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Characterization of Dkk1 gene regulation by the osteoblast-specific transcription factor Osx

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ABSTRACT

Bone formation is a developmental process involving the differentiation of mesenchymal stem cells to osteoblasts. Osterix (Osx) is an osteoblast-specific transcription factor required for bone formation and osteoblast differentiation. Previous observation that Osx inhibits Wnt signaling pathway provides a novel concept of feedback control mechanisms involved in bone formation. Wnt antagonist Dickkopf-1 (Dkk1) plays an important role on skeletal development and bone remodeling. Osx has been shown to activate the *Dkk1* promoter; however, the detailed mechanism of Osx regulation on *Dkk1* expression is not fully understood. In this study, quantitative real-time RT-PCR results demonstrated that *Dkk1* expression was downregulated in *Osx*-null calvaria at two different points of E15.5 and E18.5 in mice embryos. Overexpression of Osx resulted in upregulation of *Dkk1* expression in Tet-off stable C2C12 cell line. Inhibition of Osx expression by siRNA led to downregulation of *Dkk1* in osteoblasts. These data suggest that Osx may target Dkk1 directly. To define minimal region of *Dkk1* promoter activated by Osx, we made a series of deletion mutants of *Dkk1* promoter constructs, and narrowed down the minimal region to the proximal 250 bp by transient transfection assay. It was shown that two GC-rich binding sites within this minimal region of *Dkk1* promoter were required for the *Dkk1* promoter activation by Osx. Importantly, quantitative chromatin immunoprecipitation (ChIP) assays were performed to show that endogenous Osx associated with native *Dkk1* promoter in primary osteoblasts. Taken together, these findings support our hypothesis that Dkk1 is a direct target of Osx.

1. Introduction

Bone formation takes place through two distinct processes: endochondral ossification involving a cartilage model and intramembranous ossification by which bones form directly from condensations of mesenchymal cells without a cartilage intermediate. Bone formation is a highly regulated process involving the differentiation from mesenchymal progenitor cells into preosteoblast, then into osteoblast lineage, and finally into osteocytes [1,2]. Osteoblast differentiation is regulated by different transcription factors and signaling proteins including Indian hedgehog, Runx2, Osterix (Osx) and Wnt signaling pathway. Ihh is required for endochondral but not for intramembranous bone formation [3] and is needed for the establishment of the osteogenic portion of the perichondrium/periosteum and for the initial activation of the gene for Runx2. Runx2 is needed for bone formation since no endochondral and no membranous bones are formed in *Runx2*-null mice [4]. *Runx2* is required for the differentiation of mesenchymal cells into preosteoblasts. As a downstream gene of *Runx2*, *Osx* is required for the differentiation of preosteoblasts into mature osteoblasts. Osx is specifically expressed in all osteoblasts. In *Osx*-null embryos, cartilage is formed normally, but the embryos completely lack bone formation [2]. *Osx* expression pattern in mice indicates that the presence of *Osx* transcript begins as early as the commitment time for mesenchymal cells to enter osteoblast lineage and its signal becomes stronger as osteoblast differentiation occurs. The C terminal region of Osx contains the DNA-binding domain which can bind to specific GC-rich sequences to control target gene expression, such as osteoblast differentiation markers type 1 collagen, bone sialoprotein, and osteocalcin (OC).

Wnt signaling has been studied for its broad range of activities in cell proliferation, differentiation and cell death during both embryonic development and the adult stage in a variety of tissue types including bone [5]. Wnts are secreted glycoproteins that bind to Frizzled family receptors and low-density lipoprotein receptor-related proteins (LRP) 5/6 coreceptors. In the absence of Wnt,

Abbreviations: Osx, osterix; Dkk1, Dickkopf 1; OC, osteocalcin; E15.5, embryonic day 15.5; E18.5, embryonic day 18.5; ChIP, chromatin immunoprecipitation; Dox, Doxycycline; LRP, low-density lipoprotein receptor-related protein.

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β-catenin forms a complex with the APC, Axin and the kinases glycogen synthase kinase 3, which facilitates phosphorylation and proteosomal degradation of β-catenin. Stimulation of these receptors by Wnts leads to the intracellular molecule β-catenin to accumulate and translocate into the nucleus, where it interacts with TCF/Lef1 transcription factor to activate transcription of target genes. Wnt/β-catenin pathway has been known to play a crucial role in bone formation and bone metabolism [6]. Gain-of-function mutants of *Lrp5* cause high bone mass syndrome in patients [7] and in mice [8]. Conditional inactivation of *β-catenin* in either skeletal progenitor cells or at a later stage of osteoblast development in mouse embryos blocks osteoblast differentiation [9–12].

Inhibitors of Wnt signaling can bind to frizzled (serum frizzled-related proteins), Wnt (Wnt inhibitory factors) or LRP 5/6 (sclerostin and Dickkopf-1). These agonist proteins prevent Wnt from activating the frizzled LRP 5/6 receptor signaling pathway, leading to a decrease in signaling. The canonical Wnt pathway is regulated by a large number of antagonists, including the Dkk family and secreted frizzled-related proteins. Dickkopf (Dkk) is a Wnt antagonist. It binds to LRP5/6 receptor to form a complex with Kremen1 and 2 and inhibits Wnt signaling by reducing the availability of LRP5/6 [5]. To date, four Dkk proteins have been identified in mammals [13], among which Dkk1 and Dkk2 have been well characterized and found to act as antagonists to the canonical Wnt pathway by binding to LRP5/6 in combination with a second coreceptor designated as Kremen [14,15]. Transgenic overexpression of Dkk1 under the control of the ColA1 promoter leads to decreased bone mass [16]. In agreement with this observation, deletion of Dkk1 expression in osteoblasts results in an increase in bone formation and mass [17].

It has been discovered that in addition to its essential role in osteoblast differentiation, the osteoblast-specific transcription factor Osx also inhibits osteoblast proliferation and negatively regulates Wnt/β-catenin signaling [18]. Further data have indicated that Osx controls Wnt signaling by two different mechanisms (i) stimulates Wnt antagonist DKK1 expression and (ii) disrupts Tcf1 binding to DNA to inhibit the transcriptional activity of β-catenin/Tcf. Osx inhibition of Wnt signaling provides a feedback control mechanism involved in bone formation. Osx has been shown to activate the *Dkk1* promoter; however, the detailed mechanism of Osx regulation on Dkk1 expression is not fully understood.

In this study, our results from quantitative real time RT-PCR revealed that Dkk1 expression was downregulated at both E15.5 and E18.5 in the calvaria of *Osx*-null mouse embryos, suggesting Osx is essential for Dkk1 expression. Dkk1 gene regulation by Osx was further characterized. We provide evidences to demonstrate that Dkk1 is a direct target of Osx.

2. Materials and methods

2.1. Plasmid constructs and subcloning

The fragments of Dkk1 promoter region were generated by PCR using mouse genomic DNA as a template and subcloned into the XhoI and MluI sites of pGL-3 vector. Primers were obtained from Integrated DNA Technologies (IDT) (Coralville, IA), and the sequences were as follows: (1) Dkk1-Xho-3 5′TGG TGG AGT CTC TGG CTG CCA, (2) Dkk1-Mlu-2 k 5′GGC ATC TAT GCA AGG TTC AG, (3) Dkk1-Mlu-1 k 5′TAT TAA CCC ACC GCT GGG AAC, (4) Dkk1-Mlu-500 5′TTG ATG AAT GGC TGC TCG CA and (5) Dkk1-Mlu-250 5′TAG TGC TCT AGT GAC CCA CAC. A. Dkk1 point mutants were made with the QuickChange site-directed mutagenesis kit (Stratagene) using Dkk1-250 as a template by the following primers: (1) Dkk1-M1-1 5′GGG ACC ACA GTG CAA TTT ATT TTC GAG GGG AGA GTG TC, (2) Dkk1-M1-2 5′GA CAC TCT CCC CTC GAA AAT AAA TTG CAC TGT GGT CCC, (3) Dkk1-M2-1 5′CGA CAC ACA AAC ACT AAA AAT AAA AGC TCC TCC CAA AGC and (4) Dkk1-M2-2 5′GCT TTG GGA GGA GCT TTT ATT TTT AGT GTT TGT GTG TCG. All constructs including mutants were verified by DNA sequencing.

2.2. Cell culture and transient transfection assay

HEK293 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, CA) with 10% fetal bovine serum and 100 units/ml penicillin plus 100 μg/ml streptomycin at 37 °C. HEK293 cells were plated in 12-well plates, cultured to 60–80% confluence and transfected with FuGENE 6 (Roche) according to the manufacture's instruction. Cells were cotransfected with 300 ng of Dkk1 promoter reporter, Osx expression plasmid as indicated and 25 ng of pSV2-beta-gal. After transfection, cells were incubated for 24 h before harvest. The reporter assays were analyzed with BD Monolight system (BD Biosciences). Luciferase activity was normalized by β-galactosidase activity. Every transfection experiment was done at least three times. Values were presented as the mean ± S.D. MC3T3 cells (ATCC) were cultured in Alpha Minimum Essential Medium with ribonucleosides, deoxyribonucleosides, 2 mM L-glutamine and 1 mM sodium pyruvate, but without ascorbic acid (GIBCO), and with 10% FBS and penicillin plus streptomycin. Stable C2C12 mesenchymal cells expressing Osx were generated with pTet-off Advanced Inducible Gene Expression System (Clontech) as previously used [18]. Osx expression was induced in the absence of tetracycline. C2C12 cells were cultured in ATCC described medium with additives G418, Hygromycine, and with or without Doxycycline (Dox), a member of the tetracycline antibiotics group.

2.3. siRNA interference

MC3T3 cells were transfected by siRNA against mouse Osx with Lipofectamine 2000. siRNA oligos were purchased from Thermo Scientific Dharmacon, and siGENOME Lamin A/C Control siRNA was used as a non-specific control. Cells were cultured in 6-well plates. One day before transfection, cells were plated in 1 ml of growth medium without antibiotics. Cells were 30–50% confluent at the time of transfection. For each sample, siRNA:Lipofectamine 2000 transfection complex was prepared as follows: (1) dilute 2 μl of 50 μM siRNA in 50 μl of Opti-MEM I Reduced Serum Medium without serum, (2) mix Lipofectamine 2000 gently, then dilute 3 μl in 50 μl of Opti-MEM I medium, (3) combine the diluted siRNA with the diluted Lipofectamine 2000 and (4) add 100 μl of siRNA:Lipofectamine 2000 complex to each well. After 4 h incubation, the growth medium was replaced. Cells were cultured at 37 °C in a CO_2 incubator for 24 h before harvest.

2.4. Chromatin immunoprecipitation (ChIP) assay

ChIP assay kit was from Millipore. ChIP assays were performed according to previously described protocol [19], provided in Supplementary materials.

3. Results

3.1. Dkk1 expression is down-regulated in the absence of Osx

Our recent studies have demonstrated that Osx can inhibit Wnt signaling, a possible mechanism for Osx to inhibit osteoblast proliferation [18], and that Osx activates Dkk1 expression. In this study, we further characterized Dkk1 regulation by Osx. We carried out quantitative real time RT-PCR to compare RNA levels of Dkk1 between *Osx* wild type and knockout mice at two different points