β-catenin signaling induces the osteoblastogenic differentiation of human pre-osteoblastic and bone marrow stromal cells mainly through the upregulation of osterix expression

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Received March 19, 2015; Accepted October 13, 2015

DOI: 10.3892/ijmm.2015.2382

Abstract. Both β-catenin (β-cat) and osterix (OSX) are known to be essential for embryonic and postnatal osteoblast differentiation and bone growth. In the present study, we explored the crosstalk between β-cat signaling and OSX, and assessed its effect on the osteoblastogenic differentiation of human pre-osteoblastic cells (MG-63) and bone marrow stromal cells (HS-27A). In the HS-27A and MG-63 cells, the selective β-cat signaling inhibitor, CCT031374, and the stable overexpression of a constitutively active β-cat mutant respectively decreased and increased the cytoplasmic/soluble β-cat levels, and respectively decreased and increased TOPflash reporter activity, the mRNA levels of β-cat signaling target genes c-Myc and c-Jun, as well as the mRNA and protein expression levels of OSX. Mutational analyses and electrophoretic mobility shift assays revealed that the increased binding activity of c-Jun at a putative c-Jun binding site (-858/-852 relative to the translation start codon, which was designated as +1) in the human OSX gene promoter was required for teh β-cat signaling-induced expression of OSX in the HS-27A and MG-63 cells. During osteoblastogenic culture, stimulating β-cat signaling activity by the stable overexpression of the active β-cat mutant markedly increased alkaline phosphatase (ALP) activity and calcium deposition in the HS-27A and MG-63 cells, which was abolished by knocking down OSX using shRNA. On the other hand, the inhibition of β-cat signaling activity with CCT031374 decreased the ALP activity and calcium deposition, which was completely reversed by the overexpression of OSX. On the whole, the findings of our study suggest that β-cat signaling upregulates the expression of OSX in human pre-osteoblastic and bone marrow stromal cells by trans-activating the OSX gene promoter mainly through increased c-Jun binding at a putative c-Jun binding site; OSX largely mediates β-cat signaling-induced osteoblastogenic differentiation. The present study provides new insight into the molecular mechanisms underlying osteoblast differentiation.

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Key words: β-catenin, osterix/SP7, osteoblastogenic differentiation, osteoblast, bone marrow stromal cells, alkaline phosphatase, mineralization, promoter, c-Jun

Introduction

Osteoblasts are involved in normal skeletal growth and homeostasis (1). Bone can be produced by the direct differentiation of osteoblasts from mesenchymal progenitors (2). Osteoblast differentiation is regulated by various transcription factors and signaling proteins (3). It has been demonstrated that the transcription factors, β-catenin (β-cat) and osterix (OSX, also known as SP7), are essential for osteoblast differentiation and bone formation during embryonic development (1). During development, OSX is specifically expressed in osteoblasts, but not in osteoclast lineage cells (1). As OSX is associated with bone mineral density (BMD) in both children and adults, it is likely that OSX also plays an important role in the development of the postnatal skeleton (4,5). OSX has been found to play an essential multifunctional role in postnatal bone growth and homeostasis (1).

β-cat is a key component of the Wnt signaling pathway (6), which is essential to osteoblast differentiation during embryonic development (3) and has been implicated in the regulation of BMD (7). In the majority of cells, β-cat is predominantly located at the plasma membrane, which is resistant to mild detergents and is referred to as the insoluble pool of β-cat (8). Small amounts of soluble β-cat are normally present in the cytoplasm (8,9). The activation of the 'canonical' Wnt pathway involves the stabilization of cytoplasmic/soluble β-cat, which interacts with the T cell factor (Tcf) family of transcription factors to activate downstream target genes, such as c-Myc and c-Jun (8-10). A previous study demonstrated that β-cat signaling in osteoblasts coordinates postnatal bone acquisition by controlling the differentiation and activity of osteoblasts (7).

Thus, it seems that both OSX and β-cat are essential for embryonic and postnatal osteoblast differentiation and bone growth. In the present study, we explored the crosstalk

between β-cat signaling and OSX, and assessed its effect on osteoblast differentiation in human pre-osteoblastic and bone marrow stromal cells.

Materials and methods

Cell culture. Human MG-63 pre-osteoblastic/osteosarcoma cell (CRL-1427) and human HS-27A bone marrow stromal cells (CRL-2496) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The HS-27A and MG-63 cells were respectively cultured in DMEM and RPMI-1640 medium (Life Technologies, Carlsbad, CA, USA) containing 10% heat-inactivated FBS (Life Technologies) and 100 U/ml penicillin-streptomycin (Sigma-Aldrich, Beijing, China) in an incubator with a humidified atmosphere of 95% air and 5% CO_2 at 37˚C. In order to induce osteoblast differentiation, the HS-27A and MG-63 cells (5,000 cells/well) were cultured in osteoblastogenic medium containing 50 mg/l ascorbic acid, 10 mmol/l β-glycerol phosphate disodium and 100 nmol/l dexamethasone. To block β-cat signaling, the cells were treated with the selective β-cat signaling inhibitor, CCT031374 (50 μM), during the entire osteoblastogenic culture period.

Plasmids and reagents. The human OSX cDNA clone (SC328709) was purchased from OriGene Technologies (Beijing, China), and the full-length OSX cDNA sequence was subcloned into the pcDNA 3.1 plasmid (Life Technologies). The human β-cat cDNA clone (SC107921) was purchased from OriGene, and the β-cat cDNA sequence lacking those encoding 151 amino-terminal residues was subcloned into the pcDNA 3.1 plasmid to generate a constitutively active (ΔN151) β-cat expression vector. The human OSX promoter/luciferase reporter (S713117) and LightSwitch luciferase assay kit (LS010) were both purchased from SwitchGear Genomics (Shanghai, China). Mutant OSX promoter/luciferase reporter constructs were generated by polymerase chain reaction (PCR) and confirmed by sequencing. OSX (sc-43984-V), c-Jun (sc-29223-V) and control (sc-108080) shRNA lentiviral particles, and goat polyclonal anti-β-cat (C-18) (sc-1496) antibody (epitope matched to the carboxyl terminal of human β-cat), rabbit polyclonal anti-OSX (Y-21) (sc-133871) antibody, mouse monoclonal anti-cJun (G-4) (sc-74543) antibody, mouse monoclonal anti-cFos (6-2H-2F) (sc-447) antibody and mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (6C5; sc-32233) antibody were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine 2000 transfection reagent, TRIzol reagent and SuperScript II reverse transcriptase were purchased from Life Technologies. The colorimetric alkaline phosphatase (ALP) assay kit (ab83369) was purchased from Abcam (Cambridge, MA, USA). The calcium (CPC) liquicolor kit (#0150-250) was purchased from Stanbio Laboratory (Boerne, TX, USA). The selective β-cat signaling inhibitor, CCT031374, was purchased from Tocris Bioscience (Bristol, UK). Puromycin and G418 were purchased from Sigma-Aldrich. Putative transcription factor binding sites in the human OSX gene promoter sequence were identified using online PROMO software (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3), as previously described (11,12).

Stable transfection and lentiviral transduction. The constitutively active (ΔN151) β-cat and the OSX expression vectors were respectively transfected into the cells using Lipofectamine 2000 transfection reagent (Life Technologies) according to the manufacturer's instructions. Pools of stable transductants were generated via selection with G418 (800 μg/ml) according to the manufacturer's instructions. The OSX and c-Jun shRNA lentiviral particles produce target-specific shRNA designed to specifically knockdown OSX and c-Jun expression, respectively, whereas control shRNA lentiviral particles contain a scrambled shRNA sequence that will not lead to the specific degradation of any cellular mRNA. Lentiviral transduction was performed, and pools of stable transductants were generated via selection with puromycin (5 μg/ml) according the manufacturer's instructions (Santa Cruz Biotechnology). Cells stably transfected with the constitutively active (ΔN151) β-cat expression vector were stably transfected with lentiviral OSX shRNA to knock down OSX. Cells stably transfected with the OSX expression vector were treated with the selective β-cat signaling inhibitor, CCT031374 (50 μM), during the entire osteoblastogenic culture period to block β-cat signaling.

Western blot analysis. For whole cell lysates, the cells were lysed with a hypotonic buffer containing 2% Nonidet-P-40 and a protease inhibitor cocktail (Sigma) by sonication 3 times for 3 sec on ice. The supernatant obtained following centrifugation at 2,000 x g for 15 min at 4˚C was used to determine the protein concentration by the Coomassie blue method and also for subsequent steps. For the detection of soluble β-cat, the cells were lysed in 0.1% Nonidet P-40 lysis buffer (0.1% Nonidet P-40, 10 mM HEPES, pH 7.5, 142.5 mM KCl, 5 mM $MgCl_2$ and 1 mM EGTA). The lysates were centrifuged at 14,000 x g for 10 min, and the supernatants were saved as soluble cell lysate, as previously described (8,10). Equal amounts of proteins for each sample were separated by 8-15% SDS-polyacrylamide gel and blotted onto polyvinylidene difluoride microporous membranes (Millipore, Billerica, MA, USA). The membranes were incubated for 1 h with a 1:1,000 dilution of primary antibody, and then washed and revealed using secondary antibodies with horseradish peroxidase conjugate (1:5,000, 1 h). Peroxidase was revealed using an ECL kit (GE Healthcare, Shanghai, China). Three independent experiments were performed.

Transient transfection and luciferase reporter assay. The cells were transfected with the human OSX promoter/luciferase reporter or TOPflash or FOPflash plasmids (Upstate Cell Signaling Solutions, Billerica, MA, USA) using Lipofectamine 2000 transfection reagent (Life Technologies). The luciferase assays were performed 30 h after transfection using the LightSwitch Luciferase assay kit (SwitchGear Genomics) following the manufacturer's instructions. The pRL-CMV plasmid (Promega, Madison, WI, USA) encoding *Renilla reniformis* luciferase (at one fifth molar ratio to test plasmids) was co-transfected with the test plasmids in each transfection as an internal control for data normalization. Each experiment was repeated 3 times in duplicate.

Reverse-transcription-quantitative PCR (RT-qPCR). RNA was prepared from the cells using TRIzol reagent, and cDNA was synthesized using SuperScript II reverse transcriptase

(Life Technologies). Quantitative (real-time) PCR (qPCR) was performed on an ABI PRISM 7700 Sequence detection system, with the fluorescent dye SYBR-Green Master Mix (Applied Biosystems, Beijing, China), according to the instructions provided by the manufacturer. The primers used were as follows: for OSX, 5'-TGCTTGAGGAGGAAGTTCAC-3' (forward) and 5'-AGGTCACTGCCCACAGAGTA-3' (reverse); for c-Myc, 5'-GCAAACCTCCTCACAGCCCACT-3' (forward) and 5'-AACTTGACCCTCTTGGCAGCA-3' (reverse); for c-Jun, 5'-CAAAGTTTGGATTGCATCAAGTG-3' (forward) and 5'-TAACATTATAAATGGTCACAGCACATG-3' (reverse); for GAPDH, 5'-GACTCATGACCACAGTCCATGC-3' (forward) and 5'-AGAGGCAGGGATGATGTTCTG-3' (reverse). Relative quantification of the mRNA levels was determined using the $2^{-\Delta\Delta Ct}$ method, which normalizes the expression levels of genes of interest against that of GAPDH in the same samples, as previously described (13). Each experiment was repeated 3 times in duplicate.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from the HS-27A and MG-63 cells, as previously described in the study by Johnson *et al* (14). EMSA was performed with ^{32}P-labeled double-stranded oligonucleotides incubated with nuclear extract in EMSA buffer [10 mM Tris, pH 7.5, 5% glycerol, 1 mM EDTA, pH 7.1, 50 mM NaCl, 1 mM DTT, 1 mM EDTA and 0.1 mg/ml poly(dI-dC)]. For oligonucleotide competition analysis, a 100-fold molar excess of unlabeled competitor oligonucleotides was also added to the mixture and incubated at room temperature for 30 min. For antibody supershift assays, 1 μl monoclonal antibodies to c-Jun or c-Fos (Santa Cruz Biotechnology) was added to the mixture. The reaction was then incubated on ice for 1 h. Protein-DNA complexes and free DNA were fractionated on 5% polyacrylamide gels in 1X Tris-glycine EDTA buffer at 4˚C and were visualized by autoradiography.

Quantitative assessment of osteoblast differentiation and mineralization. In order to induce osteoblast differentiation, the HS-27A and MG-63 cells (5,000 cells/well) were cultured in osteoblastogenic medium. The cells were not passaged during the experiment (maximum 28 days), but the culture medium and supplements were changed twice each week. Osteoblast differentiation was determined quantitatively by measuring ALP activity on day 14 of culture with a colorimetric ALP assay kit (Abcam), as previously described (15,16). Data are presented relative to the total protein concentration. Osteoblast differentiation was also assessed by *in vitro* mineralization. On day 14 (HS-27A cells) and on day 28 (MG-63 cells), calcium was extracted from the monolayers by incubating the cells overnight in 0.6 N HCl and measured quantitatively as μg/well using a calcium (CPC) liquicolor kit (Stanbio Laboratory), as previously described (17). Each experiment was repeated 3 times in duplicate.

Statistical analysis. Statistical analyses were performed using SPSS for Windows 19.0 (IBM, Inc., Chicago, IL, USA). All continuous variable values are expressed as the means ± SD. A comparison of the means between 2 groups was performed using the Student's t-test. Comparisons of the means between multiple groups were performed with one-way ANOVA followed by post hoc pairwise comparisons using Tukey's tests. A two-tailed value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

β-cat signaling upregulates the expression of OSX in human pre-osteoblastic and bone marrow stromal cells. To explore the potential crosstalk between β-cat signaling and OSX in human pre-osteoblastic and bone marrow stromal cells, we stably overexpressed a constitutively active β-cat mutant, which lacks 151 amino-terminal residues (ΔN151), in the HS-27A human bone marrow stromal cells and MG-63 human pre-osteoblastic cells. We also used CCT031374, a selective β-cat signaling inhibitor, which decreases the cytoplasmic/soluble β-cat level (8,18) in order to inhibit β-cat signaling in the cells. In addition, we stably transduced lentiviral shRNA to knock down OSX in the cells overexpressing the ΔN151/active β-cat mutant, and stably overexpressed OSX in the cells treated with CCT031374. As shown in Fig. 1, ΔN151/active β-cat was overexpressed in the HS-27A and MG-63 cells compared with the levels of wild-type soluble β-cat in the controls. Compared with the controls, CCT031374 decreased the soluble β-cat level by approximately 60 and 75% in the HS-27A and MG-63 cells, respectively (Fig. 1). In the cells overexpressing active β-cat, the protein level of OSX increased by approximately 2.9- and 3.4-fold in HS-27A and MG-63 cells, respectively; in cells treated with CCT031374, the protein level of OSX decreased approximately 55 and 64% in the HS-27A and MG-63 cells, respectively (Fig. 1). In addition, the overexpression and knockdown of OSX had no significant effect on the soluble β-cat levels. These results suggest that β-cat signaling upregulates the expression of OSX in human pre-osteoblastic and bone marrow stromal cells.

We then examined whether β-cat signaling induces the expression of OSX through the transcriptional activation of the OSX gene. The transcriptional activity of β-cat signaling in the HS-27A and MG-63 cells was measured with TOPflash, a synthetic luciferase reporter for β-cat/Tcf signaling activity (8,10). As shown in Fig. 2A, compared with the controls, the active β-cat and the inhibitor, CCT031374, markedly increased and decreased the luciferase activity of TOPflash, respectively; little change was observed with FOPflash, a negative control reporter (8,10). In agreement with the results of the TOPflash/FOPflash experiments, RT-qPCR revealed that active β-cat and the inhibitor, CCT031374, markedly increased and decreased the mRNA levels of the established β-cat signaling target genes, c-Myc and c-Jun (8-10), respectively (Fig. 2B). The mRNA levels of OSX followed a similar trend as those of c-Myc and c-Jun, under the effects of the active β-cat and the inhibitor, CCT031374 (apart from the cells in which OSX was overexpressed or knocked down, which served as positive controls in the experiment) (Fig. 2B), suggesting that the OSX gene is a target of β-cat signaling.

β-cat signaling transactivates the human OSX gene promoter. We then investigated whether β-cat signaling transactivates the human OSX gene promoter, as well as the possible mechanisms involved. We employed a commercial human OSX promoter/luciferase reporter (SwitchGear Genomics),

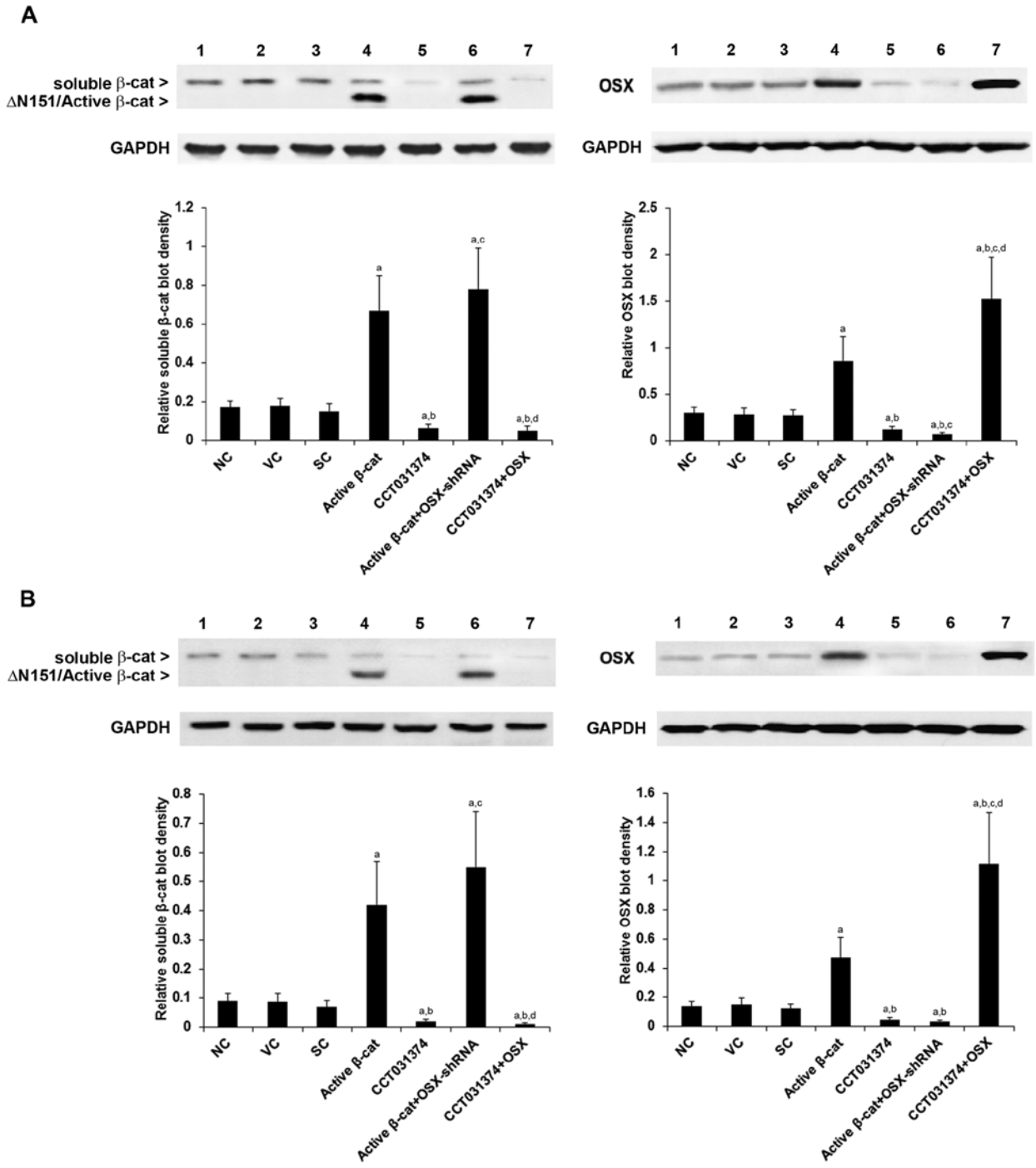

Figure 1. Protein levels of β-catenin (β-cat) and osterix (OSX) in human pre-osteoblastic and bone marrow stromal cells. In (A) HS-27A human bone marrow stromal cells and (B) MG-63 human pre-osteoblastic cells, the protein levels of cytoplasmic/soluble β-cat were measured by western blot analyses in normal control cells (NC, lane 1), cells stably transfected with the empty pcDNA3.1 vector (VC, lane 2), cells stably transduced with scramble control shRNA (SC, lane 3), cells stably transfected with constitutively active (ΔN151) β-cat (active β-cat, lane 4), cells treated with selective β-cat signaling inhibitor CCT031374 (50 μM) for 30 h (lane 5), cells stably transfected with constitutively active (ΔN151) β-cat and stably transduced with OSX-shRNA (active β-cat + OSX-shRNA, lane 6), and cells stably transfected with OSX and treated with CCT031374 (50 μM) for 30 h (lane 7). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) blotting was used as a loading control. Density of the OSX and the cytoplasmic/soluble β-cat blots was normalized against that of the GAPDH blot to obtain a relative blot density. In cells overexpressing ΔN151/active β-cat, the relative density of ΔN151/active β-cat instead of that of wild-type soluble β-cat was calculated and is shown in the bar graph. Three independent experiments were performed for each western blot analysis. Data are expressed as the means + SD. [a]P<0.05 vs. controls (NC, VC and SC); [b]P<0.05 vs. active β-cat; [c]P<0.05 vs. CCT031374; [d]P<0.05 vs. active β-cat + OSX-shRNA.

which had 865 bp of 5'-untranslated region (UTR) immediately upstream of the OSX gene translation start codon inserted in frame with the luciferase cDNA. We screened for putative transcription factor binding sites in the 865-bp human OSX promoter sequence with high stringency (factors predicted within a 5% dissimilarity margin from the consensus binding sequence) using online PROMO software (11,12). As shown in Fig. 3A, with the OSX gene ATG translation start codon designated as +1, a putative c-Jun binding site at -858/-852 and a putative AP-1/c-Jun/c-Fos binding site at -669/-657

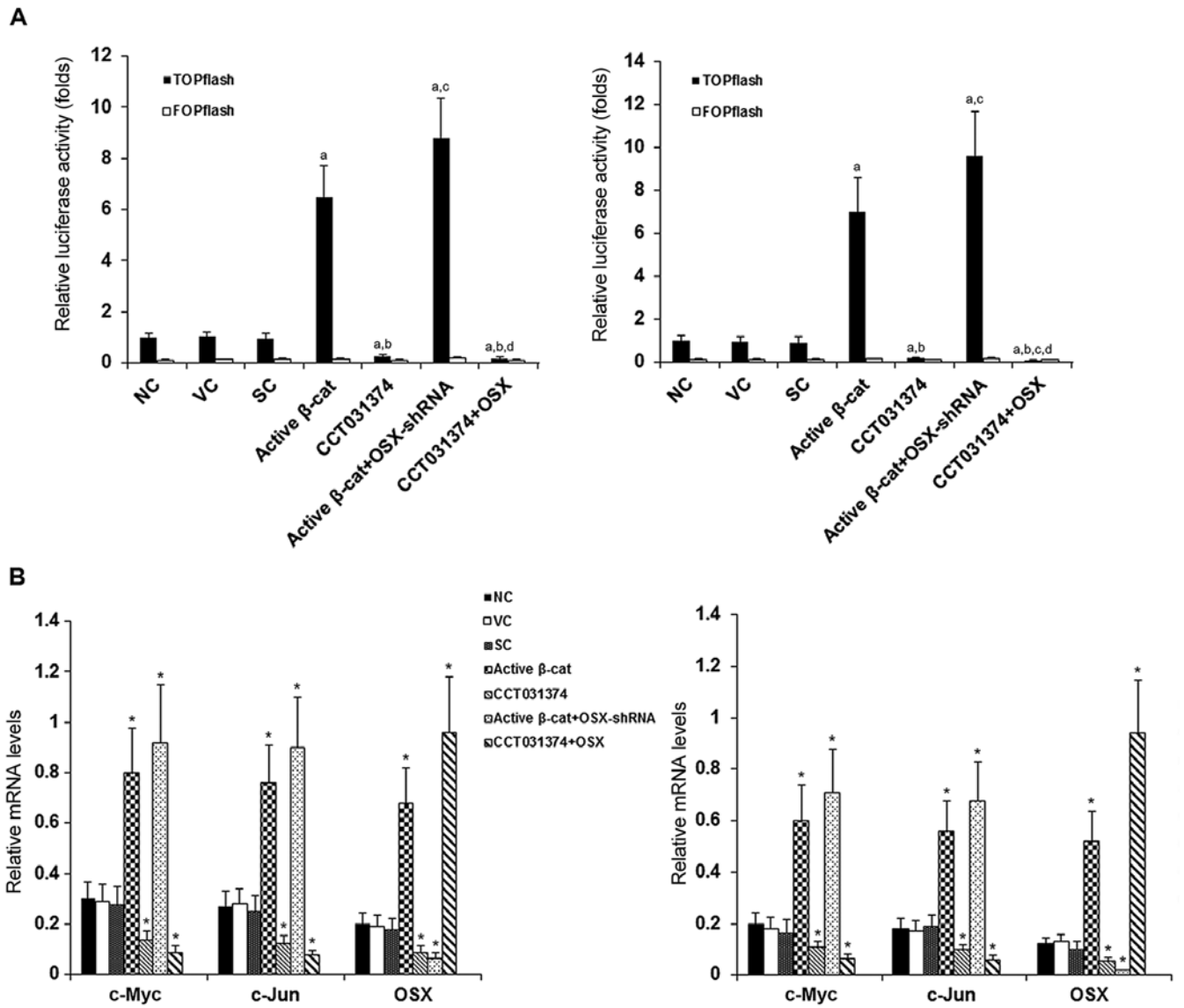

Figure 2. β-catenin (β-cat) signaling luciferase reporter activities and target gene mRNA levels in human pre-osteoblastic and bone marrow stromal cells. (A) HS-27A (left panel) and MG-63 (right panel) cells were transfected with TOPflash, a synthetic β-cat signaling luciferase reporter, or FOPflash, a negative control reporter. Thirty hours later, luciferase activity was determined in normal control cells (NC), cells stably transfected with the empty pcDNA3.1 vector (VC), cells stably transduced with scramble control shRNA (SC), cells stably transfected with constitutively active (ΔN151) β-cat (active β-cat), cells treated with selective β-cat signaling inhibitor CCT031374 (50 μM) for 30 h, cells stably transfected with constitutively active (ΔN151) β-cat and stably transduced with osterix (OSX)-shRNA (active β-cat + OSX-shRNA), and cells stably transfected with OSX and treated with CCT031374 (50 μM) for 30 h. Luciferase activity was measured 30 h after transfection and expressed as a fold change to that of NC (designated as 1). [a]P<0.05 vs. controls (NC, VC and SC); [b]P<0.05 vs. active β-cat; [c]P<0.05 vs. CCT031374; [d]P<0.05 vs. active β-cat + OSX-shRNA. (B) The mRNA levels of OSX and established β-cat signaling target genes c-Myc and c-Jun were measured by RT-qPCR in the HS-27A (left panel) and MG-63 (right panel) cells. The mRNA levels of c-Myc, c-Jun and OSX were normalized against those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *P<0.05 vs. controls (NC, VC and SC).

(AP-1, -669/-661; c-Jun, -667/-661; c-Fos, -666/-657) were identified in the -865 bp human OSX promoter region. We then introduced mutations to disrupt the putative transcription factor binding sites in the OSX promoter/luciferase reporter, respectively (Fig. 3B). As shown in Fig. 3C, compared with the controls, the overexpression of active β-cat increased OSX promoter activity 4-fold, which was completely abolished by mutation at the -858/-852, but not the -669/-657 putative transcription factor binding site in both the HS-27A and MG-63 cells. These results suggested that the -858/-852 putative c-Jun binding site was functional.

We then performed EMSAs to determine whether c-Jun specifically binds to the -858/-852 putative binding site. Oligonucleotide WT865/836 (Fig. 4A), corresponding to the human OSX promoter sequence -865/-836, was radiolabeled and used as the probe to incubate with HS-27A cell nuclear extracts in EMSAs. Unlabeled WT865/836 and Mut865/836, another oligonucleotide with the same sequence as WT865/836 apart from a mutated -858/-852 putative c-Jun binding site (Fig. 4A), were used as competitors to the probe. As shown in Fig. 4B, the nuclear extract from the cells overexpressing active β-cat exhibited significantly stronger binding activity with the probe than that from the control cells. A 100-fold molar excess of unlabeled WT865/836, but not Mut865/836, completely abolished the binding activity (Fig. 4B), suggesting specific protein binding at the -858/-852 putative c-Jun binding

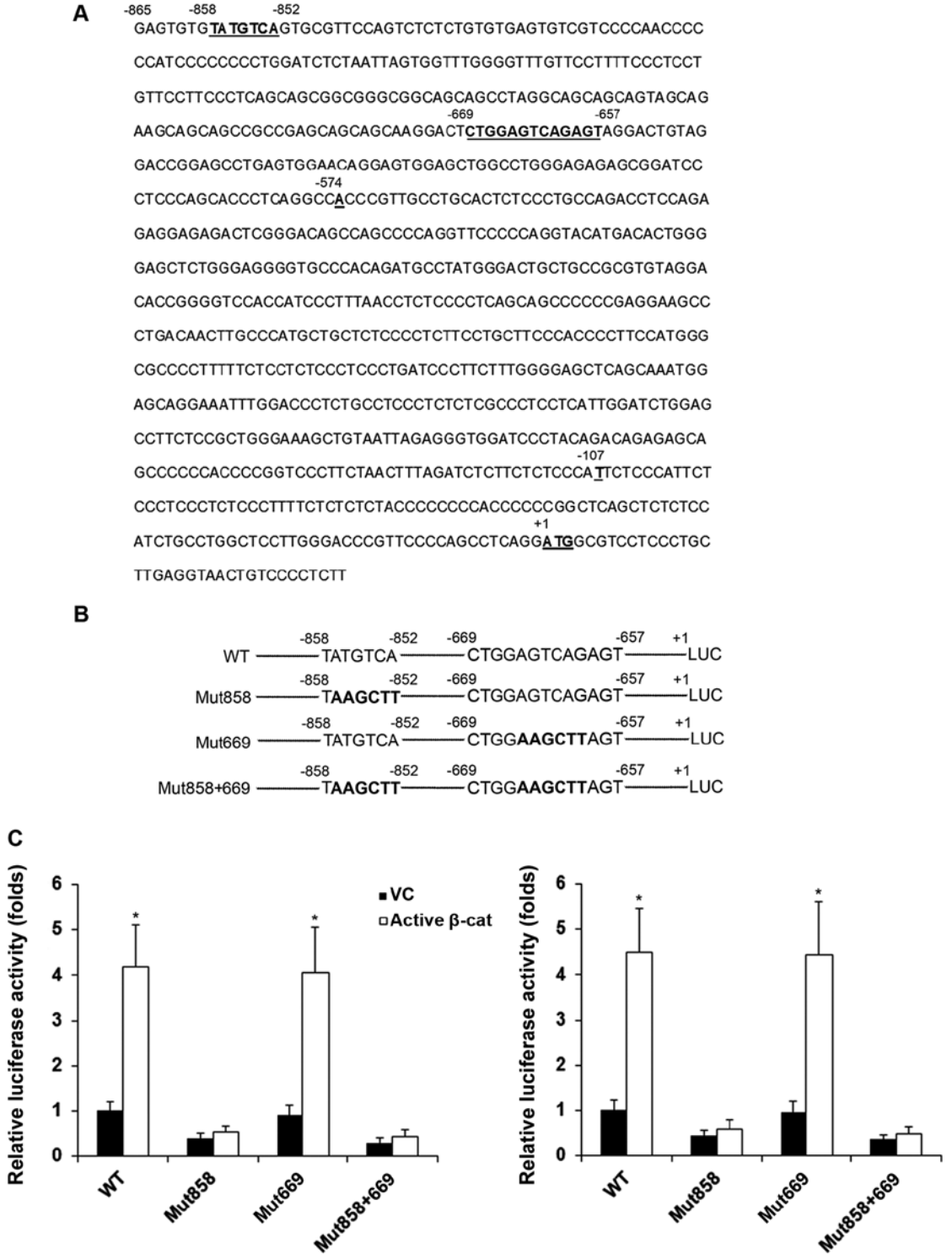

Figure 3. Mutational analysis of putative c-Jun binding sites in the human osterix (OSX) gene promoter. (A) In the OSX gene promoter sequence in a commercial human OSX promoter/luciferase reporter (SwitchGear Genomics), the ATG translation start codon is marked as +1. A putative c-Jun binding site at -858/-852 and a putative AP-1/c-Jun/c-Fos binding site at -669/-657 (AP-1, -669/-661; c-Jun, -667/-661; c-Fos, -666/-657) are underlined and in boldface. The start sites of OSX transcript 1 (Accession no. NM_001173467.1) and transcript 2 (Accession no. NM_152860.1) are underlined in boldface at -574 and -107, respectively. (B) Schematic representation of the wild-type (WT) and mutant (Mut) human OSX promoter/luciferase reporter constructs. Mutated sequence at the -858/-852 (Mut858) and the -669/-657 (Mut669) putative transcription factor binding sites are represented in boldface. (C) WT and Mut human OSX promoter/luciferase reporter constructs were respectively transfected into the HS-27A (left panel) and MG-63 (right panel) cells, which were stably transfected with the empty pcDNA3.1 vector (VC) or constitutively active (ΔN151) β-catenin (active β-cat). Luciferase activity was measured 30 h following transfection and expressed as a fold change to that of VC (designated as 1). *P<0.05 vs. VC.

site. In addition, although the control serum seemingly had no effect, anti-cJun and anti-cFos antibodies supershifted the major protein-DNA complexes to higher positions, respectively (Fig. 4B). These results suggest that c-Jun specifically

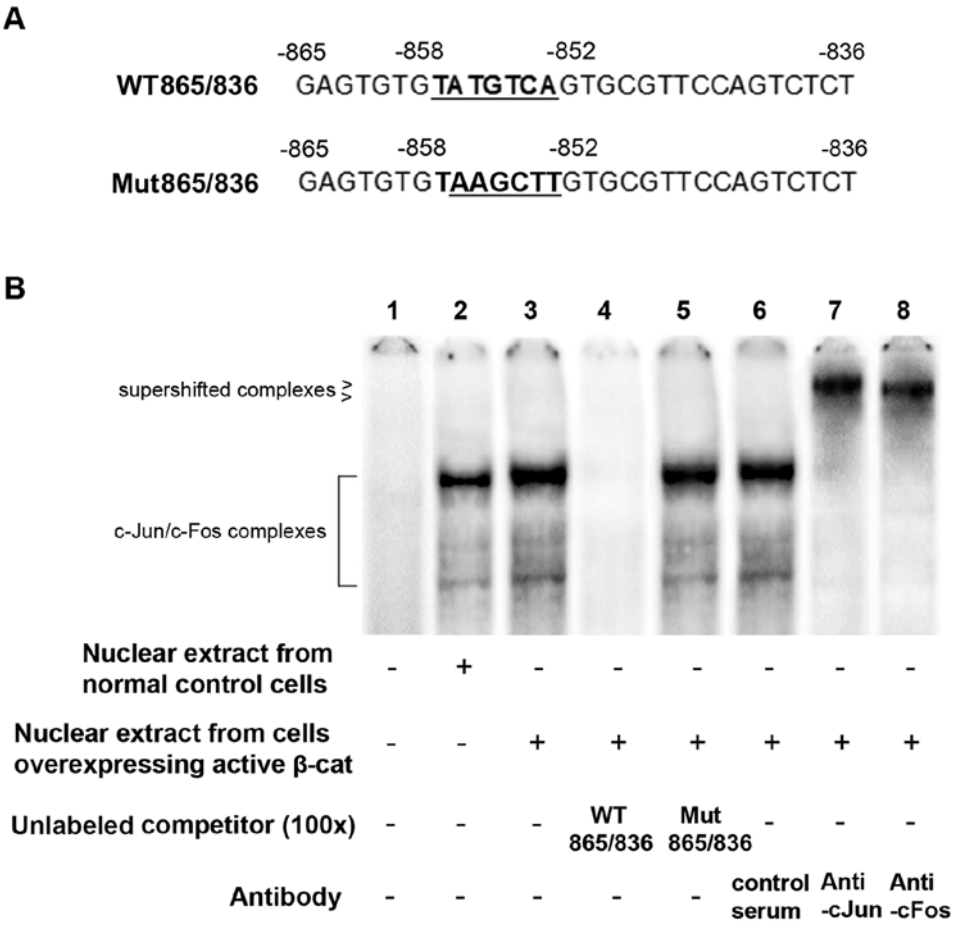

Figure 4. Specific protein-binding activity at the putative c-Jun binding site in human osterix (OSX) gene promoter. (A) Oligonucleotide WT865/836 contained the human OSX gene promoter sequence from -865 to -836 encompassing the -858/-852 putative c-Jun binding site. Oligonucleotide Mut865/836 contained the same sequence as WT865/836 except for mutations at the -858/-852 putative c-Jun binding site (underlined and in boldface). (B) Electrophoretic mobility shift assays (EMSAs) were performed using WT865/836 as the radiolabeled probe in the presence of an equal amount of nuclear extract from HS-27A cells stably transfected with the empty pcDNA3.1 vector (lane 2) or HS-27A cells stably transfected with constitutively active (ΔN151) β-cat (active β-cat) (lanes 3-8). Lane 1, radiolabeled probe only; lanes 2-3, control reaction; lane 4, a 100-fold molar excess of unlabeled oligonucleotide WT865/836 as competitor; lane 5, a 100-fold molar excess of unlabeled oligonucleotide Mut865/836 as competitor; lane 6, control serum; lane 7, anti-cJun antibody; lane 8, anti-cFos antibody. Major protein-DNA complexes and supershifted complexes are indicated.

binds to the -858/-852 putative c-Jun binding site in the form of c-Jun/c-Fos heterodimers.

To determine the functional role of c-Jun in the β-cat signaling-induced expression of OSX, we stably transduced lentiviral cJun shRNA into the HS-27A and MG-63 cells over-expressing active β-cat. As shown in Fig. 5, compared with the controls, active β-cat significantly increased the expression of OSX at both the mRNA and the protein level, which was abolished by knocking down c-Jun with shRNA.

Taken together, the above results suggest that β-cat signaling upregulates the expression of OSX in human pre-osteoblastic and bone marrow stromal cells by transactivating the OSX gene promoter mainly through increased c-Jun binding activity at the -858/-852 putative c-Jun binding site; c-Jun is an essential mediator of the β-cat signaling-induced expression of OSX in human pre-osteoblastic and bone marrow stromal cells.

Effect of β-cat/OSX signaling on osteoblast differentiation and mineralization. To examine the effects of β-cat/OSX signaling on osteoblast differentiation and mineralization, we cultured the HS-27A and MG-63 cells in osteoblastogenic medium. ALP activity, a marker of early osteoblast differentiation and the commitment of bone marrow stromal cells toward the osteoblastic phenotype (19), was measured using a colorimetric ALP assay kit (Abcam) on day 14 of osteoblastogenic culture. As shown in Fig. 6, compared with the controls, stimulating β-cat signaling activity by the overexpression of active β-cat increased ALP activity by approximately 3.0-fold in the HS-27A cells and by 3.6-fold in the MG-63 cells, which was abolished by the knockdown of OSX with shRNA. On the other hand, inhibiting β-cat signaling activity with CCT031374 decreased ALP activity by approximately 63% in the HS-27A cells and by approximately 54% in the MG-63 cells, which was abolished by the overexpression of OSX.

We also assessed the effects of β-cat/OSX signaling on osteoblast differentiation by measuring *in vitro* mineralization. During the osteoblastogenic culture period, calcium deposition was measured using a calcium (CPC) liquicolor kit (Stanbio Laboratory) on day 14 in the HS-27A cells and on day 28 in the MG-63 cells. As shown in Fig. 7, compared with the controls, the overexpression of active β-cat increased calcium deposition by approximately 2.0-fold in the HS-27A cells and by approxi-

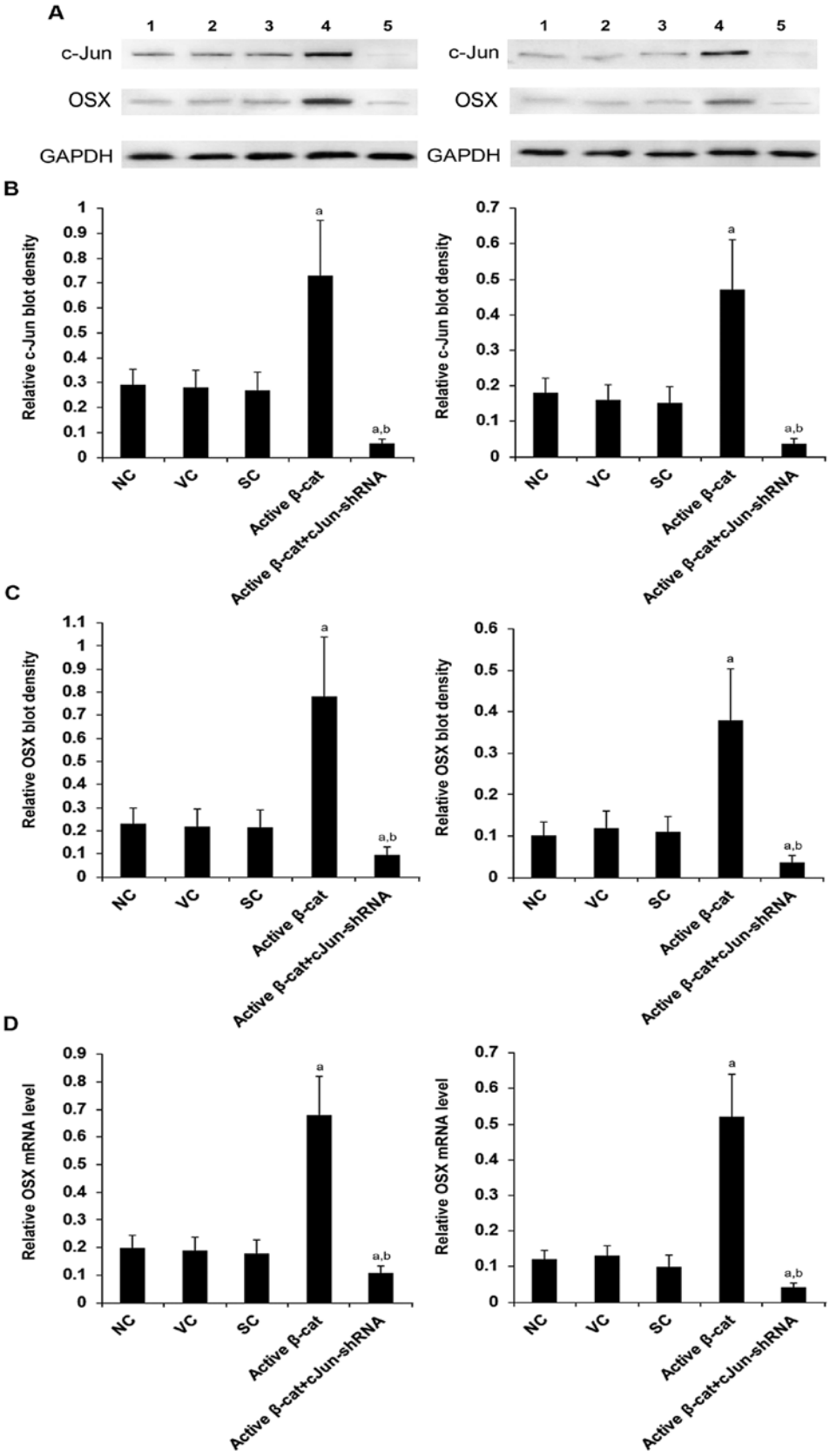

Figure 5. Knockdown of c-Jun abolished the effect of β-catenin (β-cat) on the expression of osterix (OSX) in human pre-osteoblastic and bone marrow stromal cells. (A) The protein levels of c-Jun and OSX in HS-27A (left panel) and MG-63 (right panel) cells were measurd by western blot analyses in normal control cells (NC, lane 1), cells stably transfected with the empty pcDNA3.1 vector (VC, lane 2), cells stably transduced with scramble control shRNA (SC, lane 3), cells stably transfected with constitutively active (ΔN151) β-cat (active β-cat, lane 4), and cells stably transfected with constitutively active (ΔN151) β-cat and stably transduced with lentiviral shRNA against c-Jun (active β-cat + cJun-shRNA, lane 5). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) blotting was used as a loading control. Density of (B) c-Jun and (C) OSX blots was normalized against that of the GAPDH blot to obtain a relative blot density, respectively. Three independent experiments were performed for each western blot analysis. Data are expressed as the means + SD. (D) The mRNA levels of OSX were measured by RT-qPCR assays in the HS-27A (left panel) and MG-63 (right panel) cells and normalized against those of GAPDH. [a]P<0.05 vs. controls (NC, VC and SC); [b]P<0.05 vs. active β-cat.

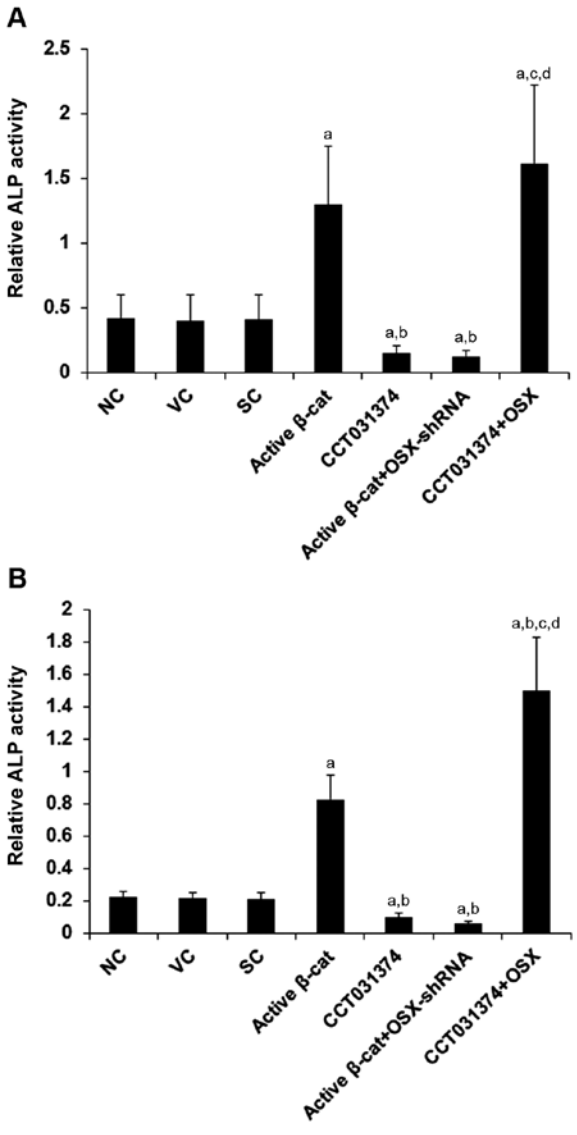

Figure 6. Effect of overexpression and inhibition of β-catenin (β-cat) and/or osterix (OSX) on alkaline phosphatase (ALP) activity in human pre-osteoblastic and bone marrow stromal cells. For osteoblast differentiation, (A) HS-27A and (B) MG-63 cells (5,000 cells/well) were cultured in osteoblastogenic medium. ALP activity was measured using a colorimetric ALP assay kit (Abcam) on day 14 of osteoblastogenic culture in normal control cells (NC), cells stably transfected with the empty pcDNA3.1 vector (VC), cells stably transduced with scramble control shRNA (SC), cells stably transfected with constitutively active (ΔN151) β-cat (active β-cat), cells treated with selective β-cat signaling inhibitor CCT031374 (50 μM) during the entire osteoblastogenic culture period, cells stably transfected with constitutively active (ΔN151) β-cat and stably transduced with OSX shRNA (active β-cat + OSX-shRNA), and cells stably transfected with OSX and treated with CCT031374 (50 μM) during the entire osteoblastogenic culture period. ALP activity was normalized against the total protein concentration in each sample. [a]P<0.05 vs. controls (NC, VC and SC); [b]P<0.05 vs. active β-cat; [c]P<0.05 vs. CCT031374; [d]P<0.05 vs. active β-cat + OSX-shRNA.

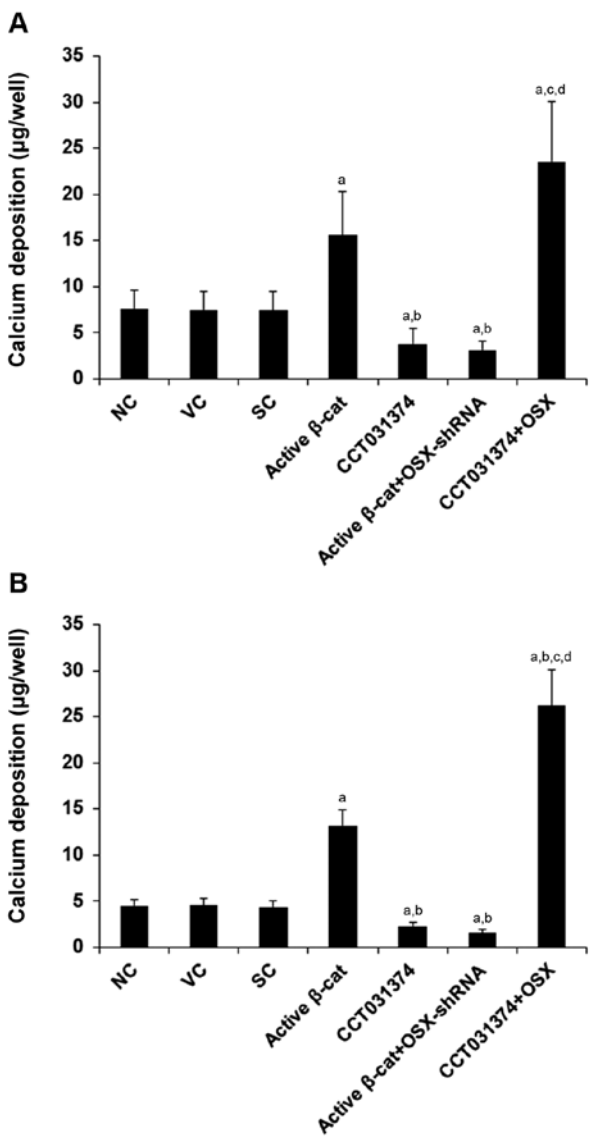

Figure 7. Effect of overexpression and inhibition of β-catenin (β-cat) and/or osterix (OSX) on calcium deposition in human pre-osteoblastic and bone marrow stromal cells. For osteoblast differentiation, (A) HS-27A and (B) MG-63 cells (5,000 cells/well) were cultured in osteoblastogenic medium. On day 14 in HS-27A cells and on day 28 in MG-63 cells, calcium deposition was measured using a calcium (CPC) liquicolor kit (Stanbio Laboratory) in normal control cells (NC), cells stably transfected with the empty pcDNA3.1 vector (VC), cells stably transduced with scramble control shRNA (SC), cells stably transfected with constitutively active (ΔN151) β-cat (active β-cat), cells treated with selective β-cat signaling inhibitor CCT031374 (50 μM) during the entire osteoblastogenic culture period, cells stably transfected with constitutively active (ΔN151) β-cat and stably transduced with Osterix (OSX)-shRNA (active β-cat + OSX-shRNA), and cells stably transfected with OSX and treated with CCT031374 (50 μM) during the entire osteoblastogenic culture period. [a]P<0.05 vs. controls (NC, VC and SC); [b]P<0.05 vs. active β-cat; [c]P<0.05 vs. CCT031374; [d]P<0.05 vs. active β-cat + OSX-shRNA.

mately 3.0-fold in the MG-63 cells, which was abolished by the knockdown of OSX. On the other hand, CCT031374 decreased calcium deposition by approximately 46% in the HS-27A cells and 48% in the MG-63 cells, which was abolished by the overexpression of OSX.

Taken together, these results suggest that OSX may be a major downstream mediator of the osteoblastogenic effect of β-cat signaling on human pre-osteoblastic and bone marrow stromal cells.

Discussion

Accumulating evidence has indicated that both OSX and β-cat are essential for embryonic and postnatal osteoblast

differentiation and bone growth (1,3-5,7). In the present study, for the first time and to the best of our knowledge, we provide evidence that β-cat signaling induces osteoblastogenic differentiation largely by upregulating the expression of OSX in human pre-osteoblastic and bone marrow stromal cells. The human MG-63 pre-osteoblastic/osteosarcoma cell line (20) and human HS-27A bone marrow stromal cell line have previously been used as cell models in osteoblastogenic differentiation studies (21,22). In addition, as shown in the present study, the two cell lines exhibited modest differences in constitutive OSX and cytoplasmic/soluble β-cat levels, with HS-27A cells demonstrating readily detectable, and MG-63 showing relatively low, OSX and soluble β-cat levels. We employed both cell lines to demonstrate generalizable findings.

The stabilization/accumulation of cytoplasmic/soluble β-cat is essential for the canonical Wnt/β-cat signaling pathway, which then leads to transcriptional activation of β-cat/Tcf-regulated genes (8-10). In the present study, we increased and decreased the cytoplasmic/soluble β-cat levels with an active β-cat mutant and a selective β-cat signaling inhibitor, respectively. The effects were reflected in the luciferase activities of TOP/FOPflash β-cat signaling reporters and the mRNA levels of the established β-cat signaling target genes, c-Myc and c-Jun (8-10). Similarly, the activation and inhibition of β-cat signaling increased and decreased the expression of OSX at both the mRNA and protein level, respectively, suggesting that OSX was a target gene of β-cat signaling. This was confirmed by subsequent findings that β-cat signaling transactivated the OSX gene promoter mainly through increased c-Jun binding activity at a putative c-Jun binding site. As c-Jun itself is a transcriptional target of β-cat/Tcf signaling, the effect of β-cat on Osx expression is likely exerted through an indirect effect of β-cat/Tcf signaling. We noted that both anti-cJun and anti-cFos antibodies supershifted the major protein-DNA complexes in EMSAs, suggesting that c-Jun specifically bound to the putative c-Jun binding site in the form of c-Jun/c-Fos heterodimers. This is in agreement with previous research that demonstrated c-Fos can only form heterodimers with c-Jun and that Jun-Fos heterodimers are more stable and have stronger DNA-binding activity than Jun-Jun homodimers (23). This was also the reason that we knocked down rather than overexpressed c-Jun to determine the functional role of c-Jun in the β-cat signaling-induced expression of OSX, as the overexpression of c-Jun increases the formation and binding of Jun-Jun homodimers to the putative c-Jun binding site and thus tends to generate artifacts.

Using a Cre-based conditional β-cat knockout mouse model, in a previous study, Rodda *et al* (2) suggested that β-cat regulates Osx downstream signaling. In agreement with their study, in our study, we demonstrated that endogenous β-cat signaling contributed to 50-55% at the mRNA level and 55-64% at the protein level of the expression of OSX in the HS-27A and MG-63 cells. Our results revealed that a 55-64% decrease in Osx expression led to significant alteration in osteoblast differentiation *in vitro*. However, whether such a decrease in Osx expression would affect osteoblast differentiation *in vivo* requires further investigation.

Osteoblast differentiation can be divided into 3 stages, namely cell proliferation, matrix maturation and matrix mineralization (24). The matrix maturation phase is characterized by maximal expression/activity of ALP. Once mineralization is complete, calcium deposition can be quantified. In this study, we induced the osteoblastogenic differentiation of HS-27A and MG-63 cells using osteoblastogenic medium, and evaluated the functional role of β-cat/OSX signaling on osteoblastogenic differentiation by measuring ALP activity and calcium deposition. Clearly, while the inducing effects of β-cat signaling on ALP activity and calcium deposition were completely abolished by the knockdown of OSX in the HS-27A and MG-63 cells, the inhibitory effect of the selective β-cat signaling inhibitor, CCT031374, was completely reversed by the overexpression of OSX, suggesting that OSX is an essential downstream mediator of the osteoblastogenic effect of β-cat signaling in human pre-osteoblastic and bone marrow stromal cells. In addition, our results revealed that the HS-27A cells had higher levels of Osx and soluble β-cat, as well as higher ALP activity and calcium deposition than the MG-63 cells, corroborating that β-cat/Osx signaling correlates with osteoblast differentiation. Nakashima *et al* (25) demonstrated that OSX functions downstream of runt-related transcription factor 2 (Runx2), another transcription factor which is required for osteoblast differentiation and bone formation during embryonic development (1). It will be interesting to explore whether and how β-cat signaling interacts with Runx2 upstream of OSX in the osteogenic program.

Zhang *et al* (3) reported that OSX inhibited β-cat/Tcf signaling activity in *Xenopus* embryos and human embryonic kidney 293 (HEK293) cells. Indeed, in this study, we noted that the knockdown of OSX enhanced the effect of active β-cat (statistically insignificant in both HS-27A and the MG-63 cells) and that the overexpression of OSX enhanced the effect of CCT031374 (statistically significant in the MG-63 cells) (Fig. 2), suggesting that OSX inhibits β-cat/Tcf signaling in human pre-osteoblastic and bone marrow stromal cells. Based on these findings, it is likely that the inhibitory effect of OSX on β-cat signaling is a feedback mechanism for β-cat signaling-induced expression of OSX and that the dynamic equilibrium between the two reverse processes may help determine the direction of the osteogenic program. Verification of the feedback mechanism and further exploration of its underlying molecular mechanisms needs to be elaborated on in future studies.

In conclusion, the findings of the present study suggest that β-cat signaling upregulates the expression of OSX in human pre-osteoblastic and bone marrow stromal cells by transactivating the OSX gene promoter mainly through increased c-Jun binding at a putative c-Jun binding site. In addition, our data indicate that OSX largely mediates β-cat signaling-induced osteoblastogenic differentiation. This study provides new insight into the molecular mechanisms underlying osteoblast differentiation.

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