

Upregulation of *smpd3* via BMP2 stimulation and Runx2

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Deletion of *smpd3* induces osteogenesis and dentinogenesis imperfecta in mice. *smpd3* is highly elevated in the parietal bones of developing mouse calvaria, but not in sutural mesenchymes. Here, we examine the mechanism of *smpd3* regulation, which involves BMP2 stimulation of Runx2. *smpd3* mRNA expression increased in response to BMP2 treatment and Runx2 transfection in C2C12 cells. The Runx2-responsive element (RRE) encoded within the -562 to -557 region is important for activation of the *smpd3* promoter by Runx2. Electrophoretic mobility shift assays revealed that Runx2 binds strongly to the -355 to -350 RRE and less strongly to the -562 to -557 site. Thus, the *smpd3* promoter is activated by BMP2 and is directly regulated by the Runx2 transcription factor. This novel description of *smpd3* regulation will aid further studies of bone development and osteogenesis. [BMB reports 2009; 42(2): 86-90]

INTRODUCTION

Sphingomyelinases (SMases) belong to the phosphodiesterase family. Three well-characterized SMases (*smpd1*, *smpd2*, and *smpd3*) hydrolyze sphingomyelin to ceramide and phosphorylcholine (1-3). The acid sphingomyelinase, *smpd1*, is localized in the endolysosomal compartment, while the neutral SMases, *smpd2* and *smpd3*, are localized in the endoplasmic reticulum membrane and the Golgi apparatus, respectively (4). Sphingomyelin phosphodiesterase 3 (*smpd3* or neutral sphingomyelinase-2) is a member of a large superfamily of magnesium-dependent phosphohydrolases (4-6). This enzyme hydrolyzes sphingosylphosphocholine in the plasma membrane to produce ceramide and was originally identified as a brain-specific neutral sphingomyelinase (2). However, deletion of *smpd3* induces osteogenesis and dentinogenesis imperfecta in mice.

Bone morphogenetic protein 2 (BMP2) is a member of the transforming growth factor (TGF)- β family (7). BMP2 was originally identified in demineralized bone matrix as a factor that induces ectopic bone formation, and a purification method for

large-scale production has been developed (8). BMP2 is one of the most potent bone-inducing agents in osteoblast differentiation and induces the osteogenic trans-differentiation of fibrogenic, myogenic, and adipogenic cells *in vitro* and *in vivo* (6, 9). Several transcription factors are induced by BMP signaling and participate in osteoblast differentiation (9-11).

Runx2, previously known as Cbfa1/Pebp2 α A/AML3, plays an essential role in osteoblast-mediated bone formation and contributes to BMP2-induced trans-differentiation of C2C12 cells by diverting them from the myogenic pathway to the osteogenic pathway (11, 12). CBF β is required for the function of Runx2 and regulates Runx2-isoform dependently in skeletal development (13).

Runx2-deficient (*i.e.*, Runx2^{-/-}) mice experience a complete failure in bone formation due to arrested osteoblast maturation (14). Here, to explore the regulatory mechanism of *smpd3* by BMP2 and Runx2, we studied *smpd3* mRNA expression changes by real-time RT-PCR analysis and analyzed the *smpd3* promoter by luciferase assays and electrophoretic mobility shift assays (EMSA).

RESULTS

***smpd3* is upregulated by Runx2 downstream of BMP2**

To determine whether BMP signaling controls *smpd3* expression, C2C12 cells were treated with 300 ng/ml BMP2 and mRNA levels were measured by real-time PCR. BMP2 stimulation increased *smpd3* mRNA levels (Fig. 1A). Runx2 transfection also dose-dependently resulted in increase of *smpd3* expression (Fig. 1B), with *smpd3* expression increasing 13-fold in the presence of 1.5 ng/ml Runx2 and CBF transfection. Thus, *smpd3* expression is modulated by BMP2 and Runx2 appears to contribute to the upregulation of *smpd3*.

Runx2 directly up-regulates the SMPD3 promoter

To determine how Runx2 regulates the *smpd3* promoter, we first cloned a 1.1 kb region flanking the 5' portion of *smpd3*. A luciferase vector was constructed that contained a DNA fragment that included -1,100 nt to the first exon (*i.e.*, 25 base pairs) of the *smpd3* promoter region, including the *smpd3* gene (Fig. 2A). To determine if Runx2 directly activates the *smpd3* promoter, we co-transfected cells with the *smpd3* promoter construct and/or Runx2 and CBF β cDNA. *smpd3* promoter activity increased approximately 7-fold in Runx2-trans-

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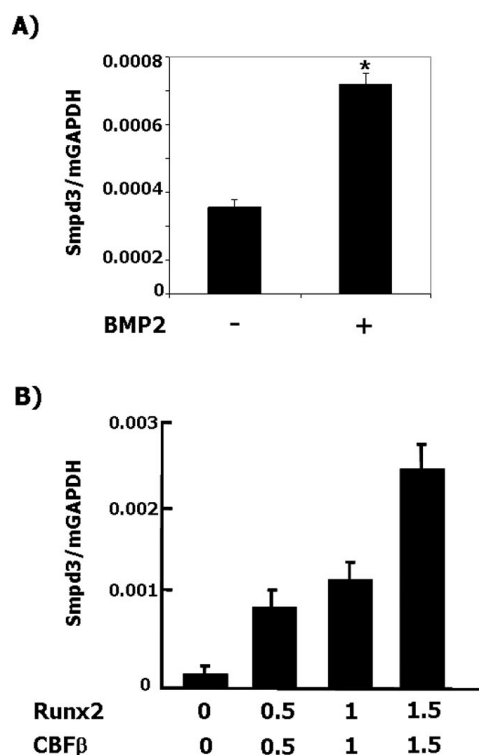


Fig. 1. Expression of *smpd3* mRNA in C2C12 cells in response to BMP2, Runx2, and CBFβ treatment. (A) Confluent C2C12 cells were cultured for 24 hours in media without FBS and were then treated with 300 ng/ml BMP2. C2C12 cells were harvested 12 hours after BMP2 treatment and total RNA was isolated for real-time RT-PCR analysis. (B) Confluent C2C12 cells were treated with medium alone (0% FBS) as a control or with the indicated concentration (0.5, 1, 1.5 μg/well) of Runx2 and CBFβ. Total RNA was isolated at the indicated times. Expression of *smpd3* was analyzed by real-time RT-PCR (A,B). Expression levels relative to mGAPDH are presented. The experiments were repeated independently at least three times. Asterisks (*) indicate $P < 0.05$ versus the control.

ected C2C12 cells (Fig. 2B, lanes 2 and 4).

We then generated deletion constructs of the *smpd3* promoter (Fig. 2A). The transactivating potential of Runx2 decreased in the Del-*smpd3*-1 and Del-*smpd3*-2 deletion constructs (Fig. 2C). COS7 cells co-transfected with 1.1 kb-*smpd3*-Luc and Runx2 showed higher luciferase activity than Del-*smpd3*-1 and Del-*smpd3*-2-Luc transfectants. Thus, Runx2-responsive elements (RRE) in the -562/-557 region are important for activation of the *smpd3* promoter by Runx2 (Fig. 2C).

Identification of Runx2-binding sites within the *smpd3* promoter region

We performed EMSA analysis to determine which Runx2-responsive elements within the *smpd3* promoter were involved in Runx2 binding. Three sets of double-stranded oligonucleotides were designed, corresponding to the three putative Runx2-responsive elements (RRE) in the 1.1 kb *smpd3* promoter, termed

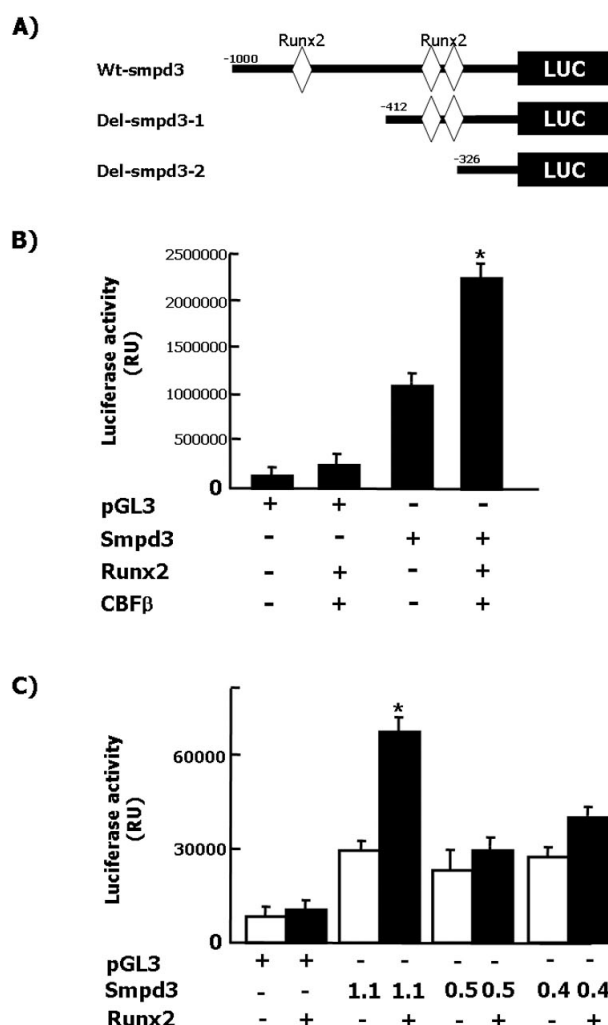


Fig. 2. Regulation of *smpd3* promoter activity. (A) The 1.1 kb 5'-flanking promoter region of *smpd3* and serial deletion constructs. Three potential Runx2-binding sites (RRE) were identified within the 1.1 kb *smpd3* promoter. Deletion constructs were designated as WT-*smpd3* (1.1) (spanning nucleotides -562 to -557), Del-*smpd3*-1 (0.5) (spanning nucleotides -355 to -350), and Del-*smpd3*-2 (0.4) (spanning nucleotides -342 to -337). (B) Cos7 cells were transiently co-transfected with the WT-*smpd3* reporter construct and Runx2, CBFβ cDNA, or the pcDNA3.1 control vector. A total of 0.5 μg DNA was used, by compensating with the pcDNA3.1 empty vector. Luciferase activity was measured 24 hours after transfection. Each assay was performed at least three times in triplicate. (C) Cos7 cells were transiently co-transfected with the WT-*smpd3* reporter or a deletion construct, and either Runx2 cDNA or the pcDNA3.1 control vector. Luciferase activity was measured 24 h after transfection. Each assay was performed at least three times in triplicate. The experiments were repeated independently at least three times. Asterisks (*) indicate $P < 0.05$ versus the pcDNA3.1 control.

P1 (spanning nucleotides -562 to -557), P2 (spanning nucleotides -355 to -350), and P3 (spanning nucleotides -342 to -337). *In vitro* transcribed and translated Runx2 proteins were

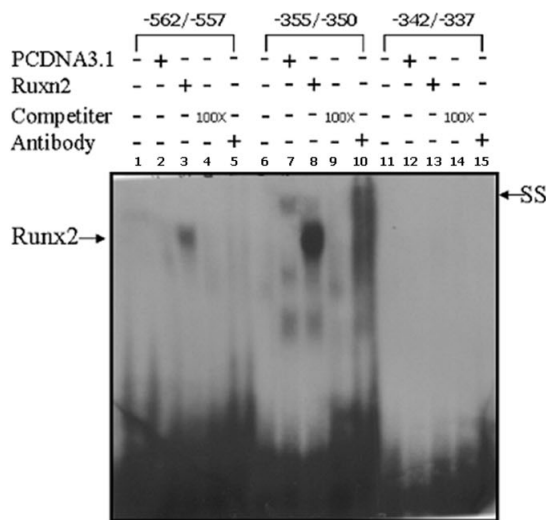


Fig. 3. Runx2 binds to the *smpd3* promoter. [γ - P^{32}]ATP-labeled P1 (spanning nucleotides -562 to -557), P2 (spanning nucleotides -355 to -350), and P3 (spanning nucleotides -342 to -337) probes were incubated with *in vitro*-translated proteins. Lanes 1, 6, and 11 contain free probe; the remaining lanes contain proteins incubated with the labeled P1, P2, P3 probes alone (lanes 2, 3, 7, 8, 12, 13) or with 100-fold molar excess of unlabeled probe (lanes 4, 9, 14) or anti-Flag antibody (for supershift assays, lanes 5, 10, 15). Arrowheads indicate the binding of Runx2 to each probe.

incubated with labeled P1, P2, or P3 oligonucleotides, with or without cold competitors. The Runx2 protein weakly bound to the P1 probe (Fig. 3), but bound well to P2. Each cold competitor efficiently out-competed the radiolabeled probes, implying that binding was specific. To further confirm the specificity of Runx2 binding to P1, P2, and P3, we performed supershift assays using an anti-Flag antibody. The complex band disappeared with the addition of the anti-Flag antibody, even though supershifted bands were not clearly detected. These data reveal that, although the P2 region was bound most strongly by Runx2 (Fig. 3), transcriptional activation by Runx2 predominantly occurs at the -562/-557 site.

DISCUSSION

The identification of genes that are differentially regulated in osteogenesis will shed light on the basic developmental processes in bone. Our previous microarray analysis of developing mouse calvarial bones confirmed data regarding bone-specific gene expression and revealed several candidate bone-related genes, including *smpd3* (15). Here, we show that *smpd3* expression is upregulated by BMP2 and that the *smpd3* promoter is modulated by the transcription factor, Runx2, downstream of the BMP2 signaling pathway.

smpd3 mRNA expression in C2C12 cells increases in response to BMP2 treatment and Runx2 transfection (Figs. 1A and 1B, respectively). The metabolic and chemical character-

istics of *smpd3* have been previously reported (1, 2, 16, 17). SMases enhance BMP4-induced synthesis of osteocalcin by ceramide upstream of p44/p42 MAP kinase in MC3T3-E1 osteoblast cells (18). Furthermore, p38 MAPK acts as an upstream regulator of *smpd3*, which plays a role in the TNF- α -stimulated expression of VCAM and ICAM (adhesion proteins) (19). BMP2 signaling could activate *smpd3* transcription via MAPK signaling.

The luciferase activity of the 1.1 kb *smpd3* promoter increased in C2C12 cells transfected with Runx2 (Fig. 2B) but was lower after transfection with Del-*smpd3*-1-Luc, suggesting that the -562/-557 region of the *smpd3* promoter is important for activation by Runx2 (Fig. 2C). EMSA analyses revealed that the RREs within the -355 to -350 region bind strongly to Runx2 (Fig. 3), with weak Runx2 binding to the -562 to -557 region despite the strong transactivating power of this region. *smpd3* appears to play a role in the mineralization of bone and dentine (20).

Neutral SMases are ubiquitously expressed but are expressed at higher levels in the brain and in the embryonic bone growth plate (21). The phenotype of *smpd3*^{-/-} mice mimics that of human chondrodysplasia, which manifests as a genetic disorder with clinical heterogeneity (2, 12). In addition, defects in *smpd3* are responsible for developmental defects of the bone and altered regulation of ceramide levels (22). Growth retardation of *smpd3*^{-/-} mice primarily manifests as a delay in long bone ossification (1, 3, 12). Also, deletions in *smpd3* induce osteogenesis and dentinogenesis imperfecta in mice (2). The mitogenic effects of TNF- α on mesenchymal cells is mediated by the sphingolipid pathway, and *smpd3* (the initial step of the sphingolipid pathway) is regulated by a proteinase cascade that involves furin, MT1-MMP, and MMP2 (23). SMases are activated by the BMP2-Runx2 signaling pathways and function as key intercellular messengers in osteoblast differentiation.

In conclusion, *smpd3* is a downstream target of Runx2, which increases *smpd3* expression in premyoblastic cells. Thus, BMP2 stimulates Runx2 expression, which induces *smpd3* expression by directly binding to the *smpd3* promoter.

MATERIALS AND METHODS

Materials

Bioactive recombinant human BMP2 protein was purchased from Wyeth, Inc. (Cambridge, MA), and fetal bovine serum was purchased from GIBCO-BRL (Grand Island, NY). The RevertAIDTM first strand cDNA synthesis kit for reverse transcription was purchased from Fermentas (EU). We purchased DNA polymerase from Invitrogen (Carlsbad, CA), and Taq polymerase and dNTP mixtures were purchased from Promega (Madison, WI). The ECL plus reagent and T4 enzyme were purchased from Amersham Pharmacia Biotech (Arlington, IL). The PVDF membranes were purchased from Schleicher & Schuell (Dassel, Germany).

DNA construction

A plasmid containing *Runx2* was constructed by cloning *Runx2* cDNA into the BamHI and XhoI sites of pcDNA3.1-FLAG or into the HindIII and XhoI sites of pcDNA3.1-Myc. A 1120 bp fragment from the 5'-flanking region of the *smpd3* gene was amplified by PCR using specific primers: 5'-CGGGTACCCATCAAGG AAGTCGTCACAC-3' (i.e., forward/kpnI) and 5'-CCGCTCGAG CGGCTCTGGCATCCGGAGCC-3' (i.e., reverse/XhoI). The pGL3-basic vector, which contains a polyadenylation signal upstream of the luciferase gene, was used to construct the expression vectors by subcloning a PCR-amplified fragment of the *smpd3* promoter into the KpnI/XhoI site. The PCR products were confirmed by electrophoresis and DNA sequencing. The -562/-557, -355/-350, and -342/-337 bp regions were predicted to be Runx2-binding sites. Two deletion constructs were cloned from the 1.1 kb *smpd3* promoter. The first deletion construct contained the -412 to +103 region (Del-*smpd3*-1-luc), which removed the -562 to -557 Runx2-binding sites. The second deletion construct contained the -326 to +103 region (Del-*smpd3*-2-luc), which removed the -355 to -350 and -342/-337 Runx2-binding sites. The Del-*smpd3*-1 and Del-*smpd3*-2 deletion constructs from 1.1 kb-*smpd3*-luc were generated by PCR using specific primers. All cloned DNA fragments were confirmed by DNA sequencing.

Cell culture

Premyoblastic C2C12 cells were cultured in Dulbecco's Modified Eagle's Medium DMEM (Gibco/BRL) with 10% fetal bovine serum (FBS). When cells were 80% to 90% confluent, they were starved by culturing in DMEM media without FBS for 48 hours to induce quiescence. COS7 cells were maintained in DMEM/high glucose media (Hyclone) supplemented with 10% FBS and 1% antibiotic/antimycotic. DMEM containing 2% FBS and 1% antibiotic/antimycotic was used to starve the COS7 cells.

RNA isolation and real-time RT-PCR

Total cellular RNA was extracted from the cells and the concentration was measured by spectrophotometry. To measure the *smpd3* mRNA levels, real-time PCR was performed using an *smpd3* sense primer (5'-TGTAAGTCCGACAGTACCAG-3') and an *smpd3* antisense primer (5'-GAAGCCGAGTTCTCCAG GTAGC-3'). Fluorescence-based real-time PCR was performed using the DNA Engine OPTICON[®] 2 system (MJ Research, Waltham, MA) and SYBR Green I (Molecular Probes, Eugene, OR). Samples were normalized to mGAPDH cDNA using mGAPDH primers.

Transient transfection and luciferase assays

Cell transfections and luciferase assays were performed as previously described (9). Briefly, the day before transfection, COS7 cells were plated in six-well plates at a density of 1.2×10^5 cells/well. The cells were transfected with Lipofectamine Plus reagent

(Invitrogen) according to the manufacturer's instructions. Each transfection was performed using the same amount of Runx2 and CBF β cDNA or pcDNA3.1 (Mock) and an *smpd3* promoter construct [WT-*smpd3* (1.1), Del-*smpd3*-1 (0.5), or Del-*smpd3*-2 (0.4) deletion luciferase reporter]. Total DNA (0.5 μ g/well) was used in each transfection. Twenty-four hours after transfection, cells were harvested for luciferase assays or RNA isolation. Supernatants from the cell lysates were examined using the luciferase assay system (Promega). The results are presented as representative data from at least three independent experiments, with triplicate wells in each experiment.

Electrophoretic mobility shift assays

We designed double-stranded DNA probes specific to *smpd3* promoter sequences containing the Runx2-responsive element [Probe 1: 5'-ACGCTCAGGATAGCGCAA-3', Probe 2: 5'-AAA GGCAGGAAACACTGA-3', Probe 3: 5'-ATCTGCAGGATGGG AGCA-3']. The double-stranded DNA probes were end-labeled with [γ -³²P]ATP using T4 polynucleotide kinase (Promega). The remaining EMSA procedures were performed as previously described (9). The Runx2 proteins were produced by *in vitro* transcription and translation using TNT-Coupled Reticulocyte Lysate (Promega). The FLAG-Runx2 protein was then bound to the labeled, double-stranded DNA probes in the presence or absence of unlabeled competitor (100-fold molar excess) for 20 minutes at room temperature. Supershift assays were performed by pre-incubating the FLAG-Runx2 protein with an α -FLAG antibody (Sigma) for 20 minutes at room temperature prior to incubation with the labeled probe. The protein-DNA complexes were then separated at 4°C on a 6% polyacrylamide gel containing 0.5x TBE buffer.

Statistical analysis

All experiments were repeated independently at least three times. Statistical significance was assessed using one-way ANOVA followed by the Bonferroni test. Results are presented as mean \pm S.E.M. Significant differences were defined as $P < 0.05$.

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