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GATA2 and Sp1 Positively Regulate the *c-kit* Promoter in Mast Cells

Keiko Maeda, Chiharu Nishiyama, Hideoki Ogawa, and Ko Okumura

The *c-kit* gene is expressed in hematopoietic stem cells and lineage progenitor cells but is downregulated during cell development in most lineages, except for mast cells. In mast cells, high expression of *c-kit* is maintained during development, and c-Kit signaling is essential for mast cell development. To analyze the mechanisms by which *c-kit* gene expression are regulated in mast cells, we examined mast cell type-specific regulation of the *c-kit* promoter region. We observed that a GC-box in the *c-kit* promoter was critical for transcriptional activity and was bound to the transcription factor Sp1 as assessed using reporter assay and electrophoretic mobility assay. Chromatin immunoprecipitation assay and coexpression analyses showed that the transcription factor GATA2, which was recruited to the *c-kit* promoter in a mast cell-specific manner, in addition to Sp1, transactivated the *c-kit* promoter via the GC-box. Electrophoretic mobility assay and rechromatin immunoprecipitation assay indicated that GATA2 binds to the GC-box by forming a complex with Sp1. Introduction of Sp1 small interfering RNA significantly reduced the amount not only of Sp1 but also of GATA2 binding to the *c-kit* promoter in mast cells, resulting in suppression of *c-kit* transcription. Knockdown of GATA2 suppressed the recruitment of GATA2 toward the *c-kit* promoter, subsequently suppressing cell surface expression of c-Kit. These findings indicate that GATA2 and Sp1 play crucial roles in expression of the *c-kit* gene in mast cells. *The Journal of Immunology*, 2010, 185: 000–000.

The *c-kit* gene encodes a receptor tyrosine kinase that is essential for hematopoiesis and is known as stem cell factor (SCF) receptor and is one of the specific markers of mast cells. In the hematopoietic system, *c-kit* is expressed in stem cells and lineage progenitor cells, but is downregulated upon terminal erythrocyte and monocyte/macrophage development. In mast cells, *c-kit* is expressed throughout the developmental pathway to mature mast cells from hematopoietic stem cells, including mast cell progenitors (Lin[−], c-Kit⁺, Sca1[−], Ly6c[−], FcεRIα[−], CD27-b7⁺, T1/ST2⁺) in mouse adult bone marrow (1), and basophil and mast cell bipotent progenitors (Lin[−], c-Kit⁺, FcγRII/III⁺, β7⁺) in mouse spleen (2). The c-Kit/SCF signaling is essential for mast cell development in both mice and humans as follows. *W* and *SI* mice, which have mutations in the chromosomal loci encoding c-Kit and SCF, respectively, have severely decreased numbers of mast cells (3, 4), and numerous *W* mutations affecting c-Kit structure and function have been identified and characterized. In the *W* mutant *Wsh*, *c-kit* expression is diminished in hematopoietic progenitors in the bone marrow (BM) and lost in BM-derived mast cells (BMMCs) (5). Mastocytosis is characterized by accumulation of mast cells in various organs and release of mast cell mediators, caused by active mutations in the *c-kit* gene (6, 7). The D816V-mutated active variant of c-Kit, which is most frequently detected in

patients with systemic mastocytosis, induces cluster formation and expression of several molecules involved in mast cell differentiation and adhesion, including microphthalmia transcription factor, IL-4 receptor, histamine, CD63, and ICAM-1 (8), demonstrating that c-Kit-mediated signaling is critical for differentiation and stimulation of mast cells. Therefore, determination of the mechanism of *c-kit* expression in mast cells will be useful for prevention and/or treatment of *c-kit*-mediated mast cell disorders.

The role of MITF as a transcription factor that regulates *c-kit* expression in mast cells has been extensively studied by Kitamura's group using *mi/mi* mice that carry a serious mutation in *Mitf* (9, 10). Their reports demonstrate that MITF is critical for the expression of *c-kit* in mast cells but not in erythroid precursors, testicular germ cells, or neurons. Their studies also suggest the presence of other unknown transcription factors that regulate *c-kit* expression in mast cells based on a number of observations including an increase in the proportion of c-Kit positive mast cells in *mi/mi* mice after birth.

The GATA family is comprised of six zinc-finger transcription factors, GATA1–6. Among them, GATA1, 2, and 3 are indispensable for hematopoiesis. GATA3 is considered to be essential for T cell differentiation from the earliest stages of development, and is a master regulator of Th2 differentiation (11, 12). In addition, GATA3 is upregulated by Notch2 signaling in myeloid progenitors, which facilitates development toward mast cell-producing progenitors (13). GATA1 is expressed in erythroid cells, megakaryocytes, eosinophils, and mast cells, and is involved in the development of these lineages (14–16). Furthermore, GATA1 regulates the expression of mast cell-specific molecules, including FcεRI α-, β-chains (17–20), and carboxypeptidase A genes (21). GATA2 is expressed at particularly high levels in progenitors of the several hematopoietic lineages, and is required for mast cell development but is dispensable for the terminal differentiation of erythroid cells and macrophages (22). *Gata2* knockout mice exhibit panhematopoietic defects and embryonic lethality (23), whereas enforced expression of GATA2 in mouse embryonic stem cells enhances proliferation of hematopoietic progenitors (24). In a

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Abbreviations used in this paper: BM, bone marrow; BMDC, BM-derived dendritic cell; BMMC, BM-derived mast cell; ChIP, chromatin immunoprecipitation; Ct, threshold cycle number; SCF, stem cell factor; shRNA, short hairpin RNA; siRNA, small interfering RNA.

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mouse embryonic cells and OP9 stroma cell system, GATA2 not only inhibits macrophage differentiation, but also induces lineage distortion to megakaryocyte or erythroid cell differentiation by suppressing the expression and function of the transcription factor PU.1, depending on differentiation status (25). Both GATA1 and GATA2 are expressed in mast cells. Although GATA2 is dominantly expressed in immature cells (16), GATA1 is mainly expressed in mature cells. The repression of GATA1/2 leads to the impairment of cell survival, degranulation and cytokine production, due to defective signal transduction in mast cells (26, 27). These observations suggest that GATA1 and 2 play important roles in gene expression in mast cells. However, the role of GATA1 and 2 in *c-kit* expression in mast cells is largely unknown.

In the current study, we analyzed the regulation mechanisms of *c-kit* expression in mast cells and found that *c-kit* expression of mast cells is maintained by GATA2 and Sp1 recruitment to the promoter region.

Materials and Methods

Cell culture

For BMDCs, lineage negative BM cells from BALB/c mice were prepared using the MACS lineage negative cell preparation system (Miltenyi Biotec, Tübingen, Germany), and cultured in RPMI 1640 (Sigma-Aldrich, St Louis, MO), supplemented with FBS (10%, Sigma-Aldrich), nonessential amino acid solution (1%, Invitrogen, Carlsbad, CA), SCF (100 ng/ml, Wako Junyaku, Osaka, Japan), and IL-3 (10 ng/ml, Wako Junyaku) for at least 4 wk based on previously established methods (28, 29).

For BM-derived dendritic cells (BMDCs), BM cells from BALB/c mice were cultured in RPMI 1640, supplemented with FCS (10%), nonessential amino acid solution (1%), and 10 ng/ml GM-CSF (Peprotech, London, U.K.). After at least 10 d, we harvested the loosely adherent cells.

The PT18 mouse mast cell line was cultured in RPMI 1640, supplemented with FCS (10%) and nonessential amino acid solution (1%), and the CV1 simian kidney cell line was cultured in DMEM (Sigma-Aldrich), supplemented with FCS (10%) and nonessential amino acid solution (1%).

Plasmid constructs

The mouse *c-kit* promoter (−622/+22) was subcloned into the reporter plasmid pGL3-Basic (Promega, Madison, WI) at the XhoI/HindIII sites and deletion mutants were generated using an endonuclease/exonuclease deletion kit (Takara Bio, Otsu, Japan) in a reporter assay.

Plasmids in which several bases were replaced were generated by site-directed mutagenesis using a QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. All mutations were verified by sequencing analysis.

GATA2 open reading frame cDNA was obtained from PT18 mRNA by RT-PCR using SuperScript First-Strand synthesis system (Invitrogen) and Advantage 2 DNA polymerase (Clontech, Mountain View, CA), and Sp1 and GATA1, described in a previous study (17, 30), GATA2 was subcloned into pCR3.1 expression plasmids (Invitrogen), and the generated GATA1/pCR, GATA2/pCR, and Sp1/pCR were used to produce GATA1, GATA2, and Sp1, respectively.

EMSA

PT18 nuclear extracts were prepared as described in our previous report (19). Binding reactions were allowed to proceed at room temperature for 15 min in 10 mM HEPES, 100 ng poly(IIdC) (Amersham Pharmacia Biotech, Piscataway, NJ), FITC-labeled probes (Invitrogen), and 5 µg nuclear extract. Abs and competitor DNA were added to the reaction mixtures before the probes. Samples were analyzed by Fluoroimager (Amersham Pharmacia Biotech) after 4% PAGE. The oligonucleotides used were mouse *c-kit* −108/−77, 5'-CCC GGG CGG GAG AAG GGA GGG GCG TGG CCA CG-3', *c-kit* GC-box competitor of *c-kit*, −108/−77 (oligo mGC) 5'-CCC GGG CGG GAG AAG GGA GGG GAC GCG TCA CG-3', −180/−148, 5'-GGC ACA GCG CCC CCG GGA TCA GCT TAT TGC AGC-3', and −232/−197, 5'-CTA GGA GGA AGA GGA TCC AGG GTG AAG GGC CTG TGG-3'. The underlined nucleotides indicate the position of the GC-box motif in the oligonucleotide probe and nonlabeled oligonucleotides were used as self-competitors.

Abs against Sp1 (clone 1C6), GATA1 (clone N6), and GATA2 (clone H116) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA)

and isotype control mouse IgG, rabbit IgG, rat IgG, respectively, were purchased from BD Biosciences (San Diego, CA).

For in vitro transcription and translation, Sp1/pCR, GATA1/pCR, and GATA2/pCR were used with the TnT T7 Quick coupled transcription/translation system (Promega).

RNA isolation and quantitative PCR analysis

Total RNA was prepared using the RNeasy kit (Qiagen, Hilden, Germany). For quantitative analysis, cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) and analyzed with a gene specific TaqMan probe (Applied Biosystems), GATA2 (Mm 00492300_m1), Sp1 (Mm 00489039_m1), and *c-kit* (Mm 00445212_m1), using the 7500 real-time PCR system (Applied Biosystems). Quantitative values were obtained from the threshold cycle number (Ct), by subtracting the average Ct of the gene from that of GAPDH (Mm9999915_g1, Applied Biosystems) and expressed as 2^{−ΔCt} (20).

Luciferase assay

BMMCs and BMDCs (1–0.5 × 10⁶ cells) were transfected with 5 µg reporter plasmid, 3 µg expression plasmid (for coexpression analysis), and 50 ng pRL-null (Promega) by electroporation using Nucleofector II (Lonza, Basel, Switzerland) using a Macrophage Kit (Lonza) and a dendritic cell Kit (Lonza) according to the manufacturer's instructions, respectively. CV1 cells (1 × 10⁵) were transfected with 1 µg reporter plasmid, 200 ng expression plasmid, and 1 ng pRL-null by FuGene 6 (Roche, Indianapolis, IN) according to the manufacturer's instructions.

After 20 h, cells were harvested and the Dual-luciferase assay kit (Promega) and Micro Lumat Plus (Berthold Technologies, Bad Wildbad, Germany) were used to assess luciferase activity. Firefly luciferase activity derived from the reporter plasmid was normalized to Renilla luciferase activity derived from pRL-null.

Chromatin immunoprecipitation and rechromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed as described previously (20) according to manufacturer's instructions (Upstate, Lake Placid, NY) using 1–2 × 10⁶ BMMCs and PT18 cells per sample. The amounts of target DNA bound to GATA1, GATA2 or Sp1 were quantified using the 7500 real-time PCR system. The ratio of a specific DNA fragment in each immunoprecipitate to the fragment in the DNA before immunoprecipitation (input DNA) was calculated from each Ct value.

Anti-GATA1 Ab, anti-GATA2 Ab, anti-Sp1 Ab, and isotype control Abs were used the same Abs for EMSA analysis. The primer and MGB probe sequences used for this analysis were as follows: for the promoter region, *c-kit_F* (5'-CCGGGATCAGCTTATTGCA-3'), *c-kit_R* (5'-GCTACAGCTCTCGCCCAAGT-3'), and MGB probe *c-kit_T* (5'-FAM-CAGCTCGTGGC-CAC-3'); for the cis control region, *c-kit -cis_F* (5'-TCAGCTGCA-TAGTGAAGTCAACTTAG-3'), *-cis_R* (5'-TCCAGCCTGGTCTACATAGGAATTA-3'), and BGM probe *-cis_T* (5'-FAM-CTACATAGCAAGAACTT-3').

The re-ChIP assay was performed as described previously (31). The primary Ab-treatment was performed as for the ChIP assay, except that the conditions were scaled up. Briefly, 1 × 10⁷ BMMCs were fixed in formaldehyde, lysed in cell lysis buffer, and sonicated to generate DNA fragments, to which 10 µg of the primary Ab, anti-GATA2 Ab, or isotype control rabbit IgG, was added. The immunocomplex was precipitated with protein G-agarose, was washed with various washing buffers, and was then released from the beads by incubation in the elution buffers at 37°C for 1 h. The eluant of the primary immunocomplex obtained using this first Ab was diluted 10-fold with dilution buffer containing 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, and 1% Triton X-100. A second Ab, anti-Sp1 Ab, or isotype control IgG, (10 µg each) was added to the diluted solution to obtain the secondary immunocomplex. After extraction of the immunocomplex, which was precipitated with protein G-agarose, in the same way as that for the first complex, the cross-linking between chromosome DNA and nuclear proteins was reversed. The chromosome DNA was then purified by phenol and chloroform extraction and was subjected to real-time PCR using the same primers and probe sets as used in the ChIP assay.

Flow cytometry analysis

For detection of cell surface expression of c-Kit, cells were incubated with FITC-labeled anti-Fc ε RI α-chain Ab and PE-labeled anti-mouse c-Kit Ab purchased from eBioscience (San Diego, CA) and BD Bioscience (Franklin Lakes, NJ), respectively, and analyzed using flow cytometry (FACS Calibur, BD Biosciences).

Retrovirus infection

An oligonucleotide designed to function as a short hairpin RNA (shRNA) against GATA2, (5'-GATCCCCCAAGTGCATGCAAGAGAATTCAA-GAGATTCTCTTGCATGCACTTGGTTTTTA-3'), was inserted into the retroviral vector for expression of shRNA, pSUPER retro-puro (OligoEngine, Seattle, WA), and retroviral supernatants were prepared using transfection of PLAT/E packaging cells. The underlined nucleotides were required as spacers. PT18 cells were cultured in growth medium with 1 μ g/ml puromycin for 10 d to obtain transfectants prior to culture in normal medium.

Western blot analysis

Cell nuclear extracts (from 1×10^6 cells) were electrophoresed through a 10% SDS polyacrylamide gel and transferred to a membrane (Immobilon-P, Millipore, Bedford, MA). The proteins on the transferred membrane were detected using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) and the density of bands analyzed. Anti-YY1 (clone H10) and anti-GATA2 Abs were purchased from Santa Cruz Biotechnology. Alexa680-conjugated anti-rabbit IgG (Invitrogen), and IRDye800-conjugated anti-mouse IgG (Rockland, Philadelphia, PA) were used as the secondary Abs.

Transfection of small interfering RNA

GATA2 small interfering RNA (siRNA) (SI01009764), Sp1 siRNA (SI1429869), and nonsilencing control siRNA (107280) were purchased from Qiagen. A 5 μ l aliquot of 20 μ M siRNA was added to 5×10^5 BMMCs with Nucleofector II. After 20 h of culture, GATA2, Sp1, and *c-kit* mRNA was analyzed by quantitative PCR.

Statistical analysis

We used Student unpaired two-tailed *t* test for all statistical analyses. Differences between groups were considered significant when $p < 0.05$, and indicated with a single asterisk ($p < 0.05$), double asterisks ($p < 0.01$), or triple asterisks ($p < 0.001$). All statistical analyses were performed with GraphPad Prism software (GraphPad Software, San Diego, CA).

Results

The GC-box is critical for *c-kit* promoter activity

To examine cell type-specific promoter regulation of the *c-kit* gene, we generated the reporter plasmid -622/pGL3-Basic (-622/B), by introducing the 5'-upstream region of the putative translational initiation codon of the mouse *c-kit* gene, including the 622 bp promoter and 22 bp 5'-UT (-622/+22), upstream of the luciferase gene of pGL3-Basic (Basic). Then, to screen *cis*-acting elements within the *c-kit* promoter region, three 5'-deletion constructs carrying various lengths of 5'-flanking region were generated from -622/B. The luciferase activity of BMMCs that had been transiently transfected with -232/pGL3-basic (-232/B) was ~12-fold higher than that of BMMCs transfected with Basic (Fig. 1A). The promoter activity was significantly reduced by the deletion of -232/-62, although the deletion of -622/-233 markedly increased the promoter activity. In contrast, *c-kit* promoter activity driven from -232/B, -61/B, and -5/B was not significantly different from that of Basic in BMDCs, in which c-Kit cell surface expression was not detected (Fig. 1B). These results suggest that the -232/-62 region contains the elements necessary for expression of the *c-kit* promoter, which is active only in c-Kit positive cells.

To identify the critical *cis*-enhancing elements in the -232/-62 region, we constructed various mutant promoters based on the -232/B plasmid with introduction of nucleotide replacements by site-directed mutagenesis. As shown in Fig. 2, several transcription factor binding motifs; two GATA motifs, the PU.1 motif, the GC-box, and the E-box, were found in the -232/+22 region based on motif analysis. The introduction of a GC-box mutation (mGC/B) markedly reduced the luciferase activity of -232/B, whereas mutation of PU.1 or the E-box (mPU/B or mE/B, respectively) did not affect the promoter activity of -232/B. In contrast, lacking

either the GATA motif of -232GA/B or -165GA/B enhanced *c-kit* promoter activity. These results suggest that the GC-box is crucial for functioning of the *c-kit* promoter in BMMCs.

Specific binding of Sp1 to the *c-kit* promoter via the GC-box

To identify the transcription factor binding to the GC-box, EMSA was performed by using FITC-labeled double-stranded oligonucleotides as the probe (-108/-77 region) with PT18 nuclear extract in the presence or absence of the competitive oligonucleotides or Abs. The intensity of the band marked with the arrow (Fig. 3A, lane 1) was decreased by addition of anti-Sp1 Ab (Fig. 3A, lane 3), whereas isotype control IgG had no effect on this band (Fig. 3A, lane 2). When Sp1 produced by an in vitro transcription/translation system was used for EMSA, the band that showed mobility identical with that observed with the nuclear protein from PT18 was detected (Fig. 3A, lane 4) and was supershifted by addition of anti-Sp1 Ab as shown by the asterisk (Fig. 3A, lane 5). Unlabeled competitive oligonucleotides were also used to confirm the binding of Sp1 to the GC-box. The band that appeared in the mixture of probe and PT18 nuclear extract disappeared by addition of the self-competitor (Fig. 3A, lane 6). In contrast, mutant competitor lacking the GC-box (oligo mGC) had no effect on this band, (Fig. 3A, lane 7). Similarly, the band caused by addition of in vitro Sp1 disappeared in the presence of self-competitor (Fig. 3A, lane 8), but remained in the presence of the oligo mGC competitor (Fig. 3A, lane 9). These results indicate that the transcription factor Sp1 binds to the GC-box of the *c-kit* promoter.

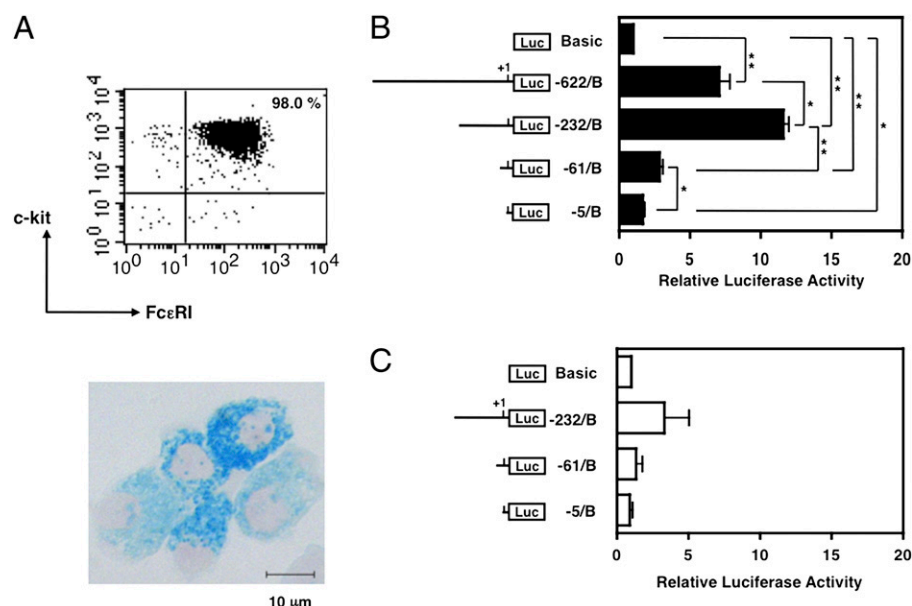
To examine the possibility that GATA1 and/or GATA2 bind to two GATA motifs in the -232/B reporter plasmid, we performed EMSA as shown in Fig. 3B. Both GATA1 and GATA2 proteins, which were produced using an in vitro transcription/translation system, bound to the -232/-197 and -180/-148 probes, and bands with GATA proteins and probes disappeared in the presence of anti-GATA1 or anti-GATA2 Ab. These findings suggest that both GATA1 and GATA2 may be recruited to GATA motifs.

To further investigate whether GATA factors and Sp1 bind to the *c-kit* promoter in vivo, a ChIP assay was performed using BMMCs and BMDCs (Fig. 4). A markedly higher amount of chromatin-containing *c-kit* promoter region (-168/+22) was immunoprecipitated by anti-GATA2 Ab (Fig. 4A) compared with control IgG in BMMCs, whereas significant binding of GATA2 was not detected in BMDCs (Fig. 4C). In addition, a significantly higher amount of *c-kit* promoter region was detected in the immunoprecipitant produced by anti-Sp1 Ab compared with isotype control IgG in BMMCs (Fig. 4A), but not in BMDCs (Fig. 4C). In contrast, a significant difference was not detected between anti-GATA1 and isotype control IgG in either BMMCs or BMDCs. Anti-GATA1 Ab, anti-GATA2 Ab, and anti-Sp1 Ab in both BMMCs and BMDCs did not immunoprecipitate the *cis*-control region of the *c-kit* promoter (Fig. 4B, 4D). These results suggest that GATA2 and Sp1, but not GATA1, are recruited to the *c-kit* promoter in a BMMC-specific manner.

The GC-box is essential for GATA2-mediated transactivation of the *c-kit* promoter

Furthermore, to elucidate the function of GATA1, GATA2, and Sp1 in the *c-kit* promoter, we introduced the GATA1/pCR, GATA2/pCR, or Sp1/pCR expression plasmid with various mutant plasmids based on -232/B into CV1 cells, and determined luciferase activity. As shown in Fig. 5A, each of the mutant reporter plasmids, lacked the GATA and/or the GC-box motifs by nucleotides replacement, was used for coexpression analysis. Enforced expression of GATA2 additionally enhanced the luciferase activities of -232/B and m-222,165GA/B to ~4-fold that of empty

FIGURE 1. *c-kit* promoter activity was reduced by deletion between $-232/-62$ in BMMCs. **A**, Surface expression of c-Kit and FcεRI, and staining with Alcian blue (original magnification $\times 630$) of BMMCs used in the current study. **B** and **C**, The 5 μ g amount of each reporter plasmid was introduced into BMMCs (**B**) or BMDCs (**C**) by electroporation. The relative luciferase activity driven by $-622/\text{pGL3-Basic}$ ($-622/\text{B}$), $-232/\text{pGL3-Basic}$ ($-232/\text{B}$), $-108/\text{pGL3-Basic}$ ($-108/\text{B}$), $-61/\text{pGL3-Basic}$ ($-61/\text{B}$), and $-5/\text{pGL3-Basic}$ ($-5/\text{B}$) are represented as the ratio to the activity driven by pGL3-Basic (Basic). Luciferase activities were normalized to the activity of pRL-null. Each experiment was conducted in duplicate for each sample, and the results expressed as the mean $\pm$ SEM for more than three independent experiments in Figs. 1, 2, and 5.



expression plasmid, but not that of mGC/B and mGA,GC/B, whereas enforced expression of GATA1 did not affect the luciferase activities derived from CV1 cells transfected with any reporter plasmids. Interestingly, overexpression of Sp1, which was identified to bind the GC-box, did not affect any luciferase activities. In addition, we introduced GATA2/pCR expression plasmid with the mutant plasmid into BMMCs by electroporation, and the luciferase activity was determined to evaluate the effect of GATA2 on the *c-kit* promoter in mast cells (Fig. 5B). The luciferase activities derived from BMMCs transfected by the plasmids, $-232/\text{B}$ and $-222,165\text{GA}/\text{B}$, were increased to ~ 2 -fold by GATA2 coexpression, whereas the promoter lacking GC-box (mGC/B and mGA, GC/B) was not significantly activated. To clarify whether GATA2 interact directly to *c-kit* promoter via Sp1 of GC-box, we performed EMSA using FITC-labeled probe containing GC-box motif ($-108/-77$) and in vitro translated Sp1 and GATA2 (Fig. 5C). When Sp1 and GATA2 were added to the probe after incubation at room temperature, the band marked with an asterisk appeared (lane 2) with lower mobility shift than that of the band containing Sp1 and the probe shown with the arrow (lane 1), suggesting that the m.w. of the complex marked with an asterisk is higher compared with that of Sp1 and probe. In addition, this band with lower mobility was supershifted by addition of anti-Sp1 as shown with double asterisks (lane 5) with showing higher m.w. than that of probe/Sp1/anti-Sp1 Ab complex as marked with an arrowhead with an asterisk (lane 4). In contrast, the band by addition of GATA2 alone to the probe mixture did not cause the specific band shift (lane 3). These results suggest that

GATA2 transactivates the *c-kit* promoter via GC-box *cis*-element, and that GATA2 binds the GC-box in the presence of Sp1.

To further confirm the interaction between GATA2 and Sp1 on the *c-kit* promoter in the BMMC chromosome, we performed a re-ChIP assay. The results of this assay indicate that GATA2 and Sp1 are simultaneously recruited to a region, including GC-box of the *c-kit* promoter, but not to a control region (Fig. 5D).

c-kit expression was reduced by knockdown of GATA2 and/or Sp1

Although a transactivating function of GATA2 was found in the coexpressing reporter assay, no effect of exogenous Sp1 was observed in the assay, which may have been due to the endogenous Sp1. Therefore, to further confirm the necessity of GATA2 and Sp1 for *c-kit* expression in BMMCs, GATA2, and/or Sp1 siRNA were introduced by electroporation. The *c-kit* mRNA levels in the resultant transfectants were determined using quantitative real-time PCR after 20 h cultivation (Fig. 6A–C), and binding of GATA2 and/or Sp1 to the *c-kit* promoter was analyzed by ChIP analysis (Fig. 6D–G). The GATA2 mRNA level in the GATA2 siRNA transfectants siGATA2/BMMC (GATA2 in Fig. 6A), and the Sp1 mRNA level in the Sp1 siRNA transfectants Sp1 siRNA (siSp1)/BMMC (Sp1 in Fig. 6B), were reduced by ~ 20 and 15%, respectively, whereas transfection of GATA2 siRNA and Sp1 siRNA had little effect on the mRNA levels of the other. The mRNA levels of *c-kit* in BMMCs were decreased to 40 or 60% by knockdown of GATA2 or Sp1, respectively (*c-kit* in Fig. 6A, 6B). These data indicate that a decrease in either GATA2 or Sp1

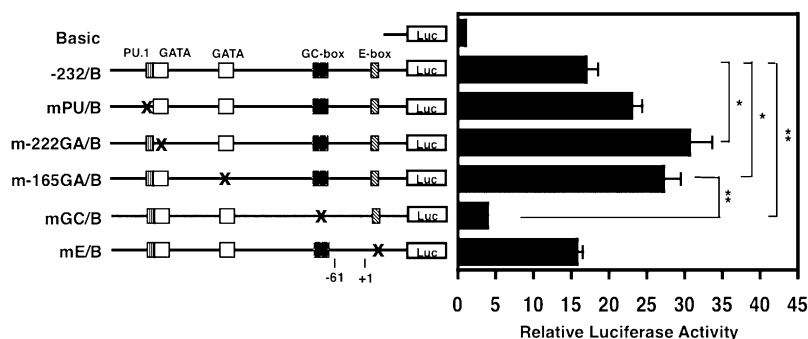


FIGURE 2. Mutation of the GC-box impaired *c-kit* promoter activity. BMMCs were transfected with 5 μ g of each reporter plasmid, Basic, $-232/\text{B}$, or the mutated $-232/\text{B}$ constructs produced by nucleotide replacement (shown as $\times$ here and in Fig. 5). The relative luciferase activity is represented as the ratio to the activity driven by pGL3-Basic (Basic).

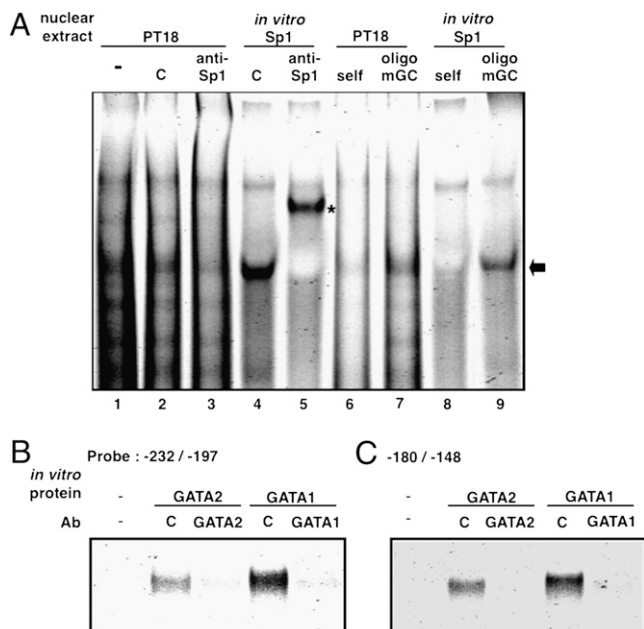


FIGURE 3. Sp1 and GATA1 and/or GATA2 are recruited to the $-107/-77$ region, and $-232/-197$ and $-180/-148$ region, respectively. *A*, EMSAs were performed with the use of 5 pmol FITC-labeled probe, 5 μ g of PT18 cell nuclear extract, and in vitro translated Sp1. The arrow indicates the position of the specific band containing probe and Sp1. Lanes 1–3, 6, and 7, Labeled probe and PT18 nuclear extract; lanes 4, 5, 8 and 9, labeled probe and in vitro translated Sp1. Additives: lane 1, none (–); lane 2, isotype control IgG (C); lane 3, anti-Sp1 Ab; lane 4, isotype control IgG (C); lane 5, anti-Sp1 Ab; lanes 6 and 8, nonlabeled probe (self); lanes 7 and 9, nonlabeled oligonucleotides containing mutated GC-box sequences introduced by nucleotide replacement (oligo mGC). Asterisk indicates the position of the supershifted band containing probe, in vitro translated Sp1 and anti-Sp1 Ab. *B*, GATA1 and GATA2 protein, prepared by an in vitro transcription/translation system, were incubated with 5 pmol of $-232/-197$ or $-180/-148$ FITC labeled probes and anti-GATA1 Ab, anti-GATA2 Ab, or isotype control IgG (C).

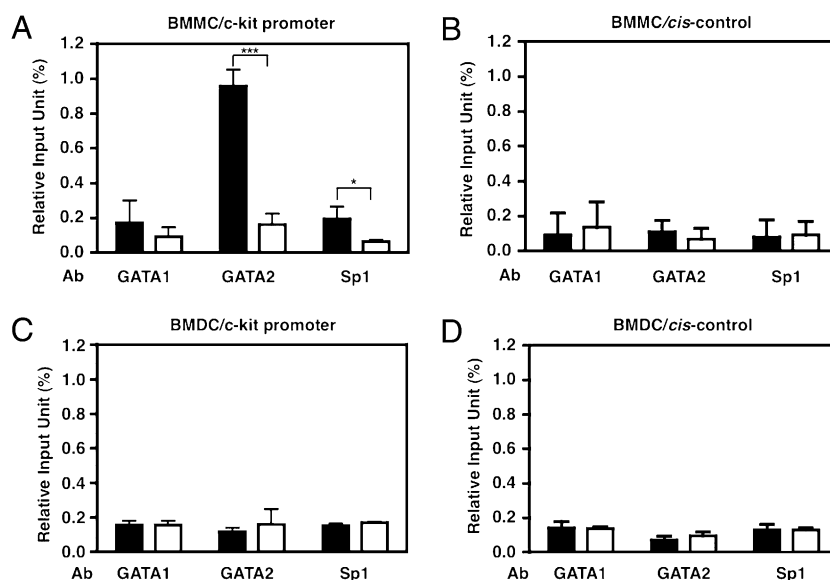
expression impairs the promoter activity of *c-kit*. Cotransfection of GATA2 siRNA and Sp1 siRNA into BMMCs, which reduced the mRNA levels of GATA2 and Sp1 to the same degree as mentioned above for single siRNA usage, downregulated the mRNA of *c-kit* by $\sim 50\%$ (Fig. 6C). To confirm whether the recruitment of GATA2

and/or Sp1 to the *c-kit* promoter region was reduced in siRNA-transfected BMMCs, a ChIP assay was performed after 20 h cultivation following siRNA transfection. As shown in Fig. 6D, binding of GATA2 to the *c-kit* promoter was not detected in BMMCs transfected with GATA2 siRNA (siGATA2) (siGATA2/BMMC), and the amount of GATA2 binding to the *c-kit* promoter in Sp1 siRNA-transfected BMMCs (siSp1/BMMC) was $\sim 20\%$ of that in nonsilencing control siRNA-transfected BMMCs (siNeg/BMMC). The binding of Sp1 to the *c-kit* promoter disappeared in siSp1/BMMCs, whereas the amount of Sp1 detected in siGATA2/BMMC was almost the same level as that of siNeg/BMMC (Fig. 6F). The promoter-specific binding of the transcription factors was confirmed by the *cis*-control ChIP assay (Fig. 6E, 6G). These findings suggest that the recruitment of Sp1 to the promoter region of *c-kit* is necessary for the binding of GATA2.

Repression of GATA2 expression by shRNA resulted in a decrease in the surface expression of *c-kit* in PT18

To further investigate the necessity of GATA2 for *c-kit* expression, GATA2 expression was knocked down in the mouse mast cell line PT18 by introduction of GATA2 shRNA/pSuper (shGATA2/PT18). PT18 cells were used for this experiment instead of BMMCs because 10 d culture of BMMCs under GATA2 knockdown condition (resulting in suppression of *c-kit* expression) to obtain shGATA2-transfectants may affect the viability and/or survival of BMMCs, which are depend on c-Kit ligand SCF, whereas PT18 are growing in SCF-independent manner. After 10 d of culture in growth medium containing puromycin to select transfectants, cells were harvested, and analyzed by flow cytometry. Surface c-Kit expression of shGATA2/PT18 was approximately one tenth lower than that obtained by transfection of empty vector (mock/PT18) (Fig. 7A). Densitometry analysis of Western blotting demonstrated that the amount of GATA2 protein in shGATA2/PT18 was reduced to $\sim 20\%$ of that in mock/PT18 (Fig. 7B), whereas the protein levels of the ubiquitously expressed transcription factor YY-1 was comparable between both transfectants. The ChIP assay showed that the recruitment of GATA2 to the *c-kit* promoter ($-168/+22$) was reduced in shGATA2/PT18 (Fig. 7C). A significant difference was not detected between anti-GATA2 and isotype control IgG in the *cis*-control region of shGATA2/PT18 and mock/PT18 (Fig. 7D). These results suggest that GATA2 is essential for the cell-surface expression of c-Kit in mast cells.

FIGURE 4. GATA2 and Sp1 are recruited to the *c-kit* promoter in mast cells. In BMMCs (*A*, *B*) and BMDs (*C*, *D*), quantitative analysis of Sp1 and GATA binding to the *c-kit* gene by the ChIP assay using real-time PCR was performed. The closed box indicates anti-GATA1, anti-GATA2, or anti-Sp1 Abs and the open box indicates isotype control IgG. *A* and *C* show the results of PCR with a *c-kit* specific probe, and (*B*) and (*D*) show that with a *cis*-control probe. The results express the mean $\pm$ SEM for two PCR with duplicate samples in each of three independent ChIP assays. Relative input units are calculated from Ct values as described in the ChIP assay method.



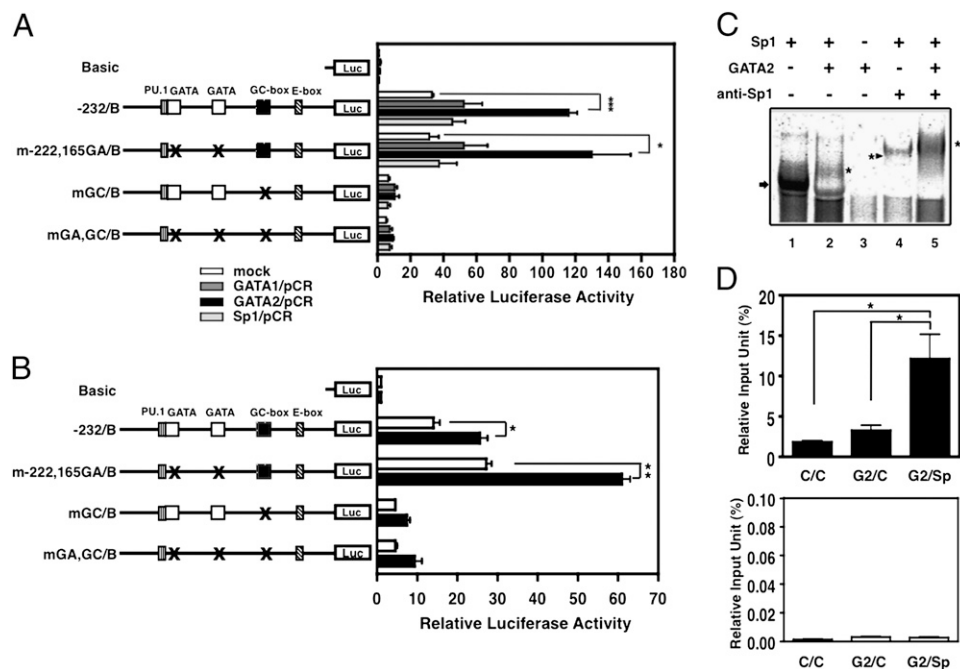


FIGURE 5. The luciferase activity of the *c-kit* promoter was enhanced by GATA2 coexpression in CV1 cells. **A**, A 1 μ g amount of each reporter plasmid and 100 ng of expression plasmid were introduced into CV1 cells using FuGene 6. The relative luciferase activity is represented as the ratio to the activity driven by pGL3-Basic (Basic) and pCR3 empty vector (mock). **B**, BMMCs were transfected 5 μ g each of reporter plasmids and 3 μ g of expression plasmid (pCR3 empty plasmid, open box, or GATA2/pCR, closed box) by electroporation. The ratio of luciferase activity of each construct to that of Basic in the presence of empty expression plasmid was represented as relative luciferase activity. **C**, EMSAs were performed with the use of 5 pmol FITC-labeled probe (–108/–77) and in vitro translated Sp1 and/or GATA2. The arrow indicated the position of the band containing Sp1 and the probe, and the asterisk indicated that of the band containing Sp1, GATA2, and the probe. Lane 1, 2, 4, and 5, in vitro translated Sp1; lane 2, 3, and 5, in vitro translated GATA2; lane 4 and 5, anti-Sp1 Ab. Double asterisks indicated the position of supershifted band containing in vitro translated Sp1, GATA2, the probe, and anti-Sp1 Ab. **D**, Re-ChIP analysis. C, isotype control IgG; G2, anti-GATA2 Ab; Sp, anti-Sp1 Ab. The result of PCR using a *c-kit* specific probe (top) or a *cis*-control probe (bottom), are shown. The results are expressed as the mean $\pm$ SEM for two PCR reactions, with duplicate samples, in each of three independent re-ChIP assays. Relative input units are calculated from the Ct values as described in the ChIP assay method.

Discussion

Although the molecular bases for the transcriptional regulation of *c-kit* in hematopoietic stem cells have been investigated, the mechanisms of *c-kit* transcription in mast cells, which continuously express *c-kit* at a high level, have been unclear. Thus, we investigated *c-kit* transcriptional regulation in mast cells using a reporter assay, EMSA, and a ChIP assay with an RNA interface. In this study, we demonstrated that the transcription factors GATA2 and Sp1 maintain *c-kit* expression cooperatively.

In the reporter assay using a series of 5'-deletion promoter constructs, the mouse *c-kit* promoter was functional only in BMMCs, but not in BMDCs, as shown in Fig. 1, indicating that cell type-specific promoter activity is assigned within the –232/–62 promoter region. Motif analysis using the program TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>) revealed the presence of one PU.1-binding motif, two GATA-binding motifs, one GC-box, and one E-box in the –232/+22 region. These motifs were candidates for *cis*-enhancing elements for the following reasons. GATA and SCL, which binds to the E-box, are predominantly expressed in hematopoietic cells, and the pentameric complex, containing GATA, SCL, E2A, LMO2, and Ldb-1, bind to DNA via the GATA or SCL/E2A or Sp1 motif, either alone or in combination (32, 33). GATA and PU.1 are essential for the generation of mast cells (34). Our reporter assay using various mutated constructs revealed that the GC-box is the essential element for functioning of the *c-kit* promoter (Fig. 2). By EMSA using anti-Sp1 Ab and competitive oligonucleotides, Sp1 was identified to bind the GC-box in the –108/–77 region (Fig. 3A). In addition, GATA1 and/or GATA2 possess the ability to bind to two GATA motifs in –232/B by

EMSA (Fig. 3B). In the ChIP assay, we detected significant binding of Sp1 and GATA2 to the –232/–62 region of the *c-kit* promoter, but GATA1 binding was not observed in BMMCs (Fig. 4A). These findings suggest that both Sp1 and GATA2 are recruited to the *c-kit* promoter region and activate the promoter in BMMCs in a cell type-specific manner (Fig. 4C). GATA has been shown to bind (A/T)GATA(A/G) or GATC sequences directly (35), and other studies have shown that the activity of GATA protein is modulated by interaction with other factors as follows. In erythrocytes, the complex of GATA1 and cofactor FOG1 coordinates cell maturation, and GATA1 represses activation of erythroid-specific gene expression by direct binding of NuRD/MeCP1 complex to FOG1 (36, 37). In megakaryocytes, the interaction of GATA1 and the SCL complex, which includes SCL, E2A, Ldb1, LMO2, and ETO2 regulate cell maturation (38). In mast cells, recruitment of FOG1 toward GATA1 represses the expression of the Fc ϵ RI β -chain, which allows mast cell-specific expression of the β -chain (20). In the current study, the E-box did not contribute to *c-kit* promoter function, suggesting that SCL is possibly not involved in *c-kit* promoter functions. In addition, we did not detect significant recruitment of FOG1 toward the *c-kit* promoter in the ChIP assay (data not shown). Previously, Tsujimura et al. (10) showed that MITF transactivates the *c-kit* promoter via binding to the CACCTG motif at –356/–351. However, in the current study, deletion of –622/–233 significantly increased *c-kit* promoter activity (Fig. 1). Although we do not have evidence to explain this discrepancy, one possible reason for the different results may be that different host cells were used for the reporter assay. In the previous studies, the cell lines, FMA3 and FDC-P1 were used for the reporter assay without exogenous MITF, and

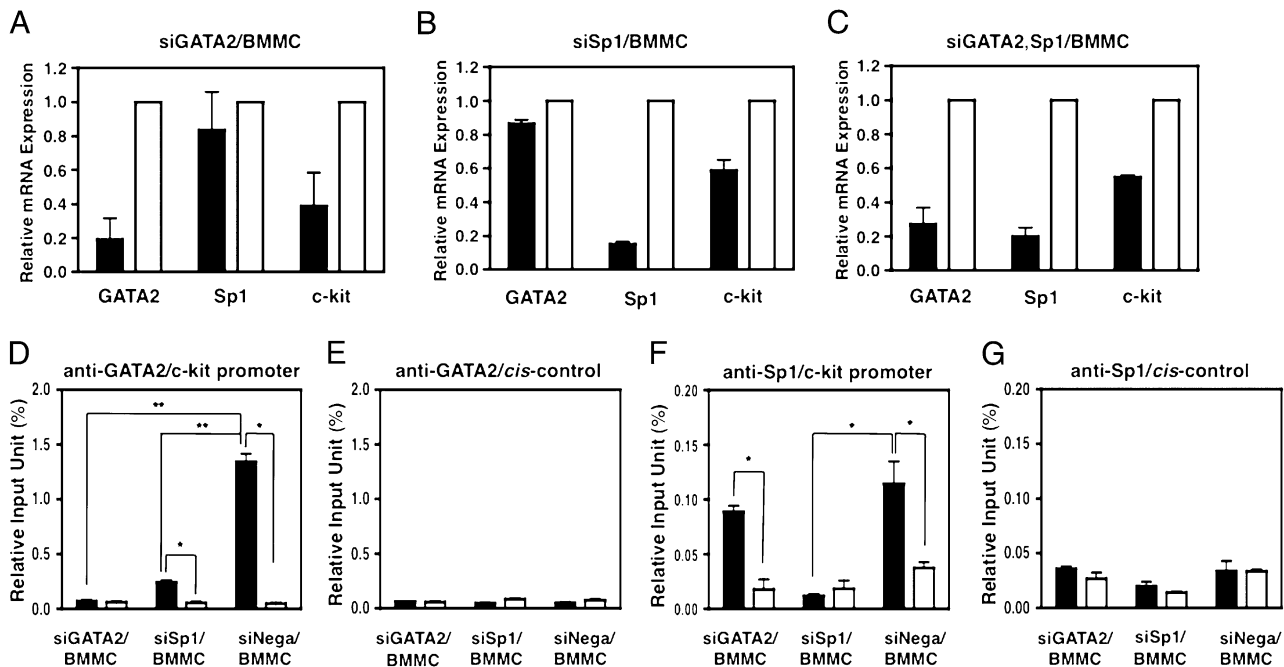


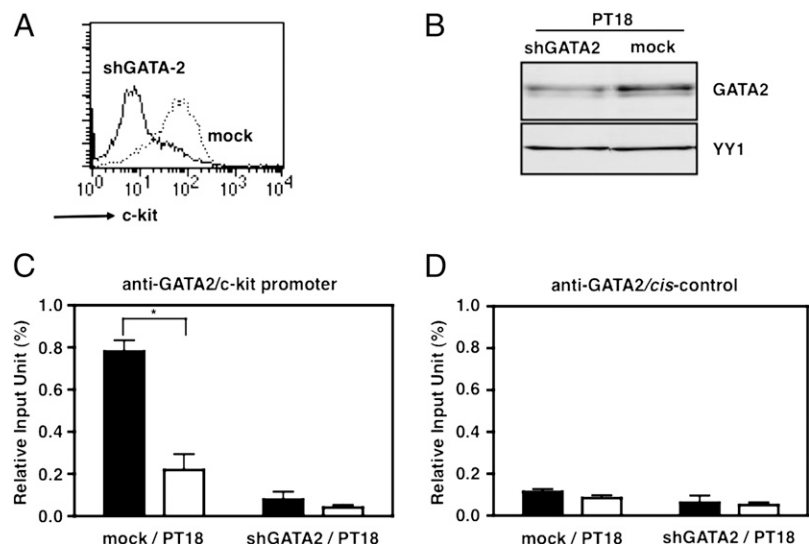
FIGURE 6. Transfection of GATA2 and/or Sp1 siRNA induced *c-kit* mRNA repression in BMMCs. *A–C*, Quantification of GATA2, Sp1, and *c-kit* mRNA in siRNA transfectants, siGATA2/BMMC (*A*), siSp1/BMMC (*B*), and siGATA2, Sp1/BMMC (*C*), using real-time PCR. The relative mRNA expression levels of siRNA transfectants (closed box) are represented as the ratio to that of control siRNA transfectants (open box). *D–G*, siRNA transfectants (siGATA2/BMMC, siSp1/BMMC, and siNega/BMMC) were analyzed with anti-GATA2 Ab (*D*, *E*) or anti-Sp1 Ab (*F*, *G*) in a ChIP assay. The amount of *c-kit* specific promoter (*D*, *F*) or *cis*-control (*E*, *G*) in each immunoprecipitate was quantified by real-time PCR. The closed box indicates the relative input unit by anti-GATA2 or anti-Sp1 Abs and the open box indicates that by isotype control IgG.

NIH/3T3 was used for the MITF-coexpression reporter assay (10), whereas BMMCs were used in the present reporter assay with deletion construct (Fig. 1). Considering that CACCTG functions as a *cis*-enhancing element in a mastocytoma, but not in another myeloid cell line, the effect of MITF may be differentially controlled in a cell type-specific manner. Furthermore, the function of MITF in BMMCs at various developmental stages may be different from its function in mast cell line. Alternatively, it is possible that an element in some other region of $-622/-233$ might affect the function of MITF. Such an element would be lost in the $-622/-233$ deletion mutant in the current study but would be retained in the point mutation of CACCTG in the previous study. In our preliminary experiments, MITF was associated with the complex containing

GATA2 and chromosome in the re-ChIP assay (data not shown). Further detailed analysis will be required to clarify the complicated roles of each transcription in the function of the *c-kit* promoter.

To identify the mechanisms of the enhancement of *c-kit* promoter activity by GATA2 and Sp1, we performed a reporter assay using various reporter constructs, introduced nucleotide replacements in the GATA and/or Sp1 binding motifs, and GATA2 or Sp1 expression plasmids, and EMSA using *in vitro* translated Sp1 and GATA2 (Fig. 5). In both of CV1 cells and BMMCs, the reporter activities of the wild-type *c-kit* promoter ($-232/B$) were enhanced by co-transfection with GATA2 expression plasmid. Although the reporter activity of $-232/B$ and $m-222,165GA/B$ were not enhanced by coexpression of Sp1 in CV1 cells, this unexpected result may

FIGURE 7. Expression of shGATA2 induced the downregulation of *c-kit* transcription and surface expression in PT18. *A*, Surface expression of c-Kit on shGATA2 transfectants. PT18 cells transfected with shGATA2/pSuper-retro-puro (shGATA2, solid line) or empty vector (mock, dotted line) were stained with PE-conjugated anti-c-Kit Ab. *B*, Western blot analysis of shGATA2-transfected PT18 cells. Cells were transfected with shGATA2 or empty vector and cultured in the presence of puromycin for at least 10 d. Cell lysates (1×10^6 cells per lane) were analyzed using anti-GATA2 or anti-YY1 Ab. *C* and *D*, Quantitative analysis of GATA2 binding to the *c-kit* promoter in a ChIP assay using real-time PCR. Chromatin of transfected PT18 cells was immunoprecipitated by anti-GATA2 Ab or isotype control IgG. Precipitated DNA molecules were analyzed by real-time PCR to test for the presence of the *c-kit* promoter (*C*) or the *cis*-control locus (*D*). The open box indicates isotype control IgG and the closed box indicates anti-GATA2 Ab.



have been due to the endogenous expression of Sp1 in CV1 cells. Regardless, coexpression analysis with CV1 indicated that GATA2 transactivates the *c-kit* promoter via the GC-box, independent of the SCL complex (33), because the hematopoietic specific factors, SCL and LMO2, are not expressed in CV1 cells. By EMSA using in vitro translated Sp1 and/or GATA2, GATA2 is suggested to the GC-box in the presence of Sp1, whereas GATA2 alone cannot bind the GC-box. Although the band containing Sp1 and GATA2 was not detected when EMSA was performed using PT18 nuclear extract and the same probe in Fig. 3A, this may have been due to dissociation of Sp1 and GATA2 during nuclear extraction, because the re-ChIP assay suggests that Sp1 and GATA2 are simultaneously recruited to the GC-box site in the *c-kit* promoter.

Mutant promoters lacking GATA motifs exhibited higher transcriptional activity compared with that of wild-type in mast cells (Fig. 2). One of the hypotheses of this enhancement may be that mutation of GATA-motif in the promoter, which plays a competitive role against the GC-box in the recruitment of GATA2, increased the amount of GATA2 binding to Sp1 on the GC-box. Alternatively, these GATA-motifs may function as silencing elements that suppress the *c-kit* promoter in other GATA1/2 positive hematopoietic lineage, including erythroids and megakaryocytes. Further analysis of GATA1/2 profiles on these GATA-motifs in various cell-types may provide interesting information regarding the mechanism of cell type-specific *c-kit* expression.

By knockdown of GATA2 and/or Sp1 in BMMCs, *c-kit* mRNA expression was reduced to ~50% (Fig. 6A–C), and GATA2 recruitment to the promoter region was reduced by the impaired recruitment of Sp1 in the ChIP assay (Fig. 6D–G). Furthermore, c-Kit surface expression of PT18 was reduced by GATA2 knockdown, accompanied with decreased recruitment of GATA2 to the promoter region (Fig. 7). These results indicate that the recruitment of GATA2 and Sp1 to the GC-box mainly enhances the *c-kit* promoter. The *c-kit* mutant promoters lacking GATA motifs was transactivated by GATA2 in the current study, suggesting that GATA2 is involved in *c-kit* promoter function without binding to the GATA motifs, although these GATA motifs possess GATA protein binding affinity. One of the possible mechanisms is indirect interaction with the GC-box via the formation of a complex with Sp1. The ChIP assay with siRNA-transfected BMMCs showed that the recruitment of GATA2 to the *c-kit* promoter is dependent on the presence of Sp1. In addition, the re-ChIP assay showed that both Sp1 and GATA2 are specifically recruited to the region of the GC-box. These data suggest that GATA2 binds to the GC-box via Sp1 binding, and that formation of a complex accelerates the binding of Sp1 to the GC-box. Sp1 physically interacts with GATA1, 2, and 3 and functions as a scaffold for GATA1 binding to the globin gene, which lacks GATA motifs (39). In addition, GATA3 regulates the dopamine β -hydroxylase gene through physical interaction with Sp1 (40). These observations support our hypothesis that GATA2 regulates the expression of the *c-kit* gene by interaction with Sp1. In any case, our data indicate that GATA2 interacts with the *c-kit* promoter region via Sp1 and maintains expression of the *c-kit* gene in a mast-cell-specific manner, although further analysis is required to clarify the involvement of other transcription factors.

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Disclosures

The authors have no financial conflicts of interest.

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