Osteoblast-specific Transcription Factor Osterix (Osx) Is an Upstream Regulator of Satb2 during Bone Formation*

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Osterix (Osx) is an osteoblast-specific transcription factor essential for osteoblast differentiation and bone formation. Osx knock-out mice lack bone completely. Satb2 is critical for osteoblast differentiation as a special AT-rich binding transcription factor. It is not known how Satb2 is transcriptionally regulated during bone formation. In this study, quantitative real-time RT-PCR results demonstrated that Satb2 was down-regulated in Osx-null calvaria. In stable C2C12 mesenchymal cells using the tetracycline (Tet)-Off system, overexpression of Osx stimulated Satb2 expression. Moreover, inhibition of Osx by siRNA led to repression of Satb2 expression in osteoblasts. These results suggest that Osx controls Satb2 expression. Transient transfection assay showed that Osx activated 1kb Satb2 promoter reporter activity in a dose-dependent manner. To define the region of Satb2 promoter responsive to Osx activation, a series of deletion mutants of Satb2 constructs were made, and the minimal region was narrowed down to the proximal 130 bp of the Satb2 promoter. Further point mutation studies found that two GC-rich region mutations disrupted the Satb2 130bp promoter activation by Osx, suggesting that these GC-rich binding sites were responsible for Satb2 activation by Osx. Gel shift assay showed that Osx bound to the Satb2 promoter sequence directly. ChIP assays indicated that endogenous Osx associated with the native Satb2 promoter in osteoblasts. Importantly, Satb2 siRNA significantly inhibited Osx-induced osteoblast marker gene expressions. Taken together, our findings indicate that Osx is an upstream regulator of Satb2 during bone formation. This reveals a new additional link of the transcriptional regulation mechanism that Osx controls bone formation.

Bone formation involves two processes: endochondral and intramembranous ossification. Most bones are formed through endochondral ossification, which needs the template of cartilage. Fewer bones are formed through intramembranous ossification, a process in which bones are formed directly from mesenchymal condensations. Transcription factors and signaling proteins affect osteoblast differentiation from mesenchymal stem cells, including Indian Hedgehog (Ihh), Runx2, Osterix (Osx), and Wnt signaling pathway proteins (1). Endochondral ossification and the activation of Runx2 require Ihh (2). Both endochondral and intramembranous ossification require Runx2, which is involved in the differentiation of mesenchymal cells into preosteoblasts (3). As a downstream gene of Runx2, Osx is specifically expressed in osteoblasts and expressed at a low level in prehypertrophic chondrocytes (4). The Osx expression pattern in mice indicates that the presence of the 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. In Osx knock-out embryos, cartilage develops normally, but there is no bone formation. These embryos do not express osteoblast differentiation markers such as osteocalcin and bone sialoprotein (Bsp). It is also reported that Osx inhibits the Wnt signaling pathway, which presents a possible mechanism for Osx to inhibit osteoblast proliferation (5). Further data have indicated that Osx controls Wnt signaling by three different mechanisms: (i) it stimulates Wnt antagonist DKK1 expression; (ii) it disrupts Tcf1 binding to DNA to inhibit the transcriptional activity of β-catenin/Tcf; and (iii) it targets Wnt antagonist sclerostin (5, 6). Osx inhibition of Wnt signaling provides a feedback control mechanism involved in bone formation. It is speculated that Osx plays an important role in coordinating both osteoblast differentiation and osteoblast proliferation during bone formation.

Satb2 is a transcription factor, which belongs to the family of special AT-rich binding proteins that binds to nuclear matrix-attachment regions. Satb2 depends on matrix-attachment region to activate gene transcription (7). Satb2 is found to be a multifunctional determinant of craniofacial patterning and osteoblast differentiation (8). Satb2 has been reported to be involved in cleft palate under conditions of haploinsufficiency (9). Haploinsufficiency of Satb2 causes craniofacial defects such as cleft palate (~25% of cases), phenocopy associated with 2q32-q33 deletions and translocations in humans. Satb2 has been considered as a critical regulator in bone formation. The bone phenotype in Satb2<sup>−/−</sup> mouse embryos includes craniofacial abnormality: a significant truncation of the mandible, a shortening of the nasal and maxillary bones, malformation of the hyoid bone, and a cleft palate (8). These mice have shorter and thinner trabeculae, a defect in bone volume, and also hind limb shortening. In addition, these mice carry defects in osteoblast differentiation and function. There was a 5-fold reduction of bone nodules in Satb2<sup>−/−</sup> osteoblasts compared with the...
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wild-type and heterozygous osteoblasts. From microarray assay, various genes have been identified to be downstream targets of Satb2 such as transcription factors, extracellular matrix proteins, bone morphogenetic protein (BMP) antagonists, matrix metalloproteinases, and their inhibitors (8). Hox genes (Hoxa2, Hoxa13, and Hoxb2), Bsp, and osteocalcin are among these Satb2 target genes. Hox genes are a cluster of genes that are critical for the correct morphogenesis of embryonic axial structures (10). Hoxa2 inhibits bone formation and regulates branchial arch patterning (8). Satb2 inhibits some Hox genes such as Hoxa2 by binding to a matrix-attachment region-like sequence. Bsp is a major structural protein of the bone matrix. Fully differentiated osteoblasts specifically express Bsp (11). Osteocalcin is only secreted by osteoblasts and thought to play a role in the metabolic regulation of the body (12). As osteoblast differentiation markers, both Bsp and osteocalcin expression levels are down-regulated in long bones and calvaria of Satb2−/− embryos. Satb2 binds to the Bsp promoter and regulates Bsp expression (8). Satb2 regulates osteocalcin via interacting directly with ATF4 and Runx2 and increasing their transcriptional activity on osteocalcin (8). Satb2 works as a protein scaffold to increase the activities of ATF4 and Runx2. Recent studies have provided direct evidence for an interdependent network connecting Runx2, Satb2, and the miR-23a∼27a∼24∼2 cluster (13). This network affects the osteoblast differentiation program. Satb2 and Runx2 are translationally suppressed by microRNA cluster during progression of osteoblast differentiation. Exogenous Satb2 can rescue the osteogenesis inhibition by the microRNA cluster. These data support the argument that Satb2 is a critical regulator in bone formation. At this time, the transcriptional regulation of Satb2 during bone formation is not well understood.

Here, our quantitative real-time RT-PCR results demonstrate that Satb2 expression is suppressed in the absence of Osx and enhanced when Osx is overexpressed. This suggests that Osx may control Satb2 expression. Additional evidence from this study indicates that Osx targets Satb2 directly. Therefore, this finding unveils the regulatory link between these two important transcription factors during bone formation.

EXPERIMENTAL PROCEDURES

Animal and Genotype—Wild-type and Osx-null mice are from a 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-bp and 395-bp PCR fragments, respectively, as described previously (4).

RNA Isolation and Real-time RT-PCR—Total RNA was purified from the calvaria of embryonic day 18.5 (E18.5) wild-type and Osx-null mouse embryos with TRIzol reagent (Invitrogen) followed by the RNeasy mini kit (Qiagen). Total RNA from C2C12 cells was isolated using an RNeasy mini kit. RNA was subjected to quantitative real-time RT-PCR, using the TaqMan

One-Step RT-PCR Master Mix reagent. Relative transcript levels were analyzed by real-time PCR in a 20-µl reaction volume on 96-well plates, using an ABI 7500 real-time PCR system (Applied Biosystems). Transcript levels were normalized to GAPDH levels. All experiments were done in duplicate and repeated at least three times.

Plasmid Constructs and Subcloning—Subcloning was performed as described previously with modifications (14). The progressive deletion fragments of the Satb2 promoter region were generated by PCR using mouse genomic DNA as a template and subcloned into the XhoI and MluI sites of the pGL-3 vector. Primers were obtained from Integrated DNA Technologies (Coralville, IA). The primer sequences were as follows: 1) Satb2-Xho-3, 5′-GGG GGG GGC GGG GGG GGT TTT TAT TAT G; 2) Satb2-Mlu-1K, 5′-GCC CCAC GGG GGG GGT TTT TAT TAT G; 2) Satb2-Mlu-1K, 5′-GCC CCAC GGG GGG GGT TTT TAT TAT G; 3) Satb2-Mlu-500, 5′-GCC CCAC GGG GGG GGT TTT TAT TAT G; 3) Satb2-Mlu-500, 5′-GCC CCAC GGG GGG GGT TTT TAT TAT G; and 4) Satb2-Mlu-130, 5′-GCC CCAC GGG GGG GGT TTT TAT TAT G. Satb2 point mutants were made with the QuikChange site-directed mutagenesis kit (Stratagene) using Satb2−/− 130 bp as a template by the following primers: 1) Satb2-M1-1, 5′-CTC TTT CAG CCC CCT ATG AAA ACC CCC TCC GCC GTC; 2) Satb2-M1-2, 5′-GAC GCC GGC GGA GGA GGT TTT TAT AGG GGG CTG AAA GAG; 3) Satb2-M2-1, 5′-CTC TTT CAG CCC CCT ATG AAA ACC CCC TCC GCC GTC; 2) Satb2-M1-2, 5′-GAC GCC GGC GGA GGA GGT TTT TAT AGG GGG CTG AAA GAG; and 4) Satb2-M2-2, 5′-GAC GCC GGC GGA GGA GGT TTT TAT AGG GGG CTG AAA GAG.

Cell Culture and Transient Transfection Assay—HEK293 cells (ATCC) were cultured in high glucose Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and 100 units/ml penicillin plus 100 µg/ml streptomycin. Cells were plated in 12-well plates, cultured to 60−80% confluence, and transfected with FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. Cells were cotransfected with 300 ng of the Satb2 promoter reporter, Osx expression plasmid as indicated, and 25 ng of pSV2-β-gal. After transfection, cells were incubated for 24 h before harvest. The reporter assays were analyzed with BD Monolight system (BD Biosciences). Luciferase activity was normalized by β-galactosidase activity. Every transfection experiment was repeated at least three times. Values were presented as the mean ± S.D. 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 pTet-Off Advanced Inducible Gene Expression system (Clontech) as used previously (5). Osx expression was induced in the absence of tetracycline (Tet). C2C12 cells were cultured in ATCC described medium with additives G418, hygromycin, and with or without doxycycline, a member of the tetracycline antibiotics group.

siRNA Interference—MC3T3 cells were transfected by siRNA against mouse Osx with Lipofectamine 2000. C2C12 cells were transfected by siRNA against mouse Satb2 with Lipofectamine 2000. siRNA oligonucleotides were purchased from Thermo

2 The abbreviations used are: E18.5, embryonic day 18; Tet, tetracycline; ALP, alkaline phosphatase.
Scientific Dharmacon, and siGENOME Lamin A/C control siRNA was used as a nonspecific control. Cells were cultured in six-well plates. One day before transfection, cells were plated in 1 ml of growth medium without antibiotics. Cells were 30–50% confluent at the time of transfection. For each sample, the siRNA-Lipofectamine 2000 transfection complex was prepared as follows: 1) 2 μl of 50 μM siRNA was diluted in 50 μl of Opti-MEM I reduced serum medium without serum; 2) Lipofectamine 2000 was mixed gently, and then 3 μl was diluted in 50 μl of Opti-MEM I medium; 3) the diluted siRNA was combined with the diluted Lipofectamine 2000; and 4) 100 μl of siRNA-Lipofectamine 2000 complex was added to each well. After 4 h of incubation, the growth medium was replaced. Cells were cultured at 37 °C in a CO2 incubator for 24 h before harvest.

**Protein Purification and Western Blot**—Protein was isolated by acetone precipitation from cell 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 rpm. Protein concentrations in the supernatant were determined using a Pierce BCA protein assay kit. 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-Satb2 rabbit polyclonal antibody (1:200, Abcam) or anti-Osx rabbit polyclonal antibody (1:200, Santa Cruz Biotechnology) and with 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 Life Sciences) was used according to the directions of the manufacturer.

**Gel Shift Assay**—Baculovirus-expressed Osx was used as the protein resource as described (5). The DNA sequences of the oligonucleotides used for gel shift assay were as follows: Satb2 wild-type, 5’-CTC TTT CAG CCC CCT CTC CCC CCC CCC TCC TCC GGC GTC and Satb2 mutant, 5’-CTC TTT CAG CCC CCT ATA AAA ACC CCC TCC TCC GGC GTC. DNA oligonucleotide was labeled using a biotin 3’ end DNA labeling kit (catalog no. 89818, Pierce.). 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 no. 20148). Protein-DNA complexes were separated on 5% polyacrylamide gels in 0.5× TBE buffer and transferred onto Biodyne B nylon membrane (catalog no. 77016). 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 no. 89880). Two hundred-fold molar excess of unlabeled Satb2 promoter oligonucleotides was used as competitor DNA.

**ChIP Assay**—The ChIP assay kit was from Millipore. ChIP assays were performed according to previously described protocol (15) 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 and 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 IP 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 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 of 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 Satb2 promoter regions were obtained from Integrated DNA Technologies, and the sequences were as follows: primer set 1, 5’-CGT GCT CAC AGC GAT TGA TTG (Satb2-1) and 5’-CTC TTT CAG CCC CCT CTC CCC CCC CCC; primer set 2, 5’-CTG CCG TGT TGT TTA TAA GAG (Satb2-D-1) and 5’-TCT GAT GAT GCC GTC ATG GTT GG (Satb2-D-2). Data were normalized by GAPDH.

**RESULTS**

**Satb2 Expression Is Down-regulated in Absence of Osx**—Osx is required for osteoblast differentiation and bone formation. Osx knock-out mice lacked bone completely. Satb2 was also critical for osteoblast differentiation as a special AT-rich binding transcription factor. To identify the possible downstream target of Osx during bone formation, we performed quantitative real-time RT-PCR to compare RNA levels of several genes between wild-type and Osx knock-out mice. RNA was isolated from calvaria of E18.5 mouse embryos. As shown in Fig. 1A, Osx expression was abolished in Osx-null calvaria, and the osteoblast marker gene Bsp was at undetected level as expected. As a negative control, Runx2 expression was unchanged as an upstream gene of Osx. Interestingly, it was observed that Satb2 expression was suppressed by 11.3-fold in Osx-null calvaria compared with that in wild-type calvaria. The significant decrease in Satb2 RNA level in Osx knock-out mice suggests that Osx regulates Satb2 gene expression.

**Overexpression of Osx Activates Satb2 Expression**—We next studied whether Osx could positively regulate Satb2 gene expression. A stable C2C12 mesenchymal cell line was used in which the expression of Osx could be induced by using the Tet-Off system as described previously (5). Expression of Osx was turned on in the absence of Tet. Total RNA was purified from C2C12 stable cell line in the presence or absence of Tet and measured by real-time RT-PCR for Satb2 expression. As shown in Fig. 1B, in the absence of Tet when Osx was overexpressed, Satb2 expression was enhanced by ~2.5 fold. Western blot was done and validated Osx protein expression in the absence of Tet (data not shown). This result demonstrates that Osx increases Satb2 expression.

**Inhibition of Osx by siRNA Leads to Repression of Satb2 Expression in Osteoblasts**—To further confirm the effect of Osx on Satb2 expression, we used siRNA transfection to knockdown Osx expression in MC3T3 osteoblast cells to determine
the possible change of Satb2 expression. Western blot and real-time RT-PCR were performed to analyze protein and mRNA expression level, respectively. The decrease of Satb2 protein expression occurred with knockdown of Osx protein expression level by siRNA as shown in Fig. 1C. When Osx RNA expression was decreased by 80% by siRNA against Osx (data not shown), Satb2 RNA expression was repressed by 64% as shown in Fig. 1D. Therefore, these data suggest that Osx regulates Satb2 positively in osteoblasts.

**Osx Activates Satb2 Promoter Activity in Dose-dependent Manner**—Results from primary mouse calvaria cells, the stable C2C12 mesenchymal cell line, and the osteoblast cell line suggest that Osx up-regulates Satb2 expression. To investigate this, we did subcloning to generate luciferase reporter construct driven by the 1kb Satb2 native promoter. This construct plasmid was cotransfected with different amounts of Osx expression plasmid in MC3T3 osteoblastic cells. Fig. 2A showed that even 100 ng of Osx plasmid transfection was able to activate the Satb2 promoter reporter. Increasing amounts of Osx plasmid led to higher expression of the Satb2 promoter reporter. This demonstrated that Osx stimulated the 1kb Satb2 promoter activity in a dose-dependent manner, suggesting that Osx activates the Satb2 gene transcriptionally. Transient transfection assay was also performed using HEK293 cells to test Satb2 promoter reporter activation, and the similar results were obtained (data not shown).
Identification of Osx-binding Site in Promoter of Satb2 Gene—
We have shown Osx can stimulate Satb2 promoter reporter activity; however, it is not known yet which region within the Satb2 promoter is responsible for Osx activation. To address this question, we generated different deletion constructs of the Satb2 promoter reporter and carried out transient transfection assay to narrow down the responsible region within the 1kb Satb2 promoter for Osx activation. Satb2 luciferase reporter constructs driven by different lengths of Satb2 promoter region were generated by subcloning. As shown in Fig. 2B, Osx was able to activate Satb2 promoter reporters of Satb2-1kb, Satb2-500bp, and Satb2-130bp in a transient transfection assay. These data suggest that there may be critical binding sites for Osx in the Satb2-130bp promoter region. It has been demonstrated in a previous report that Osx belongs to the Sp/XKLF family of transcription factors that bind to GC-rich sequence of target gene promoter to regulate gene expression (4). According to the sequence analysis of the 130-bp region of the Satb2 promoter, there are two tentative binding sites for Osx as GC-rich elements. To study which binding site is responsible for Satb2 promoter activation by Osx, two point mutants of Satb2-130bp promoter reporter were generated by the QuikChange site-directed mutagenesis kit (Stratagene) using Satb2-130bp as a template. We named the two mutants as Satb2-M1 and Satb2-M2 in which GC-rich element was replaced with A. Our results indicated that both Satb2-M1 and Satb2-M2 mutants inhibited Osx activation of Satb2-130bp promoter reporter by 50–60% as shown in Fig. 2C. Then, we asked what might be the effect of the mutations on Satb2-130bp promoter reporter if both tentative binding sites were mutated at the same time. We generated double mutants of Satb2-M1 and Satb2-M2, and named it Satb2-M12. As shown in Fig. 2C, Satb2-M12 with mutations in both tentative binding sites almost abolished Osx activation of the Satb2-130bp promoter reporter. Thus, these results revealed that two GC-rich sequences in the Satb2-130bp region were responsible for Satb2 promoter activation by Osx.

Osx Associates with Native Satb2 Promoter—To confirm Osx binding to the Satb2 promoter, we performed a gel shift assay. As shown in Fig. 3A, Osx was able to bind to Satb2 promoter oligonucleotides (lane 2), and Osx binding was abolished by both excess unlabeled Satb2 promoter oligonucleotides (lane 3) and Satb2 mutant oligonucleotides (lane 4), which were used to test the binding specificity. The data indicated that Osx bound to Satb2 promoter oligonucleotides specifically. The studies above indicate that Osx can positively regulate Satb2 expression and activate Satb2 promoter activity in vitro and that two GC-rich sequences in the Satb2 promoter are responsible for Osx activation. It is currently unknown whether endogenous Osx can associate with the native Satb2 promoter in vivo. To address this question, ChIP assay was carried out to examine whether Osx could bind to the native Satb2 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 Satb2-130bp promoter (primer set 1) or a distal upstream 3k, non-responsive region (primer set 2) as a control to demonstrate response element selectivity. Fig. 3, B and C, demonstrated that Osx was associated with the Satb2 promoter region containing the two GC-rich sequences (primer set 1) compared with the IgG control group. However, Osx was not associated with the Satb2 distal 3k promoter region without GC-rich sequence (primer set 2); therefore, the Osx-DNA association was specific. These data indicate that endogenous Osx associates with a native Satb2 promoter in primary osteoblasts in vivo.

Satb2 Is Critical Downstream Target Gene of Osx—To determine whether Satb2 is a critical downstream target gene of Osx, we tested the effects of Satb2 siRNA on Osx-induced osteoblast differentiation. In our C2C12 mesenchymal stable cell line using the Tet-off system, Osx expression was turned on in the absence of tetracycline. We observed that Osx was able to induce expressions of osteblast marker genes in this cell system,
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such as Bsp, osteocalcin, and alkaline phosphatase (ALP) as shown in Fig. 4. siRNA against Satb2 was able to knock down Satb2 expression significantly by 76% at concentration of 200 nM (data not shown). More importantly, Satb2 siRNA significantly inhibited Osx-induced osteoblast marker gene expressions, including Bsp (A), osteocalcin (OC; B), and ALP (C). In the C2C12 stable cell line using the Tet-off system, Osx expression was turned on in the absence of tetracycline. When Osx was induced in C2C12 stable cells, 200 nM Satb2 siRNA (Si-Satb2) was transfected with Lipofectamine 2000. RNA was isolated, and RNA levels were measured by real-time RT-PCR. The RNA level from control siRNA group (Si-C) was normalized to a value of 1. Values were presented as the mean ± S.D.

**FIGURE 4.** Satb2 is a critical downstream target gene of Osx. Satb2 siRNA inhibited Osx-induced osteoblast marker gene expressions, including Bsp (A), osteocalcin (OC; B), and ALP (C). In the C2C12 stable cell line using the Tet-off system, Osx expression was turned on in the absence of tetracycline. When Osx was induced in C2C12 stable cells, 200 nM Satb2 siRNA (Si-Satb2) was transfected with Lipofectamine 2000. RNA was isolated, and RNA levels were measured by real-time RT-PCR. The RNA level from control siRNA group (Si-C) was normalized to a value of 1. Values were presented as the mean ± S.D.

**DISCUSSION**

We performed in vitro and in vivo experiments to investigate the regulatory effect of osteoblast-specific transcription factor Osx on Satb2, a critical regulator of osteoblast differentiation. Satb2 expression was down-regulated in the calvaria of Osx-null mouse embryos, and Satb2 expression was up-regulated when Osx expression was induced in C2C12 mesenchymal stable cells. When Osx was suppressed by siRNA against Osx, Satb2 gene expression was repressed in osteoblasts. These results suggest that Osx positively regulates Satb2 expression. This was supported by additional observations: Osx activated 1kb Satb2 promoter reporter activity in a dose-dependent manner, and two GC-rich sequences in the Satb2 promoter region are responsible for Osx activation of the Satb2 promoter reporter. Moreover, ChIP assay showed that endogenous Osx was associated with native Satb2 promoter in primary osteoblasts in vivo. Importantly, Satb2 siRNA significantly inhibited Osx-induced osteoblast marker gene expressions. Taken together, these evidences demonstrate that Osx is an upstream regulator of Satb2 during bone formation.

Osx is an osteoblast-specific transcription factor required for bone formation (4). Osx contains a DNA-binding domain with three C2H2-type zinc fingers at its C terminus, and the sequence of this domain is similar to Sp1, Sp3, and Sp4. Osx also has a proline-rich region transactivation domain, which is responsible for inhibition of the Wnt pathway (5). Osx is essential for bone formation and mineralization in vivo. In Osx knock-out mice, there is no endochondral or intramembranous bone formation. Osx has been considered as a master regulator of osteoblast differentiation (4, 5). Nevertheless, the molecular mechanisms through which Osx controls osteoblast differentiation and function are still not well elucidated. BMP-2 and insulin-like growth factor-1 have been shown to regulate Osx in undifferentiated mesenchymal stem cells (16). Osx is known to be a downstream gene of Runx2 during osteoblast differentiation. Runx2 expression is normal in Osx-null mice, whereas there is no Osx expression in skeletal elements in Runx2-knock-out mice (4). This is confirmed through characterization of a Runx2-binding element in the Osx gene promoter (17). It is known that Osx is required for the expression of the osteoblast-specific markers such as type I collagen, osteocalcin, bone sialoprotein, osteonectin, and osteopontin. It has been demonstrated that Wnt antagonists sclerostin and DKK1 are downstream targets of Osx (5, 6), which provides possible mechanisms for Osx inhibition effect on Wnt pathway.

It is reported that Osx is associated strongly with bone mineral density and growth in children and adults (18). A genome-wide association study of BMD and related traits in 1518 children from the Avon Longitudinal Study of Parents and Children was performed to identify genetic variants affecting BMD. This study has identified associations with BMD in an area of chromosome 12 containing the Osx (SP7) locus. A meta-analysis of these existing studies demonstrated strong association between single nucleotide polymorphisms in the Osx region and adult lumbar spine BMD. Childhood and adolescent bone accumulation is a determinant of bone structural strength in later life (19). Osx is indispensable for osteoblast differentiation and bone formation during embryonic development and in postnatal bones. Osx is among the few genes that have been identified to affect postnatal bone growth. Inactivation of Osx significantly affected the properties of osteocytes, severely weakened bone structure, and caused massive accumulation of calcified cartilage (20). Most therapy for skeletal diseases with
less bone such as osteoporosis and osteonecrosis is to suppress bone resorption. To cure these diseases, it is also essential to stimulate new bone formation. Thus, there is currently great interest in developing anabolic therapies with understanding of the regulation of osteoblast differentiation and activity. It is not yet clear which transcription factors are downstream targets of Osx. The elucidation of downstream targets of Osx will significantly impact the development of anabolic therapeutic strategies to treat osteoporosis and other bone-related diseases.

Satb2 is a multifunctional transcription factor and is expressed in different tissues. At E8.5 in mice, Satb2 is in the rhombomere region. At E9.0, it is expressed in the first branchial arch, and then it develops to the medial parts of all four branchial arches, which are involved in facial structure development. Satb2 is found in the cortex of brain, in spinal cord, kidneys, the umbilical cord, and at sites of bone formation, including long bones, vertebrae, and calvaria. Satb2 is reported to influence osteoblast differentiation and craniofacial patterning (8). Satb2 is found in the cortex of brain, in spinal cord, kidneys, the umbilical cord, and at sites of bone formation, including long bones, vertebrae, and calvaria. (8). Satb2 is expressed in different tissues. At E8.5 in mice, Satb2 is in the cortex of brain, in spinal cord, kidneys, the umbilical cord, and at sites of bone formation, including long bones, vertebrae, and calvaria. (8). Satb2 is reported to influence osteoblast differentiation and craniofacial patterning (8). Satb2−/− mice display both craniofacial abnormalities that are similar to those humans with translocation in Satb2 and defects in osteoblast differentiation and function. Satb2−/− embryos have delayed bone formation or mineralization. Satb2 has been suggested as a candidate gene for human malformation syndrome of craniofacial patterning, osteoporosis, and brain development. One patient with mutation in Satb2 has craniofacial dysmorphisms: cleft palate, generalized osteoporosis, profound mental retardation, epilepsy, and a joyful personality (21). Besides its functions in bone formation, Satb2 is also associated with tumor development in different tissues (22–24). Satb2 affects chromatin-remodeling molecules in differentiating cortical neurons (25). Satb2 also contributes to the plasticity of Nanog expression and embryo stem cell pluripotency (26).

During bone formation, Satb2 has been reported to act both as a binding protein to activate or repress DNA and as a protein scaffold that increases the activity of other DNA binding proteins. Satb2 inhibits Hoxa2 by binding to its promoter. Satb2 also enhances the functions of Runx2 and ATF4 by acting as a protein scaffold. Satb2 is suspected to recruit ATF4 and Runx2 to specific subnuclear sites in osteoblasts. By acting as a protein scaffold, Satb2 can regulate the expression or activity of many important proteins in skeletal development (8). It has been reported recently that there is a translational regulatory network connecting Runx2, Satb2, and microRNA cluster 23a~27a~24-2 (13). This regulatory network demonstrated that Satb2 can function post-transcriptionally in the process of bone formation. Heterozygous nonsense mutation of Satb2 is shown to be related to osteoporosis (21). Due to the versatility of Satb2, the regulation of Satb2 by the osteoblast-specific transcription factor Osx is important for our understanding of the process of bone formation.

Bone formation is a complicated process controlled by multiple factors and pathways; it is clearly demonstrated that Osx is required for the final commitment of osteoblast lineage (1). After the lineage commitment, osteoprogenitors undergo a proliferative stage. Then, they exit mitosis and transit to express genes such as alkaline phosphatase, Bsp, and type I collagen, as they start to produce and mature osteogenic extracellular matrix. Finally, they express genes involved in mineralization of the extracellular matrix such as osteocalcin and osteopontin. This highly regulated program of gene expression and cellular differentiation is controlled by the expression and activity of different transcription factors. Ihh is the initiator of endochondral ossification. The Runx2-expressing biopotential progenitors can differentiate into either osteoblast or chondrocyte. Then cells differentiate into preosteoblasts, in which Runx2 play an essential role. In the next step, preosteoblasts differentiate into mature osteoblasts, a process in which Osx plays a critical role. In this study, we have provided evidence to support that Satb2 is one downstream transcription factor of Osx during bone formation.

Satb2 can function as a transcription factor that binds to specific DNA sequences (8). It is possible that Osx and Satb2 may share some downstream target genes in osteoblasts. For example, both Osx and Satb2 control Bsp expression. There might be two possibilities. First, Satb2 and Osx may independently bind to their corresponding DNA sequences in Bsp promoter to activate gene expression or further cooperate each other to control Bsp expression; Secondly, Osx may regulate Bsp expression indirectly, probably through controlling Satb2 expression or other transcription factors. In this study, we demonstrated in Fig. 4 that Satb2 siRNA significantly inhibited Osx-induced osteoblast marker gene expressions, including Bsp, osteocalcin, and ALP. These data support our hypothesis that Satb2 is a critical downstream target gene of Osx, suggesting that Satb2 participates at least partially in Osx-induced osteoblast marker gene expressions. This study cannot rule out the possibility that other Osx downstream transcription factors may also contribute to Osx-induced osteoblast marker gene expressions. The detailed mechanisms of these regulations on downstream osteoblast marker genes deserve further investigation.

In summary, we show that Osx is an upstream regulator of Satb2 during bone formation. Our findings indicate a direct regulation between two important transcription factors and add a new part to the complex transcriptional network that controls bone formation. Identification of Satb2 as a direct target of Osx will help to better understand the molecular mechanism of Osx effect on bone formation.

REFERENCES
9. Britanova, O., Depew, M. J., Schwarz, M., Thomas, B. L., Mileitch, L.
Osx Is Upstream Regulator of Satb2