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Critical Roles for PU.1, GATA1, and GATA2 in the Expression of Human FcεRI on Mast Cells: PU.1 and GATA1 Transactivate *FCER1A*, and GATA2 Transactivates *FCER1A* and *MS4A2*

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The high-affinity IgE receptor, FcεRI, which is composed of α-, β-, and γ-chains, plays an important role in IgE-mediated allergic responses. In the current study, involvement of the transcription factors, PU.1, GATA1, and GATA2, in the expression of FcεRI on human mast cells was investigated. Transfection of small interfering RNAs (siRNAs) against PU.1, GATA1, and GATA2 into the human mast cell line, LAD2, caused significant downregulation of cell surface expression of FcεRI. Quantification of the mRNA levels revealed that PU.1, GATA1, and GATA2 siRNAs suppressed the α transcript, whereas the amount of β mRNA was reduced in only GATA2 siRNA transfectants. In contrast, γ mRNA levels were not affected by any of the knockdowns. Chromatin immunoprecipitation assay showed that significant amounts of PU.1, GATA1, and GATA2 bind to the promoter region of *FCER1A* (encoding FcεRIα) and that GATA2 binds to the promoter of *MS4A2* (encoding FcεRIβ). Luciferase assay and EMSA showed that GATA2 transactivates the *MS4A2* promoter via direct binding. These knockdowns of transcription factors also suppressed the IgE-mediated degranulation activity of LAD2. Similarly, all three knockdowns suppressed FcεRI expression in primary mast cells, especially PU.1 siRNA and GATA2 siRNA, which target FcεRIα and FcεRIβ, respectively. From these results, we conclude that PU.1 and GATA1 are involved in FcεRIα transcription through recruitment to its promoter, whereas GATA2 positively regulates FcεRIβ transcription. Suppression of these transcription factors leads to downregulation of FcεRI expression and IgE-mediated degranulation activity. Our findings will contribute to the development of new therapeutic approaches for FcεRI-mediated allergic diseases. *The Journal of Immunology*, 2014, 192: 3936–3946.

The high-affinity IgE receptor, FcεRI, plays a crucial role in various IgE-mediated responses, including allergic diseases and host defense against parasites. Mast cells and basophils are the major effector cells expressing FcεRI, whose cross-linking by IgE and multivalent Ags causes stimulation of cells, including rapid degranulation, immediate eicosanoid generation, and transcription of cytokine genes. FcεRI expression levels on the cell surface affect the magnitude of IgE-mediated responses. In the case of allergic patients carrying high serum IgE value, FcεRI on the cell surface is occupied by an IgE Ab a priori. In addition to its role as a trigger of allergen-induced stimulation of cells, the interaction between IgE and FcεRI is involved in the enhancement of allergic responses, even in the absence of cross-linking by allergens, by at least two mechanisms, as follows: 1) the binding of monomeric IgE to FcεRI increases the amount of cell surface FcεRI (1); and 2) the binding of monomeric IgE to FcεRI enhances cell viability (2, 3). Uncovering the mechanism of FcεRI expression will be important for finding ways to control FcεRI expression, with a view to suppressing allergic responses.

FcεRI is composed of three subunits, as follows: α, the IgE-binding subunit; and β and γ, the signal transduction subunits. In our previous studies regarding the transcriptional regulation of FcεRI subunit genes, several transcription factors have been identified (4–9). Among them, hematopoietic cell-specific transcription factors are known to be involved in mast cell/basophil-specific gene regulation. Briefly, cooperation between PU.1 and GATA1 in FcεRI-positive cells (5), and the suppressive effect of FOG-1 on GATA1 in FcεRI-negative cells (7), determines the cell type–specific expression of human α and mouse β genes, respectively. In these previous studies, reporter assays and EMSAs were mainly used to identify and to analyze the role of the transcription factors regulating the promoter activation. In contrast,

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Abbreviations used in this article: BMMC, bone marrow–derived mast cell; ChIP, chromatin immunoprecipitation; NP, nitrophenyl; siRNA, small interfering RNA.

the involvement of GATA1 and GATA2 in the expression of FcεRI was denied in some studies using mouse bone marrow–derived mast cells (BMMCs) and/or the rat basophilic leukemia cell line, RBL-2H3 (10, 11). Therefore, in the current study, we analyzed the involvement of PU.1, GATA1, and GATA2 in FcεRI expression in the human mast cell line LAD2 and primary human mast cells using small interfering RNA (siRNA) and the chromatin immunoprecipitation (ChIP) assay to verify our previous findings. This report demonstrates that PU.1, GATA1, and GATA2 are positive regulators of the expression and function of FcεRI in human mast cells. We newly identified GATA2 as a critical transactivator of the human *FcεRIβ* promoter, whose cell type–specific transcription factors have not been reported to date. Additionally, we showed that FcεRIα expression was induced by GATA2 as well as PU.1 and GATA1.

Materials and Methods

Cells

The human mast cell leukemia cell line, LAD2, which was provided by Dr. A. Kirshenbaum (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD), was maintained in the presence of recombinant human stem cell factor (PeproTech, London, U.K.), as described previously (12). Human primary mast cells were generated from peripheral blood $CD34^+$ cells (Frozen Mobilized Peripheral Blood $CD34^+$ cells, mPB015F; ALLCELLS, Emeryville, CA) as progenitors based on the previously established method (13). In three independent experiments (Figs. 6, 7), three independently generated primary mast cells were used. This study was approved by the ethics committee of the Juntendo University School of Medicine.

Knockdown of PU.1, GATA1, GATA2, and c-kit expression with siRNA

PU.1 siRNA (Stealth Select RNAi, HSS144058, HSS144060, and HSS186060), GATA1 siRNA (HSS142150, HSS142151, and HSS142152), GATA2 siRNA (HSS104003, HSS104004, HSS178122), c-kit siRNA (HSS105821), and control siRNA (Stealth RNAi Negative Universal Control Lo GC, Med GC, and Hi GC [12935-200, 300, and 400]) were purchased from Invitrogen (Carlsbad, CA). Transfection of siRNA was performed, as described previously (14). Briefly, 10 (or 1) μl 20 μmol/L siRNA was introduced into 2×10^6 (or 2×10^5) LAD2 cells or primary mast cells with a Neon 100 μl kit (or a Neon 10 μl kit) using a Neon transfection system (Invitrogen) set at Program 16 (for LAD2) or 5 (for primary mast cells).

Flow cytometry

At 48 h after siRNA transfection, LAD2 cells (1×10^6) or primary mast cells (2.5×10^5) were incubated with 1 μg FITC-conjugated anti-human FcεRI Ab (clone AER-37; eBioscience, San Diego, CA) or FITC-conjugated mouse IgG2b (BD Biosciences). To stain c-kit, PE-conjugated anti-human CD117 Ab (clone TB5.B8; BD Biosciences) was used. Stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR).

Quantification of mRNA by real-time PCR

Extraction of total RNA from cells and reverse transcription to synthesize cDNA were performed, as described previously (15). The amount of mRNA for PU.1, GATA1, GATA2, and FcεRIα, β, and γ was determined using a Step-One Real Time PCR system (Applied Biosystems) with TaqMan gene expression systems (Hs02786711_m1 for SPI1 [PU.1], Hs01085823_m1 for GATA1, Hs00231119_m1 for GATA2, Hs00758600 for FCERIA [FcεRIα], Hs00999628 for MS4A2 [FcεRIβ], Hs00175408 for FcεRIγ, and Hs00174029_m1 for c-kit) and TaqMan Universal Master Mix (Applied Biosystems). mRNA levels were evaluated as a ratio against that of GAPDH (4326317E) as the housekeeping gene by calculating cycle threshold values, as previously described (7, 16).

ChIP assay

The ChIP assay was performed as described in our previous reports (7, 15, 17). Anti-PU.1 (T21, sc352), anti-GATA1 (N6, sc-265), and anti-GATA2 (H-116, sc-9008) Abs purchased from Santa Cruz Biotechnology (Santa Cruz, CA) were used for immunoprecipitation, and rat IgG (BD Biosciences) and rabbit IgG (02-6102; Invitrogen) were used as control Abs. The amount of chromosomal DNA, including promoters of FcεRIα, β, and γ, was determined by the TaqMan system using the primers and TaqMan probes listed in Supplemental Table E1.

Luciferase assay

MS4A2 promoter regions were amplified from human genomic DNA by using PCR and inserted into the multicloning site of pGL-4 Basic (Promega, Madison, WI) to generate reporter plasmids. The nucleotide sequences of primer sets are listed in Supplemental Table E2. The expression plasmids pCR-GATA1 (4) and pCR-GATA2 (18), which were generated in our previous studies, and their empty vector pCR3.1 (Invitrogen), were used in this study. HEK293T cells (human embryonic kidney cell line) were transfected with 500 ng reporter plasmid, 500 ng expression plasmid, and 10 ng pRL-null (Promega) with FuGENE HD (Roche Diagnostics, Indianapolis, IN). Luciferase activity was determined using Micro Lumat Plus (Berthold Technologies, Bad Wildbad, Germany) and a dual-luciferase assay kit (Promega), as described previously (7).

EMSA

EMSA was performed, as described previously (14, 15, 17, 18). Abs against GATA1, GATA2, and the control Abs were same as those used in the ChIP assay. GATA1 and GATA2 proteins were prepared by an in vitro transcription/translation system using the expression plasmids pCR-GATA1 and pCR-GATA2 as templates.

β-Hexosaminidase assay

At 24 h after siRNA transfection, LAD2 cells (5×10^5) were sensitized with 1 μg anti-nitrophenyl (NP) chimeric human IgE (clone JW8/1; AbD seroTec, Oxford, U.K.) in 1 ml medium overnight at 37°C. After washing with Tyrode's buffer (10 mM HEPES [pH 7.4], 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 0.1% BSA), cells were resuspended in Tyrode's buffer at 2×10^5 cells/100 μl and stimulated by addition of 100 μl Tyrode's buffer containing 60 ng NP (32)-BSA [NP (32)-BSA; Biosearch Technologies, Novato, CA] for 30 min at 37°C. β-Hexosaminidase activity in the supernatant was measured as follows. Culture supernatants were incubated with 1.3 mg/ml 4-nitrophenyl-N-acetyl-β-D-glucosaminide (N9376; Sigma-Aldrich, St. Louis, MO) for 60 min at 37°C. After developing the reaction with 0.2 M glycine (pH 11), absorbance was measured at 405 nm. Release was calculated as a percentage of the total β-hexosaminidase content determined after cell lysis with 1% Triton X-100 and was determined by subtracting the value of samples treated with IgE alone without NP-BSA.

Histamine release assay

Twenty-four hours after siRNA transfection, peripheral blood–derived primary mast cells (5×10^5) were sensitized with 2 μg human myeloma IgE (Calbiochem, Merck KGaA, Darmstadt, Germany) in 1 ml medium overnight at 37°C. After washing with Tyrode's buffer, cells were resuspended in Tyrode's buffer at 1.4×10^5 cells/100 μl and stimulated by addition of 100 μl Tyrode's buffer containing 0.6 μg rabbit anti-human IgE (DAKO, Glostrup, Denmark) for 40 min at 37°C. Histamine in the culture supernatant was quantified using an immunoassay kit (Immunotech A; Beckman Coulter, Marseille, France), according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using the two-tailed Student *t* test for paired or unpaired data, with p values <0.05 considered significant.

Results

Effect of siRNAs for PU.1, GATA1, and GATA2 on mRNA levels of FcεRIα, β, and γ subunits

Previously, we analyzed the transcriptional regulation of FcεRI α and β genes whose expression profiles are reflected in the cell type–specific expression of FcεRI, whereas the γ subunit is expressed in not only FcεRI-positive cells but also in FcεRI-negative cells. At that time, using reporter assays and EMSAs, we found that the human *FCER1A* (encoding FcεRIα) promoter is cooperatively transactivated by PU.1 and GATA1 (5). Although GATA1 was identified as a transactivator for the mouse *MS4A2* (encoding FcεRIβ) promoter (7, 19), cell type–specific transcrip-

tion factor that regulates human *MS4A2* has not been identified to date. It has also been revealed that GATA2, which is also known to regulate mast cell development in a synergistic manner with PU.1 (20), is necessary for the promoter function of *c-kit* (18) and *ST2/IL1RL1* (IL-33R) (14) in mast cells. In the current study, we investigated the involvement of PU.1, GATA1, and GATA2 in the expression of FcεRI using a recently established highly effective knockdown of target mRNA in mast cells to verify our previous findings. First, siRNA against PU.1, GATA1, or GATA2 was introduced into the human mast cell line LAD2, which constitutively expresses FcεRI on its cell surface, and mRNA levels of FcεRIα, β, and γ were determined in the siRNA-transfected LAD2 cells. In this experiment, we used three siRNAs each for PU.1, GATA1, and GATA2 to exclude the possibility of any off-target effects of siRNAs. Among the siRNAs, #1 and #3 of PU.1, #1 and #2 of GATA1, and #1 and #2 of GATA2 were confirmed to reduce the amount of mRNA of the target gene, whereas PU.1 #2, GATA1 #3, and GATA2 #3 did not exhibit a knockdown effect (Fig. 1A, 1E, 1I). Two effective PU.1 siRNAs, #1 and #3, dramatically suppressed α mRNA levels (Fig. 1B) but did not affect the β mRNA levels (Fig. 1C). The significant reduction of the FcεRIβ mRNA level in the PU.1 #2 transfectant is probably due to an off-target effect against FcεRIβ itself or to other molecules that regulate FcεRIβ expression. Similarly, effective GATA1 siRNAs, #1 and #2, significantly suppressed mRNA levels of the α (Fig. 1F), but not the β (Fig. 1G) subunit, even though the knockdown efficiencies of GATA1 siRNAs and the effect on FcεRIα mRNA levels were not as high as those of PU.1 siRNAs. GATA2 siRNAs, both #1 and #2, caused significant downregulation of FcεRIα and β mRNAs (Fig. 1J, 1K). As for FcεRIγ mRNA, the reduction common to effective siRNAs was not observed (Fig. 1D, 1H, 1L). These results demonstrate that PU.1 and GATA1 are involved in the expression of FcεRIα, but not β and γ, and that GATA2 positively regulates the expression of α and β, but not γ.

Effect of siRNA against PU.1, GATA1, and GATA2 on the surface expression of FcεRI in human mast cells

The above results suggest that PU.1 and GATA1 are involved in regulating FcεRIα expression and that GATA2 is involved in regulating the mRNA levels of FcεRIα and β. Therefore, to evaluate the effects of these knockdowns on cell surface expression of FcεRI protein, the expression levels of FcεRI were determined by flow cytometry after staining with an anti-FcεRIα Ab. In the following experiments using siRNAs, the #1 siRNA was selected for each, because these were judged to be effective without off-target effects. Under conditions in which all of the siRNAs significantly knocked down the target mRNA (Fig. 2A),

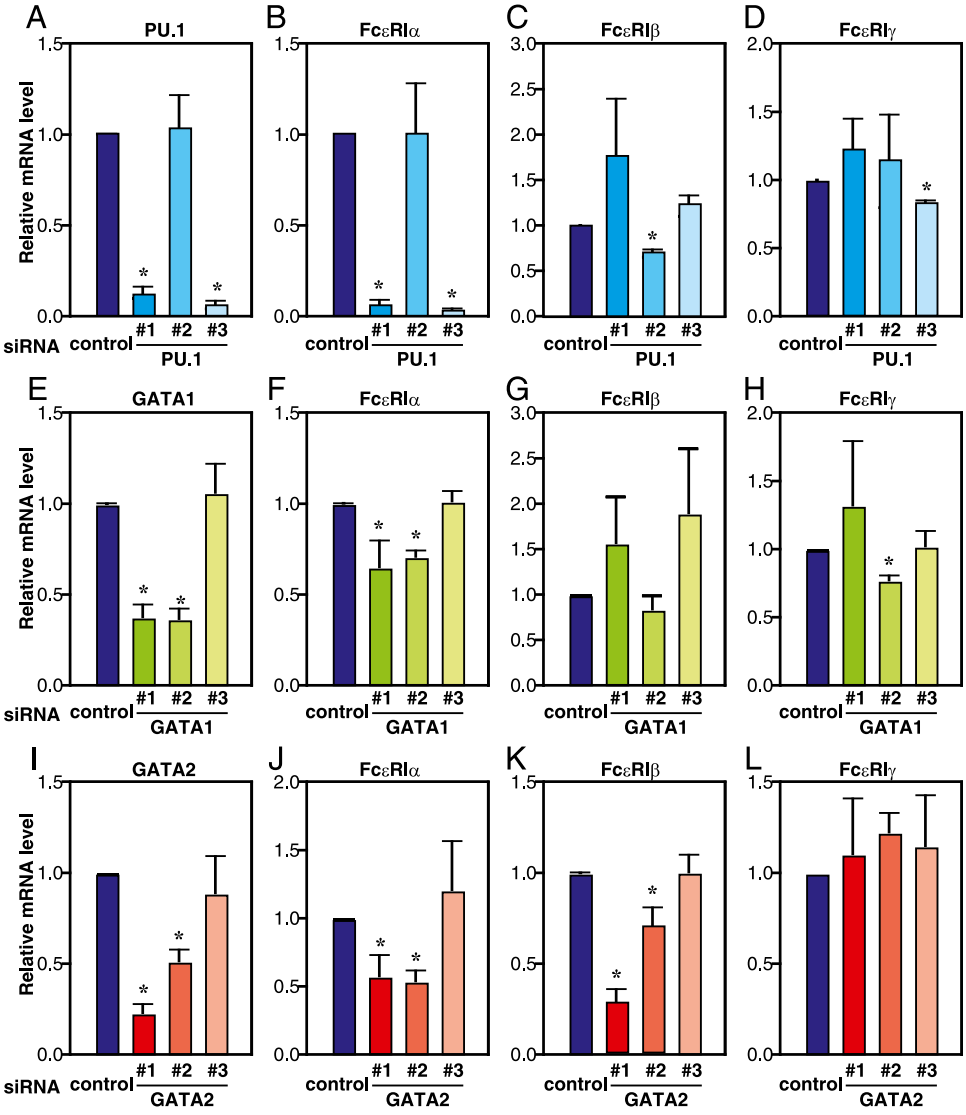

FIGURE 1. Effect of siRNA against PU.1, GATA1, and GATA2 on mRNA expression levels of FcεRIα, β, and γ. Relative mRNA expression levels of PU.1 (**A**), GATA1 (**E**), GATA2 (**I**), FcεRIα (**B**, **F**, and **J**), FcεRIβ (**C**, **G**, and **K**), and FcεRIγ (**D**, **H**, and **L**) in cells transfected with PU.1 siRNA [series of light blue bars in (A)–(D)], GATA1 siRNA [series of green bars in (E)–(H)], or GATA2 siRNA [series of red bars in (I)–(L)]. Data are expressed as the ratio of the expression level of the respective control siRNA-introduced cells (dark blue bars in each graph). The additional two siRNAs of different sequences (#2 and #3), in addition to sequence #1 corresponding to the siRNA in Figs. 2, 5, 6 and 7, were used to avoid off-target effects. The data represent the mean + SD of three independent experiments performed with duplicate samples. *$p < 0.05$.

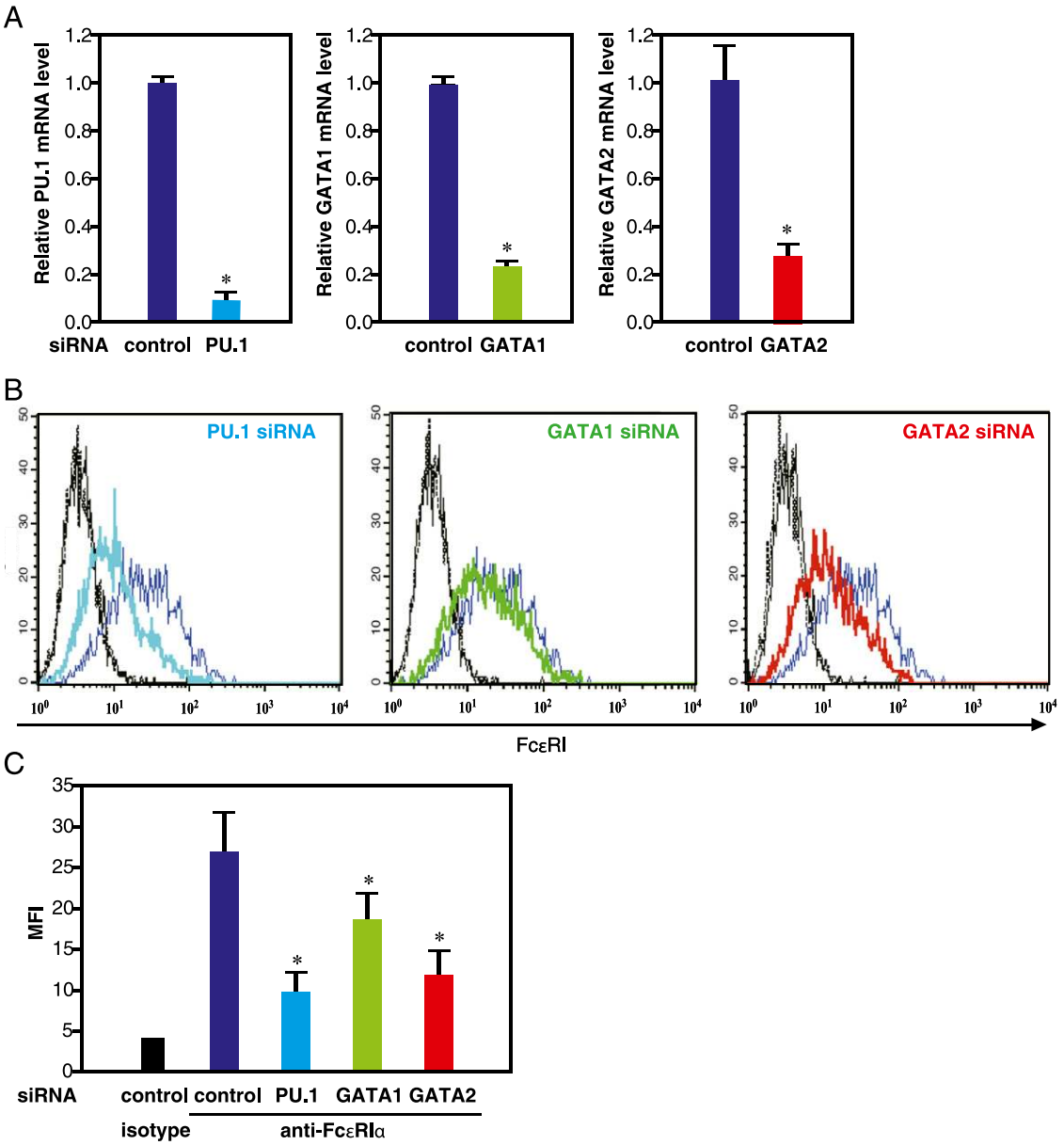

FIGURE 2. Effect of siRNAs against PU.1, GATA1, and GATA2 on cell surface expression of FcεRI in human mast cells. (**A**) mRNA levels of PU.1, GATA1, and GATA2 in siRNA transfectants. The mRNA expression levels of PU.1 (*left*), GATA1 (*center*), and GATA2 (*right*) in siRNA-transfected LAD2 cells are displayed as the ratio of mRNA levels versus those detected in control siRNA-introduced cells. Data represent the mean + SD of three independent experiments with duplicate samples. $*p < 0.05$. (**B**) Cell surface expression of FcεRI. LAD2 cells transfected with control siRNA, PU.1 siRNA, GATA1 siRNA, or GATA2 siRNA were stained with FITC-conjugated anti-FcεRIα Ab or FITC-conjugated control Ab. Dark blue line, control siRNA transfectant stained with anti-FcεRIα Ab; light blue line, PU.1 siRNA transfectant stained with anti-FcεRIα Ab; green line, GATA1 siRNA transfectant stained with anti-FcεRIα Ab; red line, GATA2 siRNA transfectant stained with anti-FcεRIα Ab; black lines, control siRNA transfectant treated with control Ab; black dotted lines, PU.1, GATA1, or GATA2 siRNA transfectant treated with control Ab. Representative results of three independent experiments are shown. (**C**) Data represent mean + SD of mean fluorescence intensity (MFI) obtained from three independent experiments. $*p < 0.05$.

FcεRI expression levels were significantly reduced by the transfection of PU.1 siRNA, GATA1 siRNA, or GATA2 siRNA compared with control siRNA-transfected cells (Fig. 2B, 2C). The extent of suppression of FcεRI expression was greater in cells transfected with PU.1 siRNA or GATA2 siRNA compared with the effect of GATA1 siRNA, which was moderate.

Recruitment of transcription factors onto the FcεRIα- and β-gene promoters in mast cells as analyzed by the ChIP assay

In a previous study, PU.1 and GATA1 were identified as transcription factors involved in transactivating the *FCER1A* promoter via binding to the critical *cis*-element at positions −52/−47 and −74/−69, respectively, by EMSA using nuclear proteins extracted from the rat basophilic leukemia cell line, RBL-2H3, and the mouse mast cell line PT18 (5). However, the binding profiles of PU.1 and GATA1 around the *FCER1A* promoter on chromosomal DNA in FcεRI-positive cells have not been elucidated. Therefore, we performed a ChIP assay to confirm whether the transcription factors, PU.1, GATA1, and GATA2, whose knockdown reduced the mRNA levels of FcεRIα (Fig. 2), are involved in the transcription of FcεRIα through their recruitment to the *FCER1A* promoter. When the amount of chromosomal DNA immunoprecipitated with Abs against PU.1, GATA1, or GATA2 was quantified with real-time PCR using a TaqMan probe and primers

targeting the −97/−30 region of the *FCER1A* gene (Fig. 3A), we found that all three Abs caused significantly higher amounts of precipitant containing the *FCER1A* promoter region compared with those of control Abs (Fig. 3B–D). From these results, it was confirmed that PU.1 and GATA1 bind directly to the *FCER1A* promoter on chromosomal DNA in LAD2, and, in addition, to our knowledge, we revealed for the first time that GATA2 binds to the *FCER1A* promoter as well.

As for the *MS4A2* promoter, it was predicted that GATA motifs may be involved in the recruitment of transcription factors, because GATA2 siRNA affected the mRNA levels of FcεRIβ (Fig. 2). When GATA motifs were searched for on the *MS4A2* promoter, two tandem GATA motifs at −476/−467 (TGATAGATAT) and one GATA motif at −331/−326 (GGATAA) were found in a region spanning ~500 bp of the promoter. Then, the amount of the *MS4A2* gene precipitated by Abs was measured using primers and probe targeting these motifs. As shown in Fig. 3H, a significantly higher amount of chromosomal DNA was immunoprecipitated by anti-GATA2 Ab, when a primer and probe set targeting the tandem GATA motifs was used (Fig. 3E), whereas significant differences were not detected between either anti-PU.1 or anti-GATA1 Ab and the respective controls (Fig. 3F, 3G). A similar result was obtained in an experiment targeting the third GATA motif at position −331/−326 (data not shown).

These results demonstrate that PU.1 and GATA1 are recruited to the *FCER1A* promoter on the chromosomal DNA and that GATA2 is recruited to the *FCER1A* promoter and *MS4A2* promoter in mast cells.

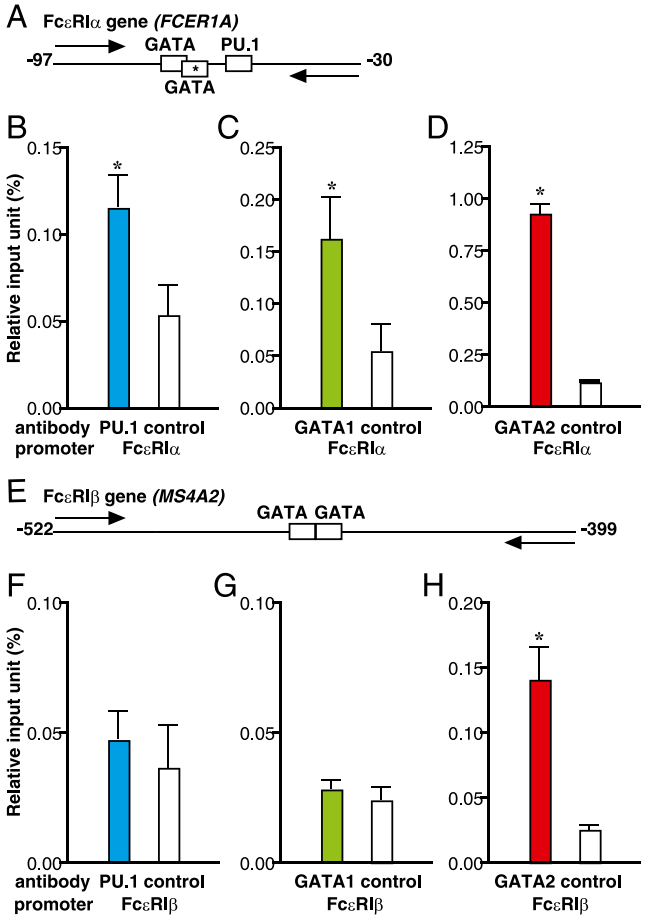

FIGURE 3. Recruitment of transcription factors to the *FCER1A* promoter and the *MS4A2* promoter in mast cells analyzed by ChIP assay. (**A** and **E**) Schematic drawings of primer sets used for ChIP assay targeting the FcεRIα gene/*FCER1A* (A) and the FcεRIβ gene/*MS4A2* (E). Binding motifs for GATA family and PU.1 in the *FCER1A* promoter were identified in our previous study (4, 5). T allele at the −66 single nucleotide polymorphism site (shown with an asterisk) in the *FCER1A* gene causes an additional GATA motif resulting in the tandem repeat of GATA motifs (33, 34). Quantitative analysis of PU.1 (**B**, **F**), GATA1 (**C**, **G**), and GATA2 (**D**, **H**) binding to *FCER1A* (B–D) and *MS4A2* (F–H). Data represent the mean + SD of three independent experiments. *$p < 0.05$.

GATA2 transactivates the human MS4A2 promoter through direct binding

Previously, we attempted to identify transcription factors that contribute to cell type–specific *MS4A2* expression and found that Oct-1 (6) and MZF-1 (8) are involved in *MS4A2* transcription. However, a positive role for GATA2 in *MS4A2* transcription has not been reported. The above results suggest that GATA2, which is recruited to the promoter region, transactivates the *MS4A2* promoter directly. To evaluate the effects of GATA2 on *MS4A2* promoter activity, we performed a reporter assay using HEK293T cells, because nonhematopoietic cell lines are useful for evaluating the transactivating activity of hematopoietic cell–specific transcription factors in coexpression analysis (7, 15, 17). Luciferase activity driven by the −783/+33 region of the *MS4A2* promoter was markedly upregulated by GATA2 coexpression, whereas the effect of coexpressed GATA1 on this promoter activity was low (Fig. 4A). Coexpression of GATA2 exhibited similar significant transactivation activity against the −585/+33 region, suggesting that GATA2 increases promoter activity through *cis*-enhancing elements in −585/+33 region. As described in the results of the ChIP assay, two candidate sites for GATA-family binding, −476/−467 (TGATAGATAT) and −331/−326 (GGATAA), are present in the −585/+33 region. Besides, we also previously found that GATA2 indirectly binds to the *c-kit* promoter via an interaction with Sp1 (18). Therefore, to clarify whether GATA2 directly binds the *MS4A2* promoter through either or both GATA sites, EMSA was performed. Among three probes, −683/−654 (probe 1 containing GGATAG, reverse), −486/−457 (probe 2 containing TGATAGATAT), and −343/−314 (probe 3 containing GGATAA), a specific band shift, which disappeared in the presence of anti-GATA2 Ab, was detected only in the case of probe 2 (Fig. 4B, marked with an asterisk, *lanes 10–12*). From these data and the results of the reporter assay, we conclude that GATA2 transactivates the *MS4A2* promoter with direct binding to the promoter via the TGATAGATAT sequence at −476/−467. Interestingly, when GATA1 protein was added to the probe mixture, a specific band shift was observed in all experiments with probes 1, 2, and 3 (Fig. 4B, marked with double asterisks). Considering that the binding of GATA1 to the *MS4A2* promoter was not detected in the ChIP assay, GATA2 dominantly binds to the *MS4A2* promoter in the nucleus of living mast cells, although GATA1 possesses the potential for binding to these GATA motifs in the in vitro condition of EMSA.

Effect of knockdown of PU.1, GATA1, and GATA2 on the FcεRI-mediated function of mast cells

FcεRI expression levels on mast cells were reduced by knockdown of PU.1, GATA1, and GATA2, suggesting the possibility that these transfectants exhibit a lower response against FcεRI-mediated stimulation, because it is well known that FcεRI expression levels are deeply associated with the degree of FcεRI-mediated mast cell function. To evaluate the effects of transcriptional modulation on the biological activity of mast cells, degranulation activity of siRNA transfectants was analyzed by β-hexosaminidase assay. As expected, degranulation activity of LAD2 cells was significantly decreased by transfection with individual siRNAs against PU.1, GATA1, and GATA2 (Fig. 5A). This result indicates that knock-

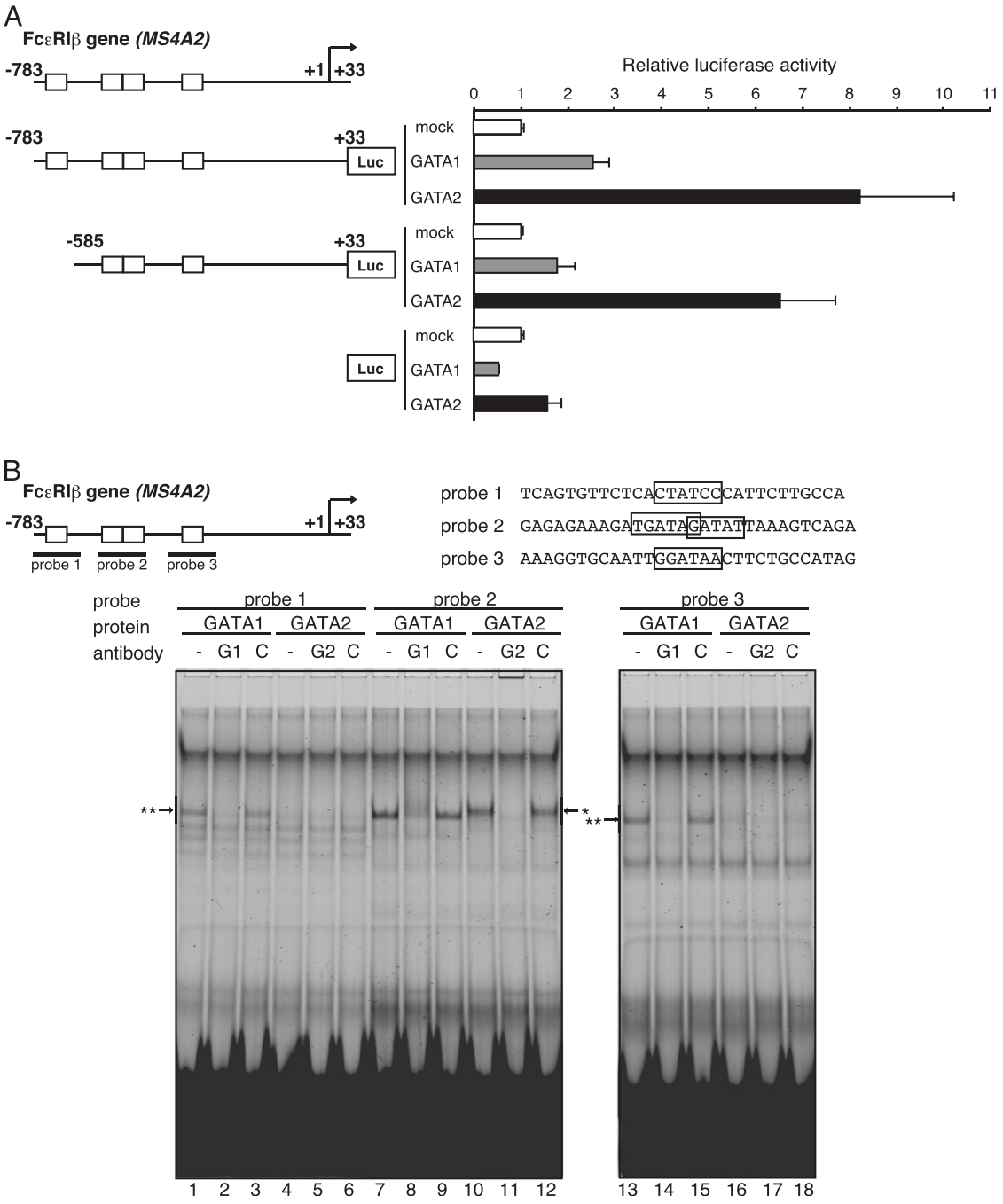

FIGURE 4. GATA2 transactivates the *MS4A2* promoter via direct binding to *cis*-enhancing elements in the promoter. (**A**) GATA-coexpressing reporter assay. A reporter plasmid carrying promoter region of the human *MS4A2* (encoding FcεRIβ) gene (−783/+33, −585/+33) or its empty plasmid pGL-4 was transfected into HEK293T cells with an expression plasmid, pCR-GATA1 (GATA1), pCR-GATA2 (GATA2), or pCR3.1 (mock). Relative luciferase activity is displayed as the ratio of luciferase activity versus that seen in cells cotransfected with mock vector. A representative result of three independent experiments performed with triplicate samples is shown as the mean ± SD. (**B**) EMSA profiles. Locations and nucleotide sequences of the three probes used in EMSAs are shown at the *top left* and *top right*, respectively. Candidate GATA motifs are boxed. In vitro transcription/translation was performed with pCR-GATA1 (GATA1) or pCR-GATA2 (GATA2) as template. Specific bands corresponding to complexes of GATA2 and the probe are marked with an asterisk, and those of GATA1 and probe are marked with double asterisks. −, without Ab; C, with control Ab; G1, with anti-GATA1 Ab; G2, with anti-GATA2 Ab.

down of PU.1, GATA1, or GATA2 or a combination thereof could be a target for antiallergic medication due to suppression of the biological function of mast cells.

The effects of GATA2 knockdown on c-kit expression and of c-kit knockdown on FcεRI expression

We previously reported that knockdown of GATA2, which transactivates the mouse *c-kit* promoter, reduces the c-kit expression in mouse BMMCs (18). Considering that LAD2 is a stem cell factor–dependent cell line, we cannot exclude the possibility that the above effects of GATA2 siRNA on FcεRI expression and its function may be dependent on c-kit expression. Therefore, first we examined whether GATA2 knockdown affects human c-kit expression in LAD2 cells. When the mRNA level of GATA2 was <20% of the control level following introduction of GATA2 siRNA (Fig. 5B, *top right*), c-kit mRNA levels in GATA2 siRNA

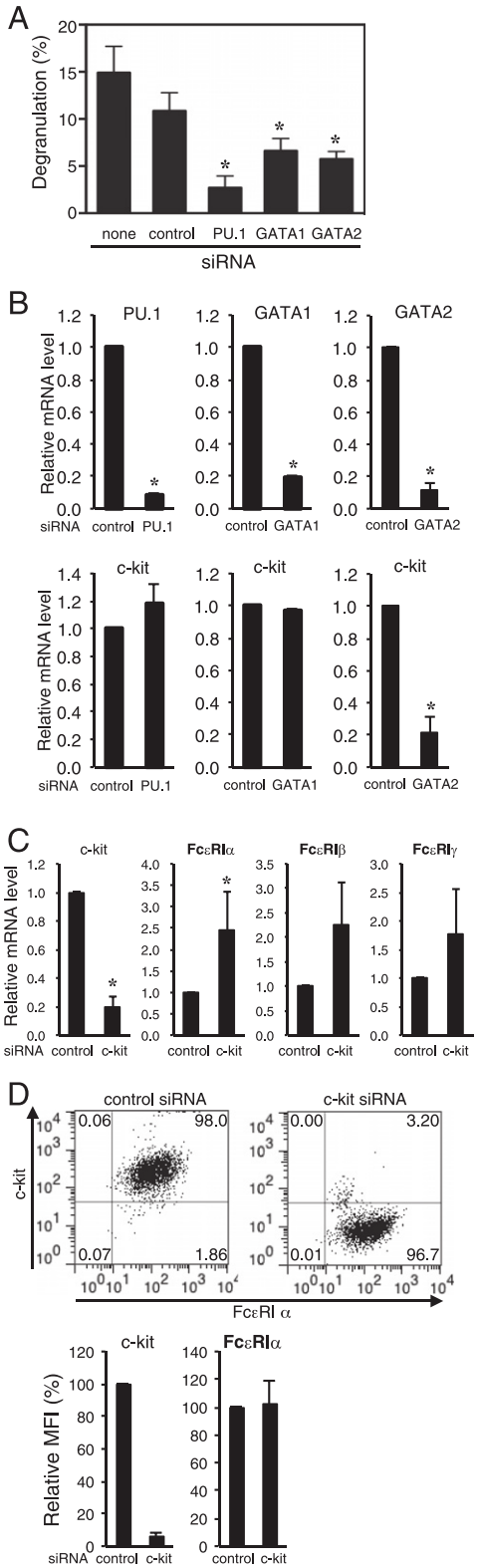

FIGURE 5. Effects of siRNAs on degranulation and the expression of c-kit. (**A**) Effect of siRNA for PU.1, GATA1, or GATA2 on the FcεRI-mediated degranulation of mast cells. The data are the mean + SD of four independent experiments performed with duplicate samples. *$p < 0.05$. (**B**) Effect of siRNA for PU.1, GATA1, or GATA2 on the c-kit mRNA level. mRNA levels of target molecules (*top*) and c-kit (*bottom*) in transfectants in which PU.1 siRNA (*left*), GATA1 siRNA (*center*), or GAT2 siRNA (*right*) was introduced. *$p < 0.05$. (**C**) Effects of c-kit siRNA on mRNA levels of FcεRIα, β, and γ. *$p < 0.05$. (**D**) Effects of c-kit siRNA on cell surface expression of c-kit and FcεRI. Dot-plot profiles of control siRNA transfectant (*top left*) and c-kit siRNA transfectant (*top right*) double stained with anti-FcεRIα and anti–c-kit Abs. Relative mean fluorescence intensity (MFI) (*bottom*) was calculated in the same way as in a previous study (14). control, control siRNA transfectant; GATA1, GATA1 siRNA transfectant; GATA2, GATA2 siRNA transfectant; None, without electroporation; PU.1, PU.1 siRNA transfectant.

transfectants decreased to ~20% compared with control siRNA transfectants (Fig. 5B, *bottom right*). In contrast, transfection of siRNAs for PU.1 and GATA1 did not affect c-kit mRNA levels (Fig. 5B, *bottom left and center*). These data suggest that GATA2 is a positive regulator of c-kit transcription in human mast cells, as previously observed in mouse BMMCs (18), whereas PU.1 and GATA1 are not involved in c-kit expression.

The quantification of c-kit mRNA levels (Fig. 5B) demonstrated that the effects of siRNAs for PU.1 and GATA1 on FcεRI expression were independent of c-kit expression, but GATA2 siRNA may indirectly affect FcεRI by suppressing c-kit expression in addition to direct effects on FcεRIα and β. Therefore, to confirm whether FcεRI expression is downregulated by suppression of c-kit expression, we analyzed FcεRI expression levels in c-kit siRNA transfectants. Introduction of c-kit siRNA significantly reduced c-kit mRNA levels (Fig. 5C) and the amount of cell surface c-kit protein (Fig. 5D). In this experimental condition, cell surface FcεRI levels in c-kit siRNA transfectants were comparable to those in control transfectants (Fig. 5D), and mRNA levels of FcεRIα, β, and γ were slightly higher (Fig. 5C). From these results, we conclude that downregulation of c-kit expression in LAD2 cells does not reflect FcεRI expression levels.

Involvement of PU.1, GATA1, and GATA2 in the expression and function of FcεRI in peripheral blood–derived primary mast cells

Finally, to confirm the involvement of PU.1, GATA1, and GATA2 in FcεRI expression in primary cells, we analyzed the effect of these siRNAs on FcεRI expression in primary mast cells generated from human peripheral blood $CD34^+$ cells following a previously established protocol (13). As shown in Fig. 6A and 6B, all siRNAs for PU.1, GATA1, and GATA2 exhibited significant suppressive effects on cell surface FcεRI expression levels in primary mast cells; in particular, the effects of PU.1 siRNA and GATA2 siRNA were striking. By quantitative analysis of mRNA level, it was confirmed that transcripts of target molecules were effectively knocked down by siRNA (Supplemental Fig. E1). Quantification of mRNA for each subunit in siRNA-transfected cells revealed that FcεRIα mRNA was dramatically reduced by knockdown of PU.1 and slightly reduced by knockdown of GATA1 (Fig. 6C), whereas FcεRIβ mRNA was markedly reduced by transfection of GATA2 siRNA (Fig. 6D).

The effect of gene knockdown on the function of primary mast cells was examined by IgE-mediated histamine release assay of siRNA-transfected cells (Fig. 7). The histamine-releasing activity was shown as the ratio (Fig. 7A), and PU.1 siRNA and GATA2 siRNA significantly suppressed histamine release from primary mast cells. The results of five independent experiments are also shown as independent graphs (Fig. 7B–D), because the potential for degranulation activity varies among independently generated different lots of cells.

Discussion

PU.1, GATA1, and GATA2 are hematopoietic cell–specific transcription factors, and there is a negative crosstalk between PU.1 and GATA1/GATA2. Briefly, several studies reported that PU.1

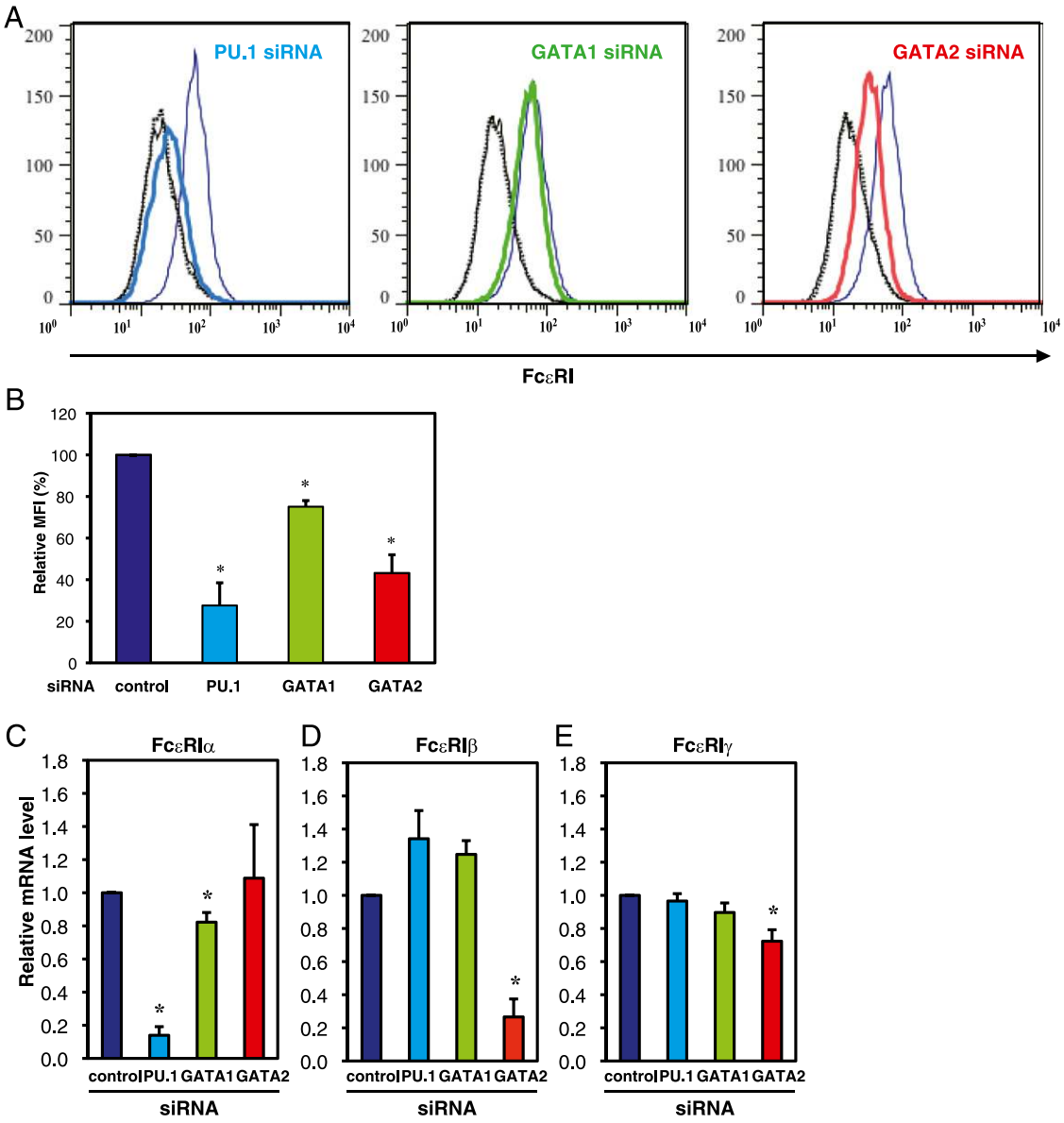

FIGURE 6. Involvement of PU.1, GATA1, and GATA2 in the expression of FcεRI on primary mast cells. Cell surface protein expression levels of FcεRI (**A**, **B**) and mRNA levels of FcεRIα, β, and γ (**C**–**E**). A representative histogram of three independent experiments. $CD34^+$ cell-derived primary human mast cells were transfected with control siRNA, PU.1 siRNA, GATA1 siRNA, or GATA2 siRNA and were stained with FITC-conjugated anti-FcεRIα Ab or FITC-conjugated control Ab. Dark blue line, control siRNA transfectant stained with anti-FcεRIα Ab; light blue line, PU.1 siRNA transfectant stained with anti-FcεRIα Ab; green line, GATA1 siRNA transfectant stained with anti-FcεRIα Ab; red line, GATA2 siRNA transfectant stained with anti-FcεRIα Ab; black lines, control siRNA transfectant treated with control Ab; black dotted lines, PU.1, GATA1, or GATA2 siRNA transfectant treated with control Ab. (B) Data represent the mean + SD of relative mean fluorescence intensity (MFI) obtained from three independent experiments. $*p < 0.05$ versus control. Relative MFI was calculated in the same way as in a previous study (14). The mRNA expression levels of FcεRIα (C), FcεRIβ (D), and FcεRIγ (E) in primary mast cells are displayed as the ratio of mRNA levels versus those detected in control siRNA-introduced cells. Data represent the mean + SD of three independent experiments with duplicate samples. $*p < 0.05$. In the primary mast cell experiments (Fig. 6), three independently generated mast cell populations were used.

(GATA) inhibits the function of GATA (PU.1), resulting in dominion by itself, during the development from hematopoietic stem cells toward the monocyte (erythrocyte/megakaryocyte) lineage (21–28). In contrast, PU.1 and GATA1/GATA2 exhibit a cooperative function in mast cells, as follows: 1) PU.1 and GATA2 cooperatively induce mast cell development (20); 2) PU.1 and GATA1 synergistically transactivate cell type–specific gene regulation in mast cells (5, 29); and 3) PU.1 is required for GATA1 expression in mast cells (30). In our previous studies, PU.1, GATA1, and GATA2 were identified as specific transcription factors that participate in the transcriptional regulation of certain genes expressed in mast cells, including human *FCER1A* (FcεRIα), mouse *Ms4a2* (FcεRIβ), mouse *c-kit*, and human *ST2*/*IL1RL1* (4, 5, 7, 14, 18, 19). However, it has also been reported that knockdown of GATA1 and GATA2 by siRNA does not affect FcεRI expression in rodent mast cells and/or a basophilic cell line (10, 11).

Therefore, to clarify the involvement of these transcription factors in the expression of FcεRI, we transfected siRNAs against PU.1, GATA1, and GATA2 into the human mast cell line, LAD2, using a recently established method (14). Knockdown of PU.1 and GATA1 reduced the cell surface expression of FcεRI due to suppression of FcεRIα transcripts, and GATA2 knockdown reduced both FcεRIα and FcεRIβ mRNA levels (Figs. 1, 2). These results

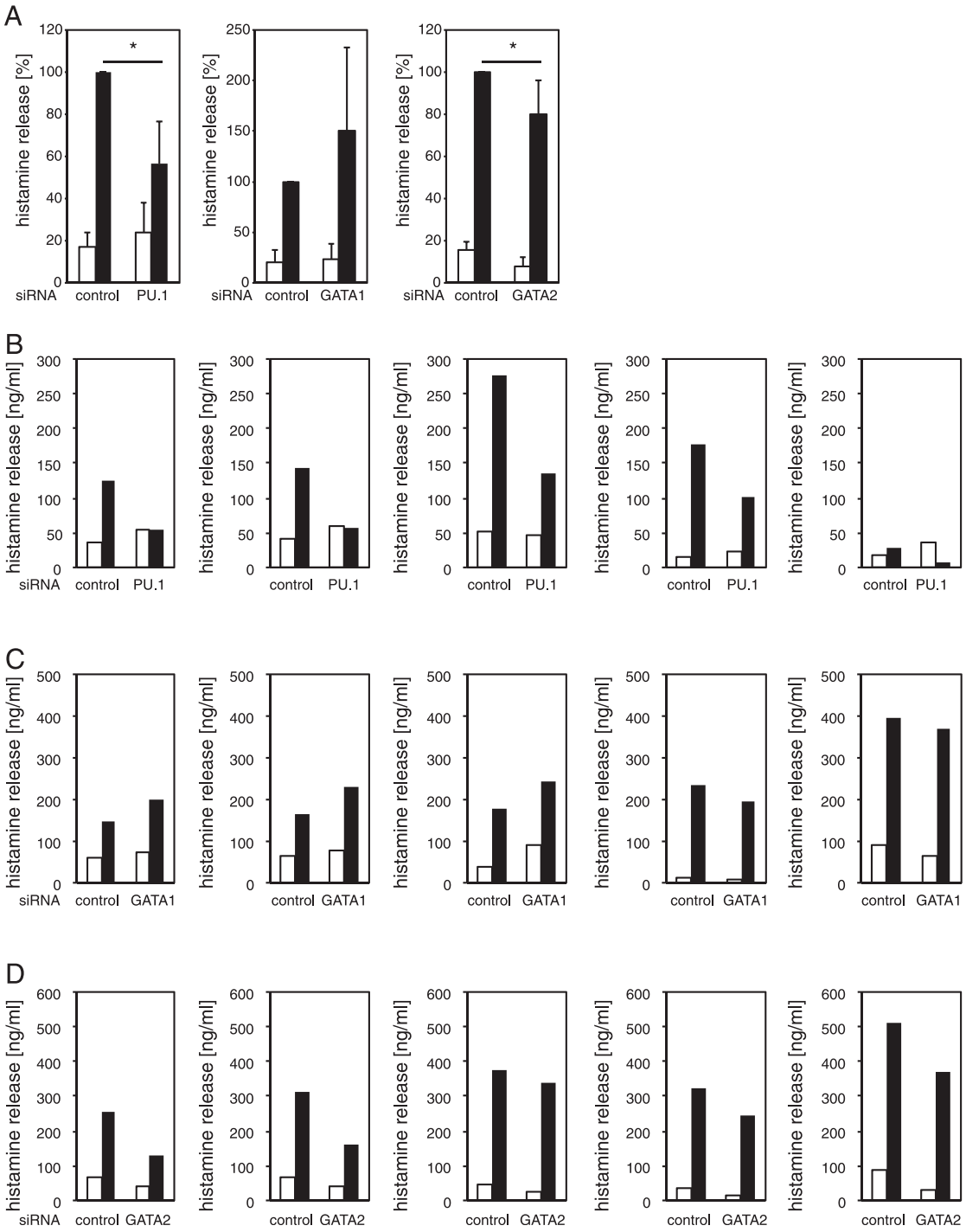

FIGURE 7. Effect of siRNA transfection on IgE-mediated histamine-releasing activity of primary mast cells. (**A**) The degree of degranulation is displayed as the ratio of the released histamine levels versus those released from Triton X-100–treated cells (100%). The concentration of the released histamine in the supernatant of PU.1 siRNA transfectants (**B**), GATA1 siRNA transfectants (**C**), and GATA2 siRNA transfectants (**D**). The results of five independent experiments ($n = 1$) using four independently generated mast cell populations are shown. Filled bars, cross-linked with IgE plus anti-IgE Ab; open bars, IgE alone without anti-IgE Ab. control, control siRNA-introduced cells; GATA1, GATA1 siRNA-introduced cells; GATA2, GATA2 siRNA-introduced cells; PU.1, PU.1 siRNA-introduced cells; $*p < 0.05$.

are consistent with the ChIP assay data showing that PU.1, GATA1, and GATA2 bind to the *FCER1A* promoter in mast cells, whereas GATA2, but not PU.1 or GATA1, binds to the *MS4A2* promoter (Fig. 3). These results also demonstrate that PU.1 and GATA1 are involved in FcεRI expression by transactivation of *FCER1A* and that GATA2 transactivates *FCER1A* and *MS4A2*. This observation does not concur with the data from previous studies using siRNA, in which neither GATA1 siRNA nor GATA2 siRNA affected FcεRI expression in BMMCs and/or RBL-2H3 cells (10, 11). This discrepancy may be due to specific differences between humans and rodents. Alternatively, considering that BMMCs from GATA1 knockdown mice showed delayed expression of FcεRI dependent on the knockdown level (31, 32), the developing stage of mast cells and/or extent of knockdown of the target molecule may be the cause of these differences. We hope to analyze the effect of these siRNAs on FcεRI expression using

mast cells of various subtypes and/or at different developmental stages to clarify this point in the near future.

Finally, we used primary human mast cells to evaluate the involvement of PU.1, GATA1, and GATA2 in FcεRI expression (Figs. 6, 7), because we need to eliminate, as much as possible, any artificial effects due to using a cell line. After repeating the experiments on three independently generated primary mast cells, we conclude that the involvement of PU.1 and GATA1 in FcεRIα transcription, and that of GATA2 in FcεRIβ transcription, is common to both LAD2 cells and primary mast cells. The role of GATA2 in FcεRIα expression differed between these two types of cells. In essence, GATA2 participated in the transcription of FcεRIα in LAD2 cells, but not in primary mast cells. The knockdown of PU.1 and GATA2 was effective in suppressing the degranulation of primary mast cells as was the case for LAD2 cells. In contrast, the suppressive effect was not observed when GATA1 siRNA was used in primary cells. The effect of GATA1 siRNA on the levels of FcεRI protein expression and FcεRIα mRNA in primary cells was lower compared with these effects in LAD2 cells. This difference may explain the discrepancy between LAD2 and primary cells. Alternatively, the expression of some molecules related to signal transduction may be enhanced by knockdown of GATA1, which functions as suppressive transcription factors in certain situations (14) in primary cells in a more sensitive manner. Regardless, we can conclude that the knockdown of PU.1, GATA1, and GATA2 significantly downregulated FcεRI expression in primary human mast cells as well as in human mast cell lines.

In the current study, involvement of GATA2 for *FCER1A* and *MS4A2* was observed as novel findings. Identification of GATA2 is impressive in the field of *MS4A2* promoter analysis, because the cell type–specific transcriptional mechanism of *MS4A2* has been largely unknown, although Oct-1 and MZF-1 have been identified to be transactivators for human *MS4A2* gene via the promoter and intron, respectively, which are not sufficient to explain the cell-type specificity of FcεRIβ expression (6, 8). A reporter assay and EMSA indicated that GATA2 transactivates the *MS4A2* promoter via direct binding to GATA motifs in the promoter (Fig. 4). Interestingly, although GATA1 possesses the potential to bind to all GATA motifs in the *MS4A2* promoter in vitro (Fig. 4B), GATA1 protein binding to the *MS4A2* promoter was not detected in living cells (Fig. 3). Previously, we found that the amount of endogenous GATA1 protein in LAD2 cells is quite low compared with the GATA2 protein level (14), which may explain the higher occupancy of GATA2 on the *MS4A2* promoter (Fig. 3) and the *IL-1RL1/ST2* promoter (14). Alternatively, other molecules may control the recruitment of GATA1 and GATA2 to the target sites in the nucleus. In future studies, we will analyze the binding profiles of GATA1 and GATA2 on chromosomal DNA in mast cells using ChIP-on-chip and/or ChIP-sequence analyses to reveal the mechanism that determines the dynamics and function of GATA1 and GATA2. FcεRIβ expression is limited in mast cells and basophils, whereas FcεRIα is detected in additional hematopoietic lineages, including dendritic cells, monocytes, and activated eosinophils. Therefore, further detailed analysis on the human *MS4A2* promoter may reveal mast cell/basophil-specific transcriptional mechanisms.

Knockdown of PU.1, GATA1, and GATA2 repressed IgE-mediated degranulation of LAD2 cells (Fig. 5A). Because the degranulation degree parallels the FcεRI expression level, the decreased expression of FcεRI in siRNA-introduced cells can explain this repression of degranulation. Furthermore, it is reported that GATA1 and GATA2 are involved in the expression of several molecules related to the IgE-induced signal transduction of terminally differentiated mast cells (10, 11). Therefore, siRNAs for GATA1 and GATA2 may have additional effects targeting the gene expression of intracellular molecules. In the current study, we confirmed that introduction of GATA2 siRNA suppressed c-kit expression in LAD2 cells (Fig. 5B). Considering that knockdown of c-kit did not suppress FcεRI expression (Fig. 5C, 5D) and that degranulation of PU.1 siRNA transfectants, which exhibit normal expression level of c-kit, is more strongly suppressed than GATA2 siRNA transfectants (Fig. 5A), it is suggested that the effect of GATA2 siRNA on FcεRI expression is the main cause of the inhibition of degranulation and that the effect on c-kit expression partly affects the degranulation degree even though the viability of LAD2 cells may be reduced in the presence of GATA2 siRNA. The suppressive effect of PU.1 siRNA on degranulation degree is in no way inferior to the effects of GATA1 and GATA2 (Fig. 5A), suggesting that PU.1 may participate in the expression and function of other molecules as is the case for GATA1 and GATA2, although the target genes of PU.1 in mast cells remain largely unknown. Further detailed analysis will be required to clarify the role of PU.1 in IgE-mediated activation of mast cells.

As described above, the effect of transcription factor knockdown is generally complicated, because any transcription factors target several molecules. In the current study, we found that all of the siRNAs against PU.1, GATA1, and GATA2 suppressed IgE-mediated activation of mast cells. This observation demonstrates that inhibition of these transcription factors may be useful in the development of a new therapeutic approach to allergic diseases.

In conclusion, we have demonstrated that PU.1, GATA1, and GATA2 are involved in the expression of FcεRI in a human mast cell line and primary human mast cells using siRNA with high transfection efficiency, and by ChIP assay. The findings support and elaborate upon our previously published data that were derived using reporter assays and EMSAs.

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Disclosures

The authors have no financial conflicts of interest.

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