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Mitochondrial function contributes to oxysterol-induced osteogenic differentiation in mouse embryonic stem cells

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ABSTRACT

Oxysterols, oxidized derivatives of cholesterol, are biologically active molecules. Specific oxysterols have potent osteogenic properties that act on osteoprogenitor cells. However, the molecular mechanisms underlying these osteoinductive effects on embryonic stem cells (ESCs) are unknown. This study investigated the effect of an oxysterol combination of 22(S)-hydroxycholesterol and 20(S)-hydroxycholesterol (SS) on osteogenic differentiation of ESCs and the alterations to mitochondrial activity during differentiation. Osteogenic differentiation was assessed by alkaline phosphatase (ALP) activity, matrix mineralization, mRNA expression of osteogenic factors, runt-related transcription factor 2, osterix, and osteocalcin, and protein levels of collagen type IA (COLIA) and osteopontin (OPN). Treatment of cells with SS increased osteoinductive activity compared to the control group. Intracellular reactive oxygen species production, intracellular ATP content, mitochondrial membrane potential, mitochondrial mass, mitochondrial DNA copy number, and mRNA expression of peroxisome proliferator-activated receptor-γ coactivators 1α and β, transcription factors involved in mitochondrial biogenesis, were significantly increased during osteogenesis, indicating upregulation of mitochondrial activity. Oxysterol combinations also increased protein levels of mitochondrial respiratory complexes I–V. We also found that SS treatment increased hedgehog signaling target genes, Smo and Gli1 expression. Inhibition of Hh signaling by cyclopamine suppressed mitochondrial biogenesis and ESC osteogenesis. Subsequently, oxysterol-induced Wnt/β-catenin pathways were inhibited by repression of Hh signaling and mitochondrial biogenesis. Transfection of β-catenin specific siRNA decreased the protein levels of COLIA and OPN, as well as ALP activity. Collectively, these data suggest that lipid-based oxysterols enhance differentiation of ESCs toward the osteogenic lineage by regulating mitochondrial activity, canonical Hh/Gli, and Wnt/β-catenin signaling.

1. Introduction

Embryonic stem cells (ESCs) are characterized by two essential functions, unlimited proliferation and differentiation into specific lineages [1]. These properties make them valuable for cell therapy applications and tissue engineering. ESCs are particularly good candidates for regenerative medicine research aimed at repairing bone defects with low numbers of osteoprogenitor cells because they are capable of differentiating along the osteoblastic lineage [2]. For these applications, differentiation should occur in well-defined conditions with specific stimulatory culture medium supplements. However, very few molecules have been reported as being osteoinductive [3]. Thus, a number of groups have focused on establishing differentiation protocols using novel factors and strategies, which improve the differentiation of ESCs into osteoblastic cells, particularly for applications requiring mass production [4,5].

Oxysterols are oxidized derivatives of cholesterol, which are important in many biological processes including cholesterol homeostasis, inflammation, apoptosis, and cellular differentiation [6–9]. Recently, oxysterol combinations, 22(R)- or 22(S)- and 20(S)-hydroxycholesterol, were reported to possess potent osteogenic activity [10]. These oxysterol combinations enhanced in vitro osteogenic differentiation and in vivo bone formation by activating specific signaling molecules [11,12]. However, the molecular details of this activity and many other osteogenic processes are not well understood. In particular, little attention has been paid to the effect of oxysterols on mitochondrial activation and biogenesis during ESC differentiation.

Mitochondria, the powerhouse of cells, play an important role in the metabolism of nutrients. They can vary in number and function in different cell types in response to altering energy demands [13]. Mitochondrial biogenesis and oxidative phosphorylation, as well as replication of mitochondrial DNA, are key regulatory events in cell proliferation, differentiation, and death [14,15]. These mitochondrial functions are significantly involved in early embryonic development [16]. Recently, researchers reported that stem cells utilize specific metabolic pathways and that mitochondrial function is involved in regulation of stem cell maintenance and differentiation [17,18]. In fact, differentiating stem cells show increases in mitochondrial mass and oxygen consumption, which contributes to spontaneous ESC

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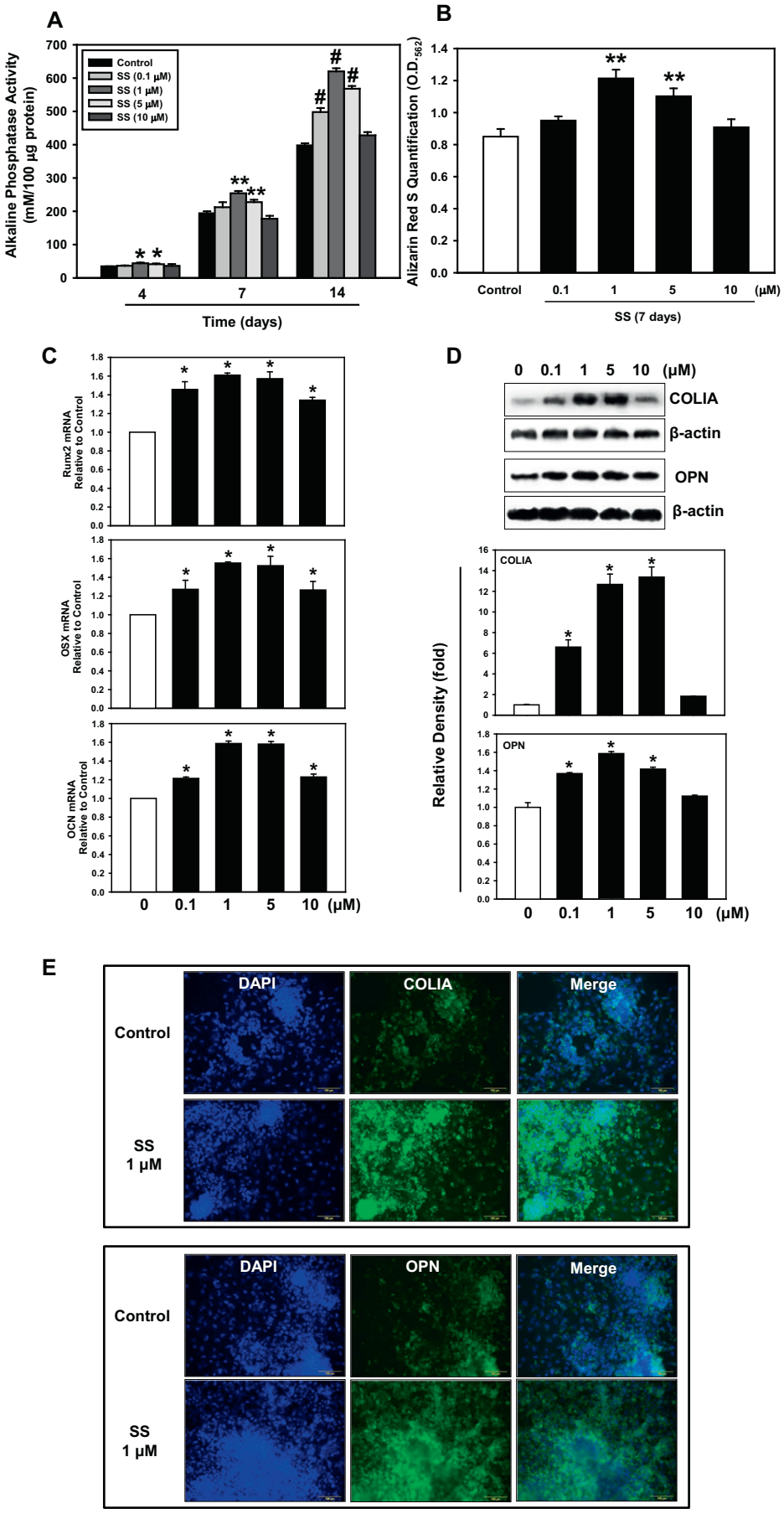

A
Alkaline Phosphatase Activity (mM/100 μg protein)
Control
SS (0.1 μM)
SS (1 μM)
SS (5 μM)
SS (10 μM)
Time (days)
B
Alizarin Red S Quantification (O.D562)
Control
SS (7 days)
(μM)
C
Runx2 mRNA Relative to Control
OSX mRNA Relative to Control
OCN mRNA Relative to Control
D
COLIA
β-actin
OPN
Relative Density (fold)
E
DAPI
COLIA
Merge
Control
SS 1 μM
OPN

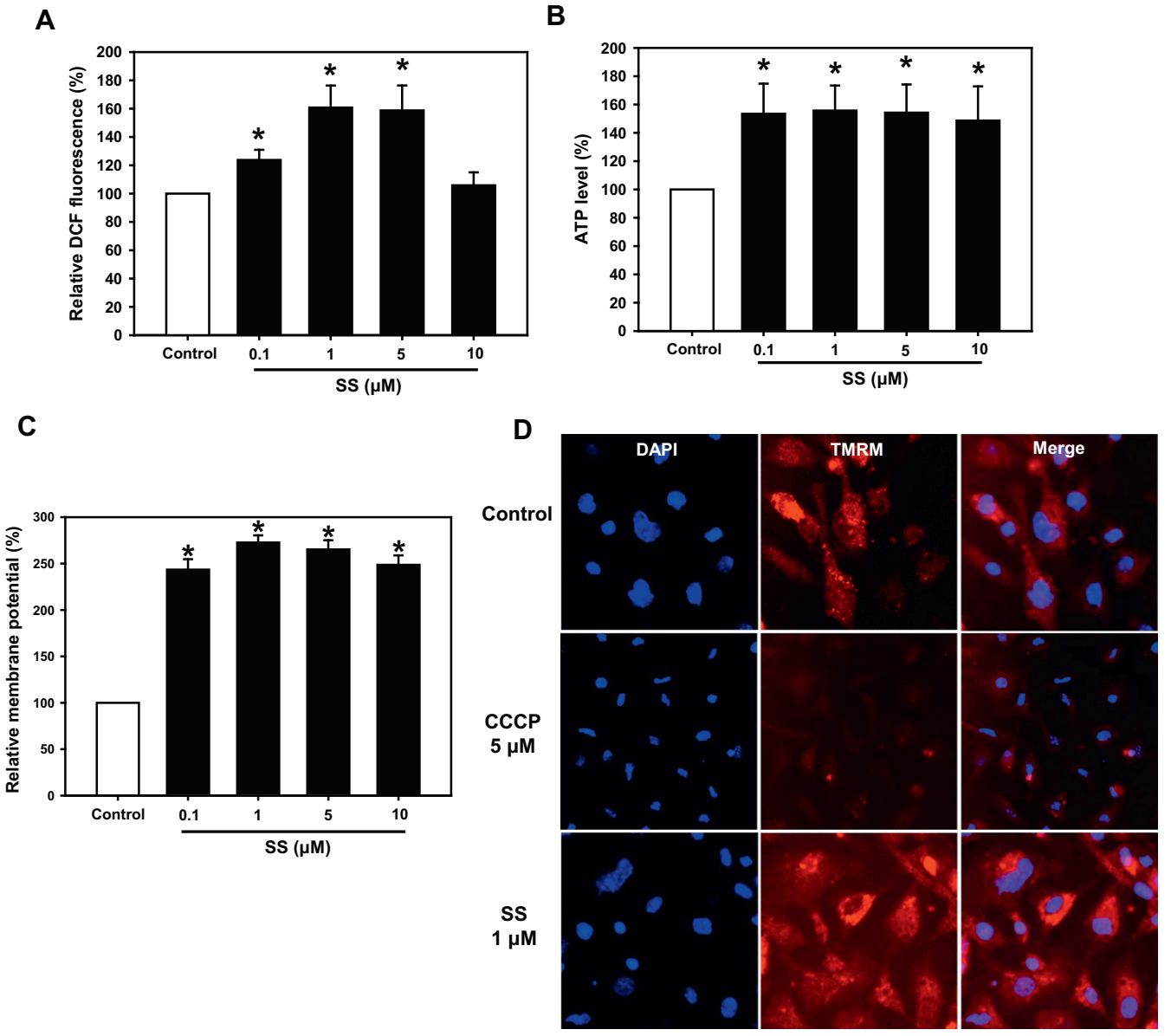


Fig. 2. Oxysterols affect intracellular ROS, ATP levels, and mitochondrial membrane potential during osteogenic induction. (A) Cells were incubated with SS at different concentrations for 2 h and then 10 μM CM-H_2DCF-DA was added. After 40 min, DCF fluorescence was determined by spectrofluorophotometer. (B) Cellular ATP concentration, (C) relative membrane potential, and (D) mitochondrial morphology were assessed as described in the Materials and methods. The treatment of CCCP was used as a positive control. Values are reported as mean ± S.D. of four independent experiments, $^{*}P < 0.05$ vs. the control value.

differentiation [19]. Moreover, upregulation of mitochondrial biogenesis has been shown to be involved in mesenchymal stem cell differentiation in response to osteoinductive signals [20]. While mitochondrial function and biogenesis are important in stem cell homeostasis, little is known about the biological significance of these factors and about the molecular mechanisms responsible for regulating stem cell fate.

Emerging evidences indicate the important role of hedgehog (Hh) signaling in bone homeostasis by regulating bone formation and resorption as well as in bone repair by modulating of cell behaviors [21,22]. Hh signaling is activated by receptors including the G protein-coupled receptor smoothened (Smo) and the twelve-pass membrane protein patched (PTCH). The receptor PTCH represses Smo activation in the absence of Hh stimulation, but this inhibition is released when Hh is introduced. Eventually, the end point of Hh signaling, the Gli (glioma-associated oncogene family members) transcription factor modulates Hh signaling transduction referred to as canonical Hh/Gli pathway [23]. Oxysterols were recently implicated as crucial activators of Hh signaling in pluripotent mesenchymal cells [12]. However, whether Hh signaling controls osteogenic specification of ESCs, and this mechanism arises from oxysterols, remain unclear. Thus, it is important to determine how Hh signaling pathway can regulate processes that lie on either oxysterol-induced intracellular signaling or the osteogenic spectrum of ESCs.

Fig. 1. Oxysterols affect ESC osteogenesis. Cells were incubated in osteogenic medium at different oxysterol combinations (SS; 0.1, 1, 5, 10 μM) for 4, 7, or 14 days. (A) ALP activity and (B) Alizarin Red quantification were assessed as described in the Materials and methods. (C) The mRNA levels of Runx2, OSX, and OCN were analyzed after 7 days of osteogenic induction. Moreover, (D) the protein levels of COLIA and OPN were determined by Western blot analysis or (E) immunostaining. Panels (bars) denote the mean ± S.D. of five experiments for each condition determined from densitometry relative to β-actin, $^{*}P < 0.05$ vs. the control value. The nuclei were stained with DAPI, shown in blue. Values are reported as means ± S.D. of five independent experiments, $^{*}P < 0.05$; $^{**}P < 0.01$; or $^{\#}P < 0.001$ vs. the control value at each day.

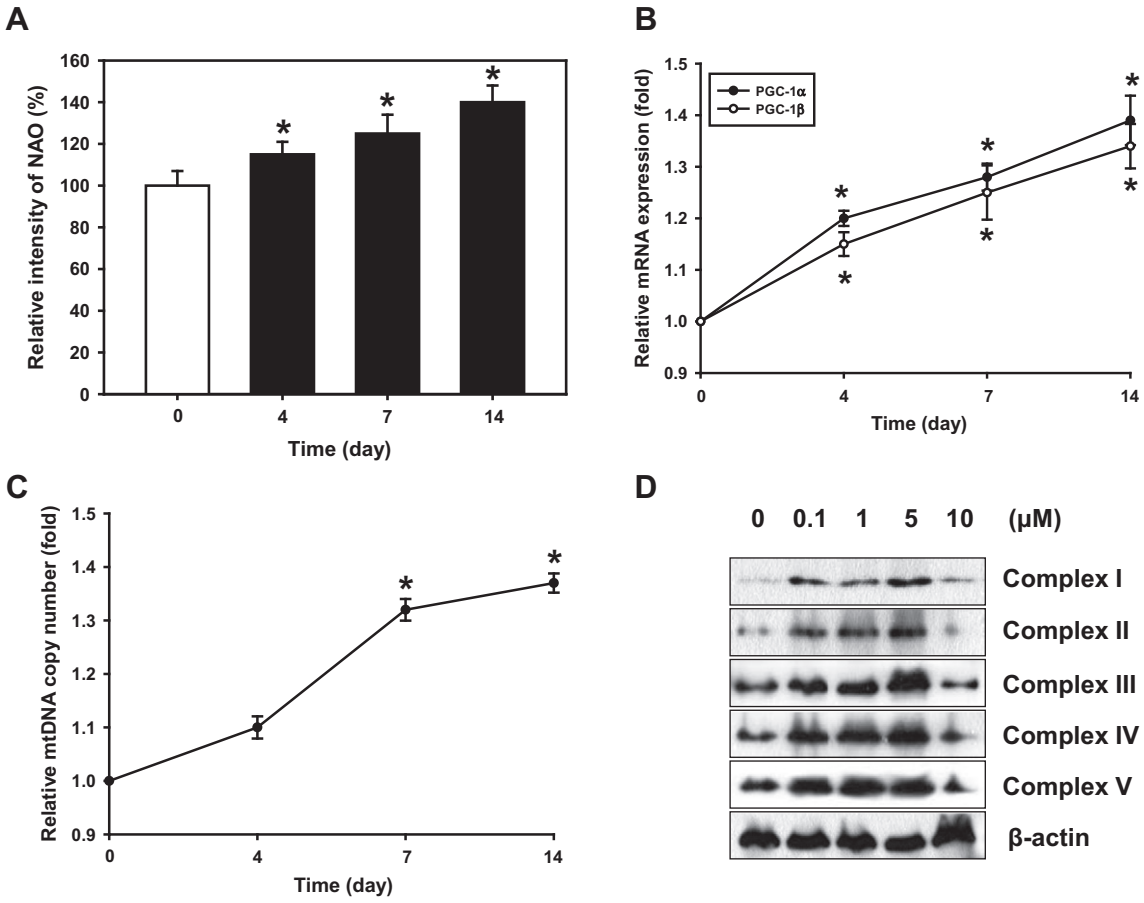


Fig. 3. Oxysterols affect mitochondrial biogenesis and respiratory enzyme complexes. Cells were cultured in the absence or presence of 1 μM SS for 4, 7, and 14 days. Next, (A) mitochondrial mass, (B) mRNA expression levels of PGC-1α and PGC-1β, (C) mtDNA copy number were measured as described in the Materials and methods. (D) The protein levels of mitochondrial respiratory enzyme complexes after 4 days of osteogenic induction were detected by Western blot analysis. All data were obtained from three independent experiments, $^{*}P < 0.05$ vs. the control value.

The Wnt signaling pathways have been implicated to regulate multiple biological events such as morphogenesis, cell fate determination, and differentiation during embryonic development [24,25]. Wnt signaling is divided into the canonical and the non-canonical pathways, depending on the β-catenin, which act as a coactivator of the T-cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors, resulting in cellular responses via the increased expression of target genes [26]. It is well known that canonical Wnt/β-catenin signaling is considered as a key regulator of bone biology [27,28]. Increasing evidences suggest that activation of β-catenin through Wnt signaling enhances differentiation of stem/progenitor cells into osteogenic lineage and subsequent plays an important role in bone formation and regeneration [29–31]. Thus, exploring the mechanism of Wnt pathway and its related signaling pathways can suggest a practical strategy for controlling ESCs during osteogenic differentiation.

In the present study, we demonstrate the importance of mitochondrial function during ESC osteogenic differentiation in response to oxysterols. We also provide evidence for the presence of mitochondria-coordinated Hh/Gli and Wnt/β-catenin signaling pathways, which provides new potential cell therapy approaches for oxysterol-induced bone regeneration.

2. Materials and methods

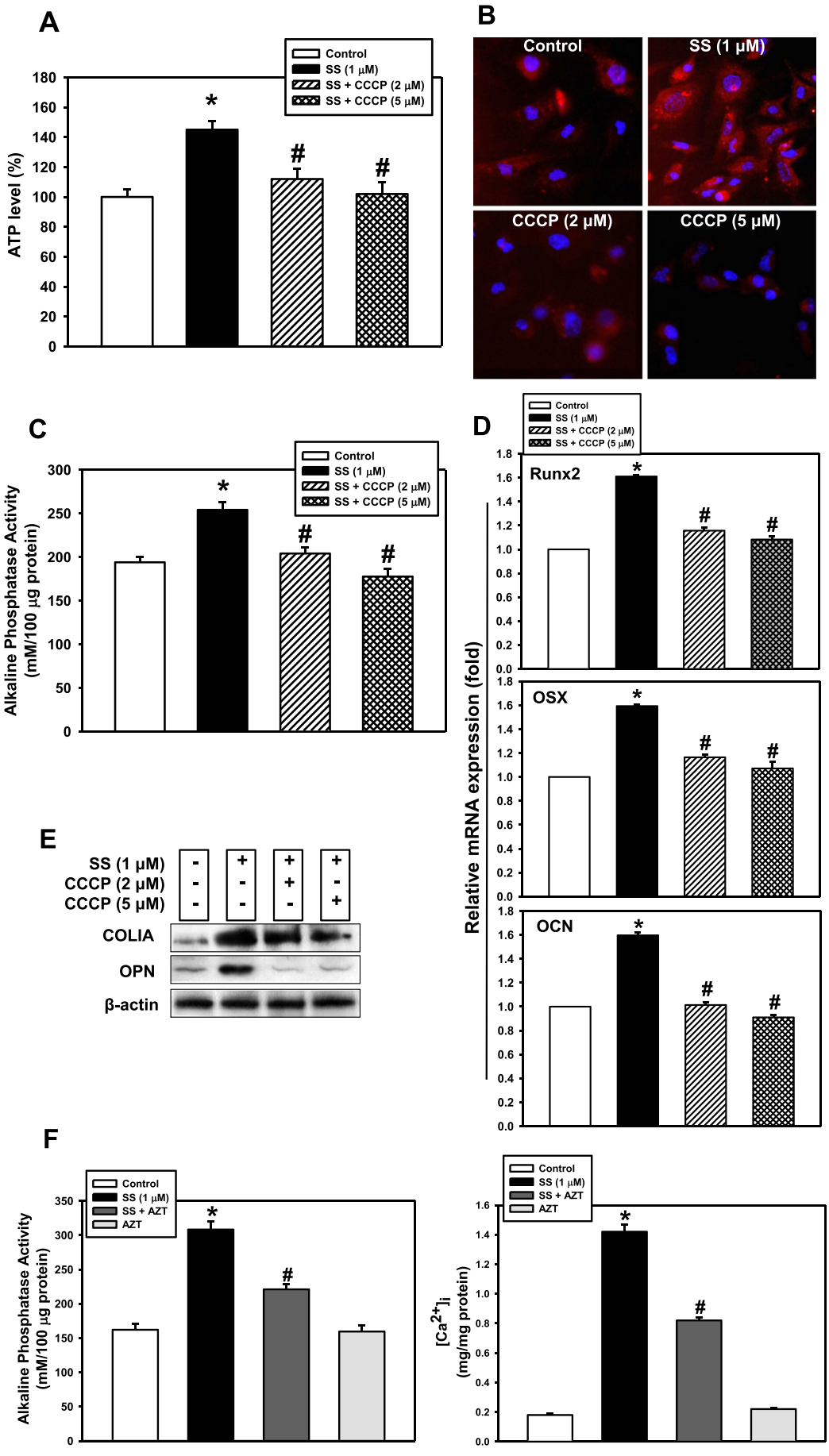
2.1. Materials

The mouse ESC line D3 was obtained from the American Type Culture Collection (Rockville, MD, USA). Fetal bovine serum (FBS) was purchased from Gibco-BRL (Gaithersburg, MD, USA). Unless otherwise specified, chemicals and laboratory wares were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA).

2.2. ES cell culture and embryoid body formation

The ESCs were prepared as previously described [32]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with 1.7 mM L-glutamine, 0.1 mM β-mercaptoethanol, 5 ng/mL mouse leukemia inhibitory factor (LIF), 15% FBS, and 1% penicillin and streptomycin, without a feeder layer at 37 °C in an atmosphere containing 5% CO_2. To form embryoid bodies (EBs), the cells were dissociated by 0.05% trypsin/EDTA. Then 2000 cells were hung from the lids of 100-mm culture dishes for two days in 20 μL DMEM

Fig. 4. CCCP and AZT affect mitochondrial activity and oxysterol-induced osteogenic differentiation of ESCs. Cells were pretreated with 2 or 5 μM of CCCP for 2 h prior to addition of 1 μM SS. Next, (A) ATP content, (B) membrane potential, (C) ALP activity, (D) mRNA levels of Runx2, OSX, and OCN, as well as (E) the protein levels of COLIA and OPN were analyzed as described in the Materials and methods. (F) Cells were pretreated with 10 μM of AZT before SS treatment, and ALP activity and $[Ca^{2+}]_i$ were assessed. Values are reported as mean ± S.D. of four independent experiments, $^{*}P < 0.05$ vs. the control value or $^{\#}P < 0.05$ vs. the SS treatment alone.


A
Control
SS (1 μM)
SS + CCCP (2 μM)
SS + CCCP (5 μM)
ATP level (%)
B
Control
SS (1 μM)
CCCP (2 μM)
CCCP (5 μM)
C
Alkaline Phosphatase Activity (mM/100 μg protein)
D
Runx2
OSX
OCN
Relative mRNA expression (fold)
E
SS (1 μM)
CCCP (2 μM)
CCCP (5 μM)
COLIA
OPN
β-actin
F
Control
SS (1 μM)
SS + AZT
AZT
Alkaline Phosphatase Activity (mM/100 μg protein)
[Ca2+]i (mg/mg protein)

without LIF. EBs were then suspended in an additional medium for three days. Thus, EBs of the same culture age (5 days old) were used in each experiment.

2.3. Osteogenic differentiation of ESCs

ESC differentiation was performed as previously reported [33]. EBs were plated onto gelatin-coated six-well plates or 60-mm dishes (10–15 EBs per well) and maintained in an osteogenic medium consisting of a α minimal essential medium (α-MEM) containing 5% FBS, 50 μg/mL ascorbic acid, 1 μM dexamethasone, and 3 mM β-glycerophosphate for 1 day before the application of oxysterols (SS). To promote osteogenic differentiation, oxysterols at different concentrations (0.1 to 10 μM) were added to the osteogenic medium, which was changed every other day. Oxysterol was dissolved in dimethyl sulfoxide (DMSO) immediately before use, and the final concentration of DMSO did not exceed 0.1% (v/v) in any of the experiments. DMSO was used as a control; its concentration was always 0.1%.

2.4. Alkaline phosphatase activity

Cells were lysed using a 50 mM Tris–HCl buffer (pH 7.0) containing 1% (v/v) Triton X-100 and 1 mM PMSF and the total protein was quantified by the Bradford procedure [34]. ALP activity was analyzed with the total cell lysate by adding 200 μL of *p*-nitrophenylphosphate (pNPP) as a substrate (Sigma, USA) for 30 min at 37 °C. The reaction was paused by adding 3 N NaOH and the absorbance was read with spectrophotometer at 405 nm. The enzyme activity was validated as mM/100 μg of protein.

2.5. Alizarin Red staining

The cells were fixed for 30 min in 4% paraformaldehyde fluid, washed three times with ice-cold phosphate-buffered saline (PBS), stained for 5 min with Alizarin Red (Sigma, USA). To quantify mineralization, bound dye was solubilized in 10 mM sodium phosphate containing 10% cetylpyridinium chloride and quantified spectrophotometrically at 562 nm.

2.6. Intracellular calcium assay

Cells were washed three times with PBS and lysed in 50 mM Tris–HCl buffer (pH 7.0) containing 1% (v/v) Triton X-100 and 1 mM PMSF without EDTA after they were incubated with oxysterol combination for 14 days. The protein content was then quantified according to the method by Bradford [34]. The intracellular calcium content was measured using a calcium assay kit according to the manufacturer's instructions (BioAssay Systems, Hayward, CA, USA.), and the absorbance was read spectrophotometrically at 602 nm. The calcium content level was expressed as mg/mg protein.

2.7. RNA isolation and Real Time RT-PCR

The RNA extraction was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. The real-time RT-PCR was then performed in the Rotor-Gene 2000 real-time thermal cycling system (Corbett Research, NSW, Australia) using a QuantiTect SYBR Green RT-PCR kit (QIAGEN, CA, USA). The primers used were 5′-CCAACTTCCTGTGCTCCGTG-3′ (sense), 5′-TCTTGCCTCGTCCGCTCC-3′ (antisense) for Runx2, 5′-ACCAGGTCCAGGCAACAC-3′ (sense), 5′-GGGCAGTCGCAGGTAGAA-3′ (antisense) for OSX, and 5′-CAGGAGGG CAATAAGGTAGT-3′ (sense), 5′-GAGGACAGGGAGGATCAAG-3′ (antisense) for OCN, 5′-AAAGGGCCAAGCAGAGAGA-3′ (sense), 5′-GTAAAT CACACGGCGCTCTT-3′ (antisense) for PGC-1α, 5′-CTCCAGGCAGGTTC AACCC-3′ (sense), 5′-GGGCCAGAAGTTCCCTTAGG-3′ (antisense) for PGC-1β. The temperature of the PCR products was increased from 65 to 99 °C at a rate of 1 °C/5 s, and the resulting data was analyzed using the software provided by the manufacturer.

2.8. Western blot analysis

The extracted protein (20 μg) was separated by 10% SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were washed with TBST [10 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20], blocked with 5% skim milk for 1 h, and incubated with the appropriate primary antibodies [anti-collagen type I, anti-osteopontin, anti-β-actin (Santa Cruz Biotechnology, CA, USA), and anti-Oxphos complex kit (Invitrogen, CA, USA)] at the dilutions recommended by the supplier. The membrane was then washed, and the primary antibodies were detected with goat anti-rabbit IgG or goat anti-mouse IgG conjugated to horseradish peroxidase. The blots were developed with enhanced chemiluminescence (ECL) (Santa Cruz Biotechnology) and exposed to X-ray film (Eastman-Kodak, Rochester, NY, USA).

2.9. Immunofluorescence staining

The cells were fixed and incubated with mouse anti-collagen type I or anti-osteopontin antibody (1:100, Santa Cruz Biotechnology, Delaware, CA) for 2 h at room temperature. Subsequently, the fluorescein isothiocyanate-conjugated (FITC-conjugated) anti-mouse IgG (1:100) was treated for 2 h at room temperature. Fluorescence images were obtained using a fluorescence microscope (Fluoview 300, Olympus).

2.10. Measurement of intracellular ROS

Cellular levels of ROS were measured using 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate (CM-H_2DCF-DA; Molecular Probes, Eugene, OR, USA) according to the method described by Ali et al. [35]. EBs were incubated with oxysterols (SS) at different concentrations for 2 h and 10 μM CM-H_2DCF-DA was added. After 40 min, DCF fluorescence was determined by spectrofluorophotometer (RF-5301PC, Shimadzu, Japan) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

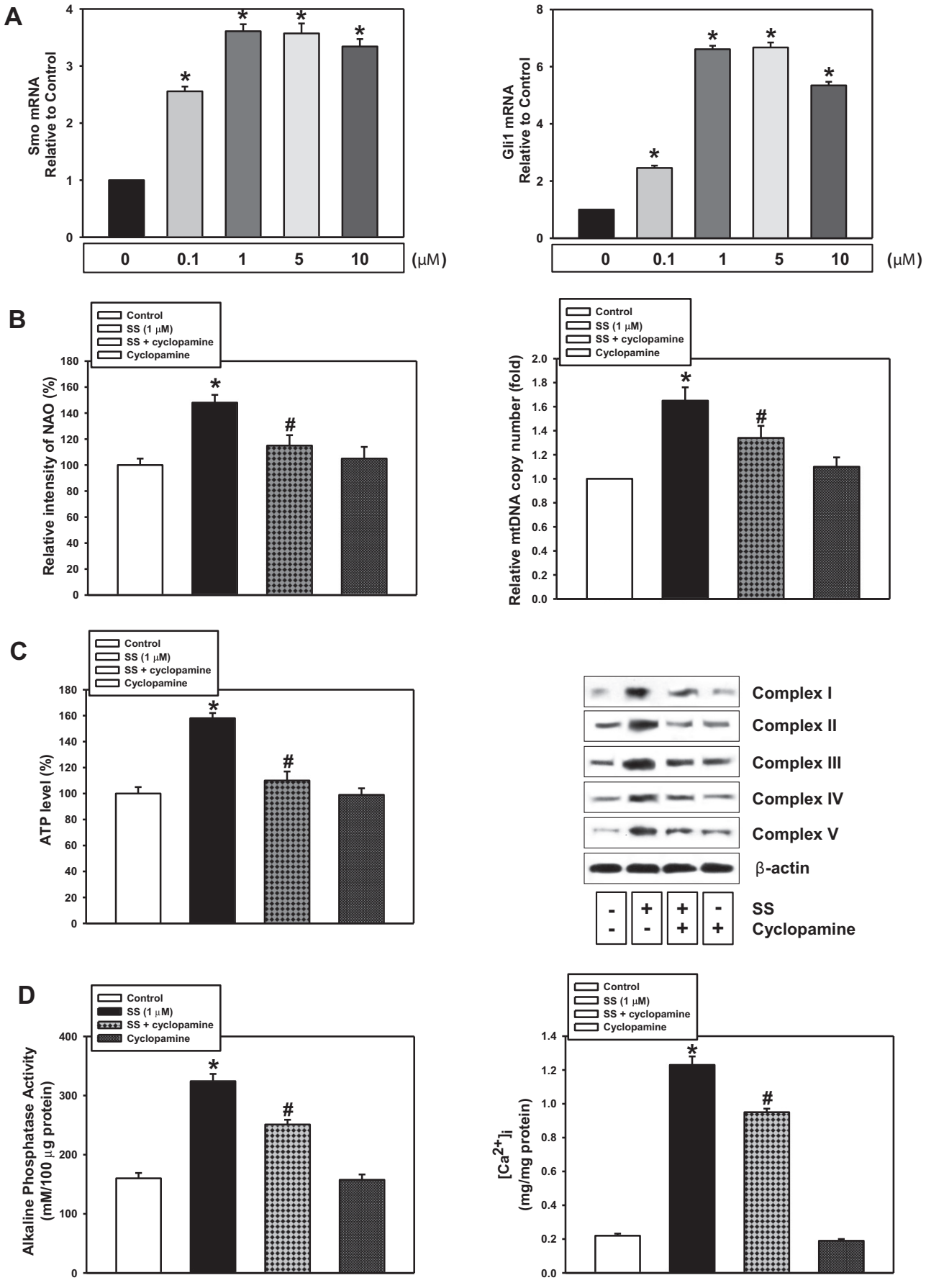
2.11. Measurement of ATP concentration

Cellular ATP concentration was determined in ESC-derived osteoblasts on fourth day of differentiation using the ATP Assay kit (BioVision, Mountain View, CA, USA), following the manufacturer's instructions. The ATP concentrations were measured by colorimetric (OD = 570 nm) method.

2.12. Fluorescent microscopic detection of mitochondrial membrane potential

The membrane-permeant red fluorescent dye tetramethylrhodamine methyl ester (TMRM, Life Technologies) was used to assess the mitochondrial membrane potential in ESC-derived osteoblasts according to a

Fig. 5. Canonical Hh signaling was evaluated during oxysterol-induced ESC osteogenic differentiation. (A) Cells were treated with different oxysterol combinations (0.1, 1, 5, 10 μM) for 7 days and then the mRNA expression of Smo and Gli1 was analyzed. Cells were pretreated with 5 μM of cyclopamine for 2 h prior to addition of 1 μM SS. Next, (B) mitochondrial mass, mtDNA copy number, (C) ATP content, and the protein levels of respiratory complexes, as well as (D) ALP activity and $[Ca^{2+}]_i$ were analyzed as described in the Materials and methods. A representative result from five independent experiments is shown, $^{*}P < 0.05$ vs. the control value or $^{\#}P < 0.05$ vs. the SS treatment alone.


A
Smo mRNA Relative to Control
0 0.1 1 5 10 (μM)
Gli1 mRNA Relative to Control
0 0.1 1 5 10 (μM)
B
Control
SS (1 μM)
SS + cyclopamine
Cyclopamine
Relative intensity of NAO (%)
Relative mtDNA copy number (fold)
C
ATP level (%)
Complex I
Complex II
Complex III
Complex IV
Complex V
β-actin
SS
Cyclopamine
D
Alkaline Phosphatase Activity (mM/100 μg protein)
[Ca2+]i (mg/mg protein)

previously described method [36]. The 4 day-differentiated cells were incubated in media containing a 100 nM final concentration of TMRM for 30 min in a 37 °C, 5% CO_2 incubator. The cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min. After another wash with PBS, cells were placed onto slides using mounting medium containing DAPI. Fluorescence images were obtained using a fluorescence microscope (Fluoview 300, Olympus). For quantitative assessment of membrane potential, the fluorescence intensity was measured by spectrofluorophotometer (RF-5301PC, Shimadzu, Japan) at ex/em 549 nm/573 nm.

2.13. Determination of mitochondrial mass

The fluorescent dye NAO (Invitrogen, CA, USA) was used to measure mitochondrial mass. Cells were trypsinized and resuspended in PBS containing 10 mM NAO. After incubation for 30 min at room temperature, the fluorescence intensity was measured by spectrofluorophotometer (RF-5301PC, Shimadzu, Japan) at ex/em 485 nm/535 nm.

2.14. Determination of mtDNA copy number

Total DNA was extracted from cells using the QIAamp DNA Mini Kit (Qiagen Technologies, USA) according to the instructions of the manufacturer. The mtDNA copy number was determined using a real-time quantitative PCR method. The primers for mtDNA were 5′-CCCA GCTACTACCATCATTCAAGT-3′ (sense), 5′-GATGGTTTGGGAGATTGGTT GATGT-3′ (antisense). The PCR products were detected by SYBR Green reagent. The mtDNA copy number was obtained using the $\Delta\Delta C_t$ method where all samples are first normalized to the level of β-actin then relative normalized values were compared between the control and treatment groups.

2.15. TCF/LEF luciferase reporter assay

Cells were transfected with Top-Flash or Fop-Flash plasmids (as a negative control) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 6 h, cells were incubated with FBS containing medium supplemented with SS or SS plus AZT. Luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's protocol.

2.16. siRNA transfection

The transfection of siRNA was performed according to the previously described method [37]. Small interference RNA (siRNA) targeting Gli1 or β-catenin was produced using a commercial kit that contains three target sequences to Gli1 or β-catenin (IDT, Integrated DNA Technologies Inc., Coralville, IA, USA). The siRNA that inhibited Gli1 or β-catenin expression the most as determined by western blotting analysis was used in further experiments. Briefly, cells were transfected for 24 h with either siRNA specific to Gli1, β-catenin (20 nM) or negative control siRNA (scrambled), using *Trans*IT-TKO transfection reagents (Mirus, Madison, WI, USA) according to the manufacturer's instructions, before being subjected to the various treatments.

2.17. Statistical analysis

All data are expressed as mean ± standard deviation (S.D.). One-way ANOVA was used for multiple comparisons (Duncan's multiple range test), using SPSS software ver. 10.0. A P value < 0.05 was considered significant.

3. Results

3.1. Induction of ESC osteogenic differentiation by oxysterols

To examine the osteogenic effect of oxysterols on ESCs, cells were exposed to 0.1–10 μM of oxysterol cocktail, 20(S)-hydroxycholesterol (20S) + 22(S)-hydroxycholesterol (22S) (SS, 1:1) for 4, 7, and 14 days. ALP activity and mineralization were then determined. Fig. 1A and B show that ALP activity and mineralization were significantly enhanced in ESCs exposed to 0.1, 1, and 5 μM of SS. To further characterize osteoblastic differentiation, we assessed mRNA expression of osteogenic target genes Runx2, OSX, and OCN using real time RT-PCR. Although the genes did not increase in a dose-dependent manner, cultures treated with SS demonstrated higher gene expression compared to the control groups (Fig. 1C). Particularly, a maximal increase in the level of each gene was observed with 1 μM SS. Slightly decreased levels were observed with higher than 5 μM treatment. We also analyzed the oxysterol effect on ESC osteogenic differentiation by determining the protein levels of osteogenic markers, COLIA and OPN at day 7 of osteogenic induction. Western blot analyses showed that the level of each protein dose-dependently increased in cells incubated with SS at a range from 0.1 to 5 μM; these levels decreased in cultures at 10 μM SS (Fig. 1D). Moreover, immunofluorescence staining with COLIA and OPN confirmed that the treatment of SS enhances ESC osteogenic differentiation (Fig. 1E).

3.2. Increased mitochondrial activity during oxysterol-induced osteogenic differentiation

To investigate oxysterol stimulation of mitochondrial activity during ESC differentiation, we first analyzed intracellular ROS production, intracellular ATP content, and mitochondrial membrane potential after 4 days of osteogenic induction. Intracellular ROS levels, ATP content, and mitochondrial membrane potential of cells incubated with SS significantly increased compared to untreated cells (Fig. 2A–C). Particularly, a maximal increase in mitochondrial content was observed with a stimulation of 1 μM SS (1.61-fold increase in ROS level; 1.56-fold increase in ATP content; and 2.7-fold increase in membrane potential; $^{*}P < 0.05$). Images of cells stained with TMRM were recorded by confocal microscopy (Fig. 2D). Fluorescence was much stronger in cells treated with SS than in the control group, whereas cells treated with 5 μM of mitochondrial uncoupler CCCP, reflected a decrease in TMRM fluorescence intensity compared with untreated cells. The applied concentration of CCCP was selected not to affect cellular function but to inhibit mitochondrial activity (Supplemental Data 1).

3.3. Effect of oxysterols on mitochondrial biogenesis and expression of respiratory enzymes during osteogenic induction

To determine mitochondrial mass after SS treatment, we used the fluorescent dye 10-n-nonyl-acridine orange (NAO). The relative NAO intensity of the SS-treated cells was significantly higher than that of the control after 4 days of osteogenic induction ($^{*}P < 0.05$; Fig. 3A). We examined mRNA expression levels of PGC-1α and PGC-1β, which are mitochondrial biogenesis-associated genes. Using real-time RT-PCR analysis, we observed a gradual increase in PGC-1α and PGC-1β expression concomitant with osteogenic differentiation of ESCs (Fig. 3B). Mitochondrial DNA (mtDNA) copy number analyzed by real-time PCR increased in a time-dependent manner ($^{*}P < 0.05$) by treatment with 1 μM SS (Fig. 3C). To further examine the enhancement of mitochondrial respiratory function during oxysterol-induced osteogenic differentiation, we assessed the expression levels of mitochondrial complex proteins by Western blot after 4 days of osteogenic induction. As shown in Fig. 3D, we found a significant increase in the protein level of each complex at 0.1, 1, and 5 μM of SS treatment. However, we also found a slight decrease in complex protein levels at 10 μM SS. These

results clearly indicate that mitochondrial respiratory enzyme complex activity is essential for oxysterol-induced osteogenic differentiation of ESCs.

3.4. Mitochondrial contribution to oxysterol-induced osteogenic differentiation

We further confirmed that the observed increased mitochondrial activity can contribute to osteogenic differentiation of ESCs using the mitochondrial uncoupler CCCP and mitochondrial biogenesis inhibitor Zidovudine (AZT). First, we evaluated the effect of CCCP on intracellular ATP content and mitochondrial membrane potential of the oxysterol-treated cell population. When cells were pretreated with 2 or 5 μM of CCCP, intracellular ATP levels and membrane potential were significantly decreased (Fig. 4A and B). Consequently, we investigated whether inhibition of mitochondrial activity by addition of CCCP would affect osteogenic differentiation of ESCs. As shown in Fig. 4C, pretreatment of cells with CCCP inhibited oxysterol-induced ALP activity. Similarly, mRNA expression of Runx2, OSX, and OCN, as well as the protein levels of COLIA and OPN were examined. We found that oxysterol-induced increases in gene and protein levels were blocked by treatment with CCCP after 7 days of osteogenic induction (Fig. 4D and E). When cells were treated with 10 μM of AZT, intracellular ATP levels, membrane potential, NAO, and mtDNA copy number were significantly decreased (Supplemental Data 2). Ultimately, the association of mitochondrial biogenesis in ESC osteogenesis was further evaluated. As shown in

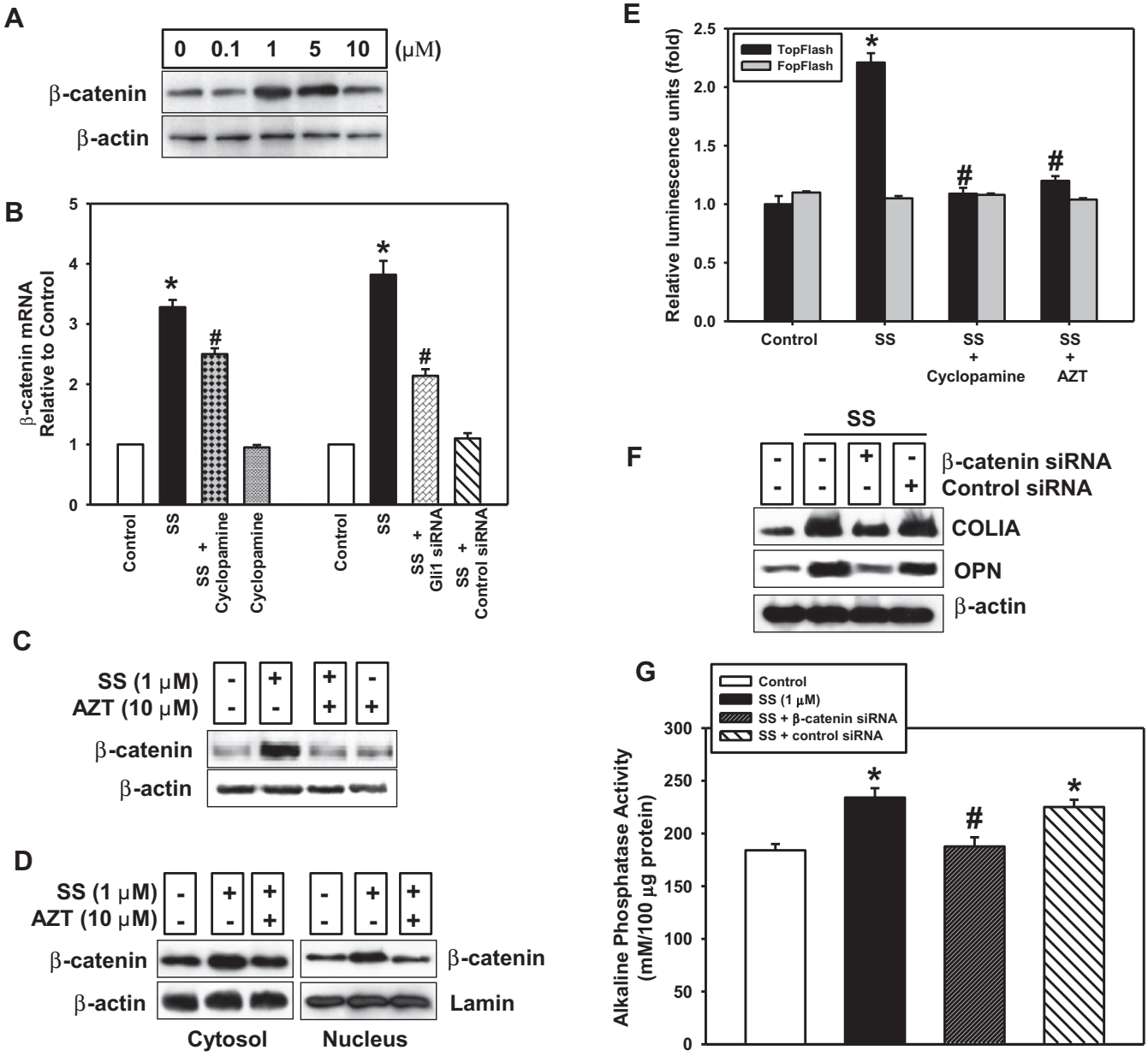


Fig. 6. Wnt/β-catenin signaling was assessed during osteogenic differentiation of ESCs. (A) Protein levels of β-catenin were determined after cells were incubated with SS at different concentrations. (B) The mRNA expression of β-catenin was measured after cells were pretreated with cyclopamine or transfected with Gli1 siRNA prior to SS treatment. Cells were treated with AZT in the presence of 1 μM SS and then (C) total protein levels of β-catenin or (D) the expression of β-catenin in the cytosol and nucleus was analyzed by Western blot analysis. (E) TCF/LEF/β-catenin transcription activity was analyzed after cells were transfected with Top-Flash or Fop-Flash plasmids and incubated with FBS containing medium supplemented with SS, SS plus cyclopamine, or SS plus AZT. (F) The protein levels of COLIA and OPN and (G) ALP activity were determined after cells were transfected with either β-catenin-specific or control siRNA for 24 h and further incubated with SS (1 μM) for 48 h. A representative result from four independent experiments is shown, $^{*}P < 0.05$ vs. the control value or $^{\#}P < 0.05$ vs. the SS treatment alone.

Fig. 4F, the increase of ALP activity and intracellular calcium concentration ($[Ca^{2+}]_i$) by oxysterols were prominently attenuated by inhibition of mitochondrial biogenesis with AZT treatment. These results suggest that mitochondrial activity and biogenesis are essential for oxysterol-mediated ESC osteogenic differentiation.

3.5. Activation of hedgehog signaling during oxysterol-mediated osteogenic differentiation

Given the role of Hh signaling in osteogenic development of pluripotent stem cells [12], we next considered whether Hh signaling is specifically associated with mitochondrial activity and osteogenic differentiation in ESCs. As shown in Fig. 5A, cells treated with SS demonstrated higher Smo and Gli1 gene expression compared to the control groups. When cells were pretreated with Smo inhibitor, cyclopamine, oxysterol-induced increases in mitochondrial mass, mtDNA copy number, ATP levels, and respiratory complex protein expression were significantly attenuated (Fig. 5B, C). Moreover, this Smo inhibition suppressed the increase of ALP activity and $[Ca^{2+}]_i$ by oxysterols (Fig. 5D). These results indicate that the canonical Hh/Gli1 pathway plays an important role in oxysterol-mediated ESC osteogenesis through positively regulating mitochondrial biogenesis and respiratory function.

3.6. Effects of hedgehog pathway and mitochondrial biogenesis on canonical Wnt signaling

Based on the validated roles of Wnt/β-catenin signaling in osteogenesis [30,31], we are encouraged to examine whether the oxysterol-induced Hh signaling and mitochondrial activity can converge at β-catenin control during osteogenic induction. As shown in Fig. 6A, when cells were treated with SS, a prominent increase in the protein level of β-catenin, which acts as an intracellular signal transducer in canonical Wnt signaling, was observed with 1 or 5 μM treatment. In order to define whether Hh signaling regulates canonical Wnt pathway, cells were pretreated with cyclopamine or transfected with Gli1 specific siRNA using *Trans*IT-TKO transfection reagents. The blocking of Hh signaling significantly decreased oxysterol-induced increases in β-catenin mRNA expression (Fig. 6B). Alternatively, inhibition of mitochondrial biogenesis by AZT resulted in decreased oxysterol-induced β-catenin, suggesting that mitochondrial biogenesis regulates Wnt/β-catenin signaling during oxysterol-induced osteogenic induction (Fig. 6C). In order to validate that oxysterols stimulate β-catenin translocation, cells were exposed to 1 μM SS. Subsequently, cytosol and nuclear proteins were isolated. Western blot analysis demonstrated that oxysterols enhanced the expression of β-catenin in the cytosol and nucleus, but that AZT suppressed oxysterol-induced expression of β-catenin (Fig. 6D). Moreover, TCF/LEF/β-catenin transcriptional activity was significantly increased by SS treatment, but the increase in reporter gene activity was abolished by cyclopamine or AZT treatment indicating that Hh signaling and mitochondrial biogenesis regulates the transcriptional activity of Wnt/β-catenin signaling (Fig. 6E). We next wanted to determine the effect of Wnt/β-catenin signaling on the regulation of oxysterol-related osteogenic factors. To confirm the efficacy of β-catenin-specific siRNA, cells were transfected with either β-catenin or negative control. Knockdown of β-catenin by siRNA transfection blocked the increase in COLIA and OPN protein levels by 1 μM SS treatment; the negative control siRNA transfection did not affect protein levels (Fig. 6F). Moreover, the pattern of ALP activity was consistent with Western blot analysis of COLIA and OPN suggesting that canonical Wnt/β-catenin signaling is involved in oxysterol-induced ESC osteogenesis (Fig. 6G).

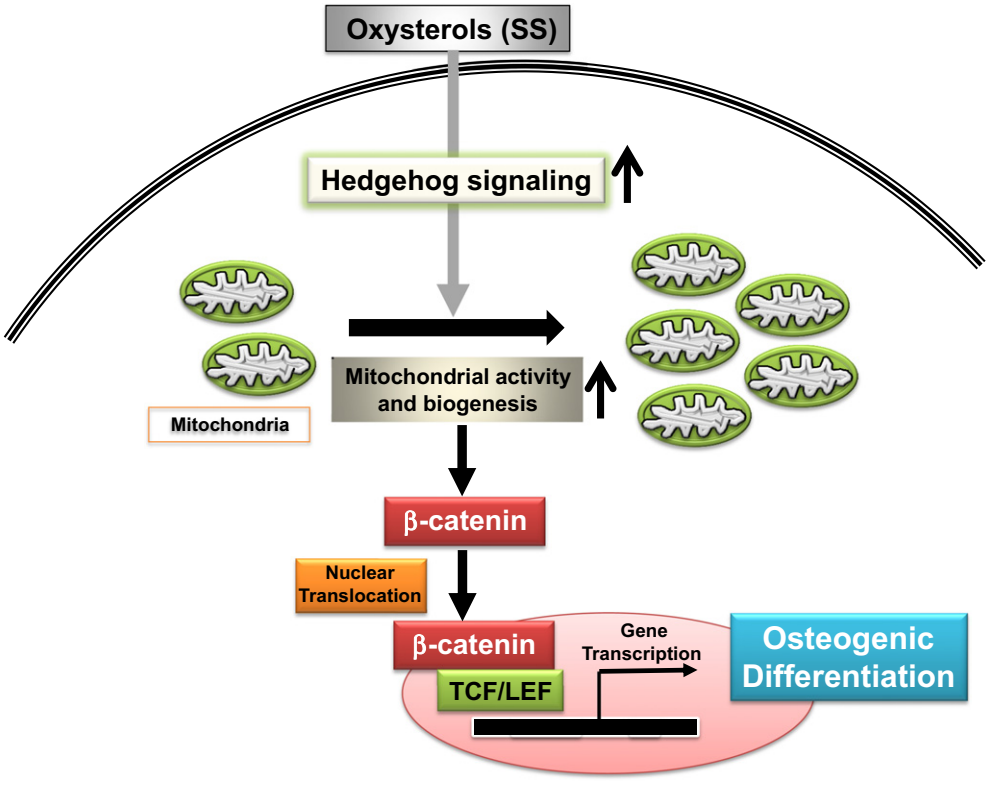


Fig. 7. Hypothesized model of the signaling pathways underlying the oxysterol effect on osteogenic differentiation of ESCs. Oxysterols (SS) stimulate Hh signaling pathways leading to increase of mitochondrial activity and biogenesis, which activate Wnt/β-catenin signaling leading to the translocation of β-catenin into the nucleus and eventually stimulates the osteogenic differentiation of ESCs.

4. Discussion

The present study demonstrated that specific oxysterols intensify ESC osteogenic differentiation. Upon differentiation, oxysterols significantly increased markers of mitochondrial activity, while coupling regulation of Hh/Gli and Wnt/β-catenin signaling. When we tried to compare the osteoinductive effect between oxysterols and BMP2 (bone morphogenetic protein 2), we found the differential action mechanism of these agents to control osteogenic specification of ESCs (Supplemental Data 3). Even though BMP2 increased the ESC osteogenesis, this factor showed a lesser extent of mitochondrial mass and mtDNA copy number referred to as mitochondria biogenesis than oxysterols. Moreover, BMP2 seems not to be involved in canonical Hh/Gli or Wnt/β-catenin signaling in osteogenic differentiation of ESCs. Thus, these findings provide experimental evidence that oxysterol-induced osteogenic differentiation of ESCs is regulated by a novel set of signal transduction pathways. Recent studies have reported that oxysterols represent naturally occurring regulatory molecules with important biological actions and play a prominent role in cell differentiation processes [38,39]. In particular, oxysterols are identified as potential agents that lead in vitro osteogenic differentiation and in vivo bone formation [11,40,41]. In mesenchymal stem cell (MSC) models, specific oxysterol combinations exert osteoinductive effects by activating critical signaling pathways [10,12,42]. Consistent with this, we observed that the individual 20S or 22S also has an osteoinductive effect on ESCs but combination of oxysterols (SS) much more increased the ALP activity and $[Ca^{2+}]_i$ than single treatment after 7 or 14 days of exposure (Supplemental Data 4). This phenomenon can be supported by previous study showing that individual oxysterols fail to induce substantial mineralization and osteogenic marker gene expression even after 14 days of treatment, despite their osteogenic potency. However, oxysterol combination significantly stimulated the osteogenic differentiation of MSCs [43]. Even though further experiments are needed to investigate the specific mechanism how individual oxysterols reciprocally enhance the osteogenic activity, based on previous and our findings, we suggest that oxysterols act as important modulators of stem cell differentiation into osteogenic lineage.

Mitochondria are the main source of ATP and act as a center for connecting extrinsic and intrinsic signals to direct cell growth, proliferation, and the differentiation process [44,45]. Increasing evidence supports the notion that mitochondria participate in regulating stem cell fate [19,46]. In this study, we provide clear evidence to suggest that oxysterols increase mitochondrial content and biogenesis during ESC osteogenic differentiation using various methods for measuring mitochondrial activity. We detected 1) augmented ATP and ROS production; 2) markers of mitochondrial biogenesis, including membrane potential, mitochondrial mass, mRNA expression levels of PGC-1α and PGC-1β, and mtDNA copy number; and 3) increased protein levels of respiratory enzyme complexes during osteogenic differentiation. Consistent with our findings, the connection between mitochondrial activity and lineage differentiation has been observed during spontaneous differentiation of ESCs [19,47]. Another study using confocal microscopy also revealed high mitochondrial membrane potential in ESC-derived cardiomyocytes compared with undifferentiated ESCs, indicating increased mitochondrial function concomitant with ESC differentiation [48]. While many studies demonstrate higher mitochondrial action during stem cell differentiation into various types, others have demonstrated that mitochondrial metabolism in human ESCs (by measuring expression levels of PGC-1α and PGC-1β) is much higher than in ESC-derived neural stem cells [49]. The reason for this discrepancy is not clear. However, we suggest that mitochondria act in important signaling pathways to regulate self-renewal and differentiation of ESCs.

In accordance with previous study showing the regulatory effect of oxysterols on Hh pathway [12], upregulation of canonical Hh/Gli1 signaling occurred during the oxysterol-induced ESC osteogenesis. Hh signaling is well known as a main regulator of osteoblast differentiation during skeletal development [50]. Interestingly, this study found the Hh pathway can mediate the oxysterol-induced mitochondrial biogenesis and respiratory function during the osteogenesis. Earlier study also demonstrated the activated Hh signaling in hepatic stellate cells (HSCs) induces glycolysis and increase of mitochondrial metabolism during the transdifferentiation of HSCs into myofibroblasts [51]. Since Hh pathway is well known to have the mitogenic functions during development [52], we consider higher levels of respiratory complexes (encoded by both nuclear and mitochondrial DNA with the exception of complex II entirely encoded by the nucleus [53]) may be due to higher Hh signaling activation by oxysterols. Thus, it can be encouraged that activation of Hh signaling is a putative critical process in enhancing mitochondrial respiratory function denoted by the increased levels of respiratory enzyme complexes and ATP content during the oxysterol-induced osteogenic differentiation of ESCs.

Moreover, interactions between Hh signaling and other key differentiation mechanism, such as Wnt, have been well documented [54]. In the present study, oxysterols stimulate Wnt signaling pathways, which inhibited by Smo inhibitor or Gli1 siRNA, suggesting that these pathways collaborate to promote the ESC osteogenesis in response to oxysterols. Wnt signaling is classified as canonical and non-canonical, referred to as β-catenin dependent and independent signaling, respectively [55]. The role of Wnt signaling in osteogenic regulation in a variety of experimental models is likely complex. We show that canonical Wnt/β-catenin signaling is activated by oxysterols during ESC osteogenesis. In contrast, a previous study showed that treatment with oxysterols increases osteogenic differentiation of marrow stromal cells mediated through a non-canonical Wnt signaling-related mechanism [42]. Despite these differences, both canonical and non-canonical Wnt pathways may play a critical role in osteoinductive activity of oxysterols. Interestingly, we also observed that inhibition of mitochondrial biogenesis, using AZT, occurs with down-regulated expression of oxysterol-induced β-catenin. A number of studies report the critical role of mitochondrial signaling pathways in ESCs; however, a detailed mechanism and the biological importance to ESC differentiation has not yet been elucidated. One previous study suggested that mitochondrial biogenesis increases β-catenin activity contributing to Wnt-mediated osteoblastic differentiation of mesenchymal cells [45]. Based on these results, we suggest that mitochondrial biogenesis may act as an upstream regulator of Wnt/β-catenin signaling. Although this is a new observation in ESCs, our findings suggest that oxysterol-induced mitochondrial biogenesis interacts with Wnt/β-catenin signaling by regulating the amount of β-catenin and its translocation into the nucleus, leading to cooperative activation of osteogenic differentiation in ESCs. Thus, oxysterol-induced ESC osteogenic differentiation is controlled by mitochondrial activity, which is linked to canonical Wnt signaling.

In conclusion, we demonstrate that oxysterols stimulate osteogenic differentiation of ESCs by increasing mitochondrial activity that is triggered through canonical Hh-regulated signaling network, as well as its downstream target, Wnt/β-catenin signaling (Fig. 7). Finally, these results in addition to enhancing stem cell differentiation, aid in the identification of osteogenic signaling networks that could help improve the efficacy of regenerative therapies.

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