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Osteoblast-specific transcription factor Osterix (Osx) and HIF-1α cooperatively regulate gene expression of vascular endothelial growth factor (VEGF)

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ABSTRACT

Bone formation is a highly regulated process involving the differentiation of mesenchymal stem cells to osteoblasts. Angiogenesis and osteogenesis are tightly coupled during bone formation. Vascular endothelial growth factor (VEGF) is involved in both processes. Relatively little is known about VEGF gene regulation in osteoblasts. Osterix (Osx) is a bone morphogenetic protein 2 (BMP-2) inducible osteoblast-specific transcription factor required for osteoblast differentiation and bone formation. Our recent study has demonstrated that Osx controls VEGF expression in osteoblasts. Here, we further characterized Osx regulation of VEGF. To address which domain of Osx is responsible for VEGF regulation, the deletion mutant analysis and transfection assay were carried out to show that proline-rich region (PRR) is required for Osx activation of VEGF promoter activity. Hypoxia-inducible factor-1α (HIF-1α) has been reported to couple angiogenesis to osteogenesis, and to upregulate VEGF. Effect of Osx on HIF-1α expression was examined in this study. Quantitative RT-PCR results revealed that HIF-1α remained unchanged between wild type and *Osx* knockout calvaria at E18.5 in mouse embryos. Overexpression of Osx in stable C2C12 mesenchymal cells using Tet-off system did not affect HIF-1α expression. HIF-1α level did not change after Osx inhibition by siRNA in osteoblasts. Moreover, BMP-2 stimulation led to upregulation of Osx and VEGF, but not HIF-1α. These results demonstrate that HIF-1α is not a downstream target of Osx in osteoblasts, suggesting that Osx regulation of VEGF is independent of HIF-1α expression level. Interestingly, synergistic interplays were observed between Osx and HIF-1α in VEGF promoter activation in transfection assay. Our findings indicate that Osx and HIF-1α cooperatively regulate VEGF expression.

1. Introduction

Endochondral ossification and intramembranous ossification are two distinct processes of bone formation. Most bones form by endochondral ossification, such as long bones, which requires a cartilage intermediate. Fewer bones, such as craniofacial bones, form directly from mesenchymal condensations without cartilage template by intramembranous ossification. Osteoblast differentiation from mesenchymal stem cells is controlled by various transcription factors and signaling proteins, including Indian Hedgehog, Runx2, Osterix (Osx), and Wnt pathway [1]. Indian Hedgehog is requisite for endochondral ossification and indispensible for the initial activation of Runx2 [2]. Runx2 is required for both endochondral and membranous ossification and needed for mesenchymal cell differentiation into preosteoblasts [3]. Osx, downstream of Runx2, is specifically expressed in osteoblasts and of low amount in prehypertrophic chondrocytes [4]. Osx was first discovered as a bone morphogenetic protein 2 (BMP-2) inducible gene in mesenchymal stem cells. *Osx* knockout embryos lack bone formation, while cartilage develops normally. 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. The discovery that Osx inhibits the Wnt pathway highlights the 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 nutrient for bone growth. Mesenchymal origin cells, like osteoblasts, respond to oxygen and nutrient supply level in bone.

Abbreviations: Osx, Osterix; VEGF, vascular endothelial growth factor; HIF-1α, Hypoxia-inducible factor-1α; PRR, proline-rich region; E18.5, embryonic day 18.5; Dox, doxycycline; BMP-2, bone morphogenetic protein 2; GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

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Replacing the avascular cartilage template with highly vascularized bone is the key step of endochondral ossification. During endochondral bone formation, chondrocytes model the growth plate at the long bone distal ends and become hypertrophic and hypoxic. Blood vessel invasion from the metaphyseal region into the avascular cartilage coincides with bone formation on the cartilaginous template. Vascular endothelial growth factor (VEGF) is involved in both angiogenesis and osteogenesis. Histological evidence indicates that osteoblasts develop together with endothelial cells in new blood vessels. VEGF enhances vascular permeability, angiogenesis, and regulates endochondral ossification [7]. It has been demonstrated that VEGF secretion increases from osteoblastic cells as osteoblastogenesis proceeds [8]. When *VEGF* was inactivated in mice, it was found that blood vessel invasion was nearly abolished, trabecular bone formation was impaired [9]. These observations provided direct evidence that VEGF plays a critical role in bone formation. The nature of the cellular and molecular mechanisms for coupling angiogenesis and osteogenesis remain poorly understood.

VEGF is a well-characterized angiogenic factor that is activated by hypoxia. Hypoxia-inducible factor-1α (HIF-1α) is a master regulator of cellular response to hypoxia. HIF-1α is a conserved transcription factor known to activate many angiogenic genes, including *VEGF*. For endochondral ossification, HIF-1α upregulates VEGF, and causes enhanced bone modeling [10]. The loss of *HIF-1α* makes bone narrow and less vascularized. Nevertheless, VEGF was still expressed in *HIF-1α* null mice, indicating that besides HIF-1α, other factors are also involved in VEGF regulation during embryonic development [11]. Transcriptional regulation of VEGF expression is not well known in osteoblasts. Our recent studies have provided the first evidence that Osx directly targets VEGF expression, involving direct binding of Osx to sequence specific, GC-rich promoter elements to activate the VEGF expression in osteoblasts [12]. The observations indicate that Osx positively regulates VEGF expression while inducing osteoblast differentiation, suggesting a potential role for Osx in coordinating osteogenesis and angiogenesis.

In this study, we further characterized VEGF regulation by Osx. We found that proline-rich region (PRR) is required for Osx activation of VEGF promoter activity, and that Osx cooperated with HIF-1α to positively regulate VEGF.

2. Materials and methods

2.1. Animal and genotype

Wild type and *Osx* knockout mice are from C57BL genetic background. All mice were bred and maintained in a specific pathogen-free facility. Mice were genotyped using genomic DNA isolated from the tails. PCR genotyping was performed with two sets of primers: Osx5 and Osx3 for the wild type allele and bpA and Osx3 for the mutant allele, producing 286 and 395 bp PCR fragments, respectively as previously described [4].

2.2. Plasmid constructs and cell cultures

pEX–Osx plasmid and mutants were subcloned using unique restriction sites of BamHI and BglII and used as previously described [5]. pGL3-VEGF-1 kb was subcloned into the XhoI and NheI sites of pGL-3 vector as previously described [12]. All deletion and mutant constructs were verified by DNA sequencing. PIP2N-HIF-1α plasmid is from Dr. Joseph Garcia in University of Texas Southwestern Medical Center. HEK293 cells (ATCC) were grown in Dulbecco's Modified Eagle Medium (GIBCO) supplemented with 10% FBS and 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were cultured in 95% air/5% CO_2 humidified incubator. Cells were trypsinized and plated before transfection. Stable C2C12 mesenchymal cells expressing Osx were generated with pTet-off® Advanced Inducible Gene Expression System (Clontech) as previously used [5]. Osx expression is 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. MC3T3 cells (ATCC) were cultured in Alpha Minimum Essential Medium with ribonucleosides, deoxyribonucleosides, 2 mM L-glutamine and 1 mM sodium pyruvate (GIBCO) and supplemented with 10% FBS and penicillin plus streptomycin. For C2C12 mesenchymal cell experiments stimulated by BMP-2 (R&D Systems), BMP-2 (300 ng/ml) was added into the medium, and RNA was isolated and analyzed after 24 h.

2.3. RNA isolation and real-time RT-PCR

Total RNA was isolated from calvaria of 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. Reaction volume is 50 μl per well on 96-well plates. Analysis was performed with ABI PRISM 7500 sequence detection system (Applied Biosystems). Primers were ordered from Applied Biosystems. Transcript levels were normalized to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) levels. All reactions were done in duplicate and all experiments were repeated at least three times.

2.4. Western blot

Proteins were isolated by acetone precipitation from C2C12 cells. Proteins were separated on 10% SDS–PAGE gels and transferred to a PVDF membrane followed by Western blot. Briefly, 3% milk in TBS containing 0.1% Tween-20 was used to block non-specific binding. The blot was incubated with an anti-Osx rabbit polyclonal antibody (1:200, Santa Cruz) or an anti-β-actin polyclonal antibody (1:200, Santa Cruz) followed by a secondary antibody (peroxidase-conjugated anti-rabbit IgG 1:5000, Sigma). After antibody incubation and washes, the ECL kit (Amersham Life Sciences) was used for detection.

2.5. siRNA interference

MC3T3 cells were transfected with siRNA directed 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. Cells were plated in 1 ml of growth medium without antibiotics 1 day prior to transfection and the cells were 30–50% confluent at the time of transfection. The final siRNA concentration was 100 nM and each well received 100 μl of the siRNA:Lipofectamine· 2000 complex in Opti-MEM I medium.

2.6. Transient transfection and VEGF promoter-luciferase reporter assay

HEK293 cells were plated in 12-well tissue culture dishes and transiently transfected with 300 ng pGL3-VEGF-1 kb promoter-luciferase reporter, Osx expression vector as indicated, various amounts of HIF-1α as indicated and 25 ng β-galactosidase plasmid, using FuGENE 6 reagent (Roche). 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.

3. Results

3.1. Proline-rich region (PRR) is required for Osx activation of VEGF promoter activity

We have shown that Osx activates VEGF promoter activity [12]; however it is not clear which domain of Osx is responsible for VEGF activation. Osx contains a proline-rich region (PRR) domain toward the N-terminal part of the protein and a three zinc finger DNA-binding motif in the C-terminal region. To determine which domain of Osx was responsible for VEGF activation, a series of deletion mutants in both N-terminal and C-terminal parts was used as indicated in Fig. 1A. 300 ng Osx wild type and mutants were transfected into HEK293 cells along with the VEGF-1 kb promoter reporter. Fig. 1B showed that the Osx mutants with the full PRR region, M1 and M4, were able to activate VEGF reporter activity like Osx wild type plasmid, whereas the Osx mutants without a complete PRR region, M2, M3, M5 and M6, disrupted Osx activation of VEGF reporter activity. Therefore, these results demonstrate that the PRR region is required for the Osx regulation of VEGF.

3.2. HIF-1α is not a downstream target of Osx in osteoblasts

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 [13]. Mechanisms of coupling angiogenesis and osteogenesis remain unclear, and one of the driving forces is hypoxia. Hypoxia activates HIF-1α in osteoblasts, and HIF-1α upregulates VEGF. Previous studies have suggested that besides HIF-1α, other factors may control VEGF expression during bone formation [11]. As an indispensible factor for bone formation, Osx has been shown to activate VEGF expression in osteoblasts [12]. Next, we asked whether HIF-1α is a downstream target of Osx.

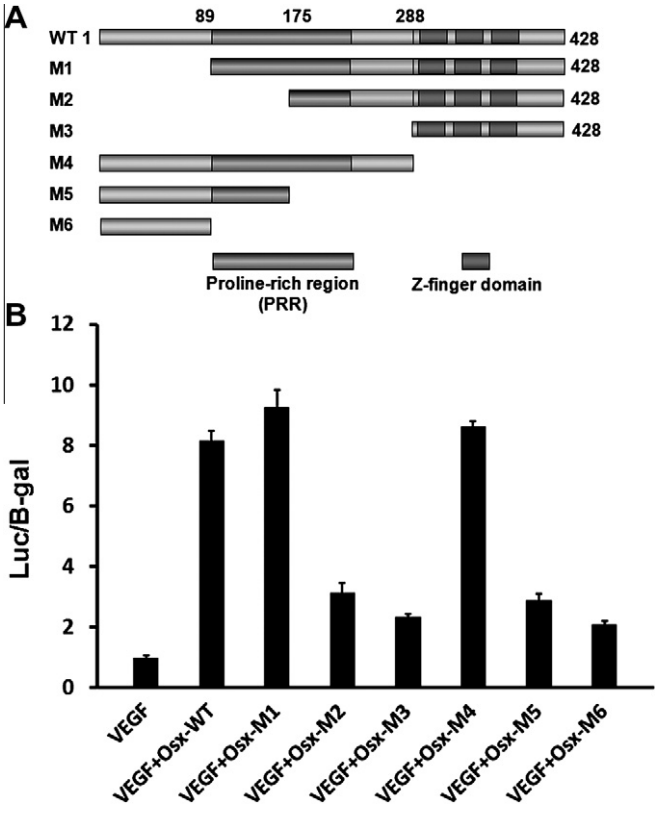

Fig. 1. Proline-rich region (PRR) is required for Osx to activate VEGF promoter activity. (A) Schematic representation of the *Osx* deletion mutants. PRR: Proline-rich region; WT: wild type; M: mutant. (B) Osx PRR domain is required for VEGF promoter activation. HEK293 cells were plated in 12-well tissue culture dishes. Each group of the cells was transiently transfected with 300 ng VEGF-1 kb promoter-luciferase reporter and 300 ng Osx wild type (WT) or mutants' expression vector. Cells were analyzed 24 h after transfection. Luciferase activity was normalized by β-galactosidase activity. Values were presented as the mean ± S.D.

RNA was isolated from both wild type and *Osx*-null calvaria at E18.5 in mouse embryos and analyzed by quantitative real-time RT-PCR. As shown in Fig. 2A, *Osx* was readily detected in wild type calvaria and predictably absent in *Osx*-null calvaria; however, *HIF-1α* gene remained unchanged. This suggests that *Osx* is not required for HIF-1α expression. To examine if Overexpression of Osx can upregulate HIF-1α, we used stable cell line in C2C12 mesenchymal cells using Tet-off system. In this cell line, Osx expression is induced in the absence of doxycycline (Dox), and Osx is not expressed when Dox is present [5]. As shown in Fig. 2B, expression of Osx is dramatically induced upon removal of Dox. Total RNA was purified from this cell line following culture in the presence or absence of Dox, and HIF-1α expression was quantitated by real-time RT-PCR. Fig. 2C showed that, in the absence of Dox (i.e., overexpression of Osx), HIF-1α expression did not change. This observation indicates that ectopic Osx expression cannot activate HIF-1α gene. To further confirm the effect of Osx on HIF-1α expression, we used siRNA transfection to knockdown Osx expression in MC3T3 osteoblast cells to determine HIF-1α level. Real-time RT-PCR was performed to analyze mRNA expression level. As shown in Fig. 2D, when Osx RNA expression was decreased by 80% by siRNA against Osx, HIF-1α RNA expression remained in the same level. Thus, these data suggest that HIF-1α is not a downstream target of Osx in osteoblasts.

3.3. BMP-2 stimulation led to upregulation of both Osx and VEGF, but not HIF-1α

Osx was first discovered as a BMP-2 inducible gene in C2C12 mesenchymal stem cells [4]. Our recent results showed that VEGF expression was enhanced when Osx was turned on in Tet-inducible stable C2C12 cells [12]. It is not known whether expressions of VEGF and HIF-1α are affected while BMP-2 induces Osx expression in C2C12 mesenchymal stem cells. To address this question, C2C12 cells were stimulated by BMP-2. Cells were harvested 24 h following the addition of BMP-2. RNA was isolated from control group and BMP-2 group followed by quantitative real-time RT-PCR analysis. As shown in Fig. 3A, Osx expression was induced by 13.5-fold after BMP-2 stimulation as expected. In this system, BMP-2 stimulation led to 2.4-fold upregulation of VEGF expression (Fig. 3B). These observations are in agreement with our previous results that ectopic Osx expression led to upregulation of VEGF in Tet-inducible stable C2C12 cells [12]. However, expression of HIF-1α did not change after BMP-2 stimulation as Osx and VEGF did (Fig. 3C). This supports our results in Fig. 2 that HIF-1α is not a downstream target of Osx. Taken together, our data demonstrate that Osx regulation of VEGF is independent of HIF-1α expression level.

3.4. HIF-1α stimulates VEGF-1kb promoter activity in a dose-dependent manner

It is known that HIF-1α upregulates VEGF for endochondral ossification [10]. Here we tested the effect of HIF-1α on VEGF promoter reporter we cloned. HEK293 cells were transiently transfected with VEGF-1 kb promoter reporter and HIF-1α expression vector. Expression plasmid p1p2n HIF-1α is a HIF-1α mutant which is constitutively active as previously used [14]. As shown in Fig. 4A, increasing amounts of HIF-1α transfection caused significantly higher expression of VEGF reporter, indicating that HIF-1α activated VEGF promoter activity in a dose-dependent manner.

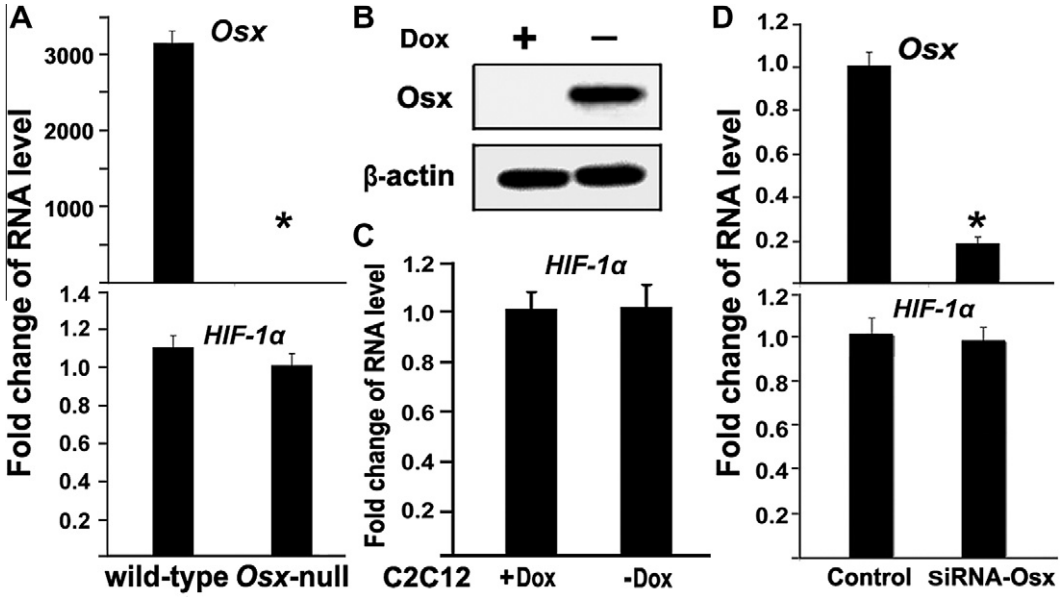

Fig. 2. Effect of Osx on HIF-1α expression. (A) Fold change in RNA level from E18.5 wild type and *Osx*-null mice embryos. Calvaria RNAs were isolated from E18.5 *Osx* wild type and *Osx*-null embryos. RNA levels for Osx and HIF-1α were analyzed by quantitative real-time RT-PCR. The level of each RNA from *Osx*-null calvaria was normalized to a value of 1. *: A star indicates statistical significance compared to *Osx* wild type group. (B) Western blot analysis of the Dox-regulated Osx expression in C2C12 stable cells. Osx expression was turned on in the absence of Dox. Beta-actin was used as a loading control. (C) Overexpression of Osx does not affect HIF-1α gene expression in C2C12 mesenchymal cells. RNA was obtained from cultures treated with or without Dox. Osx expression was induced in the absence of Dox. HIF-1α mRNA levels were quantitated by real-time RT-PCR. The HIF-1α RNA level obtained from the cells cultured with Dox was normalized to a value of 1. Values were presented as the mean ± S.D. (D) siRNA-directed knockdown of Osx does not impair HIF-1α gene expression in MC3T3 osteoblasts. MC3T3 osteoblasts were transfected with siRNA targeting Osx. RNA was isolated 24 h post-transfection. RNA expression levels were 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 ± 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.

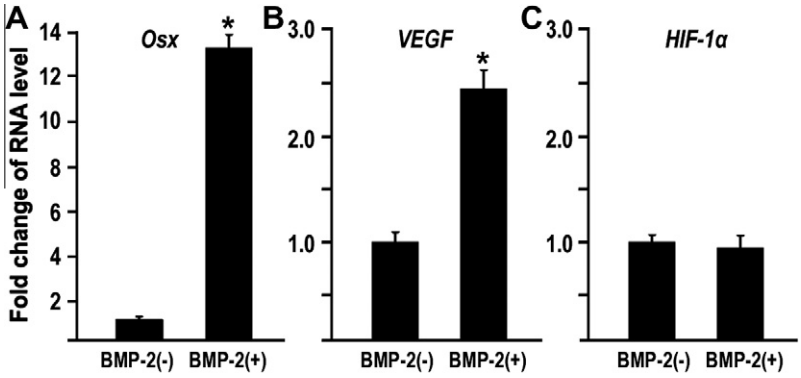

Fig. 3. BMP-2 stimulation leads to upregulation of both Osx (A) and VEGF (B), but not HIF-1α (C). C2C12 mesenchymal stem cells were stimulated by BMP-2 (300 ng/ml). Cells were harvested at 24 h following the addition of BMP-2. RNA was isolated from cell lysates of control group and BMP-2 group. RNA levels for Osx, VEGF and HIF-1α were analyzed by real-time RT-PCR. The level of RNA from control group was normalized to a value of 1. Values were presented as the mean ± S.D. A paired *t*-test was performed comparing control group and BMP-2 group. *: A star indicates statistical significance compared to control group.

3.5. Osx cooperates with HIF-1α to activate VEGF promoter activity

Osteogenesis occurs in close association with angiogenesis, and the replacement of avascular cartilage template by highly vascularized bone is the critical event in osteogenesis. During endochondral bone formation, chondrocytes model the long bone growth plate and become hypertrophic and hypoxic. It suggests that the microenvironment during transition between the cartilage template and bone is hypoxic. It is reported that HIF-1α couples angiogenesis to osteogenesis during bone development [10]. Osteoblasts can sense and respond to hypoxia by activating HIF-1α. HIF-1α increases VEGF levels in osteoblasts and hence promotes angiogenesis and osteogenesis [10]. Because the blood vessel invasion is the key step during osteogenesis, and also both Osx and HIF-1α regulate VEGF expression, we ask if there is cooperation between Osx and HIF-1α to boost the stimulation effect on VEGF. HEK293 cells were transiently cotransfected with 100 ng Osx and 200 ng HIF-1α along with VEGF promoter reporter. As shown in Fig. 4B, transfection of Osx or HIF-1α alone augmented VEGF promoter activity by 3.6- and 3.3-folds, respectively. Strikingly, cotransfection of such amounts of Osx and HIF-1α resulted in a cooperative enhancement of VEGF promoter activity by 9.3-fold. These data indicate that there is a synergistic interplay between Osx and HIF-1α in VEGF expression activation.

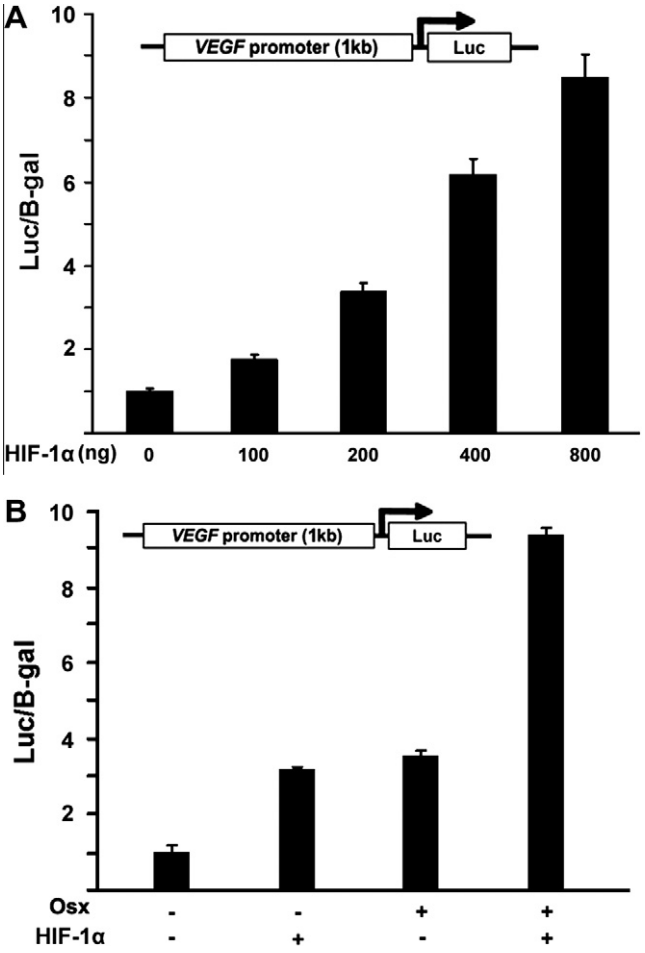

Fig. 4. Osx cooperates with HIF-1α to activate VEGF promoter activity. (A) HIF-1α activates VEGF promoter activity in a dose-dependent manner. Each group of the HEK293 cells was transfected with 300 ng VEGF-1 kb promoter reporter and indicated amounts of HIF-1α. Cells were analyzed 24 h after transfection. Luciferase activity was normalized by β-galactosidase activity. Values were presented as the mean ± S.D. (B) Osx acts synergistically with HIF-1α to stimulate VEGF promoter activity. Each group of HEK293 cells were transfected with 300 ng VEGF-1 kb promoter reporter, 100 ng Osx and 200 ng HIF-1α, or a combination of plasmids as indicated. Cells were analyzed 24 h after transfection. Luciferase activity was normalized by β-galactosidase activity. Values were presented as the mean ± S.D.

4. Discussion

Osx is an osteoblast-specific transcription factor that regulates the expression of essential genes needed for appropriate osteoblast differentiation and bone formation. Our recent study has shown that Osx controls VEGF expression while inducing osteoblast differentiation, thus suggesting a potential role for Osx in coordinating osteogenesis and angiogenesis [12]. In this study, we further characterized Osx regulation of VEGF expression. The findings presented here indicate that Osx proline-rich region (PRR) is required for the activation of VEGF gene expression and that Osx cooperates with HIF-1α to positively regulate VEGF expression.

First, we identified Osx proline-rich region as a functional domain for Osx to activate VEGF expression. This is supported by the deletion mutant analysis and transfection assay. Osx is an SP/KLF family member that functions by binding directly to DNA elements via an SP1-like DNA-binding domain consisting of three C2H2-type zinc fingers located within its C-terminus. Osx has been shown to be directly associated with the GC-rich element in proximal region of the VEGF promoter [12]. To determine the functional domain of Osx for VEGF activation, a series of deletion mutants in both N-terminal and C-terminal parts were used in this study. For example, Osx mutants M1 and M4, which cover the full PRR region, were able to activate VEGF reporter like Osx wild type plasmid as shown in Fig. 1B; however, the Osx mutants M2, M3, M5 and M6, which are without complete PRR region, disrupted Osx activation of VEGF expression. These data demonstrate that Osx PRR domain is required for VEGF regulation.

Osteogenesis is the process of depositing new bone by osteoblasts. VEGF has several important functions in osteogenesis. VEGF is required in both early and late stages of cartilage vascularization [15]. The essential step of endochondral ossification is to replace the avascular cartilage template with highly vascularized bone. Blood vessels transport mesenchymal cells to the mineralization front, where those cells differentiate to osteoblasts. VEGF is important for initial angiogenesis into the primary ossification center and keeping the blood vessel growth in developing bones [7,16]. VEGF plays an essential role in blood vessel invasion of hypertrophic cartilage, and thus affects bone formation. VEGF was found to have a direct autocrine role in osteoblast differentiation [17]. By acting via its receptor on endothelial cells, VEGF can also induce angiogenesis and hence indirectly enhance oxygen and nutrients supply for osteogenesis [13].

Molecular mechanisms responsible for coupling angiogenesis and osteogenesis remain poorly understood. As one of the driving forces, hypoxia has been reported to couple angiogenesis to osteogenesis. Hypoxia activates HIF-1α in osteoblasts. HIF-1α induces VEGF, and results in highly vascularized strong bone [10]. VEGF is still expressed in *HIF-1α* null mice, indicating that other factors can also regulate VEGF [11]. Our recent results have revealed that Osx controls VEGF expression in osteoblasts [12]. This study addresses the effect of Osx on HIF-1α expression. *Osx*-null calvaria displayed the similar expression level of HIF-1α compared to wild type calvaria in E18.5 mice (Fig. 2A). A Tet-off inducible C2C12 stable cell system revealed that ectopic expression of Osx did not result in an increase in HIF-1α level (Fig. 2C). HIF-1α expression remained unchanged in MC3T3 osteoblasts when Osx expression was knocked-down using siRNA targeting strategies (Fig. 2D). Moreover, BMP-2 stimulation led to upregulation of both Osx and VEGF in C2C12 mesenchymal stem cells (Fig. 3A and B), but the expression of HIF-1α did not change along with Osx and VEGF after BMP-2 stimulation (Fig. 3C). These observations thus indicate that HIF-1α is not a downstream target of Osx, suggesting that Osx regulation of VEGF is independent of HIF-1α expression level. Since both Osx and HIF-1α promote osteogenesis and regulate VEGF activity, it is interesting to explore the possibility that Osx and HIF-1α may work together to control VEGF. Indeed, our current results provided the evidence to support that Osx cooperated with HIF-1α to boost VEGF promoter activity. We showed that HIF-1α activated VEGF promoter activity (Fig. 4A), and further transfection assay demonstrated that Osx and HIF-1α can function together to control VEGF in a cooperative manner.

In summary, we present here that Osx has synergistic effect with HIF-1α on the regulation of VEGF expression. Osx is essential for osteogenesis. Angiogenesis is indispensible during bone formation. VEGF, as an important mediator of angiogenesis and osteogenesis, is regulated by both Osx and HIF-1α. This study revealed additional new information of the transcriptional regulation mechanism that osteoblast-specific transcription factor Osx controls VEGF expression in osteoblasts.

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