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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 3, pp. 1671–1678, January 13, 2012

Transcriptional Regulation of Vascular Endothelial Growth Factor (VEGF) by Osteoblast-specific Transcription Factor Osterix (Osx) in Osteoblasts*

Received for publication, August 1, 2011, and in revised form, November 14, 2011 Published, JBC Papers in Press, November 22, 2011, DOI 10.1074/jbc.M111.288472

Wanjin Tang[‡], Fan Yang[‡], Yang Li[‡], Benoit de Crombrugghe[§], Hongli Jiao[¶], Guozhi Xiao[¶], and Chi Zhang[‡‖1]**

*From the [‡]Bone Research Laboratory, Texas Scottish Rite Hospital for Children, Dallas, Texas 75219, the [§]Department of Genetics, University of Texas M.D. Anderson Cancer Center, Dallas, Texas 77030, the [¶]Department of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15240, and the Departments of [‖]Orthopedic Surgery and **Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75390*

Background: Osx is an osteoblast-specific transcription factor required for bone formation and osteoblast differentiation.
Results: Osx directly targets vascular endothelial growth factor expression, whereas Osx controls osteoblast marker gene osteocalcin.
Conclusion: Osx regulates vascular endothelial growth factor expression in osteoblasts.
Significance: This is the first evidence that Osx controls vascular endothelial growth factor expression, suggesting a potential role of Osx in coordinating osteogenesis and angiogenesis.

Osterix (Osx) is an osteoblast-specific transcription factor required for bone formation and osteoblast differentiation. The critical step in bone formation is to replace the avascular cartilage template with vascularized bone. Osteogenesis and angiogenesis are associated with each other, sharing some essential regulators. Vascular endothelial growth factor (VEGF) is involved in both angiogenesis and osteogenesis. Transcriptional regulation of VEGF expression is not well known in osteoblasts. In this study, quantitative real-time RT-PCR results revealed that VEGF expression was down-regulated in *Osx*-null calvarial cells and that osteoblast marker osteocalcin expression was absent. Overexpression of Osx in stable C2C12 mesenchymal cells using a Tet-off system resulted in up-regulation of both osteocalcin and VEGF expression. The inhibition of Osx by siRNA led to repression of VEGF expression in osteoblasts. These results suggest that Osx controls VEGF expression. Transfection assays demonstrated that Osx activated *VEGF* promoter activity. A series of *VEGF* promoter deletion mutants were examined and the minimal Osx-responsive region was defined to the proximal 140-bp region of the *VEGF* promoter. Additional point mutants were used to identify two GC-rich regions that were responsible for *VEGF* promoter activation by Osx. Gel shift assay showed that Osx bound to the *VEGF* promoter sequence directly. Chromatin immunoprecipitation assays indicated that endogenous Osx associated with the native *VEGF* promoter in primary osteoblasts. Moreover, immunohistochemistry staining showed decreased VEGF protein levels in the tibiae of *Osx* conditional knock-out mice. We provide the first evidence that Osx controlled VEGF expression, suggesting a potential role of Osx in coordinating osteogenesis and angiogenesis.

There are two distinct processes of bone formation: endochondral ossification and intramembranous ossification. Endochondral ossification is responsible for the formation of most bones, such as long bones, and requires a cartilage intermediate. Fewer bones, such as craniofacial bones are formed by intramembranous ossification, in which bones form directly from mesenchymal condensations. Osteoblast differentiation from mesenchymal stem cells is controlled by various transcription factors and signaling proteins, including Indian hedgehog, Runx2, Osterix (Osx),[2] and the Wnt/β-catenin signaling pathway (1). Indian hedgehog belongs to the Hedgehog family. Indian hedgehog is required for endochondral ossification and needed for the activation of Runx2 (2). Both endochondral and intramembranous ossification require Runx2, which is involved in the differentiation of mesenchymal cells into preosteoblasts (3). *Osx*, a downstream gene of Runx2, is specifically expressed in osteoblasts and at low levels in prehypertrophic chondrocytes (4). Osx is essential for the commitment of preosteoblastic cell differentiation into mature osteoblasts. Cartilage develops normally in *Osx* knock-out embryos, but no bone is formed (4). It is described that osteoblast differentiation markers such as osteocalcin are not present in these embryos. The C terminus of Osx contains its DNA-binding domain, three C2H2-type zinc fingers, which are highly identical to the motif in Sp1, Sp3, and Sp4. Our recent observation that Osx inhibits the Wnt signaling pathway highlights the

* This work was supported, in whole or in part, by National Institutes of Health Grants AR059647 and DK072230 (to G. X.), a research grant from the Arthritis Foundation to the Bone Research Laboratory (to C. Z.), and Texas Scottish Rite Hospital for Children Grant RAP01 (to C. Z.).
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[1] To whom correspondence should be addressed: Bone Research Laboratory, Texas Scottish Rite Hospital for Children, University of Texas Southwestern Medical Center, 2222 Welborn St., Dallas, TX 75219. Tel.: 214-559-8468; Fax: 214-559-7875; E-mail: Chi5.Zhang@utsouthwestern.edu.

[2] The abbreviations used are: Osx, osterix; OC, osteocalcin; Tet, tetracycline; Dox, doxycycline; HIF-1α, hypoxia-inducible factor-1α; VEGF-140bp, VEGF 140-bp promoter region; VEGF-500bp, VEGF 500-bp promoter region.

potential for novel feedback control mechanisms involved in bone formation (5).

Angiogenesis and osteogenesis are coupled spatially and temporally in bone formation (6). Blood vessels provide oxygen and nutrients for bone growth. Mesenchymal origin cells, like osteoblasts, respond to oxygen and the nutrient supply level in bone. Disruption of the blood supply surgically significantly affects bone density, tensile strength, and the modulus of elasticity (7). Replacing the avascular cartilage template with highly vascularized bone is the key step of endochondral ossification. Vascular endothelial growth factor (VEGF) is an important mediator of angiogenesis and osteogenesis. When *VEGF* was inactivated in mice, it was found that blood vessel invasion was nearly abolished, concomitant with the impaired trabecular bone formation and an expansion of the hypertrophic chondrocyte zone (8). This suggests an essential role of VEGF in endochondral bone formation. Treatment of VEGF during the calvaria organ culture led to an increase in parietal bone thickness, demonstrating a stimulatory effect of VEGF on intramembranous ossification (9). VEGF is expressed in osteoblasts, and its expression pattern during osteoblast differentiation *in vitro* suggests that VEGF plays a positive role in the regulation of osteoblast activity (10). It has been demonstrated that VEGF secretion from osteoblastic cells increases as osteoblastogenesis proceeds and that the secreted VEGF exhibits high angiogenic power as to endothelial cell proliferation (11). These findings indicate that VEGF functions as the main angiogenic factor in the early stage of osteoblastogenesis.

VEGF is regulated by hypoxia. Hypoxia-inducible factor-1α (HIF-1α) is a master regulator of cellular response to hypoxia. For endochondral ossification, HIF-1α up-regulates VEGF and causes enhanced bone modeling (12). The loss of HIF-1α makes bone narrow and is less vascularized. Nevertheless, VEGF was still expressed in *HIF-1*α null mice indicating that besides HIF-1α, other factors are also involved in VEGF regulation during embryonic development (13). Runx2 is an essential regulator of both endochondral and intramembranous ossification (3). Runx2 has been shown to control VEGF expression in chondrocytes during endochondral bone formation (14). There is no VEGF expression in the hypertrophic chondrocytes of *Runx2* knock-out mice. Overexpression of Runx2 in fibroblasts induces an increase in *VEGF* mRNA and protein levels by up-regulating VEGF transcription (14). Osx controls osteogenesis as a downstream gene of Runx2, and it is required for osteoblast differentiation and bone formation (4). Runx2 is expressed in different cells and tissues, including osteobasts, chondrocytes, epithelial cells, glioma cells, brain tissues, and different tumor tissues (15). Different from Runx2, Osx is specifically expressed in osteoblasts and at low levels in prehypertrophic chondrocytes (4). VEGF expression is regulated by Runx2 in chondrocytes; however, relatively little is known about the regulation of VEGF expression in osteoblasts.

Previous studies have indicated a similar expression pattern between Osx and VEGF during osteoblast differentiation in several *in vivo* and *in vitro* model systems. *Osx* first appears in differentiating chondrocytes, the surrounding perichondrium, and mesenchymal condensations of future membranous bones of E13.5 mouse embryos. After E15.5, *Osx* is strongly expressed in cells that are associated with all bone trabeculae and bone collar formation (4). It has been demonstrated that blood vessels were recruited to the perichondrium of the developing mouse tibia at E13.5–14.5 through the actions of VEGF (16). The VEGF expression is at low levels in the early stage of osteoblast differentiation and greatly increases during terminal differentiation of osteoblasts (10). In *Osx*-null mice as described previously (4), cortical bone and trabecular bone were intact in wild-type mice, and trabecular bone was highly vascular, but no cortical bone and no trabecular bone were formed in *Osx*-null mice, so no vascularized bone was observed in *Osx*-null mice. Given the fact that *Osx* is considered a master regulator essential for the commitment of preosteoblast differentiation into mature osteoblasts (4, 5), we hypothesize that Osx may regulate VEGF expression. We report here for the first time that Osx controls VEGF expression in osteoblasts. These data suggest a potential role for Osx to coordinate angiogenesis and osteogenesis.

EXPERIMENTAL PROCEDURES

RNA Isolation and Real-time RT-PCR—Total RNA was isolated from calvaria from E18.5 wild-type and *Osx*-null mouse embryos with TRIzol reagent (Invitrogen) followed by RNeasy mini kit (Qiagen). TaqMan One-step RT-PCR Master Mix reagent (Applied Biosystems) was used for quantitative RT-PCR. The reaction volume is 50 μl/well on 96-well plates. Analysis was performed with the ABI PRISM 7500 sequence detection system (Applied Biosystems). Primers are designed by Applied Biosystems. Transcript levels were normalized to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) levels, using primers from Applied Biosystems. The relative mRNA expression levels were calculated according to the comparative C_T ($\Delta\Delta C_T$) method as described by the manufacturer (User Bulletin number 2, Applied Biosystems). Target quantity is normalized to endogenous control and relative to a calibrator, and is calculated using formula: target amount $= 2^{-\Delta\Delta Ct}$.

Cell Cultures—HEK293 cells from ATCC were grown in high glucose Dulbecco's modified Eagle's medium (Invitrogen, catalog number 11965) supplemented with 10% FBS and 100 units/ml of penicillin and 100 μg/ml of streptomycin. Cells were cultured in a 95% air, 5% CO_2 humidified incubator. Cells were trypsinized and plated before transfection. MC3T3 cells (ATCC) were cultured in α-minimum essential medium with ribonucleosides, deoxyribonucleosides, 2 mM L-glutamine, and 1 mM sodium pyruvate but without ascorbic acid (Invitrogen) and supplemented with 10% FBS and penicillin plus streptomycin. Stable C2C12 mesenchymal cells expressing Osx were generated with the pTet-off® Advanced Inducible Gene Expression System (Clontech) as previously described (5). Osx expression is induced in the absence of doxycycline (Dox), a member of the tetracycline (Tet) antibiotics group. C2C12 cells were cultured in Dulbecco's modified Eagle's medium with the following additives to maintain selection and control Osx expression; G418 (200 μg/ml), hygromycine (150 μg/ml), and with or without Dox (20 ng/ml).

Plasmid Constructs and Subcloning—Subcloning was performed as previously described with modifications (17). The progressive deletion fragments of the *VEGF* 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). The primer sequences were as follows: 1) VEGF-Xho-3, 5′-GCG CCT CGA GCT CTG CGC TTC TCA CCG GTA; 2) VEGF-Mlu-1K, 5′-GCG CAC GCG TTT CAG TTC CCT GGC AAC ATC; 3) VEGF-Mlu-500, 5′-GCG CAC GCG TAG ATC GTA ACT TGG GCG AGC; 4) VEGF-Mlu-270, 5′-GCG CAC GCG TGT CCG CAT ATA ACC TCA CTC; 5) VEGF-Mlu-140, 5′-GCG CAC GCG TCT TTC CAT TTC GCG GTA GTG. *VEGF* 2 point mutants were made with the QuikChange site-directed mutagenesis kit (Stratagene) using VEGF-140bp as a template with the following primers: 1) VEGF-M1–1, 5′-ACC AGA CCG TCC CCG AAA CAA ATC TGG GCG GGG CTT; 2) VEGF-M1–2, 5′-AAG CCC CGC CCA GAT TTG TTT CGG GGA CGG TCT GGT; 3) VEGF-M2–1, 5′-CCG GGG CGG GTC TGA ACA AAA CTT GGG GGT GGA GCT A; 4) VEGF-M2–2, 5′-TAG CTC CAC CCC CAA GTT TTG TTC AGA CCC GCC CCG G. All deletion and mutant constructs were verified by DNA sequencing.

Transient Transfection and VEGF Promoter-Luciferase Reporter Assay—HEK293 cells were plated in 12-well tissue culture dishes and transiently transfected with the 300 ng of a *VEGF* promoter-luciferase reporter gene, various amounts of Osx expression vector as indicated, and 25 ng of β-galactosidase plasmid, using FuGENE 6 reagent (Roche Diagnostics). After transfection, cells were incubated for 24 h before harvest. The reporter assays were analyzed with the BD Monolight system (BD Biosciences). Luciferase activities were normalized by β-galactosidase activities.

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 nonspecific control. Cells were cultured in 6-well plates. One day before transfection, cells were plated in 1 ml of the growth medium without antibiotics. Cells were 30–50% confluent at the time of transfection. For each sample, a siRNA-Lipofectamine 2000 transfection complex was prepared as follows: 1) 2 μl of 50 μM siRNA in 50 μl of Opti-MEM I reduced serum medium without serum was diluted; 2) mix gently Lipofectamine 2000, then dilute 3 μl in 50 μl of Opti-MEM I medium; 3) combine the diluted siRNA with the diluted Lipofectamine 2000; and 4) then add 100 μl of siRNA-Lipofectamine 2000 complex to each well. After a 4-h incubation, the growth medium was replaced. Cells were cultured at 37 °C in a CO_2 incubator for 24 h before harvest.

Protein Purification and Western Blot—Protein was isolated by acetone precipitation from buffer RLT lysates according to the RNeasy Mini Handbook (Qiagen). The protein pellet was dissolved in 1% SDS buffer, warmed for 15 min at 55 °C, and centrifuged for 5 min at 14,000 × *g*. Protein concentrations in the supernatant were determined using a BCA Protein Assay kit (Pierce). Proteins were separated on 10% SDS-PAGE gels and transferred to PVDF membrane followed by Western blot analysis. In brief, 5% milk in TBS containing 0.1% Tween 20 was used to block nonspecific binding. The blot was subsequently incubated with anti-VEGF rabbit polyclonal antibody (1:200, Abcam) or anti-Osx rabbit polyclonal antibody (1:200, Santa Cruz Biotechnology) and the secondary antibody (peroxidase anti-rabbit 1:5000, Sigma). After each antibody incubation blots were extensively washed in TBS containing 0.1% Tween 20. For detection, the ECL kit (Amersham Biosciences) was used according to the manufacturer instructions.

Baculovirus-mediated Expression of Osx—Osx cDNA was subcloned into the pBac vector. Recombinant baculovirus was isolated and plaque purified by standard methods as previously described (17). A 500-ml culture of Sf9 cells (2×10^6 cells/ml) was infected for 48 h with the Osx-expressing recombinant baculovirus. Nuclear extracts were prepared, and recombinant Osx was purified by nickel affinity chromatography.

Gel Shift Assay—Baculovirus-expressed Osx was used as the protein resource. The DNA sequences of the oligonucleotides used for the gel shift assay were as follows: VEGF wild-type, 5′-ACC AGA CCG TCC CCG GGG CGG GTC TGG GCG GGG CTT GGG GGT GGA GCT A and VEGF mutant, 5′-ACC AGA CCG TCC CCG AAA CAA ATC TGA ACA AAA CTT GGG GGT GGA GCT A. The DNA oligonucleotide was labeled using a Biotin 3′ end DNA Labeling Kit (catalog number 89,818, Pierce Biotechnology Inc.). The Osx protein and biotin-labeled DNA probe were incubated in 1× binding buffer for 20 min at room temperature using a LightShift Chemiluminescent EMSA kit (catalog number 20,148). Protein-DNA complexes were separated on 5% polyacrylamide gels in 0.5× TBE buffer, and transferred onto a Biodyne B Nylon Membrane (catalog number 77,016). The membrane was blocked in 1× blocking buffer, washed five times with 1× wash buffer, and visualized by a Chemiluminescent Nucleic Acid detection module (catalog number 89,880).

Chromatin Immunoprecipitation (ChIP) Assay—The ChIP assay kit was from Millipore. ChIP assays were performed according to a previously described protocol (18) with some modifications. Briefly, calvarial cells were isolated from wild-type newborn mice, and were cultured in DMEM supplemented with 10% FBS. Formaldehyde was used to cross-link the cells for 10 min, and cross-linking was quenched with glycine. Cells were harvested, rinsed with PBS, and cell pellets were resuspended in 1 ml of lysis buffer. After sonication, 100 μl of sheared chromatin was diluted to 1 ml with immunoprecipitation dilution buffer for each immunoprecipitation. The chromatin solution was pre-cleared with 60 μl of protein G-agarose beads at 4 °C for 1 h. The precleared chromatin was collected and incubated at 4 °C overnight with 5 μg of an anti-Osx antibody or IgG as a negative control. The immune complexes were precipitated with 60 μl of protein G-agarose beads at 4 °C for 1 h. After washes, the antibody-protein-DNA immunocomplexes were eluted twice with 100 μl of elution buffer. Formaldehyde cross-linking was reversed by heating at 65 °C overnight with the addition of 5 M NaCl. All the samples were digested with RNase A and proteinase K. The DNA was purified using spin columns, and analyzed by PCR gel and quantitative real time PCR. The primer sets used for amplification of the *VEGF* promoter regions were obtained from IDT, and the

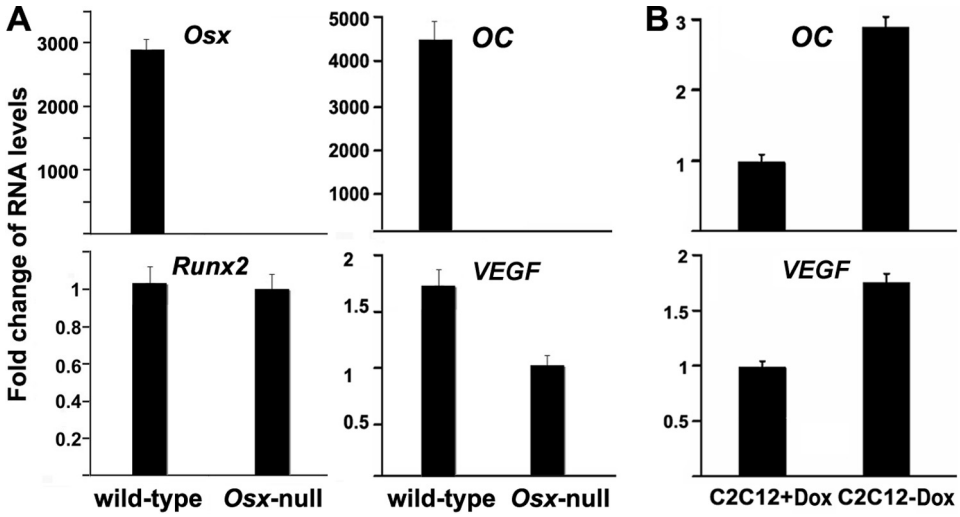

FIGURE 1. **Effect of Osx on VEGF expression.** *A*, in the absence of Osx, osteocalcin (*OC*), and VEGF mRNA levels were down-regulated. Fold-change in RNA levels is shown. Calvaria RNAs were from E18.5 *Osx* wild-type and *Osx*-null embryos. RNA expression levels for Osx, osteocalcin, Runx2, and VEGF were analyzed by real-time RT-PCR. The level of each RNA from *Osx*-null calvaria was normalized to a value of 1. Values are presented as the mean ± S.D. *B*, overexpression of Osx activates *VEGF* gene expression in C2C12 mesenchymal cells. Osx expression is induced in the absence of Dox in this cell line. RNA was obtained from cultures treated with or without Dox. VEGF and OC mRNA levels were quantitated by real-time RT-PCR. The VEGF RNA level obtained from the cells cultured with Dox was normalized to a value of 1. Fold-change in RNA levels is shown. Values are presented as the mean ± S.D.

sequences were as follows: primer set 1, VEGF-1, 5′ CTT TCC ATT TCG CGG TAG TG, and VEGF-2, 5′-CTC TGC GCT TCT CAC CGG TA; primer set 2, VEGF-D-1, 5′-TTC AGT TCC CTG GCA ACA TC, and VEGF-D-2, 5′ CGT ATG CAC TGT GTA GTC TG. Data were normalized by GAPDH.

Generation of Osx Postnatal Mutant Mice—*CAG-CreER* transgenic mice were purchased from the Jackson Laboratory. The *Osx-flox* mice were crossed with *CAG-CreER* to generate *CAG-CreER;Osx*$^{floxed/-}$ mice as previously described (19). The tamoxifen-treated *CAGCreER;Osx*$^{floxed/-}$ mice are referred to as *Osx*^{postnatal} mutant mice; the tamoxifen-treated *CAG-CreER;Osx*$^{floxed/+}$ mice are referred to as wild-type controls. *Osx*^{postnatal} mutant mice and wild-type controls were injected intraperitoneally with tamoxifen (Sigma) four times (twice with 3 mg/10 g of body weight and twice with 1.5 mg/10 g of body weight) from P16 to P26 and killed at week 6. These mice are designated 6-week mice.

Immunohistochemistry—Tibiae were fixed, decalcified, and embedded in paraffin. Sections of tibiae were stained with antibodies against VEGF using the EnVision$^+$System-HRP (DAB) kit (Dako North America, Inc., Carpinteria, CA) according to the manufacturer's instructions. Briefly, slides were baked at 55 °C for 45 min, deparaffinized in three washes of xylene, and rehydrated in a decreasing ethanol gradient. Antigen retrieval was performed using 0.1% trypsin for 10 min at 37 °C in a humidified chamber. Endogenous peroxidases were deactivated with 3% H_2O_2 in 1× PBS for 10 min, and sections were blocked in blocking solution for 30 min at room temperature. Sections were incubated with primary antibody (1:1000 dilution for rabbit anti-VEGF antibody, Santa Cruz, sc-152) in blocking solution for 2 h at 4 °C. Sections were washed in PBS three times and incubated with a donkey anti-rabbit IgG-HRP secondary antibody solution for 30 min at room temperature. After washing with PBS three times, HRP activity was detected using a dimethylaminoazobenzene substrate solution for 2 min at room temperature. Sections were counterstained with a Mayer's hematoxylin solution.

RESULTS

VEGF mRNA Levels Are Down-regulated in the Absence of Osx—Angiogenesis plays an important role in bone formation. Genetic manipulations in mice have provided evidence for a critical role of VEGF in coupling angiogenesis and osteogenesis (20). Osx is indispensable for osteoblast differentiation and bone formation (4). It would be interesting to study whether Osx is also a regulatory factor for VEGF in osteoblasts.

To identify the possible downstream targets of Osx, we performed quantitative real-time RT-PCR to compare RNA levels of several genes between wild-type and *Osx* knock-out mice. As shown in Fig. 1*A*, *Osx* expression was abolished in *Osx*-null calvaria, and the osteoblast marker gene osteocalcin (OC) expression was at an undetected level in *Osx* mutant mouse cells. This result is consistent with a previous *in vivo* finding that in Osx mutant mouse embryos, the OC mRNA expression was absent in both endochondral and membranous skeleton (4). As a negative control, *Runx2* expression was unchanged as an upstream gene of *Osx*. Interestingly, it was observed that the VEGF mRNA level was reduced by nearly 50% in *Osx*-null calvaria as shown in Fig. 1*A*. Thus, in the absence of *Osx*, both OC and VEGF mRNA levels were down-regulated in the calvaria of mice embryos.

Overexpression of Osx Results in Up-regulation of VEGF mRNA Levels—Both OC and VEGF levels were down-regulated in the absence of *Osx*, suggesting that Osx activates OC and VEGF expression. To test if OC and VEGF are positively regulated when Osx is overexpressed, we used a stable C2C12 mesenchymal cell line in which Osx expression could be induced by using the Tet-off system. The Osx expression is induced in the absence of Dox. Total RNA was isolated from two groups of cell lines grown in the presence (+) or absence (−) of Dox. Real-time RT-PCR was used to analyze the mRNA expression. As shown in Fig. 1*B*, in the absence of Dox when Osx was overex-

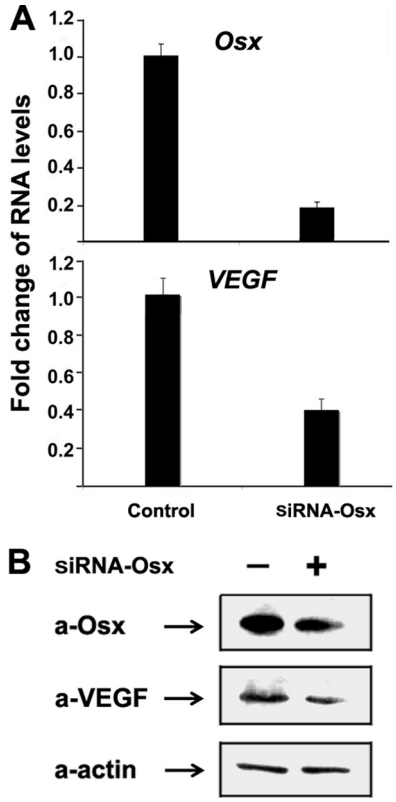

FIGURE 2. **siRNA-directed knockdown of Osx impairs *VEGF* gene expression in MC3T3 osteoblasts.** *A*, RNA expression levels were determined by quantitative real-time RT-PCR. MC3T3 osteoblasts were transfected with siRNA targeting mouse Osx. RNA was isolated 24 h post-transfection and quantitated by 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 ± S.D. *B*, Western analysis of the Osx knockdown. Protein was isolated by acetone precipitation of whole cell lysates and then analyzed by Western blot using rabbit anti-VEGF or anti-Osx polyclonal antibodies. β-Actin was used as a loading control.

pressed, there was a higher level of OC, about a 3-fold increase compared with control cells. Meanwhile, we observed higher levels of VEGF when Osx expression was induced, about a 2-fold increase compared with control cells as shown in Fig. 1*B*.

Inhibition of Osx by siRNA Leads to Repression of VEGF Expression in Osteoblasts—To further confirm the effect of Osx on VEGF expression, we used siRNA transfection to knockdown Osx expression in MC3T3 osteoblast cells to determine the possible change of VEGF expression. MC3T3 cells were chosen for this approach because they express higher, more readily detected levels of Osx and VEGF. Western blot and real-time RT-PCR were performed to analyze protein and mRNA expression levels, respectively. The decrease of VEGF protein expression occurred with knockdown of the Osx protein expression level by siRNA as shown in Fig. 2*B*. When the Osx RNA expression was decreased 80% by siRNA against Osx, VEGF RNA expression was repressed by 60% as shown in Fig. 2*A*. Therefore, these data suggest that Osx positively regulates VEGF in osteoblasts.

Osx Regulates the VEGF Promoter Activity in a Dose-dependent Manner—Results from primary mouse calvaria cells and a stable C2C12 mesenchymal cell line suggest that Osx controls VEGF expression, whereas it activates osteocalcin. To test the effect of Osx on *VEGF* promoter activity, we performed subcloning to generate a luciferase reporter construct driven by a 1-kb *VEGF* native promoter. HEK293 cells were transiently transfected with the *VEGF* promoter-luciferase reporter gene and Osx expression vector. HEK293 cells were chosen because they are very easy to grow and have high transfection efficiency for the transient transfection assay used in our current study. As shown in Fig. 3*A*, increasing amounts of Osx induced markedly higher *VEGF* promoter activities, and transfection with 800 ng of Osx resulted in a 20-fold increase of *VEGF* promoter activity, demonstrating that Osx regulates *VEGF* promoter activities in a dose-dependent manner. These results are in agreement with our data from real-time RT-PCR experiments in a C2C12 stable cell line (Fig. 1*B*), indicating that Osx regulates VEGF positively.

Identification of Osx Binding Site in Promoter of VEGF Gene—We have shown that Osx can activate *VEGF* promoter reporter activity; however, it is not known which region within the *VEGF* promoter is responsible for Osx activation. To address this question, *VEGF* luciferase reporter constructs driven by different lengths of the *VEGF* promoter region were generated by subcloning. Transient transfection assays were then performed to narrow down a responsible region within the 1-kb *VEGF* promoter for Osx activation. As shown in Fig. 3*B*, Osx was able to activate the *VEGF* promoter reporters of VEGF-1kb, VEGF-500bp, VEGF-270bp, and VEGF-140bp in a transient transfection assay. These data suggest that there may be critical binding sites for Osx in the VEGF-140bp promoter region. It was demonstrated in a previous report that Osx belongs to the SP/KLF family of transcription factors that bind to GC-rich sequences of target gene promoters to regulate gene expression (4). According to sequence analysis of the 140-bp region of the *VEGF* promoter, there are two tentative binding sites for Osx as GC-rich elements. To study which binding site is responsible for *VEGF* promoter activation by Osx, two point mutants of the VEGF-140bp promoter reporter were generated by the QuikChange site-directed mutagenesis kit (Stratagene) using VEGF-140bp as a template. We named the two mutants VEGF-M1 and VEGF-M2 in which the GC-rich element was replaced with A (Fig. 3*D*). As shown in Fig. 3*C*, our results indicated that VEGF-M1 and VEGF-M2 mutants inhibited Osx activation of the VEGF-140bp promoter reporter by 53 and 50%, respectively. Then we asked what might be the effect of the mutations on the VEGF-140bp promoter reporter if both tentative binding sites were mutated at the same time. We generated double mutants of VEGF-M1 and VEGF-M2, and named it VEGF-M12. As shown in Fig. 3*C*, VEGF-M12 with mutations in both tentative binding sites almost abolished Osx activation of the VEGF-140bp promoter reporter. Thus, these results revealed that two GC-rich sequences in the VEGF-140bp region were responsible for *VEGF* promoter activation by Osx.

Osx Associates with Native VEGF Promoter—To confirm Osx binding to the *VEGF* promoter, we performed a gel shift assay. As shown in Fig. 4*A*, Osx was able to bind to *VEGF* promoter oligos (*lane 2*), and Osx binding was abolished by VEGF mutant oligos replacing the GC-rich sequence with A (*lane 3*), which were used to test binding specificity. The data indicated that Osx bound to the *VEGF* promoter oligos specifically. The studies above indicate that Osx can positively regulate the VEGF

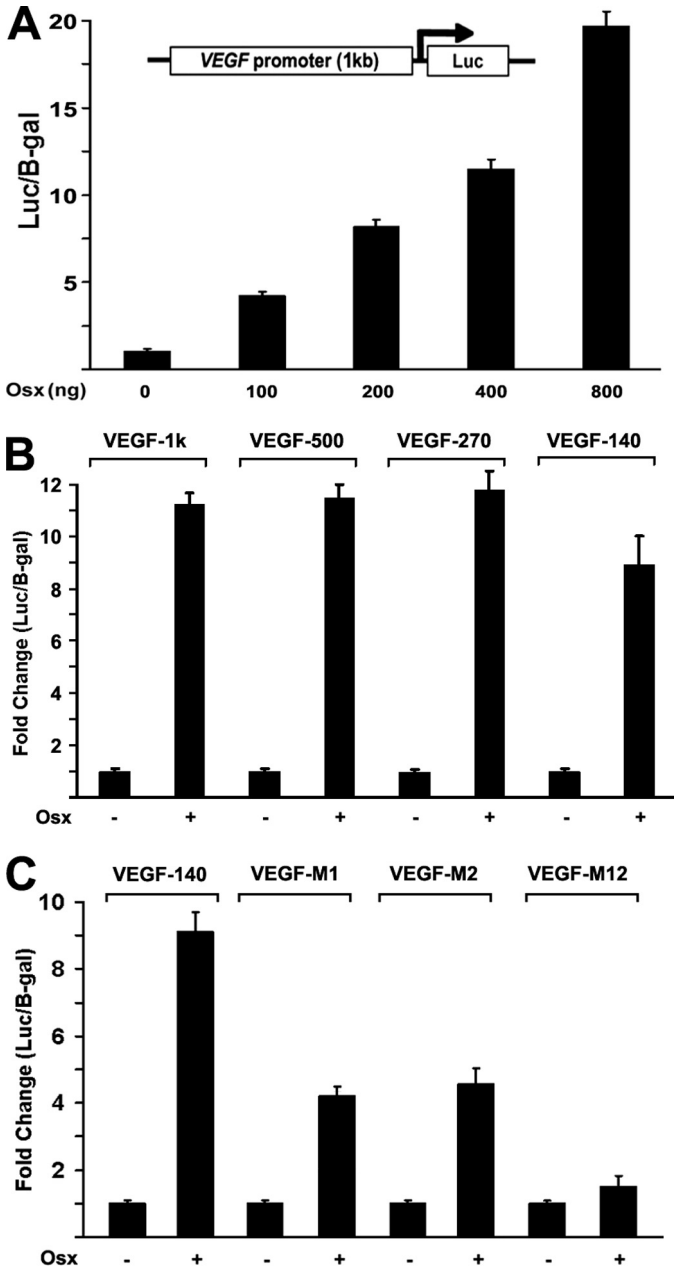

FIGURE 3. **Identification of the Osx binding site in the promoter of *VEGF* gene.** *A*, Osx regulates *VEGF* promoter activities in a dose-dependent manner. HEK293 cells were plated in 12-well tissue culture dishes. Each group of the cells was transiently transfected with 300 ng of promoter-luciferase reporter gene and the indicated amount of osterix expression vector. Cells were analyzed 24 h after transfection. Luciferase activity was normalized by β-galactosidase activity. Values are presented as the mean ± S.D. *B*, deletion analysis of the *VEGF* promoter-reporter constructs. VEGF-1kb, VEGF-500bp, VEGF-270bp, and VEGF-140bp promoter-reporter plasmids (300 ng each) were cotransfected with 400 ng of the Osx expression plasmid in HEK293 cells. After 24 h transfection, cell extracts were prepared and analyzed for luciferase activity and normalized to β-galactosidase activity. *C*, two GC-rich elements in VEGF-140bp are responsible for *VEGF* promoter reporter activation by Osx. VEGF-M1, VEGF-M2, and VEGF-M12 are individual mutants of the VEGF-140bp construct. They were transfected as described in *panel B*. Luciferase activity was normalized by β-galactosidase activity. *D*, a diagram of the proximal 140-bp region of the mouse *VEGF* promoter. *M1* refers to point mutations of VEGF-M1, and *M2* refers to point mutations of VEGF-M2. VEGF-M12 in *C* contains both M1 and M2.

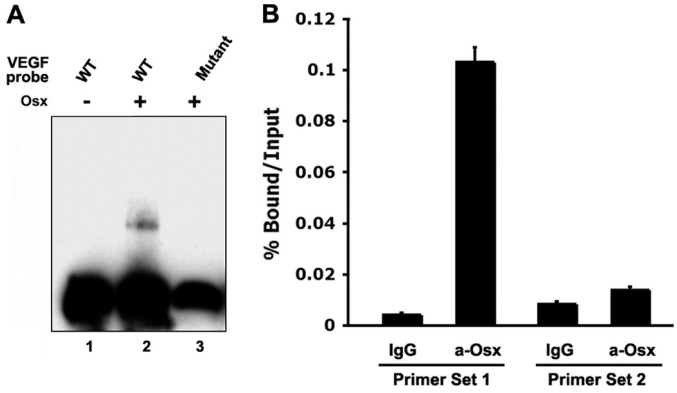

FIGURE 4. **Osx associates with *VEGF* promoter.** *A*, Osx bound to *VEGF* promoter oligos in a gel shift assay. DNA oligonucleotides of VEGF wild-type (*WT*) and mutant were labeled by Biotin. Osx protein and the biotin-labeled DNA probe were incubated. Protein-DNA complexes were separated on 5% polyacrylamide gels, and visualized by a chemiluminescent nucleic acid detection module. VEGF WT oligos were incubated with control buffer (*lane 1*) or Osx (*lane 2*). VEGF mutant oligos (*lane 3*) were used. Baculovirus-expressed Osx was used as the protein resource. *B*, endogenous Osx associated with native *VEGF* promoter *in vivo* analyzed by quantitative real-time PCR. The ChIP assay was carried out in primary calvarial osteoblasts isolated from newborn wild-type mice. Anti-Osx antibody was used for ChIP analysis, and IgG was used as a negative control. The precipitated chromatin was analyzed by quantitative real-time PCR. Primer set 1 corresponds to a segment covering two GC-rich elements within 140 bp the *VEGF* promoter. As a negative control, primer set 2 covers a distal 1-kb region of the *VEGF* promoter, which does not contain GC-rich sequence.

expression and activate the *VEGF* promoter activity *in vitro*, and that two GC-rich sequences in the *VEGF* promoter are responsible for Osx activation. It is currently unknown whether endogenous Osx can associate with the native *VEGF* promoter *in vivo*. To address this question, a chromatin immunoprecipitation (ChIP) assay was carried out to examine whether Osx could bind to the native *VEGF* promoter in primary calvarial osteoblasts isolated from newborn wild-type mice. Cross-linked extracts were immunoprecipitated with antibodies against Osx or control IgG. Following reversal of the cross-links, DNA was recovered and analyzed by quantitative real-time PCR using primers designed to amplify the Osx-responsive region covering the two GC-rich sequences of VEGF-140-bp promoter (primer set 1) or a distal upstream 1-kb, non-responsive region (primer set 2) as a control to demonstrate response element selectivity. Fig. 4*B* demonstrated that Osx was associated with the *VEGF* promoter region containing the two GC-rich sequences (primer set 1) compared with IgG control group. However, Osx was not associated with the *VEGF* distal 1k promoter region without the GC-rich sequence (primer set 2); therefore, the Osx-DNA association was specific. These data indicate that endogenous Osx associates with the native *VEGF* promoter in primary osteoblasts *in vivo*.

VEGF Protein Level Is Decreased in Osteoblasts of Conditional Osx-null Mice—To examine the effect of Osx on the VEGF protein level in osteoblasts in mice, we used *Osx* condi-

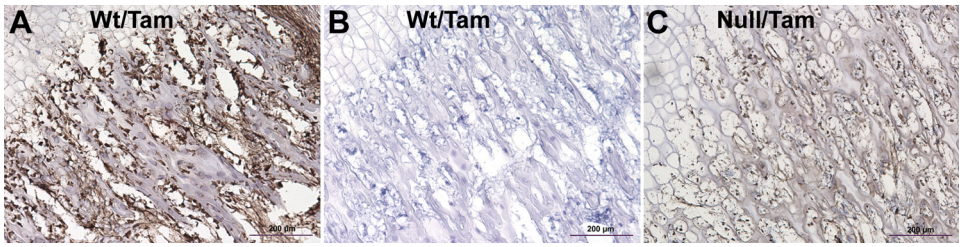

FIGURE 5. **VEGF protein level is decreased in osteoblasts of conditional *Osx*-null mice.** *A*, immunohistochemistry analysis using anti-VEGF antibody in tibiae of tamoxifen-treated wild-type mice. *B*, immunohistochemistry analysis using anti-IgG antibody in tibiae of tamoxifen-treated wild-type mice. *C*, immunohistochemistry analysis using anti-VEGF antibody in tibiae of tamoxifen-treated $Osx^{postnatal}$ mutant mice. Immunohistochemistry was performed with VEGF antibody or IgG on the decalcified paraffin sections of tibiae of tamoxifen-treated 6-week-old mice. Representative images of immunohistochemistry sections of tibiae were shown. *Wt/Tam*, tamoxifen-treated wild-type mice; *Null/Tam*, tamoxifen-treated $Osx^{postnatal}$ mutant mice.

tional knock-out mice. Osteogenic cells are completely blocked in their differentiation into osteoblasts in conventional *Osx*-null mice, which is lethal, so there is no mature osteoblast (4). Therefore, the *Osx* conditional knock-out mice model was used to address this question. The *CAG-CreER;*$Osx^{floxed/-}$ mice and wild-type controls were injected with tamoxifen from P16 to P26 and killed at week 6. We used immunohistochemistry to measure the VEGF protein level in the tibiae. The trabecular area of tibial sections was examined in the proximal metaphysis beginning immediately below the chondro-osseous junction to the mid-tibia. In the tibiae of tamoxifen-treated wild-type controls, VEGF-positive osteoblasts were identified on all surfaces of trabeculae and endosteum throughout the tibia as shown in Fig. 5*A*. The immunohistochemistry staining was highly specific because no signal was detected in the non-immune IgG control group as shown in Fig. 5*B*. In contrast, in the $Osx^{postnatal}$ mutants, there was the markedly reduced trabeculae bone in tibiae, and the trabeculae became disorganized. The VEGF signal was significantly reduced compared with the wild-type group as shown in Fig. 5*C*. These data indicated that the VEGF protein level was decreased in osteoblasts in mice in which Osx was inactivated, supporting our *in vitro* observations that Osx regulates VEGF positively.

DISCUSSION

Osx is an osteoblast-specific transcription factor required for osteoblast differentiation and bone formation. Despite the discovery of its significance in skeletal physiology a decade ago (4), relatively little is known about direct target genes for Osx and molecular mechanisms through which Osx controls gene transcription. Here we have presented evidence for one novel property of the osteoblast-specific transcription factor Osx: Osx directly targets VEGF expression. Our result showed for the first time that Osx positively regulates VEGF expression while inducing osteoblast differentiation. This is supported by the Osx-mediated gene expression studies showing coordinate expression of Osx and VEGF in several systems. For example, *Osx*-null calvaria displayed reduced *in vivo* expression of VEGF compared with wild-type calvaria in mice (Fig. 1*A*) and a Tet-off inducible cell system revealed that the ectopic expression of Osx resulted in the *VEGF* transcript level increase (Fig. 1*B*). The osteoblastic cell line MC3T3 also had markedly impaired *VEGF* gene expression when Osx expression was knocked-down using siRNA targeting strategies (Fig. 2). Importantly in further transfection experiments, a direct regulation of *VEGF* gene transcription by Osx was evident in the ability of recombinant Osx to activate the *VEGF* promoter-reporter constructs (Fig. 3), thus indicating that the RNA expression studies were likely due to the effects of Osx expression on *VEGF* gene transcription.

This study also addresses molecular mechanisms through which Osx controls VEGF gene transcription. 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 C_2H_2-type zinc fingers located within its C terminus (4). In this study, the results of the promoter sequence mutants help to support that Osx shares the SP/KLF family to bind to the GC-rich sequence of the target gene promoter. We previously reported on similar elements controlling Osx activation of the *DKK1* and sclerostin promoters (5, 21). Indeed, two such GC-rich regions exist in the most proximal regions of the murine *VEGF* promoter residing adjacent to its transcriptional start site. Our studies define these two elements as critical for mediating transcriptional activation of the *VEGF* promoter by Osx. Mutation of each element (M1 or M2) reduced Osx-directed activation of the *VEGF* promoter by ~50% and mutation of both elements nearly abolished the response (Fig. 3). A gel shift assay further supported that Osx was bound to the *VEGF* promoter sequence directly (Fig. 4*A*). 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, chromatin immunoprecipitation approaches helped substantiate the reporter gene expression studies. These studies clearly demonstrated that endogenous Osx was associated with this GC-rich proximal region of the native *VEGF* promoter in primary osteoblasts (Fig. 4*B*). These studies help establish Osx mechanisms involving direct binding of Osx to sequence-specific GC-rich promoter elements to activate the *VEGF* expression in osteoblasts. Moreover, immunohistochemistry staining showed decreased VEGF protein levels in tibiae of *Osx* conditional knock-out mice (Fig. 5), supporting our *in vitro* observations that Osx regulates VEGF expression.

During bone formation, angiogenesis and osteogenesis are tightly coupled (22). The precise mechanisms that couple osteogenesis to angiogenesis are not yet clear, but it seems to require cross-talk between osteoblast lineage cells and vascular endothelial cells (23). The significant finding of this study is that Osx controlled VEGF expression while inducing osteoblast dif-

ferentiation, thus suggesting a potential role for Osx in coordinating osteogenesis and angiogenesis. Although additional studies need to address other cell systems and the ultimate effects of Osx and VEGF regulation on the full osteoblast differentiation pathway, these early stage studies provide a sound biological context and relevance for Osx activation of *VEGF* gene expression in osteoblasts.

Acknowledgment—We thank Dr. Xin Zhou, University of Texas MD Anderson Cancer Center, for technical support in Osx conditional knock-out mice.

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