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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 250bp 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.

Keywords

Osx; Osterix; Dkk1; osteoblast; Wnt signaling; bone formation

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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, 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; 10; 11; 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 (*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.

Materials and Methods

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-2k 5'GGC ATC TAT GCA AGG TTC AG, 3) Dkk1-Mlu-1k 5'TAT TAA CCC ACC GCT GGG AAC, 4) Dkk1-Mlu-500 5'TTG ATG AAT GGC TGC TCG CA, 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, 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.

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 μ g/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 300ng of Dkk1 promoter reporter, Osx expression plasmid as indicated and 25ng of pSV2-beta-gal. After transfection, cells were incubated for 24h 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 $\pm$ 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, Hygromycin, and with or without Doxycycline (Dox), a member of the tetracycline antibiotics group.

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 1ml 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 μ l of 50 μ M siRNA in 50 μ l of Opti-MEM I Reduced Serum Medium without serum; (2) Mix Lipofectamine 2000 gently, then dilute 3 μ l in 50 μ l of Opti-MEM I Medium; (3) Combine the diluted siRNA with the diluted Lipofectamine 2000; (4) Add 100 μ l of siRNA:Lipofectamine 2000 complex to each well. After 4hr incubation, the growth medium was replaced. Cells were cultured at 37°C in a CO₂ incubator for 24hr before harvest.

Chromatin immunoprecipitation (ChIP) assay

ChIP Assay Kit was from Millipore. ChIP assays were performed according to previously described protocol [19], provided in supplementary materials.

Results

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 of E15.5 and E18.5 in mice embryos. RNA was isolated from calvaria of both E15.5 and E18.5 mouse embryos. As shown in Figure 1A, *Osx* expression was abolished at E15.5 in *Osx*-null calvaria. Osteoblast marker gene *OC* was at undetected level as expected. Interestingly, Wnt signaling antagonist *Dkk1* expression was found downregulated by about 16 folds in *Osx*-null calvaria compared with that in wild type calvaria. As shown in Figure 1B, both *Osx* and *OC* expression were abolished at E18.5 in *Osx*-null calvaria. *Dkk1* expression was found downregulated by about 64 folds in *Osx*-null calvaria compared with that in wild type calvaria. The marked decrease in *Dkk1* RNA level in *Osx* knockout mice suggests that *Osx* controls *Dkk1* gene expression.

Overexpression of *Osx* activates *Dkk1* expression

Next we asked whether *Osx* could positively regulate *Dkk1* expression. To address this question, a C2C12 stable cell line was used in which the expression of *Osx* could be induced by using the tetracycline (Tet) system as previously described [18]. *Osx* expression was tuned on in the absence of Dox (Figure 2A). Total RNA was isolated from C2C12 stable cell line in the presence or absence of Dox and measured by real time RT-PCR for *Dkk1* expression. As shown in Figure 2B, in the absence of Dox when *Osx* expression was induced, *Dkk1* expression was upregulated by about 4.8 folds. This observation indicates that *Osx* activates *Dkk1* expression.

Inhibition of *Osx* by siRNA led to repression of *Dkk1* expression in osteoblasts

To further confirm the effect of *Osx* on *Dkk1* expression, we used siRNA transfection to knockdown *Osx* expression in MC3T3 osteoblast cells to determine the possible change of *Dkk1* expression. Real-time RT-PCR was performed to analyze mRNA expression level. As shown in Figure 2C, when *Osx* RNA expression was decreased by 80% by siRNA against *Osx*, *Dkk1* RNA expression was repressed by 60%. Therefore, these data suggest that *Osx* regulates *Dkk1* positively in osteoblasts.

Identification of the *Osx* binding site in the promoter of *Dkk1* gene

We have shown *Osx* can stimulate *Dkk1* promoter activity, however it is still not clear which region within *Dkk1* promoter is responsible for *Osx* activation. To address this question, the deletion analysis and transient transfection assay were carried out to narrow down responsible region within 2kb *Dkk1* promoter for *Osx* activation. *Dkk1* luciferase reporter constructs driven by different lengths of *Dkk1* promoter region were generated. As shown in Figure 3A, *Osx* was able to activate *Dkk1* promoter reporters of *Dkk1*-2kb, *Dkk1*-1kb, *Dkk1*-500bp and *Dkk1*-250bp in transient transfection assay. Previous study has shown that *Osx* is a zinc finger-containing transcription factor and belongs to the Sp/XKLF family [2]. This family of transcription factors tends to bind to GC-rich sequence in target gene promoter region to control target gene expression. According to the sequence analysis of the region within 250bp of *Dkk1* promoter, two tentative binding sites for *Osx* were identified as GC rich elements. To test which binding site is responsible for *Dkk1* promoter activation by *Osx*, we generated two point mutants of *Dkk1*-250 promoter reporter by the QuickChange site-directed mutagenesis kit (Stratagene) using *Dkk1*-250bp as a template. The two mutants were designated *Dkk1*-250-M1 and *Dkk1*-250-M2, in which the guanines (Gs) and the cytosine (Cs) in each element were replaced with thymine (Ts) and adenines (As) respectively. As shown in Figure 3B, *Osx* activation of the *Dkk1*-250-M1 and *Dkk1*-250-M2 promoter mutants was only 55% and 58% respectively, that of the wild-type *Dkk1*-250bp promoter. Finally, we generated a double mutant in which both GC-rich elements were disrupted (*Dkk1*-250-M12). As shown in Figure 3B, *Dkk1*-250-M12 mutant almost abolished *Osx* activation of the *Dkk1*-250bp promoter reporter. Thus, these results revealed that two GC-rich sequences in *Dkk1*-250bp region were responsible for *Dkk1* promoter activation by *Osx*.

Osx associates with native Dkk1 promoter in vivo

The studies above indicate that Osx can positively regulate Dkk1 expression and activate the *Dkk1* promoter *in vitro* through two GC-rich sequences in *Dkk1* promoter. Next we tested whether endogenous Osx can bind to the native *Dkk1* promoter *in vivo*. Therefore, ChIP assay was performed to determine whether Osx could associate with *Dkk1* promoter in primary calvarial cells isolated from new born wild-type mice. Two sets of primers were designed. Primer set 1 covered GC-rich element identified within 250bp *Dkk1* promoter in Figure 3. Primer set 2 covered a sequence without GC-rich region at the distal 3kb of *Dkk1* promoter. Anti-Osx antibody was used as previously described for ChIP analysis [18]. The precipitated chromatin was analyzed by quantitative real-time PCR using Primer set 1 and 2. As shown in Figure 4, Osx was found to associate with the *Dkk1* promoter region containing GC rich sequence (Primer set 1) compared with IgG control group. Osx did not associate with the distal *Dkk1* promoter region without GC rich sequence (Primer set 2), demonstrating that the observed Osx-DNA association was specific. The data suggest that Osx associates with *Dkk1* promoter in vivo.

Discussion

Osx is an osteoblast-specific transcription factor that controls the expression of essential genes needed for appropriate osteoblast differentiation and function. Osx inhibits Wnt pathway. In this study, we further characterized Osx regulation on Wnt antagonist Dkk1 expression. The findings presented here support our hypothesis that Dkk1 is a direct target of Osx.

First, we identified Dkk1 as an Osx target gene. This is supported by the Osx-directed gene expression studies in several *in vivo* and *in vitro* model systems. For example, Osx-null calvaria displayed defective *in vivo* expression of Dkk1 at E15.5 and E18.5 compared to wild-type calvaria in mice (Figure 1). A Tet-off inducible C2C12 stable cell system revealed that ectopic expression of Osx resulted in an increase in Dkk1 transcript level (Figure 2B) and MC3T3 osteoblast cultures also had markedly impaired *Dkk1* gene expression when Osx expression was knocked-down using siRNA targeting strategies (Figure 2C). Moreover, a direct regulation of *Dkk1* gene transcription by Osx was evident in the ability of recombinant Osx to activate *Dkk1* promoter-reporter constructs (Figure 3A), thus indicating that the RNA expression studies were likely due to the effects of Osx expression on *Dkk1* gene transcription.

In terms of mechanism, Osx is an SP/KLF family member that presumably functions by binding directly to DNA promoter elements via an SP1-like DNA-binding domain consisting of three C2H2-type zinc fingers located within its C-terminus [2]. They likely are GC-rich in nature based on the similarities of DNA-binding domains and elements among this family of transcription factors. Indeed, two GC-rich regions exist in the most proximal regions of the murine *Dkk1* promoter. Importantly, our studies define these two elements as critical for mediating the transcriptional activation of the *Dkk1* promoter by Osx. Mutation of each element (Dkk1-250-M1 or Dkk1-250-M2) reduced Osx-directed activation of the *Dkk1* promoter by approximately 50% and mutation of both elements Dkk1-250-M12 nearly abolished the response (Figure 3B). Of course, limitations of this particular approach include

the heterologous expression and somewhat artificial nature of the plasmid DNA constructs used as a measure of a biologically relevant transcriptional event. Thus, the chromatin immunoprecipitation approaches (Figure 4) strongly support the reporter gene expression data. These studies clearly demonstrated that endogenous *Osx* was associated with the GC-rich proximal region of the native *Dkk1* promoter in primary osteoblastic cells. These studies help establish *Osx* mechanisms involving direct binding of *Osx* to sequence specific, GC-rich promoter elements to activate the expression of genes in osteoblastic cells.

Wnt signaling is known to have the major impact at different stages of bone formation and bone metabolism [5; 6]. Wnt signaling-mediated gene expression can promote osteoblast proliferation and differentiation. Some studies investigated the role of Wnt/ β -catenin signaling in nonunion and osteoporosis, suggesting Wnt signaling could possibly have potential to become a target of pharmacological intervention to increase bone formation [20; 21]. *Dkk1* is one of the Wnt antagonists. Our observation that *Dkk1* expression is absent in *Osx*-null calvaria of at both E15.5 and E18.5 embryos parallels the previously reported finding that *Dkk1* expression was absent in tibia of E18.5 embryos in which β -catenin was inactivated using an *Osx-Cre* transgene [12]. This suggests that both *Osx* and β -catenin are required for *Dkk1* expression in osteoblasts. Thus, the *Dkk1* gene might be a common target of these transcription factors in these cells. Due to the important role of Wnt signaling during bone formation, it will be interesting to investigate regulations of Wnt signaling. *Osx* is required for osteoblast differentiation and bone formation [2]. Previous study has demonstrated that *Osx* inhibits Wnt signaling activity during bone formation, a possible mechanism for the inhibition by *Osx* of osteoblast proliferation [18]. The findings in this study that *Osx* controls *Dkk1* expression should further reinforce the feedback control effect that *Dkk1* exerts on Wnt/ β -catenin signaling.

In summary, we performed *in vitro* and *in vivo* experiments to demonstrate that the Wnt antagonist *Dkk1* is a direct target of osteoblast-specific transcription factor *Osx* in osteoblasts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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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Research highlights

- Dkk1 expression is down-regulated in the absence of Osx
- Overexpression of Osx activates Dkk1 expression
- Inhibition of Osx by siRNA results in repression of Dkk1 expression
- GC-rich sequences of Dkk1 promoter are responsible for Osx activation
- Osx associates with native Dkk1 promoter in vivo

Figure 1.

Effect of *Osx* on *Dkk1* expression. (A) Fold change in RNA levels from E15.5 wild-type and *Osx*-null mice embryos. Calvaria RNAs were isolated from E15.5 *Osx* wild-type and *Osx*-null embryos. (B) Fold change in RNA levels from E18.5 wild-type and *Osx*-null mice embryos. Calvaria RNAs were isolated from E18.5 *Osx* wild-type and *Osx*-null embryos. RNA levels for *Osx*, *OC* and *Dkk1* were analyzed by quantitative real-time RT-PCR. The level of each RNA from *Osx*-null calvaria was normalized to a value of 1. *: A star indicates statistical significance compared to *Osx* wild type group.

Figure 2.

Osx controls Dkk1 expression. (A) Western immunoblot analysis of the Dox-regulated Osx-expressing C2C12 cells. Osx expression was turned on in the absence of Dox. Beta-actin was used as a loading control. (B) Overexpression of Osx activates Dkk1 gene expression in C2C12 mesenchymal cells. Dkk1 mRNA levels in a stable, Tet-off C2C12 mesenchymal cell line. RNA was obtained from cultures treated with or without Dox. Osx expression was induced in the absence of Dox. Dkk1 mRNA levels were quantitated by real-time RT-PCR. The Dkk1 RNA level obtained from the cells cultured with Dox was normalized to a value of 1. Values are presented as the mean $\pm$ S.D. A paired *t*-test was performed comparing Dox (-) and Dox (+) groups. *: A star indicates statistical significance compared to Dox (+) group. (C) siRNA-directed knockdown of Osx impairs Dkk1 gene expression in MC3T3 osteoblasts. MC3T3 osteoblasts were transfected with siRNA targeting mouse Osx. RNA was isolated 24 hr posttransfection. RNA expression levels as determined by quantitative real-time RT-PCR. The RNA level from the control siRNA group was normalized to a value of 1. Values were presented as the mean $\pm$ S.D. A paired *t*-test was performed comparing si-control group and si-Osx group. *: A star indicates statistical significance compared to control group.

Figure 3.

Identification of the Osx binding site in the promoter of *Dkk1* gene. (A) Deletion analysis of *Dkk1* promoter reporter activated by Osx. Dkk1-2k, Dkk1-1k, Dkk1-500bp and Dkk1-250bp *Dkk1* promoter reporter plasmids were constructed. Each plasmid was cotransfected with Osx expression plasmid in HEK293 cells. Luciferase activity was normalized by β -galactosidase activity. (B) The two GC-rich elements are responsible for *Dkk1* promoter reporter activation by Osx. Dkk1-250-M1, Dkk1-250-M2 point mutants and double mutant

Dkk1-250-M12 were constructed. Each plasmid was cotransfected with Osx expression plasmid in HEK293 cells. Luciferase activity was normalized by β -galactosidase activity.

Figure 4.

Endogenous Osx in primary osteoblasts is associated with the native *Dkk1* promoter in vivo. Chromatin Immunoprecipitation (ChIP) assays were conducted using primary calvarial osteoblasts isolated from new born wild-type mice. Anti-Osx antibody (a-Osx) was used for ChIP analysis, and IgG was used as a negative control. The precipitated chromatin was analyzed by quantitative real-time PCR. As described in the Methods, primer Set 1 corresponds to a segment covering two GC-rich elements within 250bp the *Dkk1* promoter. As a negative control, Primer Set 2 covers a distal 3kb region of the *Dkk1* promoter, which does not contain GC-rich sequences. A paired *t*-test was performed comparing IgG and a-Osx group. *: A star indicates statistical significance compared to IgG control group.