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Oxysterol-Induced Osteogenic Differentiation of Marrow Stromal Cells is Regulated by Dkk-1 Inhibitable and PI3-Kinase Mediated Signaling

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ABSTRACT

Osteoporosis and its complications cause morbidity and mortality in the aging population, and result from increased bone resorption by osteoclasts in parallel with decreased bone formation by osteoblasts. A widely accepted strategy for improving bone health is targeting osteoprogenitor cells in order to stimulate their osteogenic differentiation and bone forming properties through the use of osteoinductive/anabolic factors. We previously reported that specific naturally occurring oxysterols have potent osteoinductive properties, mediated in part through activation of hedgehog signaling in osteoprogenitor cells. In the present report, we further demonstrate the molecular mechanism(s) by which oxysterols induce osteogenesis. In addition to activating the hedgehog signaling pathway, oxysterol-induced osteogenic differentiation is mediated through a Wnt signaling-related, Dkk-1-inhibitable mechanism. Bone marrow stromal cells (MSC) treated with oxysterols demonstrated increased expression of osteogenic differentiation markers, along with selective induced expression of Wnt target genes. These oxysterol effects, which occurred in the absence of β-catenin accumulation or TCF/Lef activation, were inhibited by the hedgehog pathway inhibitor, cyclopamine, and/or by the Wnt pathway inhibitor, Dkk-1. Furthermore, the inhibitors of PI3-Kinase signaling, LY 294002 and wortmanin, inhibited oxysterol-induced osteogenic differentiation and induction of Wnt signaling target genes. Finally, activators of canonical Wnt signaling, Wnt3a and Wnt1, inhibited spontaneous, oxysterol-, and Shh-induced osteogenic differentiation of bone marrow stromal cells, suggesting the involvement of a non-canonical Wnt pathway in pro-osteogenic differentiation events. Osteogenic oxysterols are, therefore, important small molecule modulators of critical signaling pathways in pluripotent mesenchymal cells that regulate numerous developmental and post-developmental processes. J. Cell. Biochem. 105: 424–436, 2008.

KEY WORDS: OXYSTEROL; Dkk-1; PI3-KINASE; OSTEOGENIC DIFFERENTIATION; MARROW STROMAL CELLS; HEDGEHOG SIGNALING; Wnt PATHWAY

Pluripotent mesenchymal stem cells found in the bone marrow stroma, also known as bone marrow stromal cells (MSC), have the potential to differentiate into several different cell types including osteoblasts, chondrocytes, myocytes, fibroblasts, and adipocytes [Caplan, 1994; Majors et al., 1997; Prockop, 1997]. Regulation of stem cell fate down these various lineages is essential for tissue development, homeostasis, and repair [Pittenger et al., 1999; Vaananen, 2005]. Osteoporosis is a degenerative disease of the skeleton that generally occurs due to an alteration in bone turnover homeostasis and is characterized by fragile bones and increased susceptibility to bone fractures [Riggs and Melton, 1992]. Decreased bone synthesis due to reduced osteoblast formation and/or activity of progenitor cells, which occurs in parallel with increased adipocyte formation at the expense of osteoblasts, in addition to increased bone resorption from excessive osteoclast formation and/or activity are mechanisms leading to this degenerative disorder [Cummings and Melton, 2002]. In addition to anti-resorptive agents, therapeutic molecules having pro-osteogenic and anti-adipogenic effects on MSC may help intervene with osteoporosis by enhancing bone formation through a shift in the apparent imbalance in cellular differentiation in favor of osteoblasts [Rodan and Martin, 2000; Goltzman, 2002; Mundy, 2002].

Oxysterols are products of cholesterol oxidation and are formed in vivo by a variety of cell types including osteoblasts [Schroepfer,

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2000; Bjorkhem and Dicsfalusy, 2002]. We previously reported that certain oxysterols, specifically 20(*S*)-hydroxycholesterol (20S), alone or in combination with, 22(*S*)- or 22(*R*)-hydroxycholesterol, are potent inducers of osteogenic differentiation in pluripotent mesenchymal cells such as M2-10B4 (M2) MSC and C3H10T1/2 embryonic fibroblasts [Kha et al., 2004]. In addition, we showed that these oxysterols induce osteogenic and inhibit adipogenic differentiation of MSCs through activation of the hedgehog signaling pathway, which in turn regulates the master switches that control osteogenic and adipogenic differentiation, namely Runx2 and PPARγ, respectively [Dwyer et al., 2007; Kim et al., 2007a; Richardson et al., 2007]. Further elucidation of the mechanism(s) by which oxysterols regulate MSC differentiation is of great importance in the development of these compounds into potential therapeutics for intervention with osteoporosis and other musculoskeletal disorders. In more recent studies we have focused on additional mechanisms that may play a synergistic and/or cooperative role with hedgehog signaling in mediating the effects of osteogenic oxysterols on MSC differentiation.

Wnts are small (39–46 kDa) lipid-modified secreted glycoproteins that influence many aspects of embryological development, such as cell patterning, proliferation, and stem cell fate determination [Clevers, 2006; Gordon and Nusse, 2006; Willert and Jones, 2006]. Wnt proteins signal through Frizzled (Fz) molecules, which are a family of seven-pass transmembrane receptors that transduce the signal through either β-catenin-dependent (i.e., canonical β-catenin/TCF/Lef pathway) or independent (i.e, non-canonical Wnt/planar cell polarity and the Wnt/calcium pathways) mechanisms. Activation of the β-catenin-dependent pathway requires the presence of low-density lipoprotein receptor related protein (LRP)5/6 [Johnson et al., 2004]. Certain Wnts induce osteogenesis, through direct stimulation of Runx2 gene expression [Westendorf et al., 2004; Gaur et al., 2005], and inhibit adipogenesis by inhibition of PPARγ and C/EBPα [Bennett et al., 2002; Kennell and MacDougald, 2005]. Furthermore, loss of function mutations in the LRP5 gene in humans results in the osteopenic disorder osteoporosis-pseudoglioma syndrome [Gong et al., 2001], whereas gain of function mutations in this same gene results in high bone mass disorders [Boyden et al., 2002]. It is possible to specifically inhibit the β-catenin-dependent Wnt signaling pathway using the protein Dickkopf-1 (Dkk-1), which directly binds to and removes LRP5/6 from the cell surface though endocytosis, thereby preventing β-catenin-dependent Wnt signaling from occurring [Bafico et al., 2001; Westendorf et al., 2004]. Although classically thought to specifically act as an inhibitor of β-catenin dependent Wnt signaling, several reports have shown the inhibitory effects of Dkk-1 independent of β-catenin [Lee et al., 2004; Peng et al., 2006]. Interestingly, hedgehog and Wnt signaling act synergistically and/or cooperatively in regulating several physiologic and pathologic processes including osteoblast development, hair follicle morphogenesis, and cancer [Mullor et al., 2001; Taipale and Beachy, 2001; Hu et al., 2004; Silva-Vargas et al., 2005].

The PI3-kinase/Akt pathway is involved in a variety of cellular processes including cell growth, proliferation, survival, metabolism, invasion, angiogenesis, and DNA repair. More recently, the role of PI3-kinase/Akt pathway in the survival of uncommitted osteoblast precursor cells [Debiais et al., 2004; Almeida et al., 2005] and in the regulation of osteoblast differentiation and migration [Ghosh-Choudhury et al., 2002; Fujita et al., 2004; Ghosh-Choudhurry et al., 2007] has been reported. Furthermore, *Akt-/-* mice have severely delayed bone development [Peng et al., 2007], and specific deletion of Akt inhibitor, Pten phosphatase, in osteoblasts results in increased bone density throughout life in mice [Liu et al., 2007]. Recent reports have demonstrated the direct or synergistic role of PI3-kinase/Akt activation in mediating the biological effects of hedgehog signaling including cell cycle progression, neuronal and chondrogenic differentiation, and capillary morphogenesis by endothelial cells [Kanda et al., 2003; Kenney et al., 2003; Fu et al., 2006; Riobo et al., 2006].

In the present report we show that specific oxysterols exert their osteogenic effects through a Dkk-1 inhibitable and PI3-kinase-dependent mechanism(s). Although Dkk-1 is able to block the oxysterol-induced osteogenic differentiation of MSC, oxysterols appear to regulate some but not all targets of Wnt signaling.

EXPERIMENTAL PROCEDURES

MATERIALS

M2-10B4 cells were purchased from American Type Culture Collection (Rockville, MD). Oxysterols, β-glycerophosphate (βGP), and ascorbate were obtained from Sigma-Aldrich, Co. (St. Louis, MO), RPMI 1640 was from Irvine Scientific (Santa Ana, CA), fetal bovine serum (FBS) was from Hyclone (Logan, UT), recombinant mouse Shh N-terminal peptide, recombinant human BMP2, and recombinant mouse Dickkopf related protein 1 (Dkk-1) were from R&D Systems, Inc. (Minneapolis, MN), cyclopamine, LY 294002, and wortmannin were from EMD Biosciences, Inc. (La Jolla, CA). Wnt3a conditioned medium (Wnt3a CM) and empty vector conditioned medium (C CM) were generous gifts from Dr. Peter Tontonoz (UCLA, Los Angeles, CA), and recombinant human Wnt1 was purchased from Abcam (Cambridge, MA).

CELL CULTURE

M2-10B4 cells were maintained in RPMI 1640 with 10% heat-inactivated FBS, supplemented with 1mM sodium pyruvate, 100 U/ml penicillin and 100 U/ml streptomycin as previously described [Kha et al., 2004]. Treatments were performed in osteogenic differentiation medium containing 5% FBS, 50 μg/ml ascorbate, and 3mM βGP. For inhibitor studies, cells were pretreated with the appropriate inhibitor or vehicle in osteogenic differentiation medium for 2 h prior to the addition of test agents. Test agents were then added directly into the cultures containing either the inhibitor or vehicle, and the appropriate assays were performed at the times indicated. For outcomes that were measured beyond 6 days from the time of initial treatments, cells were retreated every 6 days with inhibitors and test agents. No toxicity was found in any of the experiments involving inhibitors or test agents for the duration of the studies reported.

ALKALINE PHOSPHATASE ACTIVITY ASSAY AND ^{45}Ca INCORPORATION ASSAY

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

^{45}Ca incorporation assay as a measure of matrix mineralization in cell monolayers was performed as previously described [Parhami et al., 1997].

QUANTITATIVE RT-PCR

Total RNA was extracted with the RNA isolation kit from Stratagene (La Jolla, CA) according to the manufacturer's instructions. RNA was DNase-treated using the DNA-free kit from Ambion, Inc. (Austin, TX). RNA (3 μg) was reverse-transcribed using reverse transcriptase from Stratagene (La Jolla, CA) to make single stranded cDNA. The cDNAs were mixed with Qi SYBR Green Supermix from Bio-Rad Laboratories (Hercules, CA) for quantitative RT-PCR using a Bio-Rad I-cycler IQ quantitative thermocycler. All PCR samples were prepared in triplicate or quadruplicate. Each sample was added to duplicate wells of a 96-well plate. After 40 cycles of PCR, melt curves were analyzed in order to ensure primer specificity, and the identity of all PCR products was verified by sequencing and comparing with the complete mRNA sequence obtained from PubMed's GenBank. Fold changes in gene expression were calculated using the ΔΔCt method [Richardson et al., 2007]. All primers were designed using the Beacon Designer software from Bio-Rad Laboratories (Hercules, CA). Primers used are as follows: *OCN* (5′-TCTCTCTGACCTCA-CAGATGCC-3′ and 5′-TACCTTATTGCCCTCCTGCTTG-3′), *Axin2* (5′-GAGGCAGAAGCCACACAGAGA-3′ and 5′-CTGGCCGACAGTG-CAAGAC-3′), *Cyclin D1*(5′-GACACCAATCTCCTCAACGAC-3′ and 5′-TCACAGACCTCCAGCATCC-3′), *NKD2* (5′-GAAGACAACCGC-CAAGAATG-3′ and 5′-GGAGGAGTGATTGACAGAGG-3′), *WIF-1* (5′-CAAGTGTAAGTGCCCGAAAGG-3′ and 5′-CTGGCTCCATAC-CTCTTATTGC-3′), and *GAPDH* (5′-ATTGTCAGCAATGCATCCTG-3′ and 5′-ATGGACTGTGGTCATGAGCC-3′).

TRANSIENT TRANSFECTION AND REPORTER ASSAY

M2-10B4 cells at 70% confluence were transfected for 24 h using FuGENE 6 Transfection Reagent (Roche Applied Science, Indianapolis, IN) according to manufacturer's instructions. The wild-type and mutant TCF/LEF binding site driven luciferase constructs (TBE4-luc and TBE4-luc-mut, respectively) were generous gifts from Dr. Baruch Frenkel (University of Southern California, Los Angeles, CA), and the Cyclin D1 promoter element-driven luciferase construct (Cyclin D1-luc) was a generous gift from Dr. Fanxin Long (Washington University, St. Louis, MO). Firefly luciferase values were normalized to Renilla luciferase activity and pEGFP-NI was used to evaluate transfection efficiency. Cells were then treated for 24, 48, or 72 h with test agents before measuring luciferase activity using the Dual-Luciferase Reporter 1000 Assay System (Promega, Madison, WI) according to the manufacturer's instructions.

CYTOSOLIC PROTEIN EXTRACTION AND TOTAL CELL LYSATE PREPARATION

M2-10B4 cells were dounce homogenized 25 times in HLB/P Buffer [10 mM HEPES/KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 1:100 protease inhibitor cocktail (EMD Biosciences, Inc., La Jolla, CA), 1:100 phosphatase inhibitor cocktail I and II (Sigma, St. Louis, MO)], then spun down at 2500 rpm for 5 min at 4°C. The supernatant was collected and spun down at 19,000 rpm for 30 min at 4°C. The supernatant after this spin was collected and saved as the cytosolic protein extract. For preparation of total cell lysates, M2-10B4 cells were incubated on ice for 15 min in lysis buffer (50 mM NaCl, 5 mM EDTA, pH 8.0, 5 mM EGTA, 10 mM HEPES/KOH, pH 7.9, 0.1% Triton X-100, and 1:100 protease inhibitor cocktail) (EMD Biosciences, Inc., La Jolla, CA). For phosphorylated proteins 0.1 mM sodium vanadate (Na_3VO_4) was also included in the lysis buffer. Each sample was then sonicated and spun down at 12,000 rpm for 5 min at 4°C. The supernatant after this spin was collected and saved as the total cell lysate.

WESTERN BLOT ANALYSIS

Protein concentrations were determined using the Bio-Rad protein assay (Hercules, CA), and SDS–PAGE was performed as previously described [Parhami et al., 1999]. Briefly, cytosolic extracts or total cell lysates (30 μg) were separated on a 10% Tris-HCl gel from Bio-Rad laboratories (Hercules, CA) and transferred overnight onto a nitrocellulose membrane from Amersham Biosciences (Piscataway, NJ). Blocking was performed with 5% dry skim milk (Becton, Dickinson and Company, Sparks, MD) in Tris-buffer saline containing 0.1% Tween-20 (TBS-T) for 2 h at room temperature. Blots were then incubated with the monoclonal antibody against, phospho(Ser473)-Akt, or the polyclonal antibodies against Akt, β-catenin or β-actin (Cell Signaling Technology, Danvers, MA) following the instructions of the manufacturer. Binding of the primary antibody was detected by a secondary antibody labeled with horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Ruz, CA). The blots were developed using enhanced chemiluminescence detection reagents (Perkin Elmer, Boston, MA).

STATISTICAL ANALYSIS

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) significant test. A value of $P < 0.05$ was considered significant.

RESULTS

OXYSTEROL-INDUCED OSTEOGENESIS IS INHIBITED BY THE Wnt SIGNALING INHIBITOR, Dickkopf-1 (Dkk-1)

In light of the importance of Wnt signaling in regulation of osteoblasts and bone homeostasis, we began to examine the possible role of Wnt signaling in oxysterol-induced osteogenic differentiation of MSC by treating M2 cells with Dkk-1. Several markers of osteogenic differentiation were analyzed, including ALP activity, osteocalcin (OCN) mRNA expression, and mineralization. Results showed that pre-treatment with Dkk-1 caused a partial but significant inhibition of oxysterol-induced ALP activity in M2 cells (Fig. 1a); Dkk-1 alone had no effects (data not shown). To assess if the observed inhibition of ALP activity using Dkk-1 was specific to oxysterols, we examined the effect Dkk-1 pre-treatment had on other osteoinductive factors, namely sonic hedgehog (Shh) and bone morphogenetic protein-2 (BMP-2). As with oxysterol-induced ALP activity, Dkk-1 pre-treatment partially but significantly inhibited Shh-induced ALP activity (Fig. 1b). However, BMP-2-induced ALP activity was only significantly inhibited by using the higher

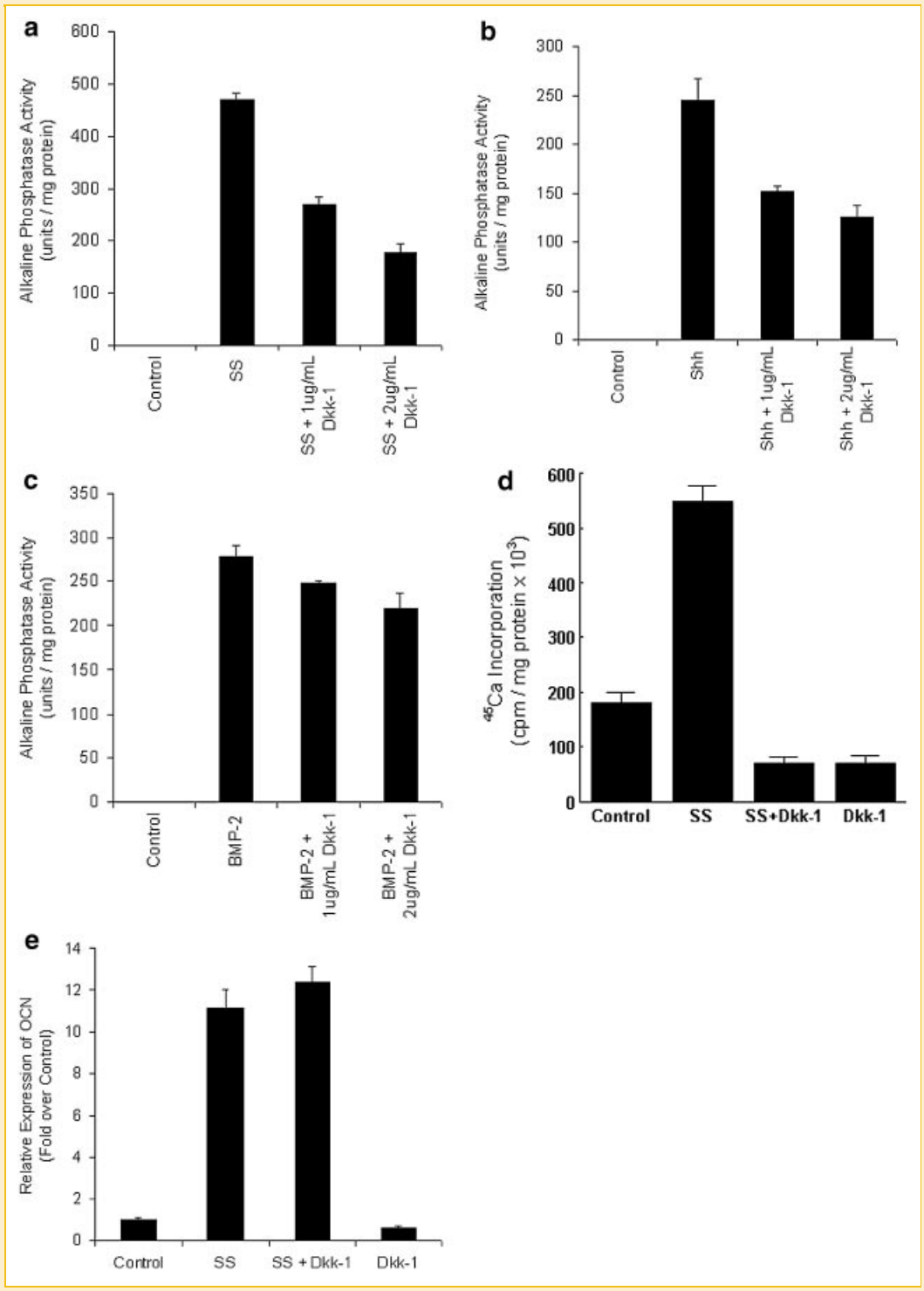

Fig. 1. The LRP5/6 inhibitor, Dickkopf-1 (Dkk-1), inhibits oxysterol-induced osteogenic differentiation in marrow stromal cells. (a–c), Alkaline phosphatase (ALP) activity assay in M2 cells pre-treated with 1 or 2 μg/ml Dkk-1 or vehicle for 2 h followed by treatment for 3 days with control vehicle or 2.5 μM of the oxysterol combination, SS (a); 200 ng/ml Shh (b); or 50 ng/ml BMP-2 (c). Results from a representative experiment are reported as the mean of quadruplicate determinations ± S.D. and normalized to protein concentrations ($P < 0.01$ for Control versus SS, Shh and BMP-2 and SS and Shh versus SS and Shh plus Dkk-1 at both concentrations; $P < 0.02$ for BMP-2 versus BMP-2 plus 2 μg/mL Dkk-1). (d) ^{45}Ca incorporation assay was used to measure mineralization in M2 cells pre-treated with 1 μg/ml Dkk-1 or vehicle for 2 h, and then treated with control vehicle or 2.5 μM SS 12 days. Data from a representative experiment are reported as the mean of quadruplicate determinations ± S.D. and normalized to protein concentrations ($P < 0.01$ for Control versus SS and SS versus SS plus Dkk-1). (e) analysis of osteocalcin (OCN) mRNA expression. M2 cells were pre-treated with vehicle or 1 μg/ml Dkk-1 for 2 h followed by treatment with control vehicle or 5 μM SS for 6 days. RNA was isolated and analyzed by Q-RT-PCR for *OCN* expression. Data from a representative experiment are reported as the mean of triplicate determination ± S.D. and normalized to *GAPDH* expression ($P < 0.003$ for Control versus SS with or without Dkk-1; $P < 0.001$ for Control versus Dkk-1).

concentration of Dkk-1, and to a lesser extent than that achieved for oxysterol- and Shh-induced ALP activity despite similar levels of ALP activity induction by all three molecules (Fig. 1c). More impressive than the partial inhibition of ALP activity was the complete and below baseline level inhibition of oxysterol-induced mineralization in M2 cells pretreated with Dkk-1 (Fig. 1d). In contrast to its effect on ALP and mineralization, Dkk-1 did not inhibit oxysterol-induced OCN mRNA expression in M2 cells (Fig. 1e), nor did it inhibit oxysterol-induced Runx2 DNA binding activity assessed by EMSA analysis (data not shown).

OSTEOGENIC OXYSTEROLS SELECTIVELY REGULATE TARGETS OF Wnt SIGNALING

To further demonstrate the role of Wnt signaling in oxysterol-induced biological effects in M2 cells, we examined the effects of oxysterols on several markers of Wnt signaling, including TCF/Lef-mediated transcriptional activity, cytosolic accumulation of β-catenin, and induced expression of several known Wnt target genes. Transcriptional activity of TCF/Lef in M2 cells treated with 5 μM SS, 200 ng/ml Shh, or 40 mM lithium chloride (LiCl) was measured using a luciferase reporter containing four wild-type or mutant TCF/Lef binding sites [Smith and Frenkel, 2005]. No significant change in reporter activity was observed in SS- or Shh-treated cells compared to untreated control cells after 24 h, whereas the positive control, LiCl significantly induced TCF/Lef reporter activity (Fig. 2a). Reporter activity was also not induced after 48 or 72 h of treatment with oxysterols or Shh (data not shown). Similar results were obtained when we used a different TCF/Lef reporter construct containing a *Cyclin D1* promoter element, which has a TCF/Lef binding site [Tetsu and McCormick, 1999] and was activated by LiCl, but not oxysterols, after 24 h of treatment (Fig. 2b). As previously shown, LiCl had no effect on pGL3basic luciferase control reporter [Tetsu and McCormick, 1999]. Furthermore, cytosolic extracts from M2 cells treated for 8, 24, and 48 h with 5 μM SS showed no significant change in β-catenin levels as measured by Western blot analysis and normalized to β-actin levels (data not shown).

In addition to the TCF/Lef reporter assays and Western blots of β-catenin described above, we also examined the effect of osteogenic oxysterols on the expression of several genes that are known targets of Wnt signaling, namely *Axin2*, *Cyclin D1*, *Naked Cuticle 2* (*Nkd2),* and *Wnt Inhibitory factor-1* (*Wif-1)* [Tetsu and McCormick, 1999; Wharton et al., 2001; Jho et al., 2002; Reguart et al., 2004; Van Raay et al., 2007]. Results showed no significant change in *Axin2* mRNA expression upon treatment of M2 cells with 5 μM SS or 200 ng/ml Shh after 8 and 48 h, whereas LiCl induced its expression at both time points (Fig. 3a,b). Oxysterols also did not significantly induce *Cyclin D1* mRNA expression, whereas Shh did cause a small but significant increase in its expression after 8 h, but not 48 h, of treatment (Fig. 3c,d). LiCl did not induce the expression of *Cyclin D1* in M2 cells at 8 h and inhibited its baseline expression after 48 h (data not shown). In contrast, mRNA expression of both *Nkd2* and *Wif-1*, which are known to be Wnt target genes that antagonize canonical Wnt signaling [Wharton et al., 2001; Reguart et al., 2004; Van Raay et al., 2007], was significantly induced by oxysterols 6 and 30-fold, respectively, after 48 h (Fig. 3e,f), but not after 8 h (data not shown). Furthermore, Shh treatment also induced *Wif-1*, but not *Nkd2* expression (Fig. 3e,f). As anticipated from the inability of Shh to induce *Nkd2* expression, the Hh pathway inhibitor, cylcopamine, at concentrations that completely abolished the induced expression of Hh target gene, *Patched* (*Ptch,* data not shown), did not inhibit oxysterol-induced *Nkd2* expression (Fig. 4a). In contrast, oxysterol-induced *Wif1* expression was completely blocked by cyclopamine (Fig. 4b). Furthermore, oxysterol-induced *Nkd2* expression was almost completely inhibited by Dkk-1 (Fig. 4c), whereas oxysterol-induced *Wif-1* was only minimally inhibited (Fig. 4d). To assess whether Wnt pathway activation might regulate oxysterol-induced Hh pathway activity, we tested the effect of Dkk-1 on oxysterol-induced *Ptch* mRNA expression, and found that Dkk-1 did not inhibit *Ptch* expression (Fig. 4e).

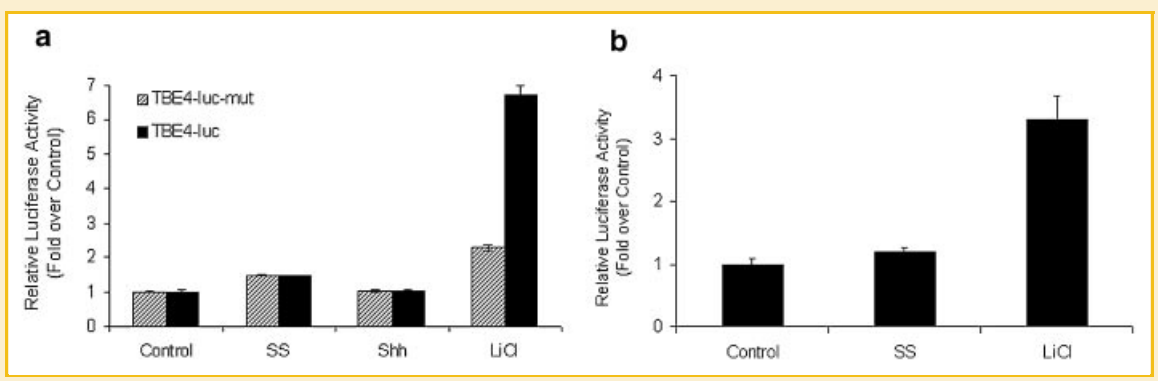

Fig. 2. Effect of osteogenic oxysterols on TCF/Lef transcriptional activity in marrow stromal cells. (a) M2 cells were transfected with a luciferase reporter driven by four wild-type or mutant TCF/Lef DNA binding sites (TBE4-luc and TBE4-luc-mut, respectively) and analyzed for their response to 5 μM SS, 200 ng/ml Shh, 40 mM LiCl, or control vehicle after 24 h of treatment. Data from a representative experiment are reported as the mean of triplicate samples ± S.D. and normalized to Renilla luciferase activity ($P < 0.001$ for Control versus LiCl with TBE4-luc expression vector). (b) M2 cells were transfected with a luciferase reporter driven by a Cyclin D1 promoter element (Cyclin D1-luc) and analyzed for their response to 5 μM SS, 40 mM LiCl, or control vehicle after 24 h of treatment. Data from a representative experiment are reported as the mean of triplicate samples ± S.D. and normalized to Renilla luciferase activity ($P < 0.01$ for Control versus LiCl).

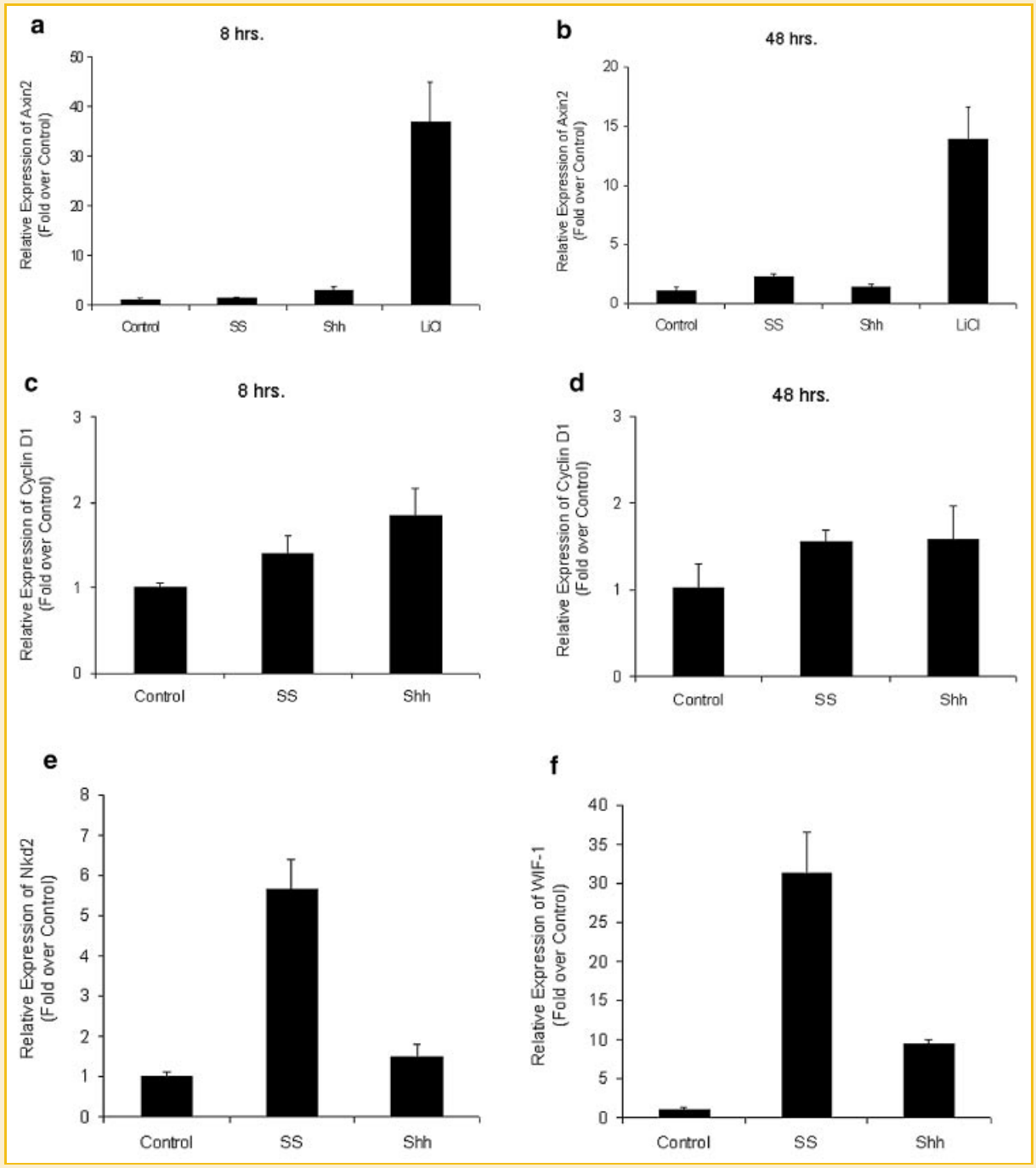

Fig. 3. Osteogenic oxysterols differentially regulate Wnt target gene expression in marrow stromal cells. (a, b) M2 cells were treated with 5 μM SS, 200 ng/ml Shh, 40 mM LiCl, or control vehicle for 8 or 48 h. RNA was isolated and analyzed by Q-RT-PCR for *Axin2* expression. Data from a representative experiment are reported as the mean of triplicate determination ± S.D. and normalized to *GAPDH* expression ($P < 0.02$ for Control versus LiCl at 8 h and 48 h, and for Control versus SS at 48 h). (c, d) M2 cells were treated with 5 μM SS, 200 ng/ml Shh, or control vehicle for 8 or 48 h. RNA was isolated and analyzed by Q-RT-PCR for *Cyclin D1* expression. Data from a representative experiment are reported as the mean of triplicate determination ± S.D. and normalized to *GAPDH* expression ($P < 0.05$ for Control versus Shh at 8 h). (e, f) M2 cells were treated with 5 μM SS, 200 ng/ml Shh, or control vehicle for 48 h. RNA was isolated and analyzed by Q-RT-PCR for *Nkd2* (e) and *WIF-1* (f) expression. Data from a representative experiment are reported as the mean of triplicate determination ± S.D. and normalized to *GAPDH* expression ($P < 0.01$ for Control versus SS for both *Nkd2* and *WIF-1* expressions; $P < 0.001$ for Control versus Shh for *WIF-1* expression).

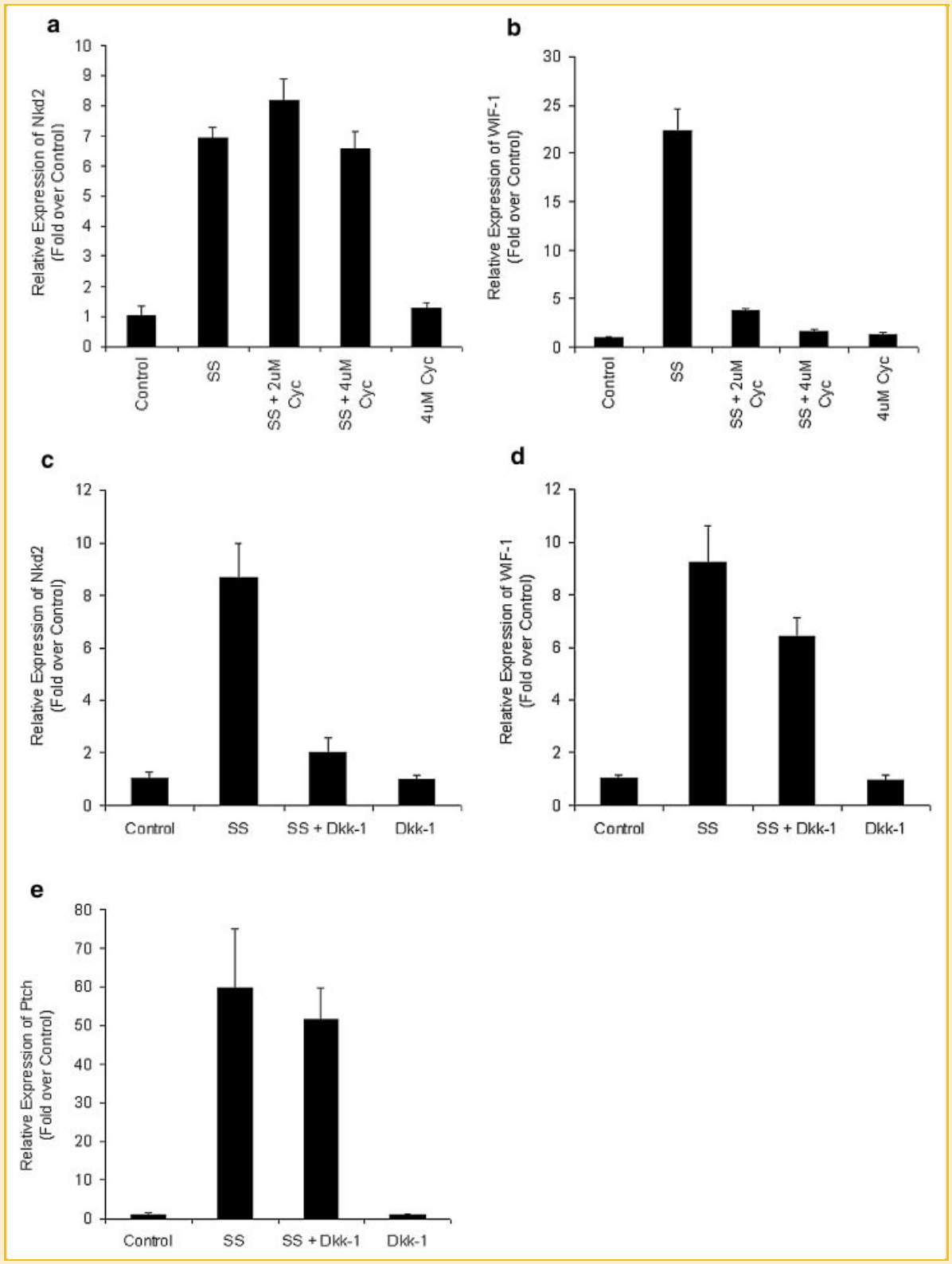

Fig. 4. Effects of various inhibitors on oxysterol-induced *Nkd2* and *Wif-1* expressions in marrow stromal cells. (a, b) M2 cells were pre-treated for 2 h with vehicle or cyclopamine (Cyc) at the concentrations indicated. Next, cells were treated with 5 μM SS or control vehicle for 48 h. RNA was isolated and analyzed for *Nkd2* (a) and *WIF-1* (b) expression by Q-RT-PCR. Data from a representative experiment are reported as the mean of triplicate determination ± S.D. and normalized to *GAPDH* expression ($P < 0.001$ for Control versus SS for both, *Nkd2* and *Wif-1*, expressions, and for SS versus SS + Cyc at both, 2 and 4 μM, for *Wif-1* expression). (c, d) M2 cells were pre-treated for 2 h with Dkk-1 or vehicle. Next, cells were treated with 5 μM SS or control vehicle for 48 h. RNA was isolated and analyzed for *Nkd2* (c), *Wif-1* (d), and *Ptch* (e) expression. Data from a representative experiment are reported as the mean of triplicate determination ± S.D. and normalized to *GAPDH* expression ($P < 0.01$ for Control versus SS for *Nkd2*, *Wif-1*, and *Ptch* expressions; $P < 0.001$ for SS versus SS plus Dkk-1 for *Nkd2* expression; $P < 0.03$ for SS versus SS plus Dkk-1 for *Wif-1* expression).

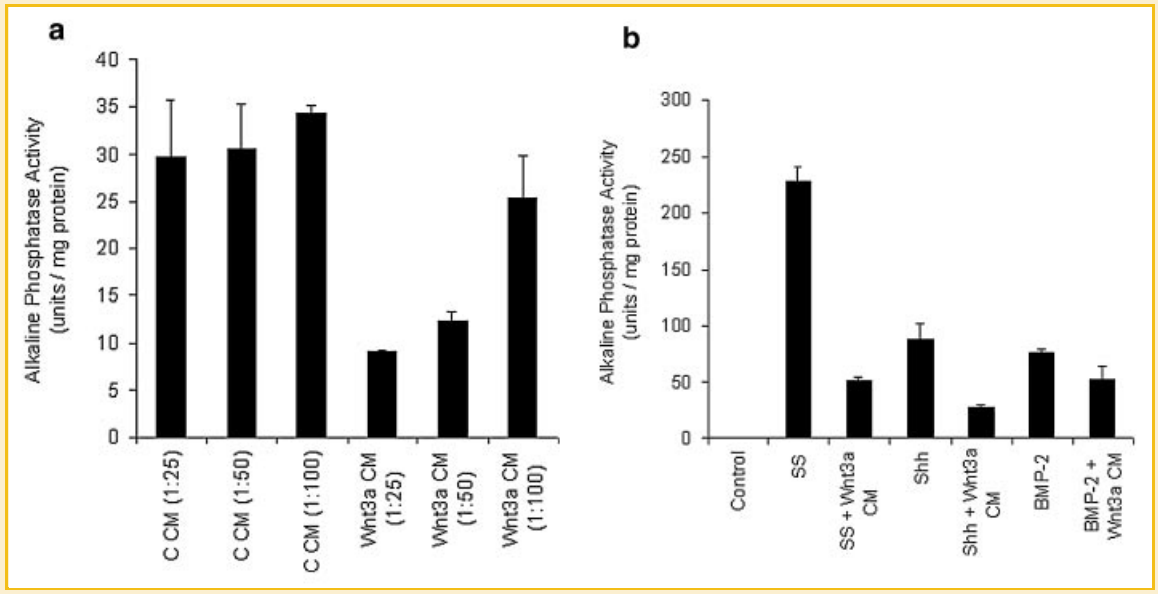

Fig. 5. Wnt3a conditioned medium inhibits oxysterol-induced alkaline phosphatase activity in marrow stromal cells. (a) ALP activity assay in M2 cells treated in osteogenic medium with various dilutions of Wnt3a conditioned medium (Wnt3a CM) or control conditioned medium (C CM) for 6 days. Results from a representative experiment are reported as the mean of triplicate determinations ± S.D. and normalized to protein concentrations ($P < 0.05$ for all C CM versus Wnt3a CM (1:25) and (1:50)). (b) ALP activity assay in M2 cells treated with 2.5 μM SS, 200 ng/ml Shh, 50 ng/ml BMP-2, or control vehicle, in combination with either C CM or Wnt3a CM at a 1:25 dilution for 3 days. Results from a representative experiment are reported as the mean of quadruplicate determinations ± S.D. and normalized to protein concentrations ($P < 0.02$ for Control versus SS, Shh and BMP-2, and for SS and Shh versus SS and Shh plus Wnt3a CM).

Wnt3a AND Wnt1 INHIBIT OSTEOGENIC DIFFERENTIATION OF M2-10B4 MARROW STROMAL CELLS

To further examine the effect of Wnt signaling on osteogenic differentiation of M2 cells, we tested the effect of Wnt3a conditioned medium (CM) on ALP activity compared to control CM (C CM). M2 cells were treated in osteogenic medium for 6 days with either C CM or Wnt3a CM at 1:25, 1:50, or 1:100 dilutions. Spontaneous increase in ALP activity that is normally seen as M2 cells differentiate in osteogenic medium was inhibited by Wnt3a CM in a dose-dependent manner compared to C CM (Fig. 5a). To test if Wnt3a had any effect on ALP activity induced by osteoinductive compounds, M2 cells were treated with 1.25 μM SS, 200 ng/ml Shh, or 50 ng/ml BMP-2, alone or in combination with a 1:25 dilution of Wnt3a CM. Wnt3a CM significantly inhibited both SS- and Shh-induced ALP activity (Fig. 5b). However, Wnt3a CM did not significantly inhibit BMP-2-induced ALP activity (Fig. 5b). Similar to the effects of Wnt3a, Wnt1 inhibited oxysterol- and Shh-induced ALP activity after 3 days of treatments (Control: 1.6 ± 0.3; SS 1.25 μM: 70 ± 7; SS 1.25 μM + Wnt1 500 ng/ml: 33 ± 3; Shh 200 ng/ml: 83 ± 5; Shh 200 ng/ml + Wnt1 500 ng/ml: 40 ± 5; Wnt1 500 ng/ml 0.2 ± 0.3 units/mg protein ± SD; $P < 0.001$ for Control vs. SS and Shh, and for SS and Shh in the presence vs. absence of Wnt1).

OXYSTEROL-INDUCED OSTEOGENESIS IS MEDIATED THROUGH THE PI3-kinase/Akt PATHWAY

To further elucidate the signaling mechanism(s) by which oxysterols regulate osteoblastic differentiation of M2 cells, we tested the effects of the PI3-kinase pathway inhibitors, LY 294002 (LY) and wortmannin (Wm) on oxysterol-induced markers of osteogenic differentiation. Pre-treatment of M2 cells with either LY or Wm significantly inhibited oxysterol-induced ALP activity in a dose-dependent manner (Fig. 6a). Similarly, Q-RT-PCR analysis showed that pre-treatment with 5 μM of either LY or Wm significantly inhibited SS-induced OCN mRNA expression after 8 days in M2 cells (Fig. 6b). Furthermore, ^{45}Ca incorporation assay showed that LY and Wm significantly inhibited oxysterol-induced mineralization after 14 days of treatment (Fig. 6c). Similar to their inhibitory effect on oxysterol-induced ALP activity, LY and Wm also inhibited Shh-induced ALP activity in M2 cells (Fig 6d). In addition, we examined the effects of LY and Wm on *Nkd2* and *Wif-1* mRNA expression in M2 cells and found that both LY and Wm caused partial yet significant inhibition of oxysterol-induced *Nkd2* and *Wif-1* mRNA expressions (Fig. 6e,f).

DISCUSSION

In the present study, we demonstrate the role of a Wnt-related signaling pathway in oxysterol-induced osteogenic differentiation of MSC. We were prompted to examine Wnt signaling due to the established role of this pathway in regulating the proliferation and differentiation of osteoblasts during bone formation, and the apparent cooperation of hedgehog and Wnt signaling in the development of osteoblasts in vivo [Hu et al., 2004; Johnson et al., 2004; Westendorf et al., 2004; Gaur et al., 2005]. Our results clearly

showed that some, but not all markers of osteogenic differentiation are blocked by Dkk-1. The fact that Dkk-1 inhibited oxysterol-induced ALP activity and mineralization but not OCN expression or Runx2 DNA binding activity is consistent with our previous reports that oxysterol-induced osteogenic differentiation of MSC is mediated by distinct mechanisms that regulate the different aspects of this process in MSC [Richardson et al., 2007]. Dkk-1-inhibitable effects of oxysterols do not appear to be β-catenin dependent due to the fact that the cytosolic levels of this protein were not affected upon treatment of MSC with oxysterols, and there was no apparent induction of TCF/Lef transcriptional activity in oxysterol-treated cells. These present results are consistent with other reports that demonstrate the antagonistic effect of Dkk-1 on various biological effects independent of β-catenin. Lee et al. reported that Dkk-1 antagonized Wnt signaling in human mesothelioma cells deficient in β-catenin [Lee et al., 2004], and Peng et al. showed that Dkk-1-induced apoptosis in human placental choriocarcinoma cells occurred independent of β-catenin [Peng et al., 2006]. However, it must be noted that since there were relatively high baseline cytosolic and nuclear levels of β-catenin in M2 cells under our experimental conditions, our findings do not rule out the potential cooperative interaction between baseline β-catenin-dependent signaling and oxysterol-induced hedgehog pathway activity, which would be inhibitable by Dkk-1. Since β-catenin/TCF/Lef dependent and independent signaling by Wnts are classically referred to as canonical and non-canonical Wnt signaling, respectively, it appears that any oxysterol-induced Wnt signaling in MSC would best associate with the latter phenomenon. Dkk-1 also partially inhibited Shh- and BMP2-induced ALP activity in M2 cells, but its inhibitory effects were less potent on BMP2-induced ALP activity than that induced by oxysterols or Shh. This difference may be in part due to a higher activation of Wnt signaling by BMP2 and greater reliance of BMP2 on Wnt signaling in inducing osteogenic differentiation [Rawadi et al., 2003; Mbalaviele et al., 2005], requiring greater concentration of Dkk-1 to inhibit this process. In contrast, oxysterols and Shh appear to induce osteogenic differentiation by hedgehog signaling as well as a Wnt signaling-related mechanism(s). We found no evidence of hedgehog signaling being induced by BMP2 in M2 cells as evidenced by the absence of Gli-1 and Ptch expression in response to BMP2 (data not shown). These results are consistent with previous reports that Dkk-1 inhibits the osteogenic effects of both Shh and BMP2 [Rawadi et al., 2003; Hu et al., 2004], although it is not clear whether such reported inhibitory effects of Dkk-1 are solely due to inhibition of β-catenin dependent mechanisms or also interference with β-catenin independent events.

Our present studies also demonstrated that Wnt signaling target genes are selectively regulated by oxysterols and Shh and through distinct mechanisms. *Axin2* was neither induced by oxysterols nor by Shh, and *Cyclin D1* was only minimally induced at 8 h but not at 48 h. In contrast *Nkd2* was only induced by oxysterols, and not by Shh, whereas *Wif-1* was induced by both oxysterols and Shh. Differential regulation of *Nkd2* expression by oxysterols and Shh is a noteworthy difference between responses induced by these osteogenic molecules. These findings, in addition to the ability of cyclopamine to completely block oxysterol-induced *Wif-1* but not *Nkd2*, and the ability of Dkk-1 to completely inhibit oxysterol-induced *Nkd2* and only minimally *Wif-1*, suggest that the effect of oxysterols on these Wnt target genes is predominantly through either Wnt- or hedgehog-dependent signaling. Given these findings, and the fact that Dkk-1 did not affect hedgehog pathway activation by oxysterols, we propose that oxysterol-induced biological responses in MSC are mediated through activation of and/or cooperation between two separate pathways: (1) the hedgehog signaling pathway and (2) the Wnt signaling pathway, with overlapping as well as distinct effects (Fig. 7).

It is interesting that PI3-kinase inhibitors LY and Wm were able to partially or completely inhibit all the above biological responses including those that appear to be mediated through hedgehog signaling as well as those mediated through Dkk-1-inhibitable signaling. LY and Wm did not inhibit oxysterol-induced hedgehog pathway activation (data not shown), suggesting that PI3-kinase acts downstream of this pathway. As a measure of PI3-kinase activation by oxysterols, we examined whether oxysterols induced phosphorylation of Akt by Western blotting. We found that despite the consistent ability of LY and Wm to inhibit oxysterol-mediated responses, increased levels of phospho-Akt normalized to total Akt in oxysterol-treated M2 cells were not substantially induced after 10 min, 30 min, 4 h, 8 h, 24 h, and 48 h of treatments (data not shown). There were significant baseline levels of phospho-Akt in M2 cells at all time points examined (data not shown). However, only in 2 out of more than 8 experiments were we able to demonstrate a modest 1.5-fold increase in phospho-Akt levels, and only after 48 h of treatment with oxysterols (data now shown). Therefore, we

Fig. 6. Oxysterol-induced osteogenesis is mediated by the PI3-kinase pathway in marrow stromal cells. (a) ALP activity assay in M2 cells pre-treated with various doses of the PI3-kinase inhibitors, LY 294002 (LY) or wormannin (Wm) or vehicle for 2 h followed by treatment for 3 days with 2.5 μM SS control vehicle. Results from a representative experiment are reported as the mean of quadruplicate determinations ± S.D. and normalized to protein concentrations ($P < 0.001$ for Control versus SS, and for SS versus SS plus LY or Wm at all concentrations). (b) M2 cells were pre-treated with 5 μM LY, 2.5 μM Wm, or vehicle for 2 h followed by treatment with 2.5 μM SS control vehicle for 6 days. RNA was analyzed for OCN expression by Q-RT-PCR and normalized to GAPDH ($P < 0.01$ for Control versus SS and for SS versus SS + LY and SS + Wm). (c) ^{45}Ca incorporation assay was used to measure mineralization in M2 cells pre-treated with various doses of LY, 1 μM Wm, or vehicle for 2 h, and then treated with 5 μM SS or control vehicle for 14 days. Data from a representative experiment are reported as the mean of quadruplicate determinations ± S.D. and normalized to protein concentrations ($P < 0.001$ for Control versus SS, and for SS versus SS + LY and SS + Wm at all concentrations). (d) M2 cells were pretreated with 5 μM LY, 1 μM Wm, or vehicle for 2 h followed by treatment with 200 ng/ml Shh or control vehicle for 3 days. ALP activity assay was performed and results from a representative experiment are reported as the mean of quadruplicate determinations ± S.D. and normalized to protein concentrations ($P < 0.001$ for Control versus Shh and for Shh versus Shh + LY and Shh + Wm). *E,f*, M2 cells were pre-treated for 2 h with 5 μM LY, 2.5 μM Wm, or vehicle followed by treatment with 5 μM SS or control vehicle for 48 h. RNA was isolated and analyzed for *Nkd2* (e) and *Wif-1* (f) expressions. Data from a representative experiment are reported as the mean of triplicate determination ± S.D. and normalized to *GAPDH* expression ($P < 0.01$ for Control versus SS, and for SS versus SS plus LY and Wm for both *Nkd2* and *WIF-1* expressions).

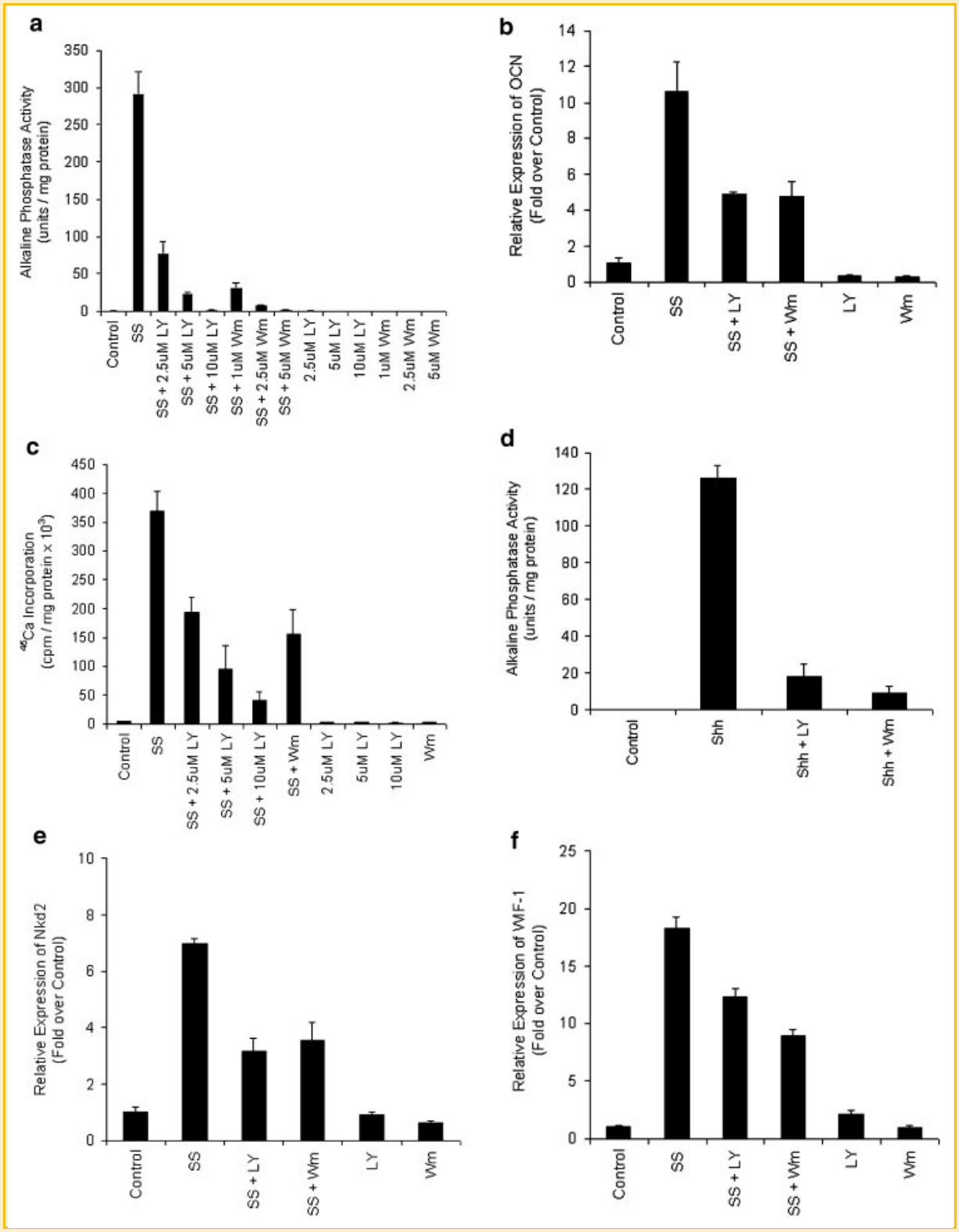
a
Alkaline Phosphatase Activity (units / mg protein)
350
300
250
200
150
100
50
0
Control
SS
SS + 2.5uM LY
SS + 5uM LY
SS + 10uM LY
SS + 1uM Wm
SS + 2.5uM Wm
SS + 5uM Wm
2.5uM LY
5uM LY
10uM LY
1uM Wm
2.5uM Wm
5uM Wm
b
Relative Expression of OCN (Fold over Control)
14
12
10
8
6
4
2
0
Control
SS
SS + LY
SS + Wm
LY
Wm
c
45Ca Incorporation (cpm / mg protein x 10^3)
450
400
350
300
250
200
150
100
50
0
Control
SS
SS + 2.5uM LY
SS + 5uM LY
SS + 10uM LY
SS + Wm
2.5uM LY
5uM LY
10uM LY
Wm
d
Alkaline Phosphatase Activity (units / mg protein)
140
120
100
80
60
40
20
0
Control
Shh
Shh + LY
Shh + Wm
e
Relative Expression of Nkd2 (Fold over Control)
10
8
6
4
2
0
Control
SS
SS + LY
SS + Wm
LY
Wm
f
Relative Expression of WF-1 (Fold over Control)
25
20
15
10
5
0
Control
SS
SS + LY
SS + Wm
LY
Wm

Fig. 6.

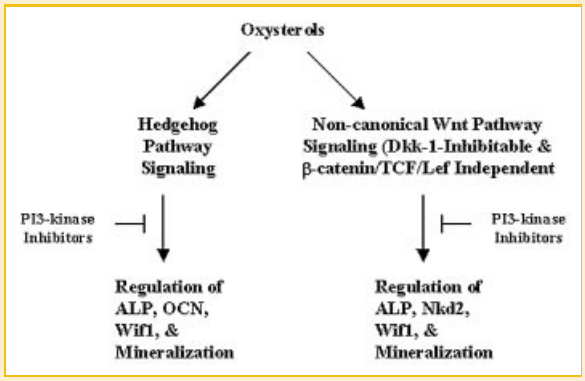

Fig. 7. Molecular mechanisms by which osteogenic oxysterols induce osteogenic differentiation of marrow stromal cells. Oxysterols activate two distinct signaling pathways: (1) the hedgehog signaling pathway and (2) a Dkk-1-inhibitable & β-catenin/TCF/Lef independent pathway. Activation of these pathways mediate osteogenic differentiation of MSC and cooperatively or distinctly regulate the markers and genes associated with osteogenic differentiation and Wnt signaling including alkaline phosphatase (ALP) activity, osteocalcin (OCN) mRNA expression, Wif-1 and Nkd2 expressions, and matrix mineralization.

speculate that rather than activation of PI3-kinase by oxysterols, its basal activity works cooperatively with oxysterols and Shh in inducing osteogenic differentiation of M2 cells. Cooperative and/or synergistic interactions between PI3-kinase and both hedgehog and Wnt signaling have been reported. Riobo et al. reported that PI3-kinase and Akt are essential for Shh signaling during neurogenic and chondrogenic differentiation and Gli activation in progenitor cells, and that their activation by insulin-like growth factor I significantly enhanced Shh-induced signaling [Riobo et al., 2006]. In their studies, Shh itself caused only a modest activation of PI3-kinase/Akt, but the baseline level of activity may have also contributed to hedgehog signaling since LY clearly reduced the baseline levels of phospho-Akt in their experimental system. Similarly, PI3-kinase/Akt signaling was shown to mediate morphological and migratory responses of endothelial cells to hedgehog signaling [Fu et al., 2006], and PI3-kinase and hedgehog signaling were found to converge on Nmyc1 to regulate cell cycle progression in neuroprogenitor cells [Kenney et al., 2003]. Furthermore, it was reported that PI3-kinase/Akt pathway mediated Wnt3a-induced proliferation of NIH3T3 cells [Kim et al., 2007b], and that prevention of apoptosis by Wnt proteins was in part mediated through PI3-kinase/Akt signaling, irrespective of their ability to stimulate canonical Wnt signaling [Almeida et al., 2005]. More recently it was reported that Wnt5a and its receptor Ror2 in Xenopus mediate gene expression in part through PI3-kinase and independently of β-catenin/TCF/Lef [Kim et al., 2007b]. The role of PI3-kinase in Runx2-mediated osteogenic and chondrogenic differentiation was clearly demonstrated in progenitor cells where inhibition by LY or a dominant-negative-Akt inhibited Runx2-dependent transcription and expression of osteogenic and chondrogenic differentiation markers [Fujita et al., 2004]. Our findings add further support to the concept of cooperative interactions between hedgehog, Wnt, and PI3-kinase signaling, specifically with respect to oxysterol-induced osteogenic differentiation of MSC (Fig. 7).

Interestingly, we found that Wnt3a and Wnt1, classic members of the Wnt family of proteins associated with the canonical Wnt signaling [Boland et al., 2004], inhibited spontaneous as well as oxsyterol- and Shh-induced ALP activity in MSC. This is consistent with reports by Boland et al. that Wnt3a suppressed osteogenic differentiation while promoting proliferation of human mesenchymal stem cells [Boland et al., 2004]. It has been suggested that canonical Wnt signaling regulates the maintenance and proliferation of progenitor cells, which may need to be suppressed in order for these cells to undergo terminal osteogenic differentiation that may be induced by a mechanism in part dependent on non-canonical Wnt signaling [Westendorf et al., 2004]. In support of this hypothesis, overexpression of *Wnt5a* as well as upregulation of *Wnt11*, both members of the non-canonical Wnt family [Schambony and Wedlich, 2007], promote and enhance the osteogenic differentiation process in osteoprogenitor cells [Boland et al., 2004]. In preliminary studies we have found a 2-fold increase in mRNA expression of *Wnt5a*, but not *Wnt3a* or *Wnt10b*, in oxysterol-treated M2 cells after 48 h of treatment (data not shown). It is also noteworthy that both Nkd2 and Wif-1 are antagonists of Wnt signaling, and their induction by osteogenic oxysterols is further support for their potential role in promoting the terminal osteogenic differentiation of progenitor cells through inhibition of canonical Wnt signaling and proliferative activity [Boland et al., 2004]. However, the role of Wnt signaling in regulation of MSC is likely to be more complex since other investigators have reported that canonical Wnt/β-catenin signaling may in fact play a pro-differentiation role when applied to a variety of osteoprogenitor cells in vitro [Bennett et al., 2005; Clement-Lacroix et al., 2005; Day et al., 2005; Hill et al., 2005]. Such variations in the reported observations may be due to differences in experimental models used by different investigators, as well as differences in commitment stage of various progenitor cells to the osteogenic lineage. Despite these differences, in vitro and in vivo data clearly demonstrate the positive role of Wnt signaling in the development and maintenance of osteoblasts and bone, either through positive regulation of proliferation and maintenance of an osteoprogenitor pool, and/or through pro-osteogenic differentiation effects on these cells. Rodda and McMahon recently demonstrated distinct roles for hedgehog and Wnt signaling in specification, differentiation, and maintenance of osteoblast progenitors [Rodda and McMahon, 2006]. The fact that specific oxysterols mediate their biological effects in part through regulation of Wnt signaling, in addition to hedgehog signaling, adds to the accumulating evidence supporting their potential role in osteoblast biology.

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