GATA4 negatively regulates bone sialoprotein expression in osteoblasts

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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, a disease characterized by defective bone formation, is caused by an imbalance in Runx2 activity. Runx2 and Sox9, members of the SOX family of transcription factors, are key regulators of bone formation and bone resorption, which are involved in the regulation of bone sialoprotein (Bsp) expression in osteoblasts (5).

Runx2 and Sox9 are involved in bone formation and bone resorption, respectively (1, 2). The balance between osteoblastic and osteoclastic activities is important for bone homeostasis. When we analyzed the expression of Runx2 or Sox9, 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 downregulated Bsp promoter activity mediated by Runx2 and Sox9. GATA4 also interacts with Runx2 and Sox9, 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]

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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 C3H10T1/2 (Fig. 2E). Collectively, these findings indicate that Sox9 might play a role in the regulation of Bsp expression during osteoblast differentiation.
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.

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 activities mediated by Runx2 and Dlx5.
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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 C3H10T1/2 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 C3H10T1/2 cells. The primer sequences are as follows: Sox9 sense: 5’-CGG GAT CCA CCA TGA ATC GCC GTG ACT TTG 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’-GGG GTC TGG ACT TTG TTA GC-3’; Runx2 sense: 5’-CCC AGC CAC CTT TAC CA-3’ and Runx2 antisense: 5’-GGT CAA CAC CAT CAT TC-3’; Bsp sense: 5’-AAA GTG AAG GAA AGC GAC GAC GA-3’ and Bsp antisense: 5’-ACT CAA CGG TGC TGG TT-3’; OCN sense: 5’-GGG GTC TGT CTC TCT GAC CT-3’ and OCN antisense: 5’-ACC TTA TGG 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 CTA CGT-3’ and GAPDH antisense: 5’-CAG GAG ACA ACC TGG TCC TCA GT-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 Tris-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 TT-3' antisense, 5'-TCC CCA TAT TCT TCT CGT 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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