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ARTICLES

Oxysterol-Induced Osteoblastic Differentiation of Pluripotent Mesenchymal Cells Is Mediated Through a PKC- and PKA-Dependent Pathway

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Abstract Oxysterols form a large family of oxygenated derivatives of cholesterol that are present in circulation, and in human and animal tissues. The discovery of osteoinductive molecules that can induce the lineage-specific differentiation of cells into osteoblastic cells and therefore enhance bone formation is crucial for better management of bone fractures and osteoporosis. We previously reported that specific oxysterols have potent osteoinductive properties and induce the osteoblastic differentiation of pluripotent mesenchymal cells. In the present report we demonstrate that the induction of osteoblastic differentiation by oxysterols is mediated through a protein kinase C (PKC)- and protein kinase A (PKA)-dependent mechanism(s). Furthermore, oxysterol-induced-osteoblastic differentiation is marked by the prolonged DNA-binding activity of Runx2 in M2-10B4 bone marrow stromal cells (MSCs) and C3H10T1/2 embryonic fibroblastic cells. This increased activity of Runx2 is almost completely inhibited by PKC inhibitors Bisindolylmaleimide and Rottlerin, and only minimally inhibited by PKA inihibitor H-89. PKC- and PKA-dependent mechanisms appear to also regulate other markers of osteoblastic differentiation including alkaline phosphatase (ALP) activity and osteocalcin mRNA expression in response to oxysterols. Finally, osteogenic oxysterols induce osteoblastic differentiation with BMP7 and BMP14 in a synergistic manner as demonstrated by the enhanced Runx2 DNA-binding activity, ALP activity, and osteocalcin mRNA expression. Since Runx2 is an indispensable factor that regulates the differentiation of osteoblastic cells and bone formation in vitro and in vivo, its increased activity in oxysterol-treated cells further validates the potential role of oxysterols in lineage-specific differentiation of pluripotent mesenchymal cells and their potential therapeutic use as bone anabolic factors. J. Cell. Biochem. 100: 1131–1145, 2007.

Key words: oxysterols; marrow stromal cells; osteoblastic differentiation; Runx2; PKC; PKA

Age-related osteoporosis and its complications are caused by increased bone resorption by osteoclasts in parallel with decreased bone formation by osteoblasts [Riggs and Melton, 1992]. It is now widely accepted that future improved treatment of osteoporosis will likely require the use of bone anabolic agents that can enhance the osteogenic differentiation and bone-forming activity of osteoblast precursor cells, and therefore increase bone mass and reduce fracture risk [Rodan and Martin, 2000; Goltzman, 2002; Mundy, 2002]. Osteoblast precursor cells exist among a population of pluripotent stem cells commonly known as mesenchymal stem cells or marrow stromal cells (MSCs) that are present in a variety of tissues, are prevalent in bone marrow stroma, and can differentiate into lineage-specific cells including osteoblasts, chondrocytes, myocytes, adipocytes, and fibroblasts [Caplan, 1994; Majors et al., 1997; Prockop, 1997].

Since MSCs appear to be present in adults and can be readily isolated and expanded in

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culture, they may be an excellent tool for interventions in many diseases that result from reduced or impaired functioning of these cells [Caplan and Bruder, 2001]. One situation in which MSCs might be used in human disease is in age-related and postmenopausal osteoporosis, where the decreased number and osteogenic activity of osteoprogenitor MSCs, in part, leads to decreased bone formation [Quarto et al., 1995; Chan and Duque, 2002; Chen et al., 2002; Moerman et al., 2004]. However, the use of these cells in the context of bone fracture healing and osteoporosis requires our ability to induce their differentiation into osteoblastic cells.

There are very few known osteoinductive factors that can drive the lineage-specific differentiation of MSCs into fully mature osteoblastic cells. The best known osteoinductive factors are the members of the bone morphogenetic protein (BMP) family of proteins that induce osteoblastic differentiation and bone formation in vitro and in vivo [Chen et al., 2004]. However, despite their potent osteoinductive properties, the use of BMPs in fracture healing is currently hampered by the large concentrations of the recombinant protein necessary to induce adequate bone formation [Lieberman et al., 2002]. Only few other candidates at the present time have been reported to possess osteoinductive properties [Manolagas, 2000; Rodan and Martin, 2000; Goltzman, 2002; Mundy, 2002]. Therefore, the rarity of potential candidate molecules that could induce the lineage-specific differentiation of precursor cells into bone-forming osteoblastic cells has created an intense area of investigation.

Identification of new targets for enhancing bone formation requires the better understanding of the molecular mechanism(s) that direct the lineage-specific differentiation of progenitor cells into osteoblasts. The first key molecule that was identified as a master regulator of osteoblastic differentiation was Runx2 [Banerjee et al., 1997; Komori et al., 1997; Ducy, 2000; Yang and Karsenty, 2002]. Extensive in vitro and in vivo investigations of the Runx2 transcription factor have demonstrated that it is necessary for the induction of osteoblastic differentiation [Ducy et al., 1997] and that it directly regulates the expression of all osteoblastic differentiation marker genes, including collagen I, osteopontin, bone sialoprotein, and osteocalcin [Otto et al., 2003]. Further studies of molecular regulation of Runx2 have shown regulation at transcriptional and post-transcriptional levels, including phosphorylation of the protein in order to regulate its binding to DNA, transactivation activity, and degradation through the proteasomal pathway [Ducy, 2000; Zhao et al., 2003]. The ability of factors such as BMP2 and Wnt to directly or indirectly regulate Runx2 is an important feature of their osteoinductive properties [Gori et al., 1999; Gaur et al., 2005].

The protein kinase C (PKC) and the protein kinase A (PKA) signaling pathways have been implicated in the regulation of osteoblastic differentiation. Both PKC- and PKA-dependent mechanisms mediate the differentiation of osteoblastic cells and gene expression in response to parathyroid hormone (PTH) [Swarthout et al., 2002; Yang et al., 2006]. PKC regulates Runx2 phosphorylation and activity in response to fibroblast growth factor [Kim et al., 2003, 2006], extracellular nucleotides and hypotonic stress [Costessi et al., 2005], a cAMP/PKA-dependent mechanism regulates the prostaglandin E2-mediated anabolic effects of a novel phosphodiesterase 4 inhibitor [Miyamoto et al., 2003], and regulation of osteocalcin gene transcription involves interaction of PKA- and PKC-dependent pathways [Boguslawski et al., 2000]. Interestingly, it appears that these important signaling pathways have overlapping, but also distinct effects on the regulation of different markers of osteoblastic differentiation.

We previously reported that specific members of the oxysterol family of cholesterol oxidation products have osteoinductive effects when applied to a variety of osteoprogenitor cells, including the pluripotent mouse MSC line, M2-10B4 (M2) [Kha et al., 2004; Shouhed et al., 2005]. Specific osteoinductive combinations of 20*S*-hydroxycholesterol (20S) with either the "*R*" or the "*S*" stereoisomer of 22-hydroxycholesterol (22R and 22S, respectively), were able to induce a full range of early and late osteoblastic differentiation markers, including alkaline phosphatase (ALP) activity, osteocalcin mRNA expression, and mineralization in M2 and other osteoprogenitor cells [Kha et al., 2004]. The osteoinductive effects of the oxysterols were in part mediated by the COX-1/PLA_2- and ERK-dependent pathways, as demonstrated by the ability of specific inhibitors of those pathways to inhibit the oxysterol-induced expression of osteoblastic differentiation markers [Kha et al.,

2004]. Osteogenic oxysterols also induced a sustained activation of MAPK in M2 cells [Kha et al., 2004]. Moreover, the osteogenic oxysterols greatly enhanced the osteoinductive effects of BMP2 in a synergistic manner [Kha et al., 2004]. Oxysterol enhancement of BMP2 effects is of potentially great importance to the clinical use of this compound, given the large doses of BMP2 that are currently necessary to induce clinically significant levels of bone healing in humans [Lieberman et al., 2002]. In addition to BMP2, other members of the BMP family have been identified and are either in limited usage or in development for use in bone healing. These include BMP7 (OP-1) [Franceschi et al., 2000; Shea et al., 2003] and BMP14 (GDF-5) [Spiro et al., 2001; Shimaoka et al., 2003].

In the present report we further elucidate the molecular mechanism(s) underlying the osteoinductive effects of the oxysterols. We demonstrate that Runx2 is an important marker of oxysterol-induced osteoblastic differentiation. We identify PKC and PKA as important mediators of the effects of the oxysterols on osteoblastic differentiation of MSC. Moreover, we report that in addition to their synergistic effects with BMP2, osteogenic oxysterols also synergize with BMP7 and BMP14 to enhance the osteoblastic differentiation of M2 cells and that this effect may be explained in part by the enhanced Runx2 DNA-binding activity. The present findings further support the fact that specific oxysterols have potent osteoinductive properties and that they may be important regulators of lineage-specific differentiation of mesenchymal stem cells.

EXPERIMENTAL PROCEDURES

Cell Culture

Pluripotent mouse progenitor cell lines M2-10B4 and C3H10T1/2 cells were purchased from American Type Culture Collection (Rockville, MD). M2-10B cells were maintained in RPMI 1640 with 10% heat-inactivated FBS, supplemented with 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 U/ml streptomycin [Kha et al., 2004]. C3H10T1/2 cells were grown in DMEM supplemented as above. Treatment media for both cell lines consisted of RPMI 1640 or DMEM containing 5% heat-inactivated FBS, 3 mM β-glycerophosphate, 50 μg/ml ascorbic acid and antibiotics as above (differentiation media) [Kha et al., 2004]. Oxysterols were obtained from Sigma-Aldrich, Co. (St. Louis, MO), and bisindolylmaleimide I (Bis), Rottlerin (Rot), H-89 and KT5720 (KT) from EMD Biosciences, Inc. (La Jolla, CA).

Electrophoretic Mobility Shift Assay

The sequences of the oligonucleotides used were OSE2—5′-AGCTGCAATCACCAACCAC-AGCA-3′ and OSE2-mutant4—5′-AGCTGCAA-TCACCAGACACAGCA-3′, originally described by Ducy et al. [1997]. Oligonucleotides were annealed to their complementary sequences by boiling and cooling. The OSE2 probe was end-labeled with γ^{32}P-ATP using polynucleotide kinase and column purified. Nuclear extracts were prepared using the modified Dignam protocol described by Osborn et al. [1989]. Nuclear extracts (10 μg) were incubated in binding buffer (10 mM Tris pH 7.5, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% glycerol), 1 μg poly(dIdC) and 0.2 ng of labeled probe for 20 min at room temperature, and complexes were resolved on a cooled, 6% acrylamide 1× TBE gel. Subsequently, the gel was dried and exposed to film overnight. Supershift analysis was performed according to the protocol by Javed et al. [2004] by pre-incubating 10 μg of nuclear extracts in KN100-binding buffer with 1 μg of Runx2 antibody (EMD Biosciences, Inc.) for 20 min at 37°C. Subsequently, 1 μg poly(dIdC) and 0.1 ng labeled probe were added for another 20 min at room temperature. Complexes were resolved on a cooled, 5% acrylamide 0.5× TBE gel. Quantification of the shifted band corresponding to the band that supershifted in the presence of Runx2 antibody was performed using a Storm840 phosphorimager and ImageQuant software (Amersham, Piscataway, NJ).

Alkaline Phosphatase Activity

Colorimetric ALP activity assay on whole cell extracts was performed as previously described [Kha et al., 2004].

Northern Blotting

Northern blotting for osteocalcin and 18S was performed as previously described [Kha et al., 2004]. Gene expression was quantified using a Storm840 phosphorimager and ImageQuant software (Amersham).

Transient Transfection and Reporter Assay

M2 cells were transfected overnight using FuGENE6 Transfection Reagent (Roche Applied Science, Indianapolis, IN) according to manufacturer's instructions. The p6OSE2-luc Runx2 responsive reporter plasmid (6XOSE2-luc) and pCMV-Osf2 Runx2 expression plasmid were kindly provided by Dr. Gerard Karsenty (Baylor College of Medicine, Houston, TX). Reporter plasmid was cotransfected with either Runx2 expression plasmid or comparable filler plasmid to maintain constant DNA levels. Firefly luciferase values were normalized to Renilla luciferase activity and pEGFP-NI was used to evaluate transfection efficiency. Cells were then treated for 24 h with test agents before measuring luciferase activity using the Dual Luciferase Reporter Assay System (Promega, Madison, WI) according to manufacturer's instructions.

Western Blotting

Nuclear extracts (30 μg) were separated on acrylamide gels and transferred overnight onto nitrocellulose membrane. Blots were blocked and incubated with Runx2 antibody (MBL International, Inc., Woburn, MA) or CREB antibody for normalization (Cell Signaling, Beverly, MA). Blots were then incubated with HRP-linked secondary antibody and binding was revealed using the Western Lightning ECL Kit (Perkin Elmer, Boston, MA) according to the manufacturer's instructions.

Statistical Analyses

Computer-assisted statistical analyses were performed using the StatView 4.5 program. All *P*-values were calculated using ANOVA and Fisher's projected least significant difference (PLSD) significance test. A value of $P<0.05$ was considered significant.

RESULTS

Increased Runx2 DNA-Binding Activity in Cells Treated With Osteogenic Oxysterols

In order to further identify potential molecular regulator(s) associated with the osteoinductive effects of oxysterols, we examined Runx2 DNA-binding activity in nuclear extracts of M2 cells treated with the osteogenic oxysterol combinations 22S + 20S (SS) and 22R + 20S (RS) by EMSA using an oligonucleotide containing the Runx2 DNA-binding consensus sequence derived from the OSE2 element as previously described [Ducy et al., 1997]. Oxysterols were added in differentiation medium containing ascorbate and β-glycerophosphate as described in the Experimental Procedures section. Nuclear extracts from M2 cells treated for 4 days with 5 μM of various oxysterols, alone or in combination, were analyzed. Results showed that nuclear extracts from cells treated with the oxysterol combination SS had a potent increase in Runx2 DNA-binding activity compared to nuclear extracts from untreated control cells (Fig. 1A). Similarly, nuclear extracts from M2 cells treated with the oxysterol combination RS showed increased Runx2 DNA-binding activity, although relatively less than that seen with SS treatment. In contrast, nuclear extracts from cells treated with 5 μM of individual oxysterols, 20S, 22R, 22S, 7-ketocholesterol (7-Keto), or the lipid oxidation product 4-hydroxynonenal (4-HNE), had little or no significant Runx2 DNA-binding activity, with 20S being only minimally effective (Fig. 1A).

Dose-response studies showed that DNA binding to OSE2 oligo in cells treated with SS was stronger than in response to RS, and more similar to the pattern of the shifted bands formed in nuclear extracts from BMP-2-treated M2 cells (Fig. 1B). This is consistent with the more potent osteoinductive properties of SS than RS on inducing other markers of osteoblastic differentiation, including ALP activity and osteocalcin mRNA expression (data not shown). In addition to M2 cells, other pluripotent mesenchymal cells and osteoprogenitors, including the C3H10T1/2 embryonic fibroblasts, can be induced to undergo osteogenic differentiation when treated with osteogenic oxysterols [Kha et al., 2004]. Similar to the effects seen in M2 cells, Runx2 DNA-binding activity in nuclear extracts from C3H10T1/2 treated with osteogenic oxysterols was increased compared to control untreated cells (Fig. 1C).

In order to determine the kinetics of the increase in Runx2 DNA binding in response to oxysterols, nuclear extracts were prepared from M2 cells after 2, 4, 8, 24, 48, and 96 h of oxysterol treatment and analyzed by EMSA. Results showed that at 2, 4, and 8 h, there were similar levels of DNA-binding activity in both untreated and oxysterol-treated M2 cells (Fig. 2A). However, at later time points the level of DNA binding diminished in untreated cells, reaching nearly undetectable levels by 48 h. In contrast,

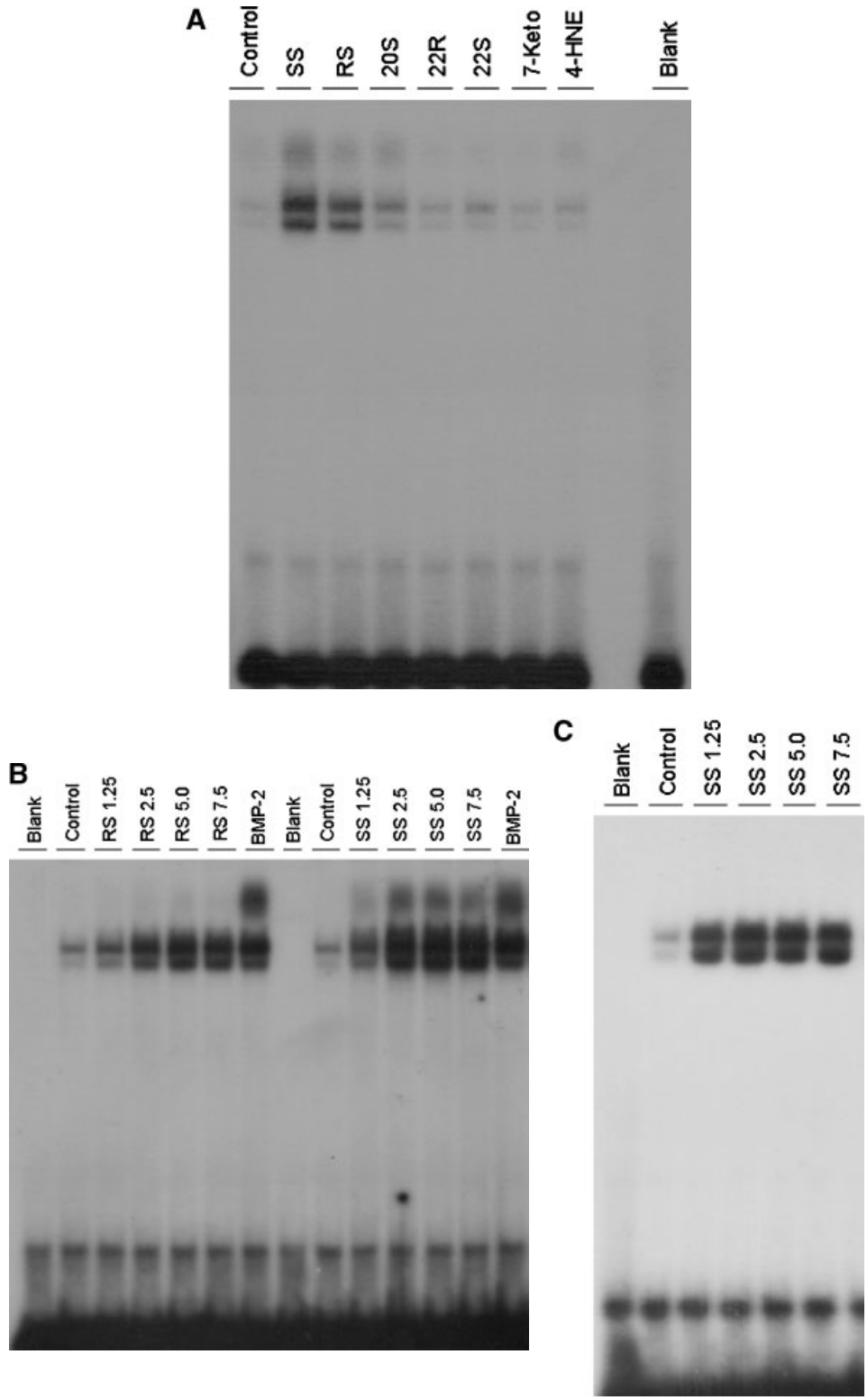

Fig. 1. Oxysterols induce Runx2 DNA-binding activity in marrow stromal cells (MSCs). **A**: M2 cells were treated at confluence with control vehicle or 5 μM of the following oxysterols or oxidized lipids, alone or in combination as indicated in differentiation medium for 4 days: 20(*S*)-hydroxycholesterol (20S), 22(*R*)-hydroxycholesterol (22R), 22(*S*)-hydroxycholesterol (22S), 7-ketocholesterol (7-Keto), 4-hydroxynonenal (4-HNE), 22S + 20S (SS), or 22R + 20S (RS). Nuclear extracts were prepared and incubated with labeled double-stranded Runx2 oligo probe and subjected to electrophoresis as described in Methods. Blank = no nuclear extract. **B**: Nuclear extracts from M2 cells treated for 4 days with various concentrations of the oxysterol combinations RS or SS or 500 ng/ml BMP-2 were subjected to Runx2 EMSA. **C**: C3H10T1/2 cells were treated in osteogenic medium supplemented with various concentrations of the oxysterol combination SS. After 2 days, nuclear extracts were prepared and Runx2 DNA-binding activity was assessed by EMSA. Data from a representative of at least three separate experiments are shown.

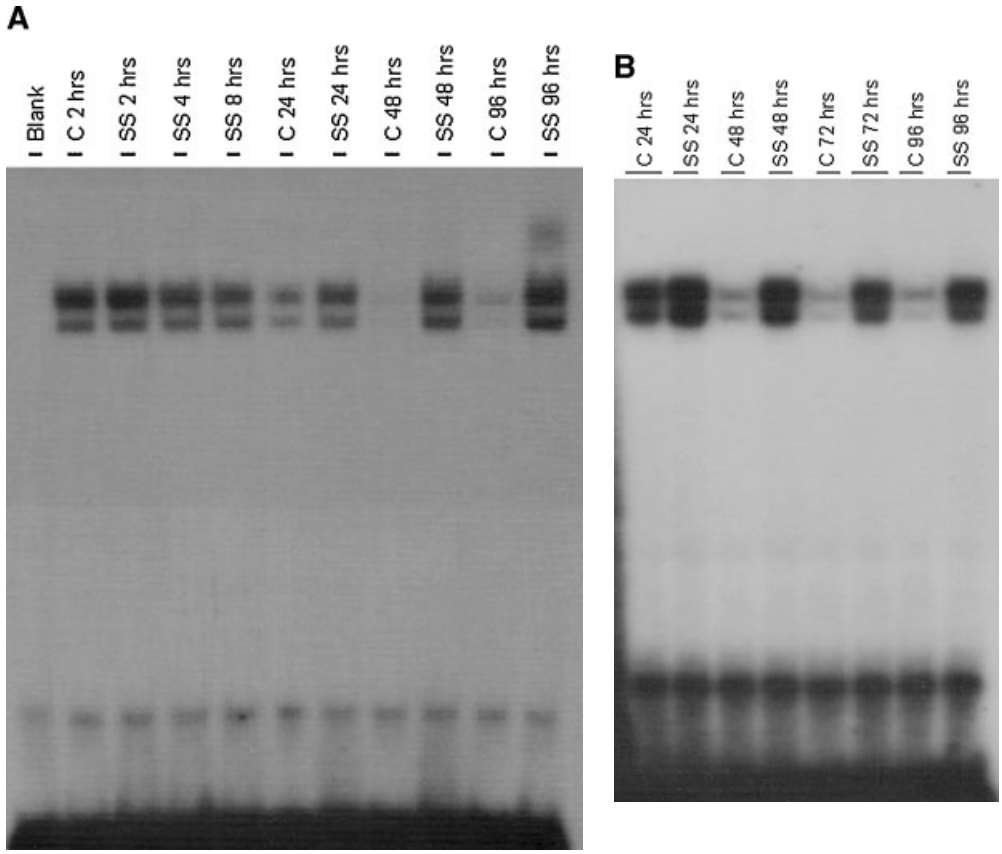

Fig. 2. Time course of Runx2 DNA-binding activity in MSCs. Nuclear extracts from (**A**) M2 cells or (**B**) C3H10T1/2 cells treated in osteogenic medium with control vehicle (C) or 5 μM of oxysterol combination SS for 2, 4, 8, 24, 48, or 96 h were analyzed by EMSA to assess Runx2 DNA-binding activity. Data from a representative of at least three separate experiments are shown.

DNA-binding activity remained elevated in nuclear extracts from oxysterol-treated cells. Similar results were obtained in oxysterol-treated C3H10T1/2 cells (Fig. 2B).

In order to verify the identity of the OSE2 DNA-binding protein in oxysterol-treated cells, competition and supershift analyses were performed, using nuclear extracts from M2 cells treated for 4 days with 5 μM SS. Competition studies with unlabeled cold OSE2 oligo demonstrated that the addition of 10- to 200-fold excess of unlabeled oligonucleotide dose-dependently inhibited binding to the labeled OSE2 probe. (Fig. 3A). In contrast, addition of a 200-fold excess of cold oligonucleotide containing a mutated Runx2 binding site (OSE2-mut) did not compete for binding to Runx2 in nuclear extracts from oxysterol-treated cells. Furthermore, EMSA analyses showed that in the presence of a Runx2-specific antibody, but not a SMAD1-specific antibody, a supershifted band was formed that was stronger in oxysterol-treated cells than in untreated control cells (Fig. 3B).

The transcriptional activity of Runx2 in untreated control and SS-treated C3H10T1/2 cells was measured using the 6XOSE2-luc reporter in the presence or absence of Runx2 expression vector. Reporter activity was minimally, but significantly, increased in the presence of oxysterols compared to untreated control cells (20%, $P = 0.004$) (Fig. 4). However, in the presence of Runx2 expression vector, baseline reporter activity was increased 11-fold and oxysterol treatment further increased this induction to 24-fold.

In order to determine whether the oxysterol-induced Runx2 DNA-binding activity is at least in part due to increased Runx2 protein levels, Western blotting was performed on nuclear extracts from cells treated with control vehicle or 5 μM SS for 2 or 4 days. Results showed

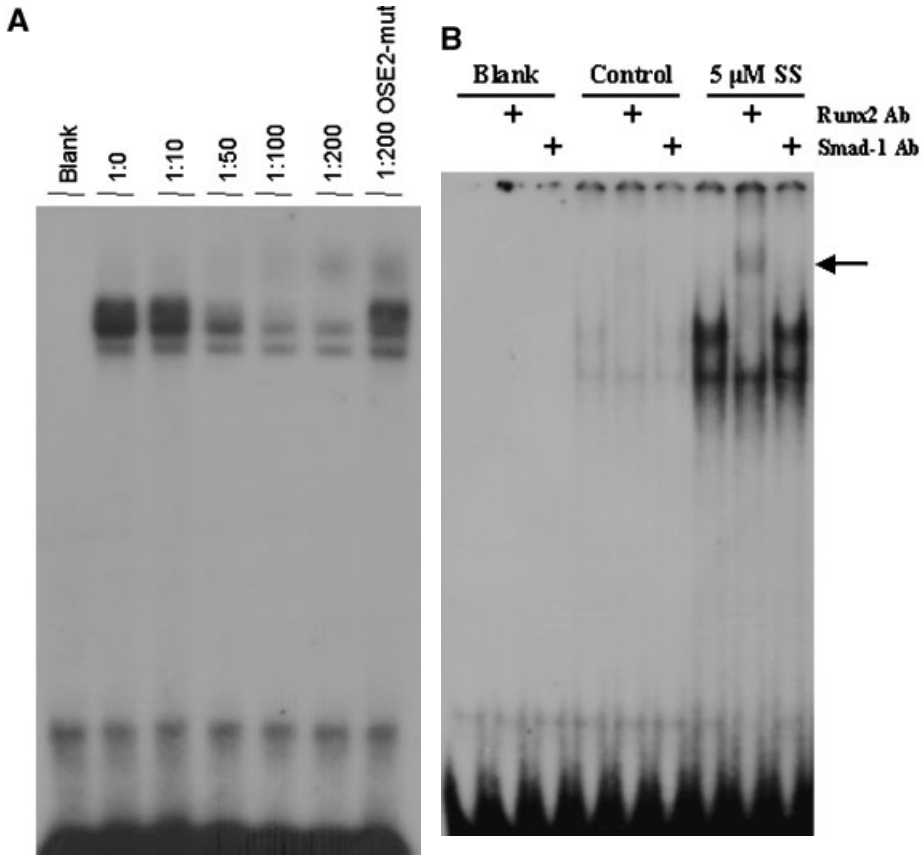

Fig. 3. Competition and supershift analysis of oxysterol-induced Runx2 DNA binding. **A**: Nuclear extracts from M2 cells treated with 5 μM of oxysterol combination SS were pre-incubated with 10- to 200-fold excess of cold OSE2 oligonucleotide or 200-fold excess of cold mutant OSE2 oligonucleotide (OSE2-mut) and subjected to EMSA analysis as described in Experimental Procedures. **B**: Nuclear extracts from M2 cells treated with control vehicle or 5 μM of oxysterol combination SS were incubated with 1 μg of anti-Runx2 or anti-SMAD1 antibody (Ab) before the addition of labeled probe. EMSA analysis for binding to OSE2 oligonucleotide was performed. Arrow points to the supershifted band. Data from a representative of at least three separate experiments are shown.

that substantial increases seen in DNA-binding activity of Runx2 in nuclear extracts from oxysterol-treated cells were not associated with increased Runx2 protein levels in the same nuclear extracts (data not shown). In fact, minimal to no increases in Runx2 protein levels in response to oxysterol treatment were seen in face of robust induction of DNA-binding activity.

Role of Protein Kinase C and Protein Kinase A in Oxysterol-Induced Osteoblastic Differentiation

In order to elucidate the signaling mechanism(s) by which oxysterols regulate osteoblastic differentiation of M2 cells, we tested the effects of selective inhibitors of several signaling molecules that are known to mediate the effects of other anabolic factors, namely PKC and PKA pathways. Bisindolylmaleimide (Bis) and Rottlerin (Rot) were used as inhibitors of PKC pathway, and H-89 and KT5720 (KT) were used as inhibitors of PKA pathway. EMSA using nuclear extracts from M2 cells treated for 4 days with 5 μM SS in the presence or absence of inhibitors showed that Bis at 7.5 μM and Rot at 0.5 μM caused a nearly complete inhibition of Runx2 DNA-binding activity (Fig. 5A,B). However, relative to PKC inhibitors, 5 and 7.5 μM H-89 had a much less potent inhibitory effect on Runx2 DNA binding in oxysterol-treated cells (Fig. 5C,D). KT at 1.5 and 3 μM did not inhibit Runx2 DNA-binding activity in SS-treated cells (data not shown). The inhibitors when used alone had minimal, insignificant, or no effect on the baseline levels (data not shown).

In addition to Runx2, we also examined the effect of PKC and PKA inhibitors on other markers of osteoblastic differentiation that are

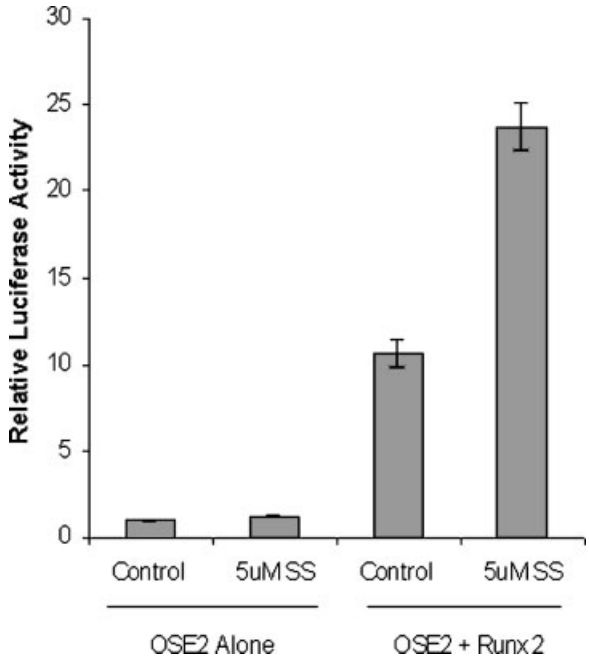

Fig. 4. Effect of osteogenic oxysterols on Runx2 transcriptional activity. C3H10T1/2 cells were transfected with 6XOSE2-luc reporter alone (OSE2 alone) or in combination with Runx2 expression plasmid (OSE2 + Runx2) or comparable filler plasmid and treated with control vehicle or the oxysterol combination 20S + 22S (SS) for 24 h. Data from a representative of three experiments are reported, expressed as the mean of triplicate samples ± SD, normalized to Renilla luciferase activity. ($P = 0.004$ for Control vs. SS in cells containing OSE2 alone, and $P < 0.0001$ for Control vs. SS in cells containing OSE2 + Runx2, and for Control vs. Control in OSE2 alone and OSE2 + Runx2).

increased by oxysterol treatment of M2 cells, namely ALP activity and osteocalcin mRNA expression. Results showed that pre-treatment with 5 and 7.5 μM Bis or 0.5 and 0.75 μM Rot significantly reduced ALP activity in SS-treated cells (Fig. 6A). Pre-treatment with 5 and 7.5 μM H-89 or 2.5, 5, and 7.5 μM KT demonstrated similar inhibitory effects on ALP activity in oxysterol-treated cells (Fig. 6B). Moreover, oxysterol-induced osteocalcin mRNA expression in M2 cells was in part inhibited by PKC and PKA inhibitors (Fig. 6C,D). The inhibitors when used alone had no significant effects on the baseline levels in these studies (data not shown).

Oxysterols Enhance BMP7- and BMP14-Induced Osteoblastic Differentiation

We previously reported that the osteogenic oxysterols synergize with BMP2 to induce osteoblastic differentiation of M2 cells [Kha et al., 2004]. Similar to BMP2, treatment of M2 cells for 2 days with 25–500 ng/ml of BMP7 or BMP14 induced ALP activity in M2 cells in a dose-dependent manner (data not shown). We found that the osteogenic oxysterols also synergized with BMP7 and BMP14 to induce osteoblastic differentiation of M2 cells, assessed by the synergistic induction of ALP activity (Figs. 7A and 8A) and osteocalcin mRNA expression (Figs. 7B,C and 8B,C).

In order to test if the synergistic effects of oxysterols and BMPs were in part associated with enhanced Runx2 DNA-binding activity, nuclear extracts were prepared from M2 cells after 4 days of treatment with sub-maximal doses of BMP7 and oxysterols, alone or in combination. EMSA analysis showed that treatment with BMP7 and oxysterols together further enhanced Runx2 DNA-binding activity compared to that induced by either agent alone (Fig. 9).

DISCUSSION

The present study was performed to further elucidate the molecular mechanism(s) associated with oxysterol-induced osteoblastic differentiation of mesenchymal osteoprogenitor cells. This is in follow-up to our prior observation that specific oxysterols possess osteoinductive properties on a par with those of other potent osteoinductive agents such as the BMP molecules. Since the runt-related transcription factor Runx2 is widely regarded as the master regulator of osteoblastic differentiation and mediates the osteoinductive effects of BMPs, its possible induced activity in oxysterol-treated cells would further validate the ability of these compounds to drive the lineage-specific differentiation of mesenchymal cells into osteoblastic cells. It has been reported that Runx2 is highly regulated on many levels, including transcriptional, post-transcriptional, and post-translational levels, and through interaction with cofactors that act as activators or repressors of its function [Yoshida et al., 2002; Bialek et al., 2004; Wang et al., 2004; Komori, 2005]. In many studies, osteoblastic differentiation has been correlated with increases in phosphorylation and DNA-binding activity of Runx2 and the subsequent increases in the expression of Runx2 target genes [Ducy, 2000; Xiao et al., 2000; Ziros et al., 2002]. Therefore, we examined the ability of osteogenic oxysterols to induce the DNA-binding activity of Runx2.

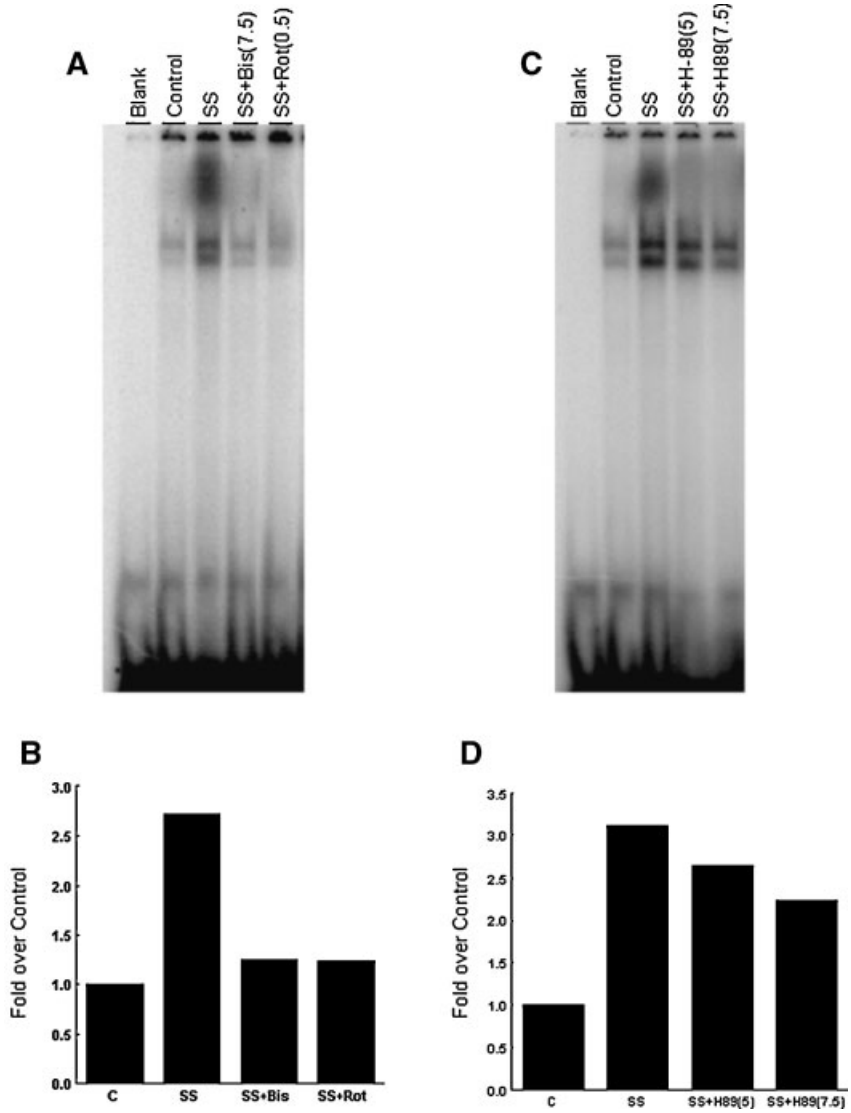

Fig. 5. Effect of PKC and PKA inhibitors on oxysterol-induced Runx2 DNA binding in MSCs. **A**: M2 cells treated with control vehicle or 5 μM of the oxysterol combination SS with or without 2 h of pre-treatment with the PKC inhibitors Bisindolylmaleimide (Bis) at 7.5 μM or Rottlerin (Rot) at 0.5 μM. After 4 days, nuclear extracts were analyzed for Runx2 DNA-binding activity by EMSA as described in Experimental Procedures. **B**: Phosphorimaging quantification of the Runx2 band. **C**: M2 cells treated as described in (A) but pre-treated with the PKA inhibitor, H-89, at 5 and 7.5 μM. **D**: Phosphorimaging quantification of the Runx2 band from (C).

Our present findings clearly demonstrate the robust activation of Runx2 DNA-binding activity in pluripotent mesenchymal cells treated with osteogenic oxysterols. The increased Runx2 DNA-binding activity was directly correlated with the osteogenic property of the oxysterols since cells treated with non-osteogenic oxysterols and oxidized lipids, including 7-Keto, 4-HNE, and individual oxysterols that alone do not induce osteoblastic differentiation [Kha et al., 2004], did not have increased Runx2 DNA binding. EMSA analysis in the presence of Runx2-specific antibody identified the DNA-binding activity to be at least in part due to the binding of Runx2 protein to the OSE2 sequence of the oligonucleotide used in our assays. However, since additional bands were also induced in the EMSAs using nuclear extracts from oxysterol-treated cells, it is possible that other nuclear factors also bind to the OSE2 sequence in response to osteogenic oxysterols. The formation of the two lower bands were consistently induced by oxysterol treatment, however the third upper band appeared to form mostly after prolonged (i.e., 4 days) treatment with oxysterols or BMP2, and much less by RS

treatment than by SS treatment (Fig. 1A,B). It is noteworthy that the SS combination of oxysterols was more potent in inducing osteoblastic differentiation of cells compared to the RS combination (data not shown). In addition, the third shifted band was only present in nuclear extracts of the oxysterol-treated M2 cells and not in nuclear extracts of C3H10T1/2 cells. Since this band was not competed out in cold competition assays with OSE2 oligonucleotide, its identity and specificity are not clear at this time.

Time course studies showed that the oxysterol-induced Runx2 DNA-binding activity was time dependent. At early time points, nuclear extracts from control M2 and C3H10T1/2 cells placed in an osteogenic medium containing fetal bovine serum, ascorbate, and β-glycerophosphate had significant levels of Runx2 DNA-binding activity. With time, this DNA-binding activity was abolished in control cells. Previous reports have demonstrated degradation of Runx2 through a proteasome-dependent pathway [Zhao et al., 2003] and we

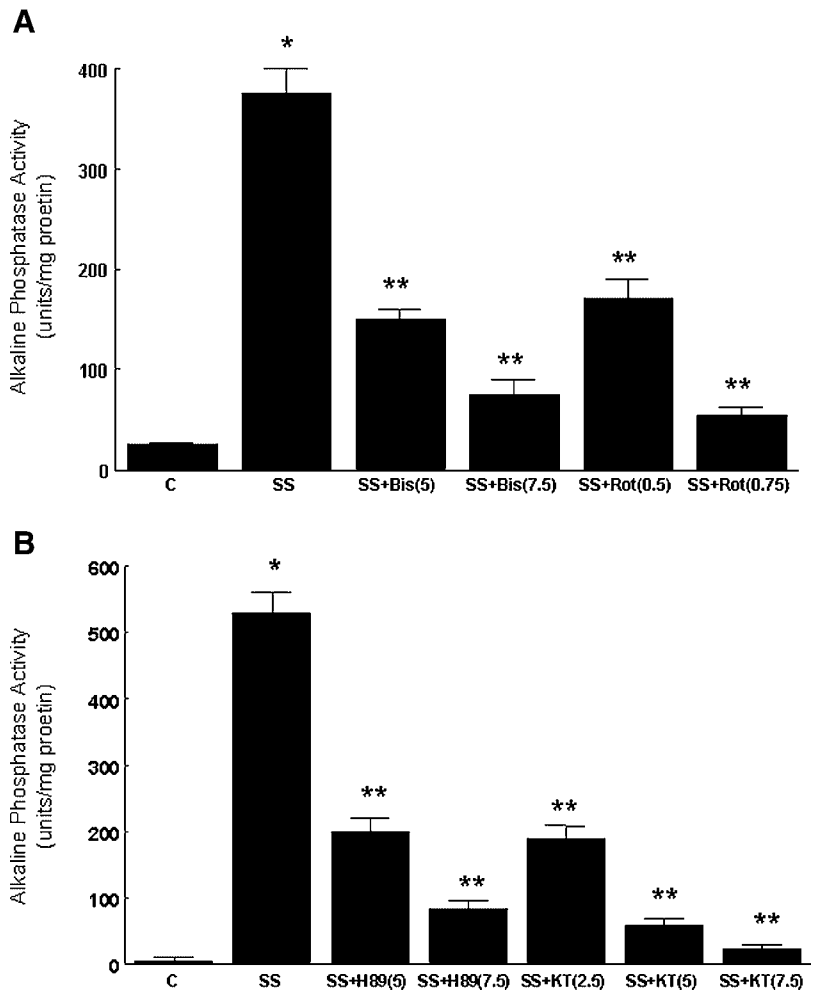

Fig. 6. Effect of PKC and PKA inhibitors on oxysterol-induced markers of osteoblastic differentiation in MSCs. **A**: M2 cells were pre-treated for 2 h with or without the PKC inhibitors Bisindolylmaleimide (Bis) at 5 and 7.5 μM or Rottlerin (Rot) at 0.5 and 0.75 μM, and then treated with control vehicle (C) or 2.5 μM of oxysterol combination SS. After 2 days of treatment, alkaline phosphatase (ALP) activity was measured. **B**: M2 cells were pre-treated as described in (A) using the PKA inhibitors H-89 at 5 and 7.5 μM and KT-5720 (KT) at 2.5, 5, and 7.5 μM. Oxysterol treatment and ALP activity were performed as described in (A). Results from a representative of three separate experiments are reported as the mean ± SD of triplicate determinations, normalized to protein concentrations (*$P < 0.001$ for C vs. SS and **$P < 0.001$ for SS vs. SS+ inhibitors). **C**: M2 cells were treated for 8 days with control vehicle (C) or 5 μM SS with or without pre-treatment with PKC or PKA inhibitors as described for (A) and (B) and analyzed for osteocalcin mRNA expression by Northern blotting. Concentrations of inhibitors used were Bis 7.5 μM, Rot 0.5 μM, H-89 7.5 μM, and KT 1.5 μM. Data from phosphorimaging analysis of the Northern blot are shown in (**D**), normalized to 18S rRNA.

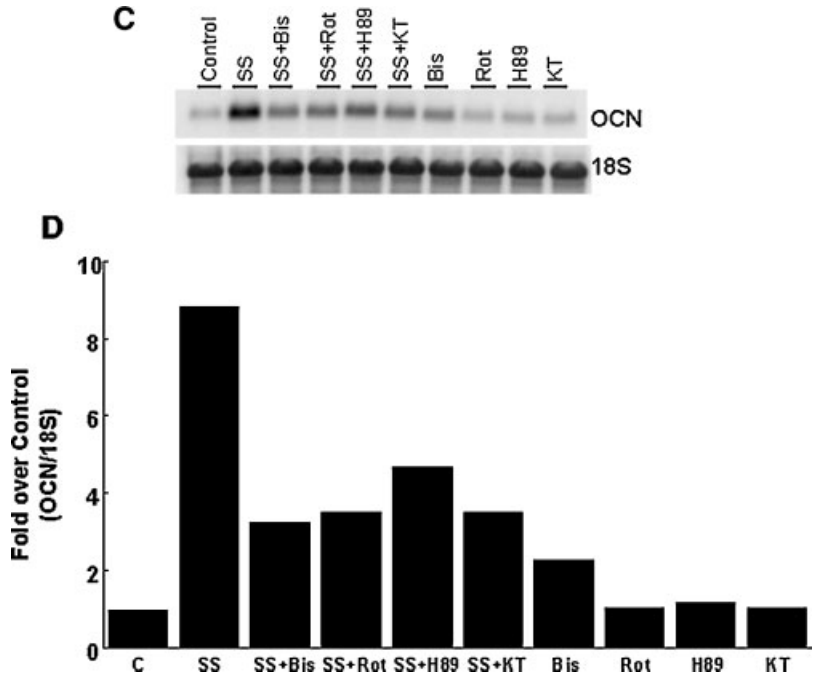

Fig. 6. (*Continued*)

speculate that a similar mechanism may be involved in this time-dependent reduction in Runx2 DNA-binding activity in our experimental system. Interestingly, oxysterol treatment inhibited the time-dependent abolishment of Runx2 DNA-binding activity seen in untreated control cells, resulting in sustained Runx2 DNA binding for as long as 4 days after treatment. It has been reported that inhibition of specific components of the proteasomal degradation pathway in osteoblasts results in increased bone formation in vitro and in vivo [Garrett et al., 2003]. However, oxysterol-induced effects on Runx2 DNA-binding activity are unlikely due to an inhibition of Runx2 protein turnover since little to no increases in Runx2 protein levels were found in response to oxysterols. Alternatively, oxysterols may induce and/or stabilize post-translational modification of Runx2, such as phosphorylation of the protein, which might be responsible for its activation and binding to DNA [Ducy, 2000].

Several signaling pathways have been shown to regulate osteoblastic differentiation of cells including PKA-, PKC-, and MAPK-dependent pathways in response to osteoinductive factors [Xiao et al., 2000; Franceschi and Xiao, 2003; Kim et al., 2003]. Our present data indicate that oxysterol-induced osteoblastic differentiation of cells is mediated in part by PKC- and PKA-dependent mechanisms. The role of these signaling pathways in regulating the different markers of osteoblastic differentiation appears to be both specific and overlapping. The increase in Runx2 DNA-binding activity in oxysterol-treated cells occurs mainly via a PKC-dependent pathway, since it is inhibited by the PKC inhibitors Bis and Rot, but only minimally by the PKA inhibitor H-89 and not by another PKA inhibitor, KT. This finding is consistent with previous reports showing PKC-dependent stimulation of Runx2 DNA-binding activity by FGF [Kim et al., 2003]. In contrast, the oxysterol-induced mRNA expression of osteocalcin, which is a downstream target of Runx2, was partially blocked to a similar extent by PKC and PKA inhibitors. In addition, both PKC and PKA inhibitors inhibited oxysterol-induced ALP activity, suggesting that the activation of this early marker of osteoblastic differentiation occurs through both PKC- and PKA-dependent mechanisms, with potentially overlapping regulation by these signaling pathways. Interestingly, despite the fact that oxysterol-induced osteoblastic differentiation is in part mediated by a MAPK- and COX-1-dependent pathway [Kha et al., 2004], these signaling molecules appear to affect the differentiation process independent of Runx2 activation since neither PD98059 nor SC-560 inhibited Runx2 DNA

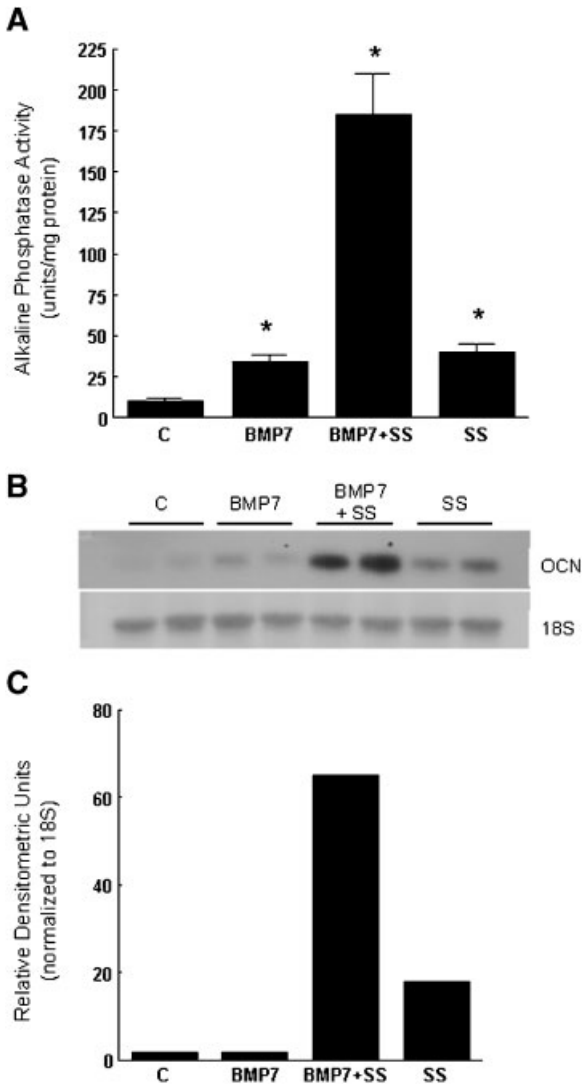

Fig. 7. Synergistic osteogenic effects of oxysterols and BMP7 in MSCs. **A**: M2 cells at confluence were treated with control vehicle (C), 50 ng/ml recombinant human BMP7, or a combination of 22S and 20S oxysterols (SS, 2.5 μM each), alone or in combination in Differentiation medium. ALP activity was measured after 2 days as previously described. Results from a representative of three separate experiments are shown, reported as the mean ± SD of quadruplicate determinations, normalized to protein concentration (*$P < 0.001$ for C vs. BMP7 or SS, and for BMP7 + SS vs. BMP7 or SS alone). **B**: M2 cells were treated under similar conditions as those described above. After 8 days, total RNA was isolated and analyzed for osteocalcin (OCN) and 18S rRNA expression by Northern blotting as previously described. Data from densitometric analysis of the Northern blot is shown in (**C**) as the average of duplicate samples, normalized to 18S rRNA.

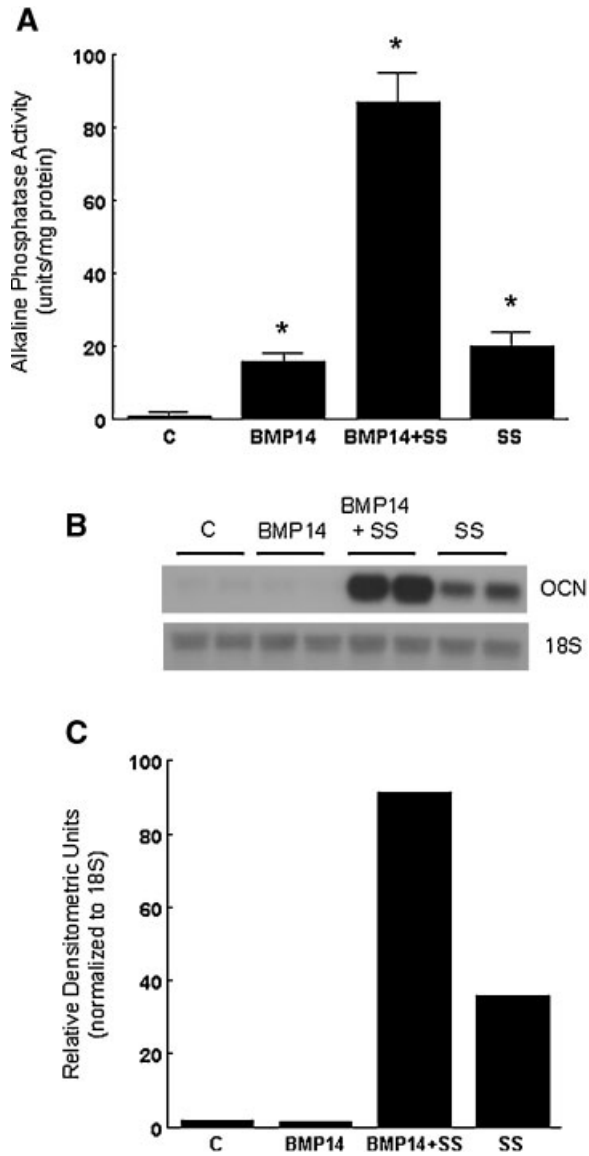

Fig. 8. Synergistic osteogenic effects of oxysterols with BMP14 in MSCs. **A**: M2 cells at confluence were treated with control vehicle (C), 50 ng/ml recombinant mouse BMP14, or a combination of 22S and 20S oxysterols (SS, 2.5 μM each), alone or in combination in Differentiation medium. ALP activity was measured after 2 days as previously described. Results from a representative of three separate experiments are shown, reported as the mean ± SD of quadruplicate determinations, normalized to protein concentration (*$P < 0.001$ for C vs. BMP14 or SS, and for BMP14 + SS vs. BMP14 or SS alone). **B**: M2 cells were treated under similar conditions as those described above. After 8 days, total RNA was isolated and analyzed for osteocalcin (OCN) and 18S rRNA expression by Northern blotting as previously described. Data from densitometric analysis of the Northern blot is shown in (**C**) as the average of duplicate samples, normalized to 18S rRNA.

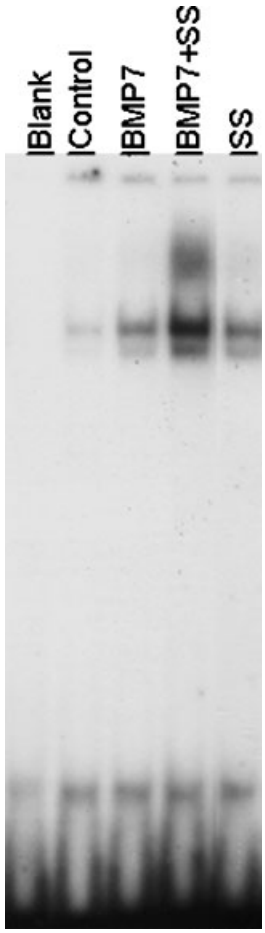

Fig. 9. Effect of oxysterols and BMP7 on Runx2 DNA-binding activity in MSCs. M2 cells were treated at confluence with control vehicle, 50 ng/ml of recombinant human BMP7, or a combination of oxysterols 22S and 20S (SS, 1.25 μM each), alone or in combination. After 4 days, nuclear extracts were prepared and analyzed by EMSA for Runx2 DNA-binding activity as previously described. Blank = no nuclear extract.

binding in oxysterol-treated cells (data not shown). These observations are consistent with the complexity of the osteogenic process, and the varying mechanisms that underlie the regulation of the different mediators of osteoblast differentiation.

The present findings demonstrate that in addition to osteoinductive proteins such as BMPs, osteoinductive oxysterols are also important regulators of osteoblastic differentiation. In vivo, oxysterols can be derived from both internal (i.e., cell-mediated synthesis) as well as external sources (i.e., through dietary intake) [Edwards and Ericsson, 1999]. Since all cells have the capacity to synthesize cholesterol, and since the metabolic machinery of mammalian cells is a highly pro-oxidant system, oxysterols are generated by most if not all cell types. Of note is the fact that other investigators have already reported the ability of osteoblastic cells to generate some oxysterols [Silva et al., 2003]. Given their potent stimulatory effect on mediators of osteogenic differentiation, we speculate that generation of osteogenic oxysterols by osteoprogenitor cells may be a yet unrecognized signal that triggers the lineage-specific differentiation of pluripotent mesenchymal cells into osteoblastic cells while inhibiting their differentiation into adipocytes. Such endogenously generated oxysterol signals might act in an autocrine and/or paracrine manner that would regulate osteoblast differentiation and bone formation, perhaps both during embryonic development of skeletal tissues as well in normal bone growth, remodeling, and repair of bone fractures.

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