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Phosphorylation of Serine422 increases the stability and transactivation activities of human Osterix

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ABSTRACT

Osterix (Osx) is an essential regulator for osteoblast differentiation and bone formation. Although phosphorylation has been reported to be involved in the regulation of Osx activity, the precise underlying mechanisms remain to be elucidated. Here we identified S422 as a novel phosphorylation site of Osx and demonstrated that GSK-3β interacted and co-localized with Osx. GSK-3β increased the stability and transactivation activity of Osx through phosphorylation of the newly identified site. These findings expanded our understanding of the mechanisms of posttranslational regulation of Osx and the role of GSK-3β in the control of Osx transactivation activity.

1. Introduction

Bone is a dynamic tissue that is constantly being reshaped by bone-forming osteoblasts and bone-resorbing osteoclasts. The differentiation of osteoblasts from mesenchymal progenitors requires the activity of specific transcription factors (such as Runx2 and Osterix) and developmental signals (including BMPs, Wnt ligands, hormones, growth factors and cytokines) for induction of osteogenesis [1]. Among which, the specific transcription factors are expressed and function at distinct time points during the differentiation process, thereby defining various developmental stages of the osteoblast lineage [2].

Osterix (Osx/sp7), a transcription factor crucial for osteoblast and osteocyte differentiation and functions, was discovered as a BMP-induced gene in C2C12 cells [3]. Osx plays an essential role in regulating the differentiation of preosteoblasts into mature osteoblasts in a step downstream of Runx2. Deletion of Osx resulted in complete absence of osteoblasts in mouse embryos [3,4]. Post-translational modifications (PTMs) play a great role of decorating proteins and driving their fate in cells by affecting multiple parameters including stability and localization [5]. Dysregulation of PTMs or mutation of modified residues are linked to disease, including cancer, Alzheimer and cardiovascular disease, highlighting the importance of these protein modifications [6–8]. Phosphorylation is the most common PTMs. Adding or removing a dianionic phosphate group somewhere on a protein often changes the protein's structural properties, its stability and dynamics [9]. Studies have shown that phosphorylation of Osx mediated by GSK-3α and Akt enhances the osteogenic activity of Osx [10,11], while p38-mediated phosphorylation of Osx at Ser-73/77 enhances the recruitment of coactivators to form transcriptionally active complexes [12]. Despite the accumulated knowledge of the phosphorylational modification of Osx,

Abbreviations: Osx, Osterix; PTMs, post-translational modifications; GSK-3, glycogen synthase kinase-3; DMEM, Dulbercco's modified Eagle's medium; α-MEM, α-minimal essential medium; FBS, fetal bovine serum; LiCl, lithium chloride; CHX, cycloheximide; RIPA, radioimmunoprecipitation assay; LC–MS/MS, liquid chromatography with tandem mass spectrometry; RT, room temperature; BSA, bovine serum albumin; ALP, alkaline phosphatase; OC, osteocalcin; BSP, bone sialoprotein

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phosphorylation sites and detailed mechanisms that account for the differential regulation of Osx remain to be explored.

Glycogen synthase kinase-3 (GSK-3) was originally identified as a Serine/Threonine kinase involved in the regulation of glycogen deposition and later functions as the key regulator of Wnt and PI3 kinase/Akt signaling [13,14]. In mammals, GSK-3 consists of two isoforms, GSK-3α and GSK-3β. GSK-3α and GSK-3β are not functionally identical, although structurally similar [15,16]. GSK-3β phosphorylates a number of substrates which are involved in embryonic development, protein synthesis, mitosis, and survival [13,17–19]. Whether GSK-3β is involved in the regulation of Osx and influences its function is not known to date.

In the present study, we identified S422, which was conserved, as a novel phosphorylation site of Osx by combining bioinformatics analysis, mass spectrometric analysis and site directed mutagenesis. Moreover, we demonstrated that GSK-3β interacted and co-localized with Osx. Our data also showed that GSK-3β increased the stability and transactivation activity of Osx through mediating the modification of the newly identified phosphorylation site. These results expanded our understanding of the mechanisms of posttranslational regulation of Osx and the role of GSK-3β in the control of Osx transactivation activity.

2. Materials and methods

2.1. Cell, regents and antibodies

HEK 293T and MC3T3-E1 cells were maintained in high glucose Dulbercco's modified Eagle's medium (DMEM) and α-minimal essential medium (α-MEM), respectively. The media were supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Lipofectamine 2000 and Alexa Fluor 594 antibodies were purchased from Invitrogen (Carlsbad, CA, USA). Lithium chloride (LiCl) was purchased from Xilong Chemical (Guangdong, China). Protein-G agarose beads and PVDF membrane were obtained from Millipore (Bedford, MA, USA). Cycloheximide (CHX) and MG-132 were purchased from Sigma (St. Louis, MO, USA). Protease inhibitors and FastStart Universal SYBR Green Master (Rox) were purchased from Roche (Indianapolis, IN, USA). RNAiso plus and PrimeScript RT reagent kit were purchased from TaKaRa Biotechnology (Dalian, China). ECL reagent was obtained from Pierce (Rockford, IL, USA). HA-tagged GSK-3β expression plasmid was obtained from Addgene (Cambridge, MA, USA). Rabbit anti-Osx antibody was obtained from Abcam (Cambridge, MA, USA). Mouse anti-Flag antibody and anti-HA antibody were obtained from Sigma. Mouse anti-β-actin antibody, anti-rabbit and anti-mouse IgG antibody were obtained from Santa Cruz (Santa Cruz, CA, USA).

2.2. Plasmid construction and mutagenesis

Osx cDNA was inserted into pRKI vector at the *EcoR* I and *Hind* III sites to generate wide type pRKI-Flag-Osx plasmid (WT-Osx). WT-Osx was used as a template to generate the phosphorylation site mutant constructs using PCR-driven overlap extension. cDNA of WT-Osx and S422R-Osx was amplified and subsequently subcloned into pRKI-GFP vector at the *EcoR* I and *Bam* HI sites to generate GFP-Osx or GFP-S422R-Osx fusion constructs. HA-tagged GSK-3α expression plasmid was constructed by inserting the GSK-3α cDNA into *EcoR* I and *Bam* HI sites of the pRK2H vector. The inserted fragments were confirmed by sequencing. GSK-3β expression plasmid was obtained from Addgene (Cambridge, MA, USA). hOc-Luc reporter plasmid and β-gal expression plasmid were described previously [20]. The primer sequences used in the plasmid construction were shown in the Supplementary material.

2.3. LC–MS/MS assays of phosphorylation sites

HEK 293T cells transfected with Flag-tagged Osx expression plasmid were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease and phosphatase inhibitors. Osx was immunopurified from the cell lysates with anti-Flag antibody before being resolved by 8% PAGE. After silver staining, the protein band corresponding to the molecular weight of Osx was excised and subjected to liquid chromatography with tandem mass spectrometry (LC–MS/MS) analysis.

2.4. Western blot

Western blot was performed as previously descripted [20]. Briefly, cells were lysed with RIPA buffer plus protease inhibitors, separated via SDS–PAGE, transferred to PVDF membrane, immunoblotted, and detected with ECL reagent.

2.5. Co-immunoprecipitation (Co-IP) assay

HEK 293T cells co-transfected with HA-tagged GSK-3β and Flag-tagged WT-Osx or S422R-Osx expression plasmids were lysed with RIPA buffer plus protease and phosphatase inhibitors. 400 μl of the cell lysates were incubated overnight at 4 °C with anti-HA antibody. To precipitate the immunocomplexes, 50 μl of protein-G agarose beads were added and incubated at 4 °C for 6 h. The agarose beads were pelleted by centrifugation and then washed with RIPA buffer. The agarose slurry was collected by centrifugation and precipitated proteins were detected by Western blot as above.

2.6. Immunofluorescence staining

HEK 293T cells were plated onto the six-well plate with glass bottom inserts. The cells were co-transfected with HA-tagged GSK-3β and GFP-Osx or GFP-S422R-Osx fusion constructs. 30 h after transfection, the cells were fixed in 4% paraformaldehyde for 20 min at room temperature (RT). After washing twice with PBS, cells were permeabilized with PBS containing 0.5% Triton X-100 for 10 min at 4 °C, then treated with blocking buffer containing 4% bovine serum albumin (BSA) for 45 min at RT. The cells were incubated with anti-HA (1:1000) antibody overnight at 4 °C, followed by incubation with Alexa Fluor 594 antibodies (1:200) for 1 h at RT. Cells were then mounted in Vectashield containing DAPI. Confocal images were taken on a Zeiss LSM710-NLO meta.

2.7. Luciferase reporter assay

HEK 293T cells were co-transfected with the hOc-Luc reporter, β-gal construct, HA-tagged GSK-3β, Flag-tagged WT-Osx or S422R-Osx expression plasmids. 36 h after transfection, the cells were treated with or without LiCl. 24 h after treatment, luciferase activity was measured with the Luciferase Reporter System (Promega) using a GloMax™ Base Instrument. Relative luciferase activity was calculated by normalizing to the corresponding β-gal activity for transfection efficiency.

2.8. RNA isolation and Real-time PCR

RNAiso plus was used for total RNA isolation from cultured cells according to the manufacturer's protocol. The ribosomal bands were visualized on a 1% TAE agarose gel to assess RNA integrity.