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Prolyl isomerase Pin1 regulates the osteogenic activity of Osterix

Sung Ho Lee [a], Hyung Min Jeong [a], Younho Han [a], Heesun Cheong [b], Bok Yun Kang [a], Kwang Youl Lee [a,*]

[a] College of Pharmacy & Research Institute of Drug Development, Chonnam National University, Gwangju 500-757, Republic of Korea
[b] Research Institute, National Cancer Center, 323 Ilsan-ro, Ilsandong-gu, Goyang-si, Gyeonggi-do 410-769, Republic of Korea

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ABSTRACT

Osterix is an essential transcription factor for osteoblast differentiation and bone formation. The mechanism of regulation of Osterix by post-translational modification remains unknown. Peptidyl-prolyl isomerase 1 (Pin1) catalyzes the isomerization of pSer/Thr-Pro bonds and induces a conformational change in its substrates, subsequently regulating diverse cellular processes. In this study, we demonstrated that Pin1 interacts with Osterix and influences its protein stability and transcriptional activity. This regulation is likely due to the suppression of poly-ubiquitination-mediated proteasomal degradation of Osterix. Collectively, our data demonstrate that Pin1 is a novel regulator of Osterix and may play an essential role in the regulation of osteogenic differentiation.

1. Introduction

Osterix, a zinc finger-containing transcription factor, plays a critical role in osteoblast differentiation and bone formation. No bone formation occurs in Osterix-knockout mice (Baek et al., 2009, 2010; Nakashima et al., 2002; Zhou et al., 2010). Osterix is required for bone morphogenetic protein 2 (BMP2) and Runx2-induced osteoblast differentiation and bone growth in both postnatal and adult mice (Lee et al., 2003; Matsubara et al., 2008). Runx2 expression level in osteogenic cells of Osterix-null mutants is comparable to that in wild-type osteoblasts. In contrast, Runx2-deficienct embryos do not express Osterix, suggesting that Osterix acts downstream of Runx2, which in turn regulates the expression of many osteoblast-specific differentiation markers, including alkaline phosphatase, osteocalcin, osteonectin, osteopontin, and Runx2 (Fu et al., 2007; Nakashima et al., 2002; Zhang et al., 2008). The functional roles of Osterix in bone and cartilage development have only recently been investigated. Osterix is thought to regulate the transcription of several osteoblast marker genes containing GC-rich and Sp-binding sites on their promoters. Forced expression of Osterix *in vitro* has been reported to induce the expression of osteocalcin; collagen, type 1, alpha 1 (Nakashima et al., 2002); collagen, type 11, alpha 2 (Goto et al., 2006); and osteopontin (Kim et al., 2006). Osterix can also inhibit osteoblast proliferation through the inhibition of the Wnt signaling pathway (Zhang et al., 2008) and act as a negative regulator of chondrogenesis. Therefore, understanding the regulation of Osterix gene expression will be beneficial in modulating osteoblast differentiation and bone formation.

Pin1 is a highly conserved and relatively smaller enzyme (18 kDa) (Lu et al., 1996), which contains an N-terminal WW domain that acts as a phosphoprotein-binding module (Lu et al., 1999) and a C-terminal catalytic domain that is distinct from other conventional peptidyl–prolyl cis–trans isomerases (PPIases) (Ranganathan et al., 1997; Yaffe et al., 1997). By virtue of its unique WW and catalytic domains, Pin1 isomerizes specific phosphorylated Ser/Thr-Pro bonds and regulates the function of a defined subset of phosphoproteins (Yaffe et al., 1997). The isomerization of Ser/Thr-Pro motifs is especially important because kinases and phosphatases can specifically recognize the *cis* or *trans* conformation of the prolyl peptide bond of their substrates (Werner-Allen et al., 2011; Zhou et al., 2000), and phosphorylation further slows down the isomerization rate of proline (Lu and Zhou, 2007; Yaffe et al., 1997). Pin1 activity controls a subset of protein functions in diverse cellular processes such as cell cycle and cell growth (Liou et al., 2002, 2003; Lu and Zhou, 2007; Lu et al., 1999, 2007; Ryo et al., 2002, 2003; Wulf et al., 2002, 2003; Zhou et al., 2000). Importantly, Pin1 is tightly regulated by several mechanisms (Lu et al., 2002; Ryo et al., 2002), and its deregulation can contribute to a plethora of human diseases, including aging, cancer, neurological disorders, and autoimmune and inflammatory diseases (Lee et al., 2011; Liou et al., 2002; Lu and Zhou, 2007). However, the underlying mechanism of how Pin1 is involved in bone metabolism, particularly osteogenesis, remains unknown.

In this study, we investigated the functional roles of Pin1 during osteoblast differentiation. We found that Pin1 increases BMP2-induced osteoblast differentiation in C2C12 cells, and induces the protein stability and transcriptional activity of Osterix. This

* Corresponding author. College of Pharmacy & Research Institute of Drug Development, Chonnam National University, Gwangju 500-757, Republic of Korea. Tel.: +82-62-530-2939; fax: +82-62-530-2949.
E-mail address: kwanglee@chonnam.ac.kr (K.Y. Lee).

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up-regulation of Osterix function is likely due to the reduction of ubiquitin proteasome-mediated degradation of Osterix. Taken together, our results suggest a novel regulatory mechanism of Pin-1-mediated Osterix function that can subsequently induce osteoblast differentiation.

2. Materials and methods

2.1. Plasmids, antibodies, and reagents

Plasmids for the expression of Myc-Osterix, HA-Osterix, and GFP-Osterix were constructed in a CMV promoter-derived expression vector (pCS4+). The Xpress-tagged Pin1 wild-type, S16A, K63A, and M130F mutant plasmids were generously provided by Dr. Hong Seok Choi (Chosun University, Gwangju, Korea). For Pin1-knockdown, the following oligonucleotides targeting the mouse Pin1 (accession number: NP_006221) sequence (in capital letters) were annealed and inserted into pSUPER. Puro: sense, 5′-gat ccc cGC CGG GTG TAC TTC AAT tca aga gAT TGA AGT ACA CCC GGC ttt ttg gaa a-3′ and antisense, 5′-agc ttt tcc aaa aaG CCG GGT GTA CTA CTT CAA Tct ctt gaA TTG AAG TAG TAC ACC CGG Cgg g-3′. For inhibition of MEK activity, 10 mM stock solution of the MEK inhibitor, U0126 (Calbiochem, Cat#662005), was used at 5 μM final concentration. Antibodies against the following epitopes were used: Myc (9E10) and HA (12CA5) from Roche Applied Science; Flag (M2) and α-Tubulin (B-5-1-2) from Sigma-Aldrich; Pin1 (SC-46660), GFP (FL), and Osterix (A-13) from Santa Cruz Biotechnology; Anti-Xpress (R91025) antibody from Invitrogen. Pin1 inhibitor Juglone (420120) and MG132 (Calbiochem), cycloheximide (Sigma), calf intestinal alkaline phosphatase (CIAP; Invitrogen), and recombinant human BMP2 (335-BM, R&D Systems) were used.

2.2. Cell culture and transient transfections

Human embryonic kidney (HEK) 293 cells, C2C12 mouse myoblast cells, and $Pin1^{-/-}$ mouse embryonic fibroblasts (MEFs), which were kindly provided by Dr. Kun Ping Lu (Beth Israel Deaconess Medical Center, Harvard Medical School), were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS. All these cell lines were cultured and maintained at 37 °C in humidified air containing 5% CO2. DMEM, FBS, and the antibiotics were purchased from Life Technologies. Transient transfection was performed using polyethyleneimine (PEI; Polysciences, Inc.). The total amount of transfected plasmids in each group was normalized by adding an empty vector.

2.3. Immunoblotting (IB) and immunoprecipitation (IP)

HEK293 cells were lysed in an 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 Na_3VO_4, 250 μM PMSF, 10 μg/mL leupeptin, and 10 μg/mL aprotinin). After centrifugation, the supernatants were used as cell lysates. For immunoprecipitation, the cell lysates were incubated with the appropriate antibodies and protein A- or G-Sepharose beads. Cell lysates containing 30 μg of total protein or immunoprecipitated proteins were subjected to SDS–PAGE and the proteins were transferred to a PVDF membrane. Proteins were detected using the relevant primary antibodies, horseradish peroxidase-coupled secondary antibodies (GE Healthcare Life Sciences), and enhanced chemiluminescence (ECL) reagent (Millipore). The signals were detected and analyzed by an LAS4000 luminescent image analyzer (Fuji Photo Film Co.).

2.4. Luciferase reporter assay

C2C12 cells were seeded on 24-well plates 1 day before transfection. The cells were transfected with a luciferase reporter plasmid [containing the regulatory sequences of osteoblast differentiation marker *ALP, BSP*, or *OC* genes], CMV promoter-driven β-galactosidase reporter (pCMV-β-gal), and the indicated combinations of the expression plasmids. Luciferase activity was measured after 36 h, using the Luciferase Reporter Assay Kit (Promega) and normalized with the corresponding β-galactosidase activity for transfection efficiency. All the experiments were performed in triplicate and repeated at least three times. The average and S.D. of the representative experiments are shown.

2.5. RT-PCR analysis

C2C12 cellular RNA was prepared using TRIzol reagent (Life Technologies), according to the manufacturer's instructions. Random hexamer-primed cDNAs were synthesized from 1 μg of total RNA using SuperScript III First-Strand Synthesis System (Life Technologies). The following conditions were used for PCR amplification: initial denaturation at 94 °C for 5 min; 28–30 cycles of denaturation at 94 °C for 1 min, annealing at a temperature optimized for each primer pair for 1 min, and extension at 72 °C for 1 min; and final extension at 72 °C for 10 min. The following PCR primers were used: mouse *ALP* (accession number: NP_031457) forward 5′-ATT GCC CTG AAA CTC CAA AAC C-3′ and reverse 5′-CCT CTG GTG GCA TCT CGT TAT C-3′; mouse *BSP* (accession number: NP_032344) forward 5′-CAG AAG TGG ATG AAA ACG AG-3′ and reverse 5′-CGG TGG CGA GGT CCC AT-3′; mouse *COL1A1* (accession number: NP_031768) forward 5′-TCT CCA CTC TTC TAG GTT CCT-3′ and reverse 5′-TTG GGT CAT TTC CAC ATG C-3′; and mouse *GAPDH* (accession number: XP_001476757) forward 5′-ACC ACA GTC CAT GCC ATC AC-3′ and reverse 5′-TCC ACC CTG TTG CTG TA-3′.

2.6. Alkaline phosphatase (ALP) staining

For ALP staining, C2C12 cells were seeded in 24-well plates, transfected, and then incubated in the differentiation medium (2% FBS/DMEM) with BMP2 (10 ng/mL) for 72 h. The cells were then fixed in 4% paraformaldehyde for 10 min at room temperature (RT), rinsed with PBS, and then incubated with 300 μg/mL BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) solution (Sigma-Aldrich) for 20 min at RT. ALP-positive cells were stained blue/purple.

2.7. Glutathione-S-transferase (GST) pull-down assay

Recombinant GST-tagged empty vector and Pin1 (WT, Y23A, Y24A, and Y94A) proteins were expressed in *E. coli* and purified using glutathione–sepharose beads. For each GST-pull down assay, beads carrying 10 μg of GST-Osterix protein were equilibrated with cell lysis buffer and incubated with the cell lysates. The retained proteins were subjected to SDS–PAGE and IB.

2.8. Immunocytochemical analysis

C2C12 cells grown on microscope coverslips were transfected with the GFP-Osterix plasmids. The cells were fixed with 4% paraformaldehyde for 10 min at RT and then washed with PBS. The washed slides were treated with a blocking solution (0.2% Triton X-100 in PBS) for 1 h at RT. The cells were then incubated with primary antibodies at 4 °C overnight, followed by secondary antibodies for 1 h at RT. To visualize the nuclei, the cells were counterstained with 4, 6-diamidino-2-phenylindole (DAPI; Calbiochem). The cells were washed three times with PBS, mounted