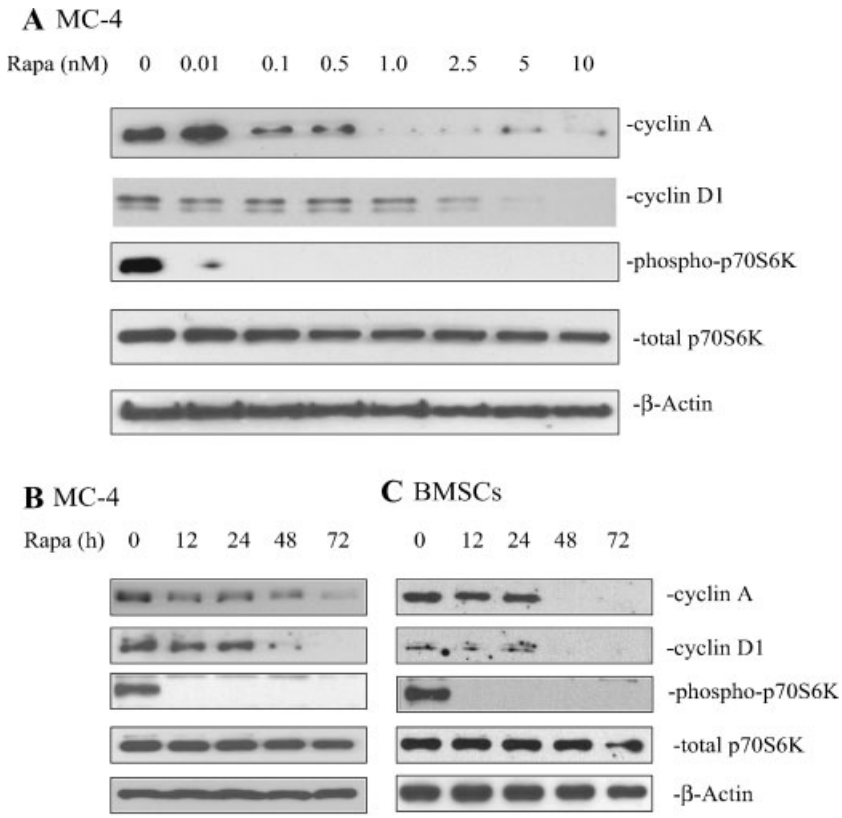

Fig. 2. Effect of rapamycin on the levels of cell cycle-related proteins. **A**: MC-4 cells were plated at 5×10^4 cells/cm^2 in 35-mm dishes ± rapamycin (0.01–10 nM) for 72 h. Whole cell extracts were prepared for Western blot analysis using rabbit or mouse antibodies against cyclin D1(1:1,000), cyclin D3(1:1,000), cyclin A(1:1,000), phospho-p70S6K(1:1,000), and total p70S6K(1:1,000). The blots were striped and re-probed with mouse b-actin antibody (1:2,000) for normalization. **B**,**C**: MC-4 cells (B) and primary BMSCs (C) were plated at 5×10^4 cells/cm^2 in 35-mm dishes ± rapamycin (10 nM) for indicated time (12–72 h) followed by Western Blot analysis as described in (A).

Bsp genes, two late markers for osteoblast differentiation, and *Osx* mRNA, an osteoblast-specific transcription factor, were determined by using quantitative real-time RT-PCR analysis. As shown in Figure 3A,B, rapamycin inhibited the expression of *Ocn* and *Bsp* mRNAs by 91% and 84%, respectively. Rapamycin reduced *Osx* mRNA by 86% (Fig. 3C). In contrast, rapamycin did not significantly affect *osteopontin* (*Opn*) mRNA level (data not shown). Separate sets of cells treated similarly were tested for mineralization capacity, a terminal parameter for osteoblast differentiation, by staining with alizarin red, a dye that directly binds to the calcium deposited in mineralized matrix. As shown in Fig. 3D, rapamycin reduced the mineralization of the extracellular matrix (ECM) by 91%. We next determined if rapamycin displays a similar inhibitory effect on osteoblast differentiation in primary BMSCs. Under these conditions, it takes approximate 15 days for primary BMSCs to differentiate and express high levels of *Ocn* and *Bsp* mRNA and high ALP activity and form mineralized nodules in the presence of Pi (data not shown). For these experiments, BMSCs were treated with rapamycin (10 nM) or drug vehicle for 15 days in differentiation media. Pi (5.0 mM) was added to the cultures for the last 4 days for the mineralization assays. One set of cells was used for RNA preparation and quantitative real-time RT-PCR analysis of *Ocn* and *Bsp* mRNA expression. The second set of cells was utilized for alizarin red staining. The third set of cells was assessed for ALP activity, an early marker for osteoblast differentiation. As shown in Figure 3E,F, the levels of *Ocn* and *Bsp* mRNA were reduced by 70% and 95%, respectively, in primary BMSCs, which was consistent with the result from MC-4 cells. Mineralization was

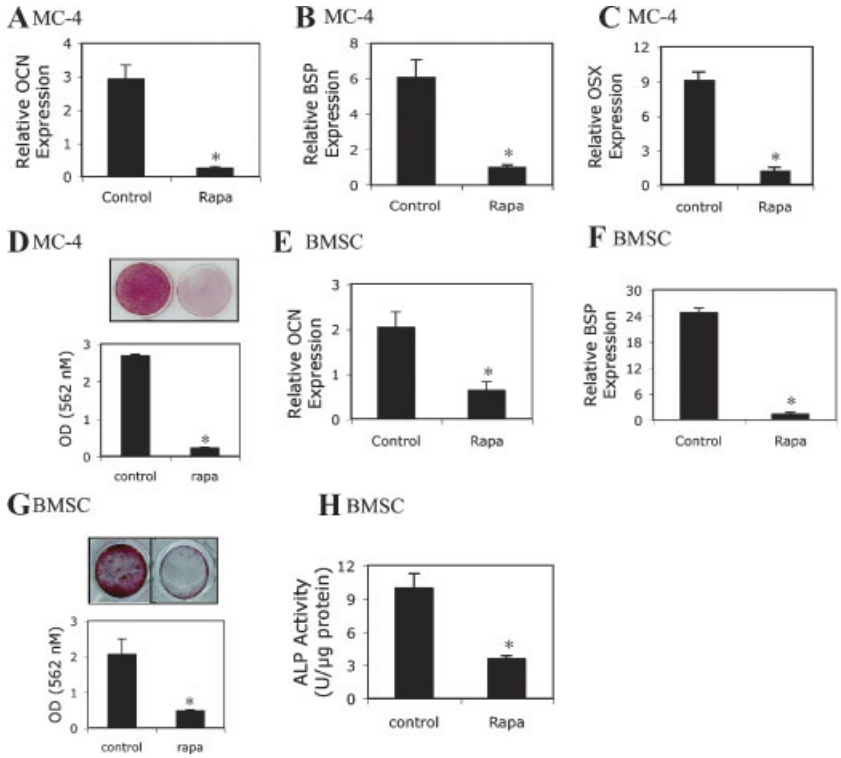

Fig. 3. Rapamycin inhibits osteoblast-specific gene expression and mineralization in differentiating osteoblasts. MC-4 cells (**A**–**D**) and primary BMSCs (**E**–**H**) were plated at 5×10^4 cells/cm^2 in 35-mm dishes in the presence and absence of rapamycin (10 nM) in differentiation medium for 10 days. Inorganic phosphate (5 mM) was added to the cultures for the last 4 days for mineralization assay groups. Cells were then assessed for osteoblast-specific Ocn, Bsp, and Osx mRNA expression by quantitative real-time RT-PCR. Levels of Ocn, Bsp, and Osx mRNA were normalized to beta-actin mRNA (A, B, C, E, and F), and alizarin red staining (D and G). ALP activity assay (H), ALP activity was normalized to total protein. Data are presented as the mean ± SD from triplicates. $^*P < 0.01$ compared to control group.

reduced by 77% (Fig. 3G). Finally, rapamycin reduced ALP enzymatic activity by 64% (Fig. 3H).

We next asked if the inhibition of osteoblast differentiation by rapamycin was due to an indirect mechanism, that is, rapamycin decreases osteoblast proliferation, consequently, inhibits cell differentiation. This is possible because cells have to reach certain density before they can differentiate. To test this possibility, MC-4 cells were plated at a saturation density (25×10^4 cells/cm^2) that is the approximate density of 10 days MC-4 cultures in proliferation medium (data not shown). Cells were then treated with and without rapamycin (10 nM) for 10 days in differentiation medium. Pi (5.0 mM) was added to the cultures for the last 4 days for the mineralization experiments. After 10 days, cells were harvested for gene expression studies or stained with alizarin red dye. As shown in Figure 4A–C, rapamycin significantly reduced the levels of *Ocn*, *Bsp*, and *Osx* mRNAs by 82%, 77%, and 80%, respectively and mineralization by 72% in these high density cells ($P < 0.01$, control vs. rapa). However, the degree of inhibition of all differentiation parameters tested in this experiment was slightly but surely lower compared to that using the normal density of cells ($P < 0.05$, normal density vs. high density). These data suggest that rapamycin inhibition of osteoblast differentiation involves both direct and indirect (i.e., through inhibition of osteoblast proliferation) mechanisms.

mTOR Signaling is not Required for Maintaining Osteoblast Differentiation State

Next, we determined if the mTOR pathway is required for maintenance of osteoblast differentiation. MC-4 cells (5×10^4 cells/cm^2) were allowed to fully differentiate by culturing them in differentiation medium for 10 days and then treated with or without rapamycin (10 nM) in differentiation medium for another 10 days followed by gene expression analysis and mineralization assays. As shown in Figure 5A–C, rapamycin was unable to inhibit either *Ocn* or

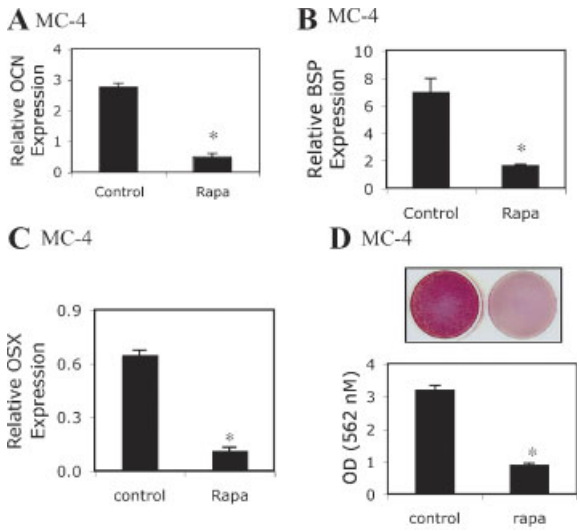

Fig. 4. Rapamycin inhibits osteoblast differentiation in high density differentiating osteoblasts. A,B: MC-4 cells were plated at very high density (2.5×10^5 cells/cm^2) in 35-mm dishes and treated in the presence and absence of rapamycin (10 nM) in differentiation medium for 10 days. Inorganic phosphate (5 mM) was added to the cultures for the last 4 days for the mineralization assay group. Cells were then assessed for osteoblast-specific Ocn, Bsp, and Osx mRNA expression by quantitative real-time RT-PCR (**A**–**C**) and for mineralization capacity by alizarin red staining (**D**). Data are presented as the mean ± SD from triplicates. $^*P < 0.01$ compared to control group.

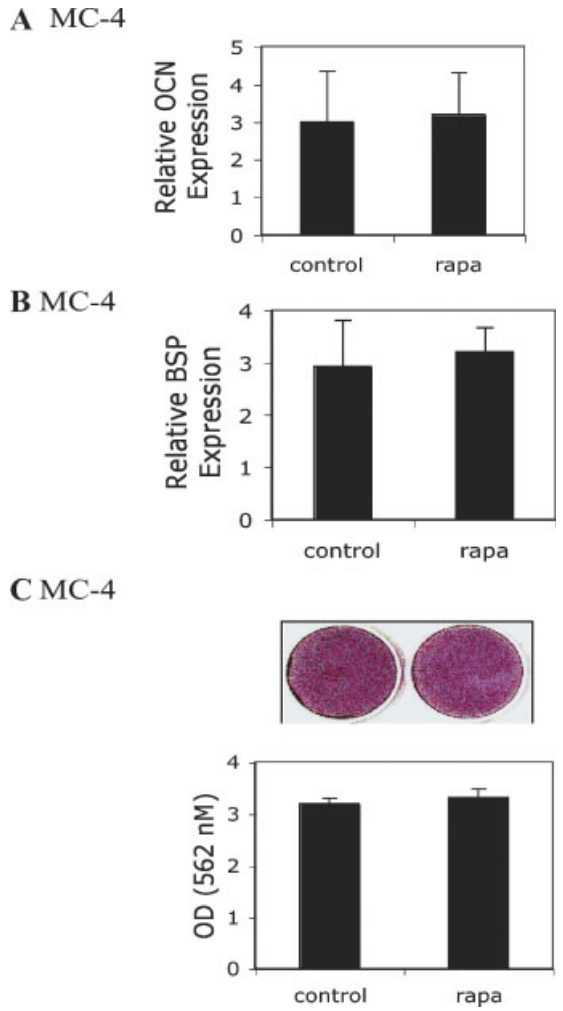

Fig. 5. Rapamycin fails to suppress osteoblast differentiation in differentiated cells. MC-4 cells (5×10^4 cells/cm^2) were first cultured in differentiation medium for 10 days and then treated ± rapamycin (10 nM) in differentiation medium for 10 days. Inorganic phosphate (5 mM) was added to the cultures for the last 4 days for the mineralization assay group. Cells were then assessed for osteoblast-specific Ocn and Bsp mRNA expression by quantitative real-time PCR (**A** and **B**) and for mineralization by alizarin red staining (**C**). Data are presented as the mean ± SD from triplicates.

Bsp mRNA expression or mineralization in differentiated MC-4 cells, which was in contrast to its effect on differentiating osteoblasts. Similar results were obtained in differentiated BMSCs (data not shown).

Rapamycin Decreases the Levels of Runx2 Protein

Because Runx2 is a master regulator for osteoblast differentiation required for osteoblast-specific gene expression and mineralization and because rapamycin inhibits all osteoblast differentiation parameters examined as shown in Figures 3 and 4, we next asked if Runx2 is involved in rapamycin inhibition of osteoblast differentiation. To test this possibility, we conducted three types of experiments. In the first, the acute effect of rapamycin treatment on level of Runx2 protein was determined in proliferating osteoblasts. MC-4 cells were treated with indicated concentrations of rapamycin for 6 h in proliferation medium followed by Western blot analysis for Runx2 levels. As shown in Figure 6A, the levels of Runx2 protein were reduced by rapamycin in a dose-dependent manner. In the second experiment, effects of rapamycin on levels of Runx2 protein were evaluated in differentiating osteoblasts. MC-4 cells were incubated with and without rapamycin for 10 days in differentiation medium. As shown in Figure 6B, rapamycin dramatically reduced the level of Runx2 protein in differentiating osteoblasts. In the third study, effect of rapamycin on level of Runx2 protein was determined in differentiated osteoblasts. In this experiment, MC-4 cells were first allowed to fully differentiate by culturing them in differentiation medium for 10 days followed by another 10 days treatment ± rapamycin in

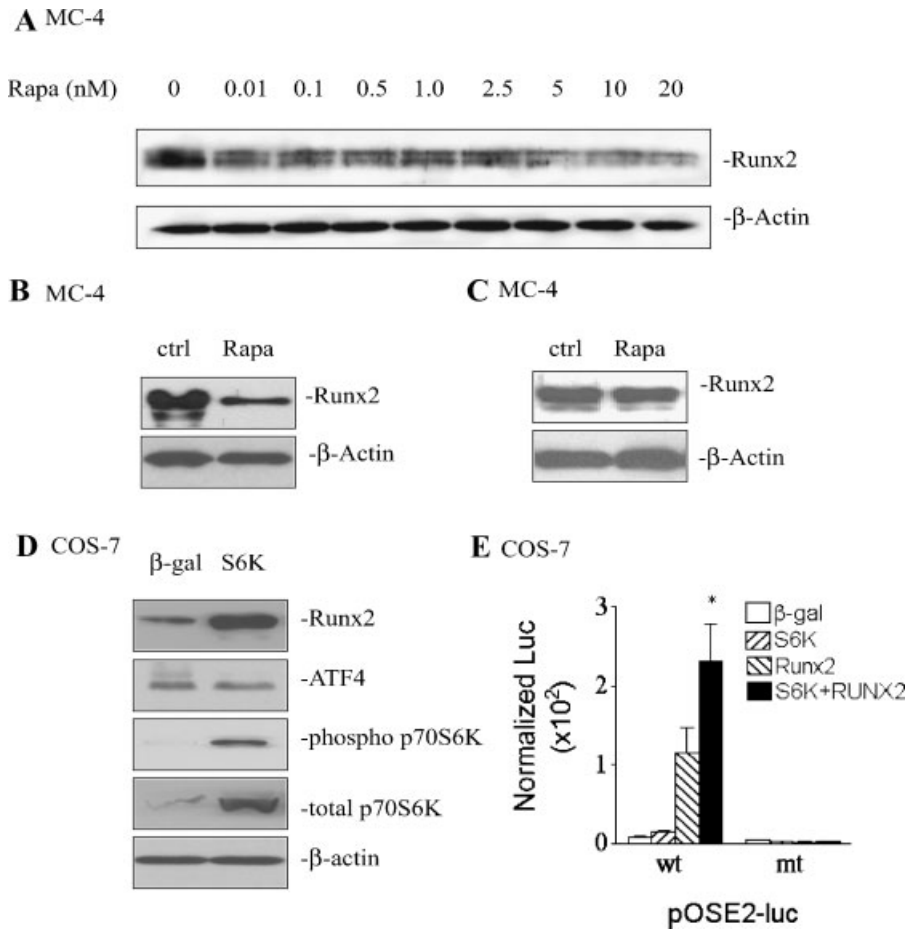

Fig. 6. mTOR signaling regulates the levels of Runx2 proteins. **A**: MC-4 cells were plated at 5×10^4 cells/cm^2 in proliferation medium ± rapamycin (0.01–20 nM) for 6 h. Whole cell extracts were prepared for Western blot analysis using rabbit antibody against Runx2 (1:1,000). The blot was striped and re-probed with mouse b-actin antibody (1:2,000) for normalization. **B**: MC-4 cells were treated ± rapamycin (10 nM) in differentiation medium for 10 days. **C**: MC-4 cells were first cultured in differentiation medium for 10 days and then treated ± rapamycin (10 nM) in the same medium for another 10 days. **D**: COS-7 cells were cotransfected with 0.5 μg of the pCMV5/Runx2 or pCMV5/ATF4 and 0.5 μg of pCMV5/b-gal or pCMV5/S6K expression vectors. After 36 h, whole cell extracts were used for Western blot analysis using antibodies against Runx2, ATF4 (1:1,000), phospho-p70S6K, p70S6K, and b-actin. **E**. COS-7 cells were transiently transfected with wild-type or mutated p60SE2-luc, pRL-SV40, containing a cDNA for Renilla Reformis luciferase to control for transfection efficiency, and expression plasmids for b-gal, S6K, Runx2, or S6K plus Runx2. After 36 h, cells were harvested for dual luciferase assays. *$P < 0.01$ compared to Runx2 alone group.

the same medium. As shown in Figure 6C, contrast to the results from proliferating or differentiating cells, the drug was unable to reduce the level of Runx2 protein in differentiated osteoblasts. Taken together, these results strongly support the notion that rapamycin inhibits osteoblast differentiation via down-regulation of Runx2 protein.

Overexpression of S6K Increases Levels of Runx2 Protein and Runx2 Activity in COS-7 Cells

S6K is one of the major downstream kinase of mTOR signaling controlling protein translation as discussed in the Introduction. We next determined the effects of S6K on the level of Runx2 protein. COS-7 cells, which lack significant amount of endogenous Runx2 protein, were cotransfected with pCMV5/Runx2 or pCMV5/ATF4 (the CMV promoter drives expression of full-length Runx2 or ATF4 cDNAs plus their 5′UTR regions) in the absence and presence of pCMV5/S6K expression vectors. As shown in Figure 6D, COS7 cells expressed very low basal levels of both phospho-p70S6K and total p70S6K proteins and the levels of both proteins were dramatically increased in S6K transfected cells compared to untransfected cells. Importantly, overexpression of pS6K in

COS-7 cells significantly increased levels of Runx2 protein. In contrast, level of ATF4, another bone-related transcription factor, was not altered by S6K overexpression. To determine the effect of S6K on Runx2-dependent transactivation, COS-7 cells were transiently transfected with p6OSE2-luc, an artificial promoter containing 6 copies of OSE2 (a specific Runx2-binding element) fused to a −34 to +13 minimal mOG2 promoter, and pRL-SV40 (for normalization) in the presence or absence of S6K and Runx2 expression plasmids. After 48 h, cells were harvested for dual luciferase assays. Firefly luciferase was normalized to Renilla Reformis luciferase to control the transfection efficiency. As previously demonstrated [Yang et al., 2003], Runx2 alone increased OSE2 activity (Fig. 6E). S6K did not significantly stimulate 6OSE2 activity in COS-7 cells in the absence of Runx2 protein. However, S6K dramatically increased OSE2 activity when Runx2 protein was co-expressed. Furthermore, introduction of a mutation of the OSE2 core sequence AACCACA to AAGAACA that abolishes Runx2 binding [Ducy et al., 1997] completely abolished S6K stimulation. These data indicate that S6K stimulation of OSE2 is dependent on the presence of Runx2 protein.

DISCUSSION

With the long-term goal of understanding the molecular mechanism underlying bone formation, we have examined the effect of rapamycin, a specific inhibitor of mTOR activity, on osteoblast proliferation, and differentiation. Several osteoblast-specific markers were examined in both MC3T3-E1 subclone 4 cells and primary mouse bone marrow stromal cells, including *Ocn*, *Bsp*, and *Osx* mRNA expression, ALP activity, and mineralization.

While the target of rapamycin, mTOR, is expressed ubiquitiously in all types of cells, the sensitivity of different cell types to rapamycin varies dramatically. This differentiated sensitivity is what permits the clinical use of rapamycin as an immunosuppressant and anti-cancer agent. It also underscores the necessity for defining the sensitivity of many dividing cells in humans to the drug, as well as mechanisms that predispose a cell to its inhibition. Experiments described in this study show that proliferation of both MC-4 cells and BMSCs is highly sensitive to rapamycin treatment. Maximal inhibition of cell proliferation was achieved at a concentration of 1 nM rapamycin. In contrast, a much higher concentration of rapamycin is required for inhibition of cell proliferation in other cell types. For examples, 10 nM of rapamycin did not significantly inhibit proliferation of 10T1/2 fibroblasts, C2C12 myoblasts, A549, and H1299 (human non-small-cell lung carcinoma cell lines), and BEAS2B (non-neoplastic bronchi epithelial cells) (data not shown). The mechanism underlying this dramatic difference in rapamycin sensitivity remains unclear.

Cyclins seem to be very critical in rapamycin-mediated antiproliferative effects. For examples, rapamycin induces a G_1 cell cycle arrest that correlates with down-regulation of cyclin D1 levels in some cell types [Albers et al., 1993; Grewe et al., 1999; Nelsen et al., 2003; Shi et al., 2005; Law et al., 2006]. Furthermore, cyclin D1 overexpression reverses rapamycin-induced cell cycle arrest [Law et al., 2006]. On the other hand, rapamycin affects cyclin A and D3 in other cells [Decker et al., 2003; Garcia-Morales et al., 2006]. Because the level of cyclin D3 is too low to be detected by Western blot analysis in both MC-4 cells and BMSCs, it is unlikely that it plays a major role in these cells for regulation of cell cycle. In contrast, rapamycin completely abolished expression of cyclin A and D1 proteins after 48 h treatment in both cells. Importantly, inhibition of p70S6K phosphorylation by rapamycin precedes the down regulation of cyclin A and D1 proteins (i.e., 12 h vs. 48 h), suggesting that the effect on cyclin A and D1 is a downstream effect of rapamycin. These data, along with their established roles in regulation of cell proliferation in other systems, suggest that cyclin A and D1 proteins play important roles in the antiproliferative effect of rapamycin in osteoblasts.

Our study also clearly demonstrates that mTOR pathway is essential for osteoblast differentiation. Inhibition of mTOR signaling by rapamycin dramatically blocked all the parameters tested for osteoblast differentiation including ALP activity (a early differentiation marker), levels of *Ocn*, *Bsp* (both late differentiation markers), and *Osx* (an osteoblast-specific transcription factor) mRNAs, and mineralization capacity (a terminal parameter for osteoblast differentiation) in both MC-4 cells and primary BMSCs. These results are consistent with previous in vitro studies from other

laboratories. For example, Lungile et al. reported that inhibition of p70S6 kinase by rapamycin blocked osteogenic protein-1-induced ALP activity in primary rat calvarial osteoblasts [Shoba and Lee, 2003]. Likewise, rapamycin suppressed BMP-4 induction of p70S6 kinase phosphorylation and subsequent vascular endothelial growth facto (VEGF) synthesis in MC3T3-E1 preosteoblastic cells [Kozawa et al., 2001].

A few lines of evidences support that rapamycin inhibits osteoblast differentiation, at least in part, by downregulation of Runx2 protein: (i) Runx2 is a key factor for osteoblast-specific gene expression and osteoblast differentiation, (ii) both osteoblast differentiation and Runx2 protein were similarly downregulated by rapamycin in differentiating osteoblasts, (iii) Runx2 protein was not reduced by rapamycin in differentiated osteoblasts, in which this drug was unable to block cell differentiation, (iv) rapamycin dramatically suppressed the levels of *Osx* mRNA, a downstream factor of Runx2 and required for osteoblast differentiation, and (v) overexpression of S6K, a major downstream component of mTOR signaling, specifically increased the levels of Runx2 proteins. It should be noted that S6K must increase Runx2 protein through posttranscriptional and/or posttranslational mechanisms because both Runx2 and ATF4 expression was driven by the CMV promoter in these experiments. The mechanism of this regulation remains to be determined. While Runx2 plays a major role in *Ocn* and *Bsp* mRNA expression and mineralization, ALP activity seems to be less dependent on Runx2. Thus, our findings do not exclude the possibility that other critical bone transcription factors may also be involved in rapamycin-mediated inhibition of osteoblasts differentiation. Finally, since rapamycin only inhibits the translation of a subset of mRNAs that contain a tract of polypyrimidine (TOP) in their 5′ untranslated region (UTR) [Jefferies et al., 1997], it does not globally suppress protein systhesis. Thus, we demonstrate that Runx2 is a novel downstream target of mTOR signaling in osteoblasts.

This study demonstrates that rapamycin inhibits osteoblast differentiation by targeting osteoblast proliferation and the early stage of osteoblast differentiation. Rapamycin blocks osteoblast specific gene expression, ALP activity, and mineralization of extracellular matrix in differentiating but not differentiated osteoblasts. In a related study, Ogawa et al. [1998] showed that rapamycin treatment slightly increased *osteopontin* and *Ocn* mRNA expression in the presence of vitamin D_3 in rat osteoblast-like ROS17/2.8 cells, a terminally differentiated osteosarcoma cell line expressing high levels of *Ocn* mRNA in the absence of ascorbic acid [Bortell et al., 1993]. Interestingly, as demonstrated in this study, the level of Runx2 protein is not reduced by rapamycin in differentiated osteoblasts, supporting the notion that Runx2 is a major downstream target of mTOR signaling in osteoblasts.

In summary, these studies clearly demonstrate an important role for mTOR signaling in osteoblast proliferation and differentiation and suggest that further studies of this critical pathway in bone formation in vivo should be explored.

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