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GATA4 negatively regulates bone sialoprotein expression in osteoblasts

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GATA4 has been reported to act as a negative regulator in osteoblast differentiation by inhibiting the Dlx5 transactivation of Runx2 via the attenuation of the binding ability of Dlx5 to the Runx2 promoter region. Here, we determine the role of GATA4 in the regulation of bone sialoprotein (Bsp) in osteoblasts. We observed that the overexpression of Runx2 or Sox9 induced the Bsp expression in osteoblastic cells. Silencing GATA4 further enhanced the Runx2- and Sox9-mediated Bsp promoter activity, whereas GATA4 overexpression down-regulated Bsp promoter activity mediated by Runx2 and Sox9. GATA4 also interacted with Runx2 and Sox9, by attenuating the binding ability of Runx2 and Sox9 to the Bsp promoter region. Our data suggest that GATA4 acts as a negative regulator of Bsp expression in osteoblasts. [BMB Reports 2016; 49(6): 343-348]

INTRODUCTION

Bone remodeling is controlled by osteoblasts and osteoclasts, which regulate the bone formation and bone resorption, respectively (1, 2). The balance between osteoblastic and osteoclastic activities is important for bone homeostasis, while an imbalance leads to bone diseases such as osteoporosis.

Runx2 is involved in mesenchymal condensation, osteoblast differentiation, chondrocyte hypertrophy, and vascular invasion of the developing skeleton from mesenchymal stem cells (3, 4). Cleidocranial dysplasia, the defective bone formation disease in humans, results from Runx2 haploinsufficiency (4, 5). Runx2 is controlled by various transcription factors such as Dlx5 and TAZ (6-8). During osteoblast differentiation, it induces the expression of osteoblast marker genes, including alkaline phosphatase (*ALP*), collagen type I, osteopontin, bone sialoprotein (*Bsp*), and osteocalcin (*OCN*) (9).

Sox9 is a member of the Sox family of transcription factors containing a highly conserved high mobility group (HMG) domain (10). To date, about 20 Sox proteins have been identified in mice and humans which are grouped into A through H, based on their structural homology outside of their HMG boxes (11). Sox9 presents a consensus DNA-binding sequence (AACAAT) and regulates various biological processes (10). Sox9 is involved in chondrogenic differentiation and extracellular matrix (ECM) deposition (12).

GATA4 is a member of the GATA family of zinc finger transcription factors and plays a role in cardiac development and adult cardiac hypertrophy. GATAs present a consensus DNA-binding sequence (A/T)GATA(A/G) and regulate various biological processes. GATA1, 2, and 3 are expressed in hematopoietic stem cells, whereas GATA4, 5, and 6 are expressed in mesoderm- and endoderm-derived tissues (13, 14). Among them, GATA4 plays various roles through interactions with regulatory proteins such as p300, RXRα, and SRF (15).

Bsp, an extracellular matrix protein, plays a critical role in osteoblast differentiation involved in cementum integrity, and is essential for periodontal function (16). Bsp belongs to the small Integrin Binding Ligand N-linked Glycoprotein (SIBLING) family, involved in mineralized tissues of the skeleton and dentition (16-18). Like other SIBLING proteins, Bsp is multifunctional, with roles in cell attachment and migration, cell signaling, collagen binding, and hydroxyapatite nucleation (16). Hunter and Goldberg demonstrated that *in vitro*, Bsp is a positive regulator of hydroxyapatite precipitation (19). Mice that were null for the *Bsp* gene (*Bsp*−/−) presented delays in bone growth, mineralization, and deficiency in acellular cementum and periodontal attachment (16, 20).

Although Bsp is one of the important genes for bone homeostasis, the regulatory mechanism of Bsp expression remains unclear. When we analyzed the *Bsp* promoter region sequence, we identified putative binding sites for Runx2, Sox9, and GATA4. Therefore, in the present study, the role of Runx2, Sox9, and GATA4 in the regulation of *Bsp* gene expression has been investigated.

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RESULTS

GATA4 regulates *Bsp* promoter activities mediated by Runx2 and Dlx5

Runx2 is important for the regulation of osteoblastic genes such as *Bsp*. Therefore, we investigated the role of Runx2 in *Bsp* expression by transient transfection assays using a Bsp luciferase reporter vector containing the 1.5 Kb promoter region of *Bsp*. This 1.5 Kb promoter region contains three putative Runx2-binding sites and one putative GATA-binding site (Fig. 1A) (7). Consistent with previous reports (21), Runx2 strongly induced the activity of the *Bsp* promoter (Fig. 1B). Runx2-mediated *Bsp* promoter activity was further enhanced by GATA4 knockdown using a shGATA4 construct, while GATA4 overexpression significantly attenuated the Runx2-mediated *Bsp* promoter activity.

Since the *Bsp* 1.5 Kb promoter region also contains a putative Dlx5-binding site, we examined the effect of GATA4 on Dlx5-mediated activation of the *Bsp* promoter. Dlx5 induced the activity of the *Bsp* promoter, although activation of the *Bsp* promoter by Dlx5 was weaker than that mediated by Runx2 (Fig. 1B, C). Similar to Runx2 results, GATA4 overexpression reduced Dlx5-mediated activation of the *Bsp* promoter, whereas GATA4 knockdown significantly increased the Dlx5-mediated *Bsp* promoter activity (Fig. 1C). Together, these results suggest that GATA4 attenuates Runx2- and Dlx5-mediated activation of the *Bsp* promoter.

Sox9 and Runx2 regulate Bsp expression

To explore the role of Sox9 in osteoblasts, we examined the expression pattern of Sox9 during osteoblast differentiation. Osteoblast-like MC3T3-E1 cells were cultured in osteogenic medium containing ascorbic acid, β-glycerophosphate, and bone morphogenetic protein 2 (BMP-2). In reverse transcription polymerase chain reaction (RT-PCR) analysis, the expression of well-known osteogenic marker genes, including *Runx2*, *Bsp*, and *OCN*, was strongly induced during osteoblast differentiation. *Sox9* was also steadily expressed in MC3T3-E1 cells during osteoblast differentiation (Fig. 2A), suggesting that Sox9 might play a role in osteoblast differentiation.

Next, we examined the effect of Sox9 on Bsp expression in osteoblasts. Sox9- or Runx2-expressing plasmid was transiently transfected in MC3T3-E1, and Bsp expression was examined by RT-PCR. *Bsp* expression was increased by overexpression of Sox9 and Runx2 (Fig. 2B). Interestingly, Sox9 and Runx2 mutually increased the *Bsp* gene expression. Similar results were observed when we investigated the effect of Sox9 on Bsp expression at the protein level. Expression levels of Bsp protein were strongly increased by Sox9 or Runx2 in a dose-dependent manner in MC3T3-E1 cells (Fig. 2C, D) and C3H10T$_{1/2}$ (Fig. 2E). Collectively, these findings indicate that Sox9 might play a role in the regulation of Bsp expression during osteoblast differentiation.

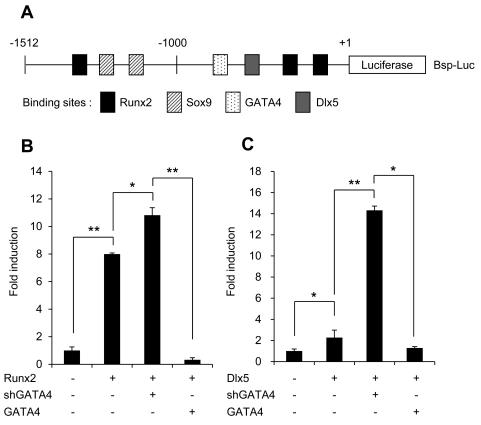

Fig. 1. GATA4 attenuates the Runx2- and Dlx5-mediated activation of the *Bsp* promoter. (A) Bsp-1.5 kb promoter luciferase reporter (Bsp-1512-Luc) contains putative binding sites for Runx2, Dlx5, Sox9, and GATA4. (B) C2C12 cells were co-transfected with Bsp-1512-Luc luciferase reporter, together with Runx2 and shRNA-GATA4 or GATA4, as indicated. (C) C2C12 cells were co-transfected with the Bsp-1512-Luc luciferase reporter together with Dlx5 and shRNA-GATA4 or GATA4, as indicated. Each well was also co-transfected with 20 ng Renilla expression vector to control for transfection efficiency. Luciferase activity was normalized to Renilla activity as expressed by the co-transfected plasmid. Data are presented as mean ± SD of triplicate samples. Results are representative of at least 3 independent sets of similar experiments. *P < 0.05, **P < 0.01 versus control.

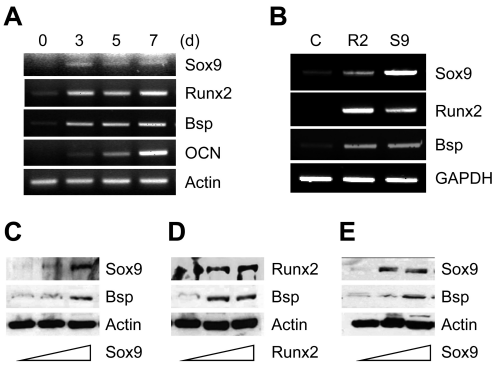

Fig. 2. Sox9 and Runx2 increase Bsp expression. (A) MC3T3-E1 cells were cultured in osteogenic medium containing ascorbic acid and β-glycerophosphate. Total RNA was collected at each time point. RT-PCR was performed for *Sox9* and osteogenic marker genes, including *Runx2*, *Bsp*, *OCN*, and β-actin for control. (B) MC3T3-E1 cells were transduced with control (pcDNA3.1; C), Runx2 (R2), or Sox9 (S9). After 48 hours, total RNA was collected from each sample. RT-PCR was performed to assess the expression of *Sox9*, *Runx2*, *Bsp*, and *GAPDH* for control. (C, D) MC3T3-E1 cells were transduced with various concentrations (200, 500, and 1,000 ng) of Sox9 (C) and Runx2 (D). (E) C3H10T$_{1/2}$ cells were transduced with various concentrations (200, 500, and 1,000 ng) of Sox9.

Sox9 and Runx2 synergistically activate the *Bsp* promoter

Since Sox9 overexpression induced Bsp expression in osteoblastic cells, we investigated whether Sox9 directly activates the *Bsp* promoter by transient transfection assay using the *Bsp* promoter plasmid. Sequence analysis indicated the presence of two putative Sox9-binding sites in the 1.5 Kb promoter region of *Bsp* (Fig. 1A). Sox9 overexpression significantly enhanced the activity of the *Bsp* promoter in a dose-dependent manner (Fig. 3A). In addition, Sox9 and Runx2 synergistically activated the *Bsp* promoter (Fig. 3B). Collectively, our data imply that Sox9 directly activates the *Bsp* promoter, and that Sox9 cooperates with Runx2 to induce Bsp expression during osteoblast differentiation.

GATA4 regulates the *Bsp* promoter activities mediated by Runx2 and Sox9

Since we observed that GATA4 attenuated the *Bsp* promoter activities mediated by Runx2 and Dlx5 (Fig. 1), we next tested whether GATA4 regulates the *Bsp* promoter activities mediated by Runx2 and Sox9. Consistent with the above mentioned data, combination of Runx2 and Sox9 enhanced the *Bsp* promoter activity. However, GATA4 overexpression down-regulated the *Bsp* promoter activity mediated by Runx2 and Sox9. GATA4 knockdown by shGATA4 overexpression strongly enhanced the *Bsp* promoter activity mediated by Runx2 and Sox9 (Fig. 3C). This finding indicates that GATA4 regulates the *Bsp* promoter activities mediated by Runx2 and Sox9.

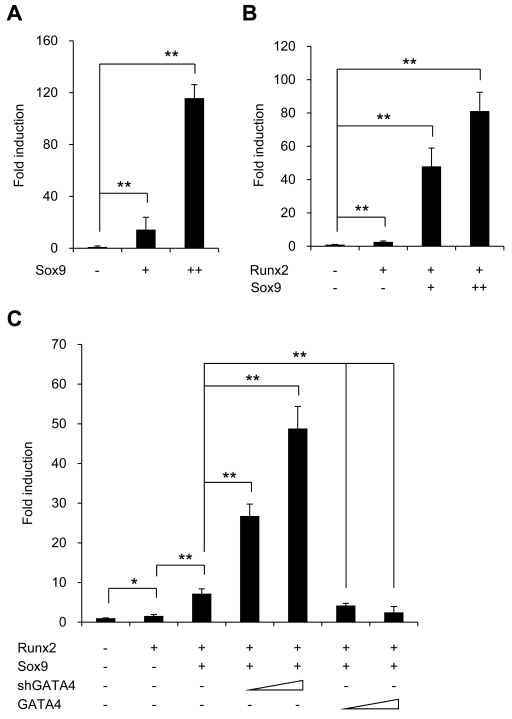

Fig. 3. GATA4 attenuates the Runx2- and Sox9-mediated Bsp activation. (A) C2C12 cells were co-transfected with the Bsp-1.5 kb promoter luciferase reporter and Sox9. (B) C2C12 cells were co-transfected with the Bsp-1512-Luc luciferase reporter and Runx2 and Sox9, as indicated. (C) C2C12 cells were co-transfected with the Bsp-1512-Luc luciferase reporter and Runx2, Sox9, and shRNA-GATA4 (25 and 50 ng) or GATA4 (50 and 100 ng) as indicated. Luciferase activity was normalized to Renilla activity as expressed by the co-transfected plasmid. Results are representative of at least 3 independent sets of similar experiments. Data are presented as mean ± SD of triplicate samples. *P < 0.05, **P < 0.01 versus control.

GATA4 attenuates the binding ability of Runx2 and Sox9 to the *Bsp* promoter region

To investigate the inhibitory mechanism of GATA4 on Runx2- and Sox9-mediated *Bsp* transcriptional activity, we performed an immunoprecipitation (IP) assay to determine the interaction between GATA4 and Runx2 or Sox9. Human embryonic kidney (HEK) 293T cells were co-transfected with Flag-GATA4 and myc-Runx2 or HA-Sox9. IP assay revealed that GATA4 could directly interact with Runx2 and Sox9 (Fig. 4A, B). These findings suggest that GATA4 might inhibit the Runx2- and Sox9-mediated *Bsp* induction through interacting with Runx2

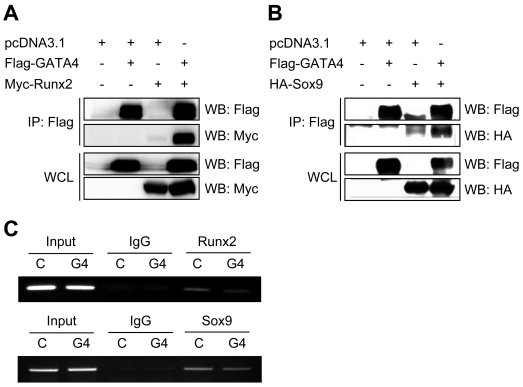

Fig. 4. GATA4 interacts with Runx2 and Sox9, and subsequently attenuates the binding ability of Runx2 and Sox9 to the *Bsp* promoter region. (A) HEK293T cells were transfected with Flag-GATA4 and myc-Runx2. Lysates were immunoprecipitated (IP) with an anti-Flag antibody. Immunoprecipitated samples (upper panel) or whole cell lysates (WCL; lower panel) were subjected to western blotting to detect GATA4 (Flag) and Runx2 (myc). (B) HEK293T cells were transfected with Flag-GATA4 and HA-Sox9. Lysates were immunoprecipitated (IP) with an anti-Flag antibody. Immunoprecipitated samples (upper panel) or whole cell lysates (WCL; lower panel) were subjected to western blotting to detect GATA4 (Flag) and Sox9 (HA). (C) ChIP assay of GATA4 binding to the *Bsp* promoter region. C2C12 cells were transfected with pMX-IRES-EGFP (C) or pMX-IRES-GATA4-EGFP (G4) plasmid. After 48 hours of transfection, samples were immunoprecipitated with control IgG or anti-Runx2 antibody (upper panel), and control IgG or anti-Sox9 antibody (lower panel); samples were subjected to PCR amplification with primers specific for the Runx2 binding sites (upper panel) or Sox9 binding sites (lower panel) in the *Bsp* promoter region.

and Sox9.

To determine whether GATA4 could modulate the binding ability of Runx2 and Sox9 to the *Bsp* promoter region, we carried out a chromatin immunoprecipitation (ChIP) assay. GATA4 was transfected in C2C12 cells and immunoprecipitated with Runx2 and Sox9 antibodies. ChIP assay revealed that the binding ability of Runx2 and Sox9 to the *Bsp* promoter region was decreased by GATA4 overexpression, than that seen in the control (Fig. 4C). Taken together, these results suggest that GATA4 can interact with Runx2 and Sox9, and attenuates the binding ability of Runx2 and Sox9 to the *Bsp* promoter region.

DISCUSSION

In the present study, we analyzed the regulatory mechanism of *Bsp* by various transcription factors, which are important for osteoblast differentiation. Our data showed that *Bsp*, one of the important genes in osteoblasts, was strongly activated by overexpression of Runx2, Dlx5, and Sox9. *Bsp* activation by these transcription factors was attenuated by GATA4, suggesting that GATA4 negatively regulates Bsp expression during osteoblast differentiation.

GATA4 had been studied in cardiovascular development such as maintenance of postnatal cardiac function and protection from stress-induced heart failure. GATA4 is responsible for pathological cardiac hypertrophy. We previously demonstrated that GATA4 plays a negative role in osteoblast differentiation by down-regulating the osteogenic genes such as *Runx2*, *ALP*, *Bsp*, and *OCN*. GATA4 interacts with Dlx5 and subsequently inhibits the binding ability of Dlx5 to the Runx2 promoter region (22). Taken together, our results suggest that GATA4 negatively regulates osteoblast differentiation via down-regulation of Runx2 and Bsp. Further studies are warranted to determine whether GATA4 regulates other osteoblastic genes.

Sox9 suppresses Runx2-mediated *OCN* expression (8). However, in this study, exogenous Sox9 increased Bsp expression in MC3T3-E1 and $C3H10T_{1/2}$ cells. Furthermore, Sox9 augmented the *Bsp* promoter activity when using the *Bsp* promoter that contained the Sox9-response elements. In addition, Sox9 further enhanced the Runx2-mediated transactivation of the *Bsp* promoter. These data indicate that Sox9 might regulate the Bsp expression together with Runx2. Interestingly, Sox9 overexpression could induce Runx2 expression in MC3T3-E1 cells, suggesting that Sox9 might also indirectly regulate Bsp expression via Runx2 induction.

In summary, our study demonstrates that GATA4 might play a negative role in osteoblast differentiation, by regulating the Bsp expression via modulating the binding activities of Runx2 and Sox9 to the *Bsp* promoter region. We recently demonstrated that GATA4 down-regulates the *Runx2* gene. Hence, our study reveals an additional layer of negative regulation of GATA4 in osteoblasts. Further elucidation of the regulatory mechanism of GATA4 for other osteoblastic genes might provide additional therapeutic approaches to various bone diseases.

MATERIALS AND METHODS

Reagents

Antibodies against Flag and hemagglutinin (HA) were obtained from Sigma-Aldrich (St Louis, MO, USA) and Roche Applied Sciences (Indianapolis, IN, USA), respectively. Anti-Runx2 and anti-Bsp were purchased from Santa Cruz Biotechnology, INC. (Dallas, TX, USA). Anti-Sox9 was obtained from Abcam (Cambridge, UK). Dynabead protein G for immunoprecipitation was obtained from Novex Life technologies (Carlsbad, CA, USA).

Constructs

GATA4 was prepared by RT-PCR using RNA from C57BL/6 mouse heart, as previously described (22). Sox9 was prepared by RT-PCR using RNA from $C3H10T_{1/2}$ cells. The primer sequences are as follows: *Sox9* sense: 5'-CGG GAT CCA CCA TGA ATC TCC TGG ACC CCT TC-3'; *Sox9* antisense: 5'-CCG CTC GAG CGC GGT CAG GGT CTG GTG AGC TG-3'. The amplified PCR fragments were cloned into the HA-pcDNA3.1 vector. Bsp-1.5 Kb promoter luciferase reporter was kindly provided by KY Lee (Chonnam National University, Gwangju, Korea).

RT-PCR

MC3T3-E1 cells were induced by 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, and 100 ng/ml BMP-2, to assess the expression of osteogenic marker genes during osteoblast differentiation. RT-PCR was performed as previously described (23, 24). The primer sequences were as follows: *Sox9* sense: 5'-TTC CTC CCT TAG CCA ACC TT-3' and *Sox9* antisense: 5'-GGC GTC TGG ACT TTA GTT GC-3'; *Runx2* sense: 5'-CCC AGC CAC CTT TAC CTA CA-3' and *Runx2* antisense: 5'-CAG CGT CAA CAC CAT CAT TC-3'; *Bsp* sense: 5'-AAA GTG AAG GAA AGC GAC GA-3' and *Bsp* antisense: 5'-ACT CAA CGG TGC TGC TTT TT-3'; *OCN* sense: 5'-GCG CTC TGT CTC TCT GAC CT-3' and *OCN* antisense: 5'-ACC TTA TTG CCC TCC TGC TT-3'; β-actin sense: 5'-AAG AGC TAT GAG CTG CCT-3' and β-actin antisense: 5'-CAC AGG ATT CCA TAC CCA-3'; *GAPDH* sense: 5'-TGA CCA CAG TCC ATG CCA TCA CTG-3' and *GAPDH* antisense: 5'-CAG GAG ACA ACC TGG TCC TCA GTG-3'.

Promoter assay

For transfection of reporter plasmids, C2C12 cells were plated on 24-well plates at a density of 2×10^4 cells/well, one day before transfection. Plasmid DNA was mixed with TransIT-2020 (Mirus, Madison, WI, USA) and transfected into the cells, as per the manufacturer's protocol. After 48 hours of transfection, the cells were washed twice with PBS and then lysed in reporter lysis buffer (Promega, Madison, WI, USA). Luciferase activity was measured with a luciferase assay

system (Promega) according to the manufacturer's instructions. Luciferase activity was measured in triplicate, averaged, and then normalized to β-galactosidase activity using *o*-nitrophenyl-β-D-galactopyranoside (Sigma-Aldrich) as a substrate.

Immunoprecipitation assay and western blot analysis

The 293T cells were transfected with Flag-GATA4 and myc-Runx2 or Flag-GATA4 and HA-Sox9 for 48 hours, washed with chilled PBS, and lysed in extraction buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, PMSF, and protease inhibitors). Cell lysates were immunoprecipitated with monoclonal anti-Flag and anti-HA antibodies. Cell lysates and immunoprecipitated samples were subsequently separated by SDS-PAGE and transferred to PVDF membrane (Millipore, Temecula, CA, USA). The membrane was blocked with TBS-T (10 mM Tri-HCl (pH 7.6), 150 mM NaCl, 0.1% Tween 20) containing 5% skim milk, and was probed with horseradish peroxidase (HRP)-conjugated antibodies, including anti-Flag-HRP (Sigma) and anti-HA-HRP (Sigma). Signals were detected with chemiluminescence (ECL) photo film.

ChIP assay

A chromatin immunoprecipitation (ChIP) assay was performed with a ChIP kit (Upstate Biotechnology, Lake Placid. NY, USA), according to the manufacturer's instructions, using antibodies against Sox9 and Runx2 with control IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The precipitated DNA was subjected to PCR amplification with specific primers for the Bsp 1.5 kb promoter region containing Sox9- and Runx2-binding sites. The following primers were used for PCR: Bsp-R3 sense, 5'-GCT TAT GGG GGT GAA TTG AA-3'; Bsp-R3 antisense, 5'-AGC TAA AGA AAA GTA TTT CAG CAT TT-3' for Runx2, and Bsp-S sense, 5'-AAA TGC TGA AAT ACT TTT CTT TAG CT-3'; Bsp-S antisense, 5'-TCC CCA TAT TCT TCT CTG TTG A-3' for Sox9.

Statistical analysis

All values are expressed as means ± SD. Statistical analyses were performed using two-tailed Student's *t*-tests. P values less than 0.05 were considered statistically significant.

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