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Glycogen synthase kinase 3 alpha phosphorylates and regulates the osteogenic activity of Osterix

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ABSTRACT

Osteoblast-specific transcription factor Osterix is a zinc-finger transcription factor that required for osteoblast differentiation and new bone formation. The function of Osterix can be modulated by post-translational modification. Glycogen synthase kinase 3 alpha (GSK3α) is a multifunctional serine/threonine protein kinase that plays a role in the Wnt signaling pathways and is implicated in the control of several regulatory proteins and transcription factors. In the present study, we investigated how GSK3α regulates Osterix during osteoblast differentiation. Wide type GSK3α up-regulated the protein level, protein stability and transcriptional activity of Osterix. These results suggest that GSK3α regulates osteogenic activity of Osterix.

1. Introduction

Osterix (Osx) is a zinc finger motif containing osteoblast specific transcription factor that is essential for osteoblast differentiation, proliferation and bone formation [1–3]. Osterix belongs to Sp subgroup of the KLF family that expresses specifically in osteoblast cells and transiently in differentiating chondrocytes [3]. During osteoblast differentiation, Osterix acts downstream of Runx2 and is critical for osteoblast differentiation. A variety of kinases, factors and pharmaceuticals modulate osteoblast differentiation by regulating Osterix activity [4–6].

Osterix, as osteoblast transcription factor, is thought to regulate the transcription of many osteoblast marker genes, such as osteocalcin, collagen type 1A1 and osteopontin [3]. Osterix inhibits osteoblast proliferation by inhibiting the Wnt signaling pathway [7]. Osterix has been reported to have anti-tumor activity [8]. The expression of Osterix is modulated by several factors including BMP-2, insulin-like growth factor-1 (IGF-1), parathyroid hormone (PTH) and 1,25 (OH)2 vitamin D3 [6,9,10]. While tumor necrosis factor-α (TNF-α) and p53 negatively regulate Osterix expression [11,12], the activity of Osterix is on the other hand regulated by kinases: p38 regulates the expression of Osterix through phosphorylation modification, protein kinase B/Akt phosphorylates Osterix and regulates the osteogenic activity of Osterix, and Erk1/2 also regulates Osterix during osteoblast differentiation [13–15].

Glycogen synthesis kinase 3 (GSK3) is a serine/threonine protein kinase that first discovered as a regulatory kinase for glycogen synthase. GSK3 comprised two isoforms: GSK3α and GSK3β. The activity of GSK3 is tightly regulated by modulation, the most widely known is that phosphorylation at tyrosine-216 in GSK3β or tyrosine-279 in GSK3α enhances the enzymatic activity of GSK-3, while phosphorylation of serine-9 in GSK3β or serine21 in GSK-3α significantly decreases the activity [16].

GSK3 is implicated in a number of diseases including type II diabetes, Alzheimer's disease, inflammation and cancer [17–20]. In addition, GSK3 is involved in skeletal development [21,22]. In this study, we demonstrated that GSK3α regulated the function of osteoblast transcription factor Osterix. We found that GSK3α increased the protein level of Osterix. The protein stability and transcriptional activity of Osterix also were up-regulated by GSK3α.

2. Materials and methods

2.1. Cell culture

The C2C12 mouse pre-myoblast cell lines were maintained at 37 °C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic. DMEM, FBS and antibiotic–antimyotic were purchased from Gibco life technologies, Invitrogen.

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2.2. Plasmids, DNA transfection antibody and chemicals

Myc-tagged Osterix, 6Myc-tagged Osterix, site mutant 6Myc-tagged S76AS80A Osterix and HA-tagged GSK3αWT (wide type)/KD (kinase dead) were constructed in a CMV promoter-derived mammalian expression vector (pCS4+). Transient transfection was performed by the calcium phosphate-mediated method or polyethyleneimine (PEI) (Polysciences, Inc.) mediated method. Antibodies against Myc (9E10, Roche Applied Science, USA), HA (12CA5, Roche Applied Science, USA) Osx (sc-22536-R, Santa Cruz Biotechnology, USA), α-tubulin (Sigma–Aldrich, St. Louis, MO, USA), phospho-serine substrate (PSR-45, Sigma–Aldrich, USA), GFP (Santa Cruz Biotechnology, USA) and GSK3α/β (sc-7291, Santa Cruz Biotechnology, USA) were used. Alkaline phosphatase (P0114) was purchased from Sigma–Aldrich, USA.

2.3. Immunoblotting and immunoprecipitation

48 h after transfection, HEK293 cells were lysed in ice-cold lysis buffer [25 mM Hepes (pH 7.5), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 1 mM EDTA, 1 mM Na3VO4, 250 μM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin]. Lysates were cleared by centrifugation and the supernatants were subjected to immunoprecipitation using appropriate antibodies and protein A or G-Sepharose beads. Immunoprecipitated proteins were resolved by SDS–PAGE and transferred to PVDF membranes. Proteins were visualized using appropriate primary antibodies, visualized using HRP-conjugated secondary antibodies and ECL reagent (Millipore Corporation, Billerica, USA).

2.4. Luciferase reporter assay

C2C12 cells were planted on 24-well plates the day before transfection. For luciferase assays, cells were transfected with the indicated plasmids along with pCMV-β-gal and were lysed 36 h after transfection. Luciferase activities were measured using a Luciferase Reporter Assay Kit (Promega) and were normalized to the corresponding β-gal activities for transfection efficiency.

3. Results

3.1. GSK3α regulated the protein level of Osterix

To study the effect of GSK3α on Osterix, C2C12 cells were overexpressed with Myc tagged Osterix (Myc-Osx) together with wide type (WT) or kinase dead (KD) HA tagged GSK3α (HA-GSK3α). As shown in Fig. 1A, WT GSK3α up-regulated the protein level of both exogenous Myc-Osterix and endogenous Osterix, while KD GSK3α did not show any significant effects on the protein level of Osterix. The increase of protein level induced by WT GSK3α was reduced by GSK3 inhibitor LiCl (Fig. 1B). These results suggest that GSK3α increased the protein level of Osterix. To identify the molecular mechanism for GSK3α induced increase of Osterix protein level, we examined whether GSK3α affected the protein stability of Osterix by using cycloheximide (CHX). C2C12 cells were transfected with Osterix with or without WT/KD GSK3α, and then the transfected cells were treated with 40 μM CHX which inhibited the translation process. The protein levels of Osterix were determined by immunoblotting. Osterix protein degraded with a half-life of approximately 4 h. However, GSK3α WT obviously blocked the degradation and prolonged the half-life of Osterix (Fig. 1C). These results indicated that GSK3α increases the protein level of Osterix partially through increasing the protein stability.

3.2. GSK3α interacted with Osterix

Since GSK3α did not affect the transcription of Osterix but the protein level of Osterix increased, GSK3α may regulate Osterix

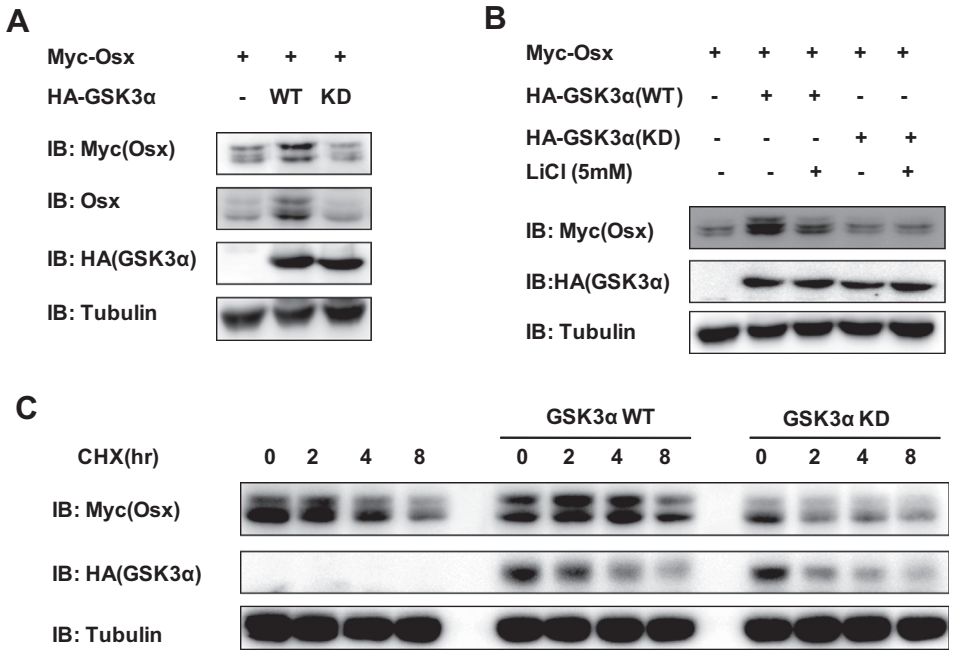

Fig. 1. The protein level of Osterix was regulated by GSK3α. (A, B) C2C12 cells were transfected with Myc-Osx together with wide type (WT) or kinase dead (KD) HA-GSK3α (A) and treated with GSK3 inhibitor LiCl at indicated concentration. The protein level of Osterix and GSK3α were determined by anti-Myc antibody and anti-HA antibody for exogenous Osterix or anti-Osx antibody for endogenous Osterix. Tubulin was used as a loading control. (C) C2C12 cells were transfected with Myc-tagged Osterix together with WT or KD GSK3α and then treated with a translation inhibitor cycloheximide (40 μM) for the indicated times. The protein levels of Dlx3 were determined by anti-Myc immunoblotting. Tubulin was used as a loading control.

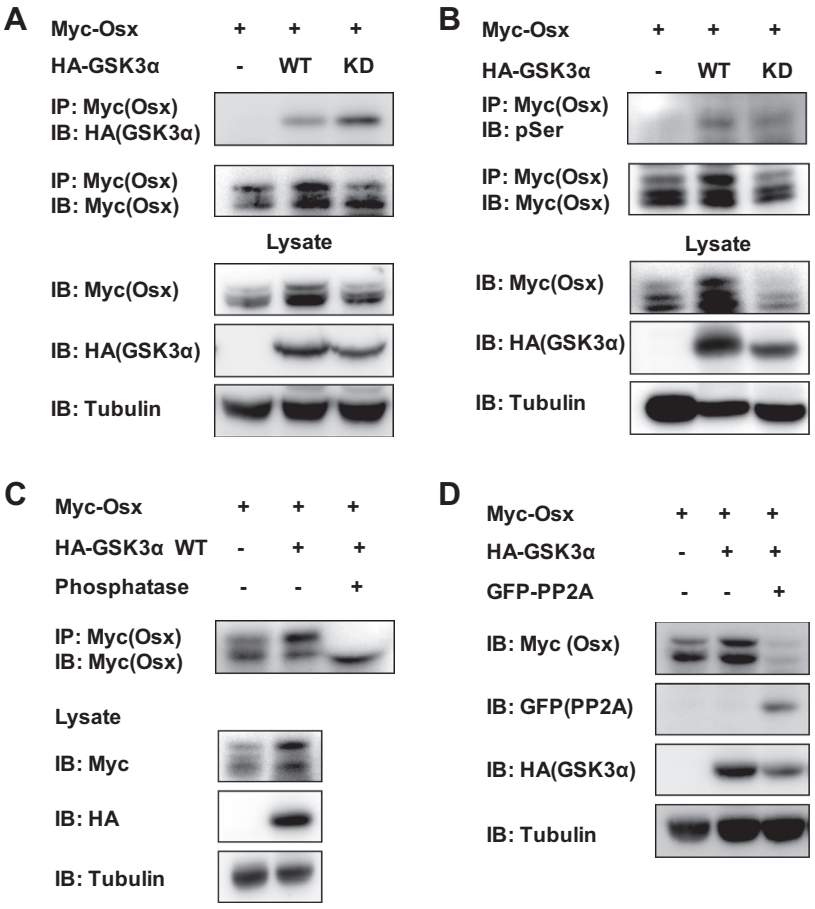

Fig. 2. GSK3α interacted with Osterix and induced the phosphorylation of Osterix. (A) C2C12 cell were transfected with Myc-Osterix WT or KD HA-GSK3α. Then cell lysate was prepared and underwent immunoprecipitation (IP) by using anti-Myc antibody. GSK3α was determined by anti-HA antibody. (B). Myc-tagged Osterix and HA-GSK3α were transfected to examine the phosphorylation of Osterix C2C12 cells. The phosphorylation of Osterix was determined by IP and anti-phospho-serine antibody. (C) Immunoprecipitates were pretreatment with alkaline phosphatase before electrophoresis. (D) C2C12 cells were over-expressed Myc-Osterix together with HA-GSK3α and GFP-PP2A. The protein levels of Osterix and GSK3α were determined by specific antibody and tubulin loading as control.

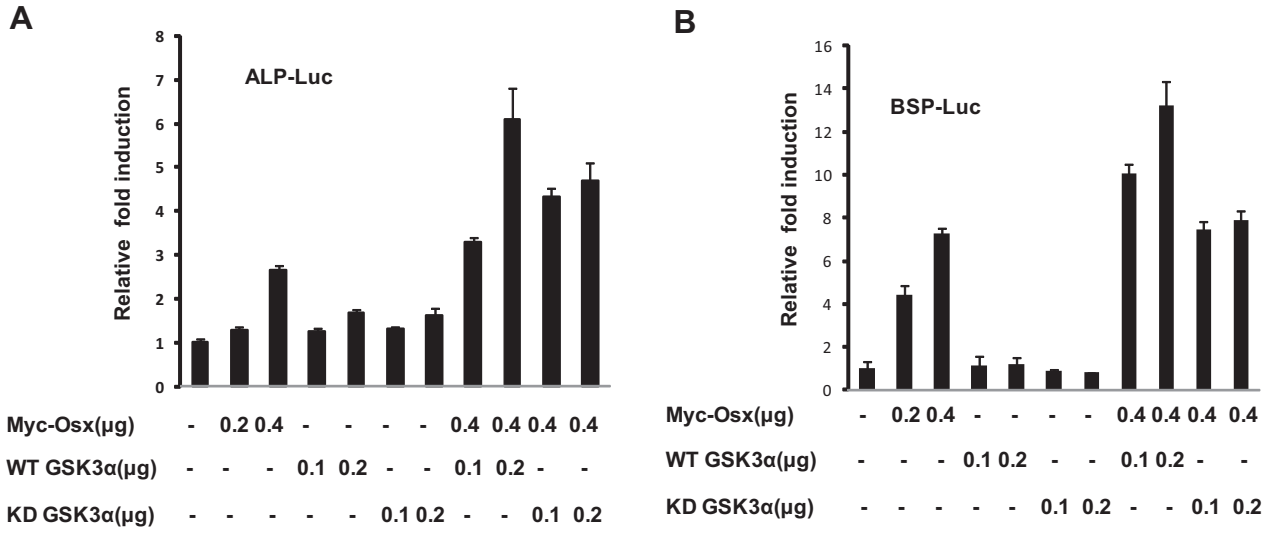

Fig. 3. The transcriptional activity of Osterix was up-regulated by GSK3α. (A, B) C2C12 cells were transfected with pCMV-β-gal, ALP-Luc or BSP-Luc luciferase reporter gene and Myc-Osterix combination with WT or KD GSK3α. The luciferase activities were measured and normalized with corresponding beta-galactosidase activities. The experiment was repeated three times and the average and standard deviations of relative luciferase activities are shown.

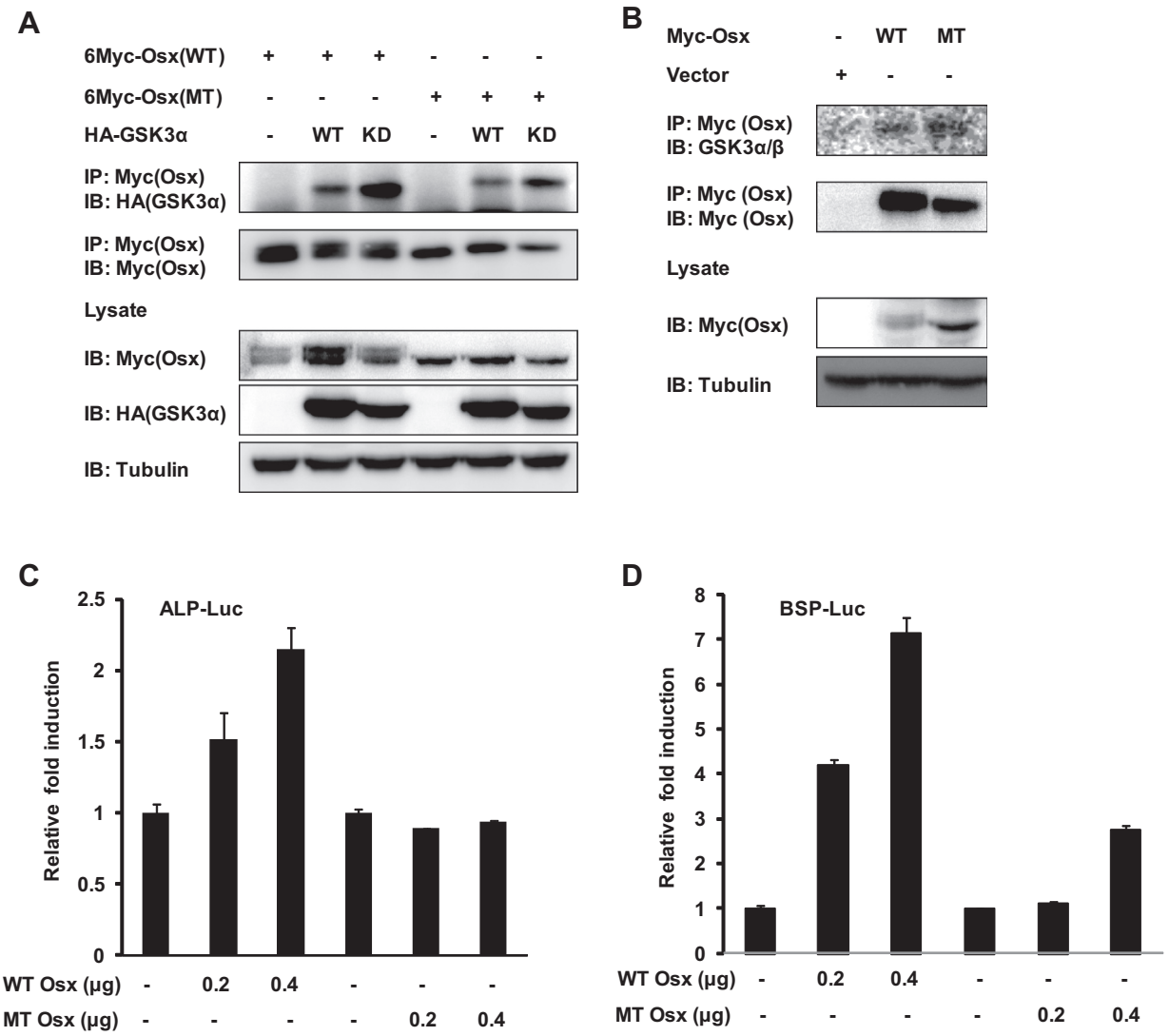

Fig. 4. GSK3α did not affect site mutant Osterix. (A). WT and MT Osterix were over-expressed together with wide type or kinase dead type GSK3α. IP was performed by using anti-Myc antibody, GSK3α was determined by anti-HA antibody. (B) C2C12 cells were over-expressed with WT and MT Osterix. IP was performed by using anti-Myc antibody and endogenous GSK3α was determined by anti-GSK3α/β antibody. (C, D) C2C12 cells were transfected with ALP-Luc and BSP-Luc osteoblast reporter gene together with WT or MT of Osterix. The luciferase activities were measured and normalized with corresponding beta-galactosidase activities. The experiment was repeated three times and the average and standard deviations of relative luciferase activities are shown.

through post-translational modification. To further examine the mechanism of GSK3α regulating Osterix, we checked whether GSK3α interacts directly with Osterix. C2C12 cells were transfected with Myc-Osterix and WT or KD HA-GSK3α. Cell lysate was prepared and underwent immunoprecipitation (IP) as shown in Fig. 2A. The result showed that over-expressed Osterix interacted with both WT and KD GSK3α (Fig. 2A). Since GSK3α acted as serine/threonine kinase, we assumed that GSK3α may induced phosphorylation of Osterix. To confirm this hypothesis, Myc-tagged Osterix was immunoprecipitated with or without WT/KD GSK3α. GSK3α may phosphorylate Osterix at serine site (Fig. 2B). This hypothesis was further supported by dephosphorylation of Osterix by using alkaline phosphatase (ALP) [23]. C2C12 cells were over-expressed with Myc-Osterix and WT HA-GSK3α, and then Osterix was purified by IP. Pretreatment with alkaline phosphatase before electrophoresis successfully induced de-phosphorylation of Osterix and changed the gel shift of Osterix (Fig. 2C). Also, external expression of phosphatase PP2A decreased the protein level of Osterix (Fig. 2D). These results indicated that GSK3α interacts with Osterix and also phosphorylated Osterix.

3.3. The transcriptional activity of Osterix was enhanced by GSK3α

Osterix acted as a pivotal transcription factor during osteoblast differentiation. To analyze whether GSK3α modulated the transcriptional activity of Osterix, alkaline phosphatase (ALP)-Luc and bone sialoprotein (BSP)-Luc osteoblast reporter genes were used. C2C12 cells were transfected with ALP-Luc, BSP-Luc along with combinations of Osterix and WT/KD GSK3α. Osterix increased the luciferase reporter gene expression, and co-transfection of Osterix and WT GSK3α further increased the expression of reporter gene (Fig. 3A and B). These results indicated that GSK3α increased the transcriptional activity of Osterix.

3.4. Site mutant Osterix almost lost the response to GSK3α

We constructed mutant type (MT) 6Myc-tagged S76A S80A Osterix which substitutes site 76 serine and site 80 serine into alanine and we then detected the effect of GSK3α on the protein level of MT Osterix. HEK 293 cells were transfected with wide type (WT) or mutant type (MT) Osterix combination with WT or KD GSK3α

and the protein levels of Osterix were determined by immunoblotting (Fig. 4A). GSK3α did not affect the protein level of MT Osterix. On the contrary, WT Osterix protein level was up-regulated by GSK3α. Immunoprecipitation revealed that site mutant of Osterix did not influence the protein–protein interaction between Osterix and over-expressed GSK3α (Fig. 4A). Both WT and MT Osterix were also interacted with endogenous GSK3α (Fig. 4B). To study the transcriptional activity of MT Osterix, C2C12 cells were transfected with ALP-Luc and BSP-Luc osteoblast reporter gene together with WT or MT of Osterix. WT Osterix increased the luciferase reporter gene expression, while MT Osterix almost lost the transcriptional activity (Fig. 4C and D). These results suggest that serine 76 and serine 80 of Osterix were important for GSK3α regulation.

4. Discussion

The balance between bone-forming osteoblasts and bone-resorbing osteoclasts is involved in bone remolding process. Zinc finger transcription factor Osterix is critical for the differentiation of pre-osteoblasts into functional osteoblasts [3]. The function of Osterix was reported regulated by post-translational modification. In the previous study, p38 regulated the expression of Osterix through phosphorylation modification [13]. We also reported before that Akt1 and Erk1/2 phosphorylated Osterix and regulated the osteogenic activity of Osterix [14,15]. In the present study, we investigated the effects of GSK3α on Osterix. We first demonstrated that the protein level of Osterix was increased by GSK3α and the increase was abolished by GSK3 inhibitor. The protein level increase did not depend on the transcription level since mRNA of Osterix did not increase by GSK3α which suggested the post-translational regulation of GSK3α on Osterix (Fig. 1). Second, we found that both endogenous and exogenous GSK3α interacted with Osterix and GSK3α also induced phosphorylation of Osterix (Fig. 2). Third, we found that the protein stability and transcriptional activity of Osterix were up-regulated by GSK3α (Figs. 1 and 3). These results suggested GSK3α regulated Osterix through post-translational modulation and further proved that GSK3α involved in skeletal development as introduced before.

GSK3 is a serine/threonine selective kinase that recognizes and phosphorylates the consensus sequence SXXXS(P) in certain proteins [24]. Sequence analysis of Osterix indicated the primary sequence 70-TNGLL**S**PAG**S**PPAPT-85 contain the consensus sequence which can be phosphorylate by GSK3. So we constructed the site serine 76 and serine 80 double mutants Osterix (6Myc-S76AS80A Osterix) and tested the effect of GSK3α on the MT Osterix. We found that GSK3α did not increase the protein level of mutant Osterix even though the protein–protein interaction was still existed (Fig. 4A and B). This result further certified the phosphorylation of Osterix by GSK3α. Except in a special case GSK3 induced phosphorylation of β-catenin did not appear to require priming [25]. Normally, most of the substrates phosphorylated by GSK3 require prior phosphorylation of a serine residue at C-terminal to the target site corresponding to GSK3 [26]. However, in this study, the mechanism of GSK3α phosphorylate Osterix was not well understood. Thus, further detailed studies about GSK3α are still needed.

Serine/threonine protein phosphatase PP2A is a heterotrimeric phosphatase, and ubiquitously expresses in cells. PP2A has diverse cellular function and plays roles in signaling cascades such as Raf, MEK and Akt signaling pathways. GSK3 isoforms are regulated by PP1 on GSK3β and PP2A on GSK3α [27]. In our current study, we assumed that GSK3α phosphorylates Osterix, so we introduced PP2A to de-phosphorylate Osterix, and we found that GFP-tagged PP2A decreased the protein level of GSK3α as well as Osterix (Fig. 2B). Since PP2A is not specifically dephosphorylated GSK3α, we thought that PP2A decreased the protein level of Osterix through two ways: first, PP2A dephosphorylated Osterix, second, PP2A induced the protein level decrease of GSK3α down-regulated the effects on up-regulation for Osterix by GSK3α. However, the detailed mechanism is still not clear. More research about this process is still needed.

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