

# Involvement of PU.1 in Mast Cell/ Basophil-Specific Function of the Human *IL1RL1/ST2* Promoter

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## ABSTRACT

**Background:** The human *IL1RL1/ST2* gene encodes IL33 receptor. Recently, IL33 has been recognized as a key molecule for the development of Th2 response. Although mast cells and basophils are major targets of IL33 and play important roles in IL33-mediated Th2-type immune responses, the expression mechanism of ST2 in mast cells and basophils is largely unknown. In the present study, we analyzed regulation mechanism of the human *ST2* promoter in the human mast cell line LAD2 and basophilic cell line KU812.

**Methods:** Promoter activity was determined by reporter assay with plasmids carrying the wild-type *ST2* promoter obtained from human genomic DNA and its mutant. The transcription factor binding to the identified *cis*-element was identified by an electrophoretic mobility shift assay (EMSA). The effect of candidate transcription factor on *ST2* expression was confirmed by analyzing *ST2* mRNA level in siRNA-introduced cells.

**Results:** Reporter assay demonstrated that a *cis*-element of typical Ets-family binding sequence was critical for promoter activity in LAD2 and KU812. An Ets-family transcription factor PU.1 bound to this element in an EMSA. When PU.1 expression was suppressed by siRNA, *ST2* mRNA level was significantly reduced in KU812.

**Conclusions:** These observations indicated that PU.1 positively regulates the *ST2* promoter as a transcription factor that directly transactivates the *ST2* promoter via Ets-family-related *cis*-element in mast cells and basophils.

## KEY WORDS

basophils, *IL1RL1/ST2*, IL33 receptor, mast cells, PU.1

## INTRODUCTION

IL33, which was identified as a ligand for *IL1RL1/ST2* in 2005,<sup>1</sup> is expressed in various cell types including fibroblasts, epithelial cells, and endothelial cells with localization in the nucleus in a steady state.<sup>2</sup> IL33 is released upon cell lysis following pro-inflammatory stimulation and causes Th2-type immune responses through binding to *IL1RL1/ST2*. Several studies have demonstrated that not only Th2 but also mast cells and basophils are key target cells of IL33.<sup>3-8</sup>

The human *IL1RL1/ST* gene is constitutively

transactivated in mast cells/basophils and the transcript is mainly driven from the distal promoter in mast cells/basophils, whereas another proximal promoter at 10.5 kb downstream is activated in fibroblasts.<sup>9,10</sup> In previous studies, a transcription factor, GATA1, transactivated the *ST2* distal promoter via GATA-motifs in this region in mouse mast cells,<sup>10</sup> whereas another GATA-family transcription factor GATA3 transactivated the same promoter in mouse T cells.<sup>11</sup> In contrast, another transcription factor PU.1 belonging to the Ets-family regulates mast cell/basophil-specific gene regulation in a synergistic

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manner with GATA1/2.<sup>12,13</sup> However, the involvement of PU.1 in the human *ST2* promoter is largely unknown. These observations prompted us to analyze the role of PU.1 in the regulation of the human *ST2* promoter.

## METHODS

### CELLS

Human mast cell leukemia, LAD2, which was kindly provided by Dr. Arnold Kirshenbaum,<sup>14</sup> and human basophilic leukemia, KU812, were maintained as described previously.<sup>12,15</sup> This study was approved by the ethics committee of Juntendo University School of Medicine.

### PLASMIDS

The human *IL1RL1/ST2* gene promoter was amplified by PCR using the following primers and human genomic DNA purified from peripheral blood using a QIAamp DNA Blood Midi Kit (QIAGEN, Hilden, Germany) as template. Synthesized oligonucleotides 5'-C AAATAGGGAGAATcTcGaGAAAACCTGCAGTTAAC-3' (nucleotide replacement and introduced *Xho*I sites are shown with lower-case letters and italic, respectively) and 5'-ccaagcTTCTGCCACAGTTTCACAAC TCAGAAAGCCA-3' (nucleotide replacement and introduced *Hind*III sites are shown with lower-case letters and italic, respectively) were used as forward and reverse primers, respectively, to obtain -100/+84. Amplified DNA was inserted into a reporter plasmid, pGL4-Basic (Promega, Madison, WI, USA) after digestion with appropriate restriction endonucleases. A mutant reporter plasmid lacking Ets-sequence was generated using a QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) with 5' -CAGCTTATCAGTAACCTGGTTaacGTCTCTTAA CTG-3' (nucleotide replacements are shown with lower-case letters). All nucleotide sequences of inserted DNA were confirmed by sequencing analysis.

The expression plasmids, pCR-PU.1<sup>12</sup> and pCR-GATA2,<sup>16</sup> were generated in our previous study.

### REPORTER ASSAY

Each reporter plasmid (5 µg) and an internal control plasmid pRL-null (25 ng; Promega), or reporter plasmid (5 µg) and co-expression plasmid (totally 6 µg) were introduced into LAD2 and KU812 by electroporation using Genepulser II (BioRad Laboratories, Hercules, CA, USA). At 20-24 h after transfection, cells were harvested and relative luciferase activity in cells was determined using a luminometer, Micro Lumat Plus (Berthold Technologies, Bad Wildbad, Germany) with a Dual-luciferase assay kit (Promega) as described previously.<sup>12,17</sup>

### ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

The synthesized oligonucleotide 5'-GTAACCTGGTT

CCTGTCTCTTAAC-3' (-48/-25 of the *ST2* promoter) and an oligonucleotide complementary to it were 5'-FITC-labeled and were annealed to obtain double-stranded probe DNA. Nuclear extract was prepared from KU812 cells according to a previously reported method<sup>18</sup> with some modifications. Briefly, 1 × 10<sup>7</sup> cells were suspended in 500 µl of Buffer A (10 mM HEPES-KOH pH7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 1 mM DTT) and kept on ice for 30 min. The suspended cells were then passed through 30 G needles 30 times. After centrifugation at 700 × *g* for 10 min, the pellet was resuspended in 100 µl of Buffer B (20 mM HEPES-KOH pH7.9, 25% glycerol, 450 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM DTT). A Halt Protease Inhibitor Cocktail Kit (PIERCE, Rockford, IL, USA) was contained in both Buffer A and Buffer B. PU.1 protein was produced using a TNT T7 quick-coupled transcription/translation system with pCR-PU.1 as template. Wild-type competitor (same sequence as the probe DNA), and mutant Ets competitor (5'-GTAACCTGGTctCTGTCTCTTAAC-3', mutated nucleotides are shown with lower-case letters) were used in competition assay. Electrophoresis of probe-protein mixture and detection of fluorescence were performed as described previously.<sup>19-21</sup>

### INTRODUCTION OF SMALL INTERFERING (si) RNA

Ten microliters of 20 µM PU.1 siRNA (Stealth Select RNAi, #HSS144058, Invitrogen, Carlsbad, CA, USA) or control siRNA (Stealth RNAi Negative Universal Control, #46-2000, Invitrogen) was introduced to 2 × 10<sup>6</sup> cells with a Neon 100 µl kit using an electroporator, Neon transfection system (Invitrogen), set at program #16. The transfection efficiency of this condition was confirmed to be over 98% by monitoring BLOCK-iT Alexa Fluor Red Fluorescent Oligo (#14750-100, Invitrogen) using flow cytometry.

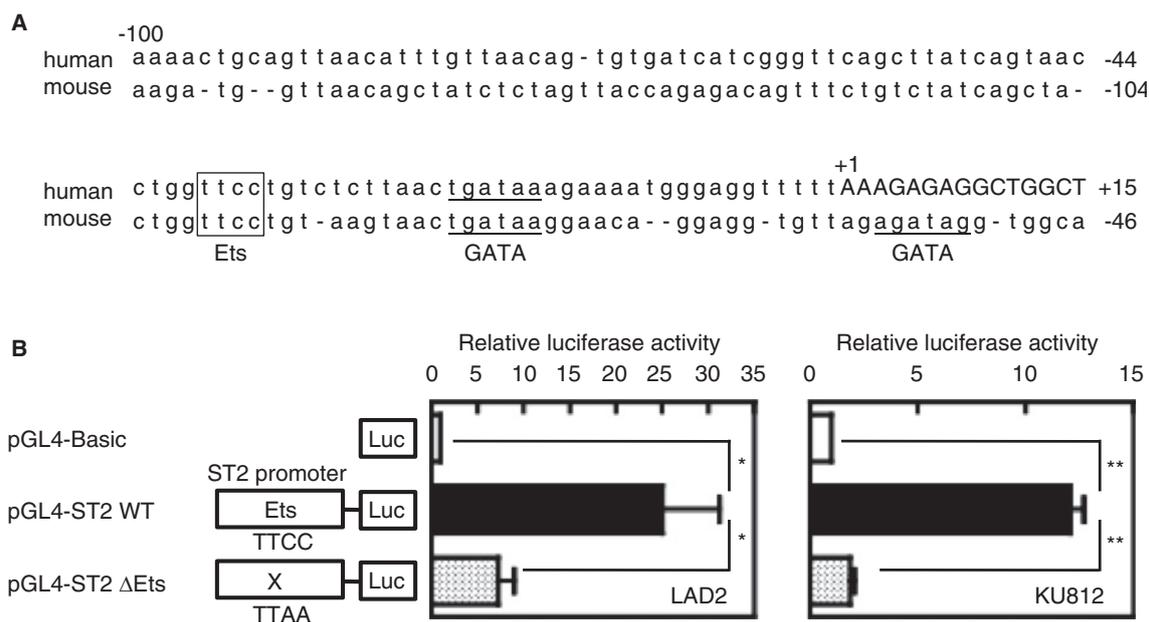
### QUANTIFICATION OF mRNA

Total RNA was prepared from LAD2 and KU812 using an RNeasy Micro Kit (QIAGEN) and was reverse transcribed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantification of *ST2* mRNA and PU.1 mRNA was performed using TaqMan system with TaqMan Gene Expression Assays (Applied Biosystems; #Hs00545033\_m1 for *IL1RL1/ST2*, and #Hs02786711\_m1 for *Spi1/PU.1*) with human GAPDH #4326317E as a housekeeping gene.

## RESULTS

### AN Ets-MOTIF IN THE HUMAN *ST2* PROMOTER IS INVOLVED IN TRANSCRIPTION ACTIVITY

In the previous studies, the essential region of the hematopoietic cell-specific distal promoter was identified to be located approximately 100 bp from the transcription start site, and two GATA-motifs in this re-



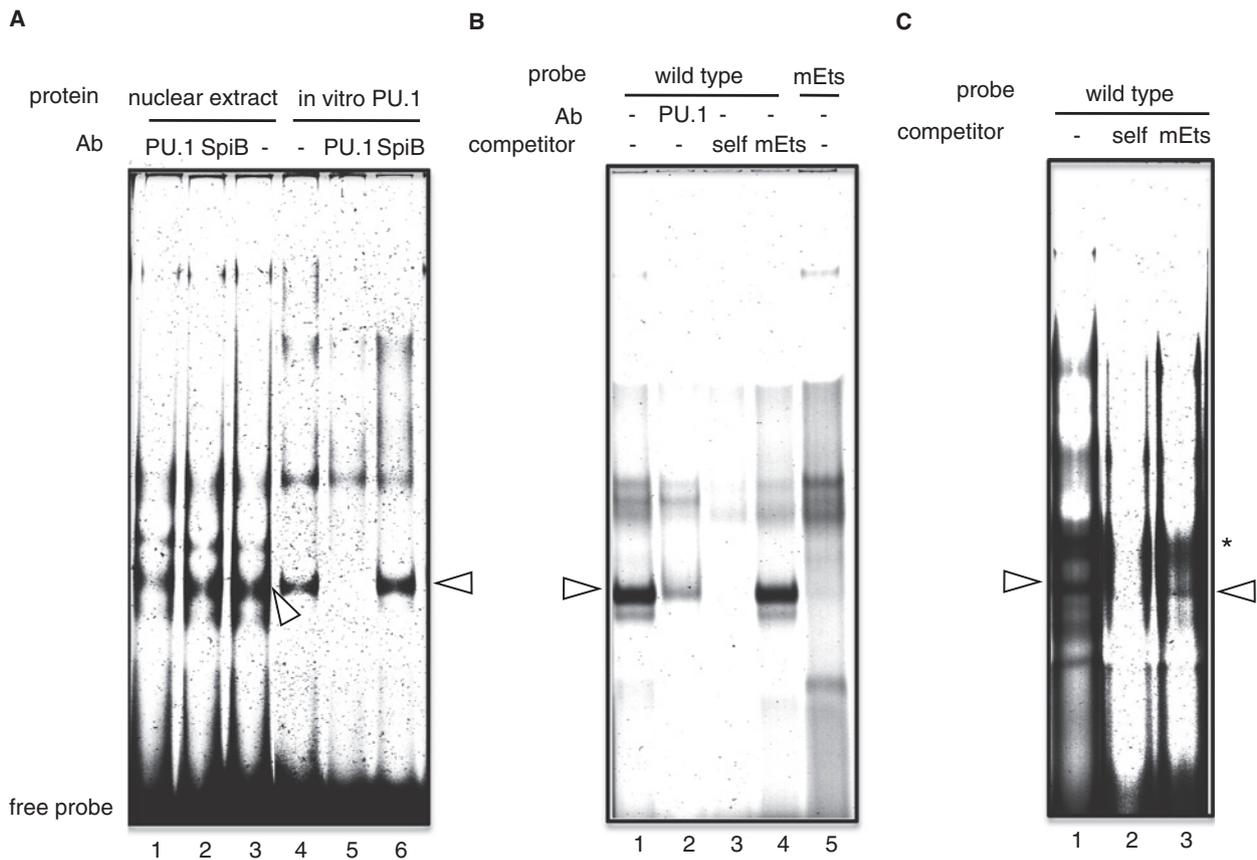
**Fig. 1** Transcription activity of the human *ST2* promoter in LAD2 and KU812. **(A)** Nucleotide sequence of the human *ST2* gene around the hematopoietic cell-specific distal promoter. The transcription start site determined by 5'-RACE in a previous study is expressed as +1.<sup>9</sup> Upper-case and lower-case letters represent the sequences in exon and in the promoter, respectively. A typical Ets-motif in the human *ST2* gene is boxed and putative GATA-binding *cis*-elements previously identified in the mouse *ST2* gene<sup>10,11</sup> are underlined. **(B)** Relative luciferase activity is displayed as the ratio of luciferase activity versus that in pGL4-Basic transfectant. Data represent means  $\pm$  SEM of six independent experiments performed with triplicate samples. Open bar, pGL4-Basic; closed bar, wild type *ST2* promoter; dotted bar, mutant *ST2* promoter lacking an Ets-motif. \* $p < 0.05$ , \*\* $p < 0.01$  in two-tailed paired Student's *t* test.

gion were identified to be *cis*-elements critical for promoter function.<sup>10,11</sup> As shown in Fig. 1A, a typical Ets-motif is located in the minimized promoter region close to a GATA-motif, at -39/-36, and this Ets-motif is conserved between human and mouse. We evaluated the effect of this Ets-motif on promoter activity by a reporter assay. The human *ST2* promoter -100/+84 exhibited significantly higher transcription activity than promoter-less pGL4-Basic (Fig. 1B) and the +16/+84 region (data not shown) in human mast leukemia cell line LAD2 and human basophilic cell line KU812. Nucleotide replacement at this Ets-motif caused significant reduction of promoter activity in both cell lines ( $\Delta$ Ets in Fig. 1B). This result demonstrates that an Ets-motif at -39/-36 is involved in the transcription activity of the human *ST2* promoter in LAD2 and KU812.

#### THE BINDING OF AN Ets-FAMILY TRANSCRIPTION FACTOR PU.1 TO THE Ets-MOTIF

To determine the transcription factor(s) binding to the *cis*-element at -39/-36, EMSA was performed using double-stranded DNA at -48/-25 of the *ST2* promoter as a probe. When nuclear proteins extracted from KU812 cells were added, several shift bands with lower mobility than that of free probe appeared

(Fig. 2A, lane 3). The density of a major band was reduced in the presence of anti-PU.1 Ab (Fig. 2A, lane 1, shown with an arrowhead), whereas Ab against Spi-B, which is another Ets-family protein, did not affect this band intensity (Fig. 2A, lane 2). The most pronounced band in the mixture of the probe and PU.1 protein that was synthesized with the *in vitro* transcription/translation system completely disappeared upon addition of anti-PU.1 Ab but not by anti-Spi-B Ab (Fig. 2A, lanes 4, 5, and 6). The mobility of the complex with the probe and *in vitro* translated PU.1 was slightly lower than that of the complex with nuclear PU.1. The difference of the mobility is likely to have been due to the fusion of Flag-tag at the N-terminus of PU.1, resulting in the higher molecular weight of *in vitro* translated PU.1. Regardless of the slight difference of mobility shift, these results suggest that PU.1 binds to the *ST2* promoter region around the *cis*-element *in vitro*. EMSAs were performed with competitive oligonucleotides to further confirm whether PU.1 specifically binds to the probe DNA via a core sequence at -39/-36 (Fig. 2B). The specific band shift, which disappeared upon addition of anti-PU.1 Ab (Fig. 2B, lane 2), was retained by addition of the mutant competitor lacking Ets-motif, mEts (Fig. 2B, lane 4), whereas the specific band dis-



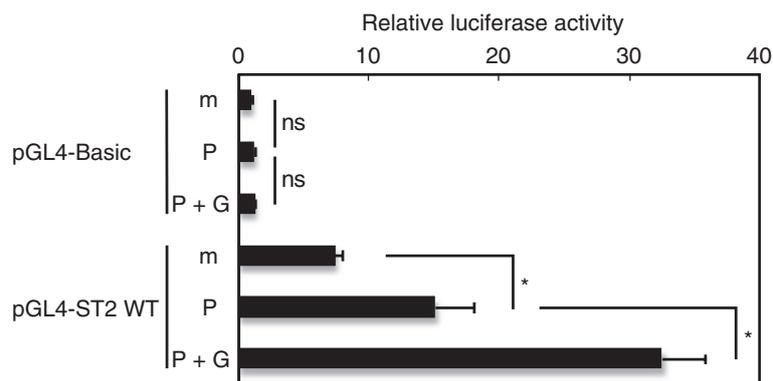
**Fig. 2** PU.1 binds to -48/-25 of the human *ST2* promoter *in vitro*. **(A)** EMSA profile with a probe of -48/-25 and proteins in the presence or absence of Ab. Nuclear extract, nuclear proteins from KU812 cells; *in vitro* PU.1, *in vitro* transcription/translocation reaction mixture with PU.1 expression plasmid; -, without Ab; PU.1, with anti-PU.1 Ab; Spi-B, with anti-Spi-B Ab. The specific band of complex with PU.1 and a probe is marked with an arrowhead. Competition assay using *in vitro* translated PU.1 **(B)** or KU812 nuclear extract **(C)**. mEts, oligonucleotide containing mutated Ets site (nucleotide sequence is shown in Methods); self, competitive oligonucleotide with wild-type sequence.

appeared in the presence of wild-type competitor (Fig. 2B, lane 3). When the mutant probe with same sequence as mEts was used instead of wild-type probe, the specific band was not detected (Fig. 2B, lane 5). Similarly, when competition assay was performed with KU812 nuclear proteins, the specific band with PU.1 was still detected in the presence of mEts competitor (Fig. 2C, lane 3), whereas this band and all other bands disappeared in the presence of wild-type competitor (Fig. 2C, lane 2). The band (shown with an asterisk) showing lower mobility than that of the band containing PU.1 (shown with an arrowhead) was also detected when mEts was used as competitor (Fig. 2C, lane 3), suggesting that another protein may bind the core sequence as well as PU.1. Taken together, these results demonstrate that PU.1 binds to the *ST2* promoter region via a core sequence at -39/-36.

### ST2 PROMOTER TRANSACTIVATION ACTIVITY OF PU.1

In order to evaluate the effect of PU.1 on *ST2* promoter activity, co-expression reporter analysis was performed using KU812. The presence of exogenous PU.1 increased *ST2* promoter activity, whereas pGL4-Basic was not affected by PU.1 co-expression (Fig. 3).

The presence of both binding elements for PU.1 and GATA has been found in mast cell-specific enhancer regions.<sup>12,22</sup> PU.1 and GATA1/2 cooperate in mast cells,<sup>12,13</sup> in contrast, PU.1 and GATA1/2 inhibit each other in other hematopoietic lineages.<sup>23-26</sup> Then, the effects of PU.1 and GATA2 on the human *ST2* promoter were analyzed to confirm whether the *ST2* promoter is one of the models of this cooperation system. As shown in Figure 3, co-expression of PU.1 and GATA2 caused significantly higher transcription activity of the *ST2* promoter compared with co-expression of PU.1 alone.



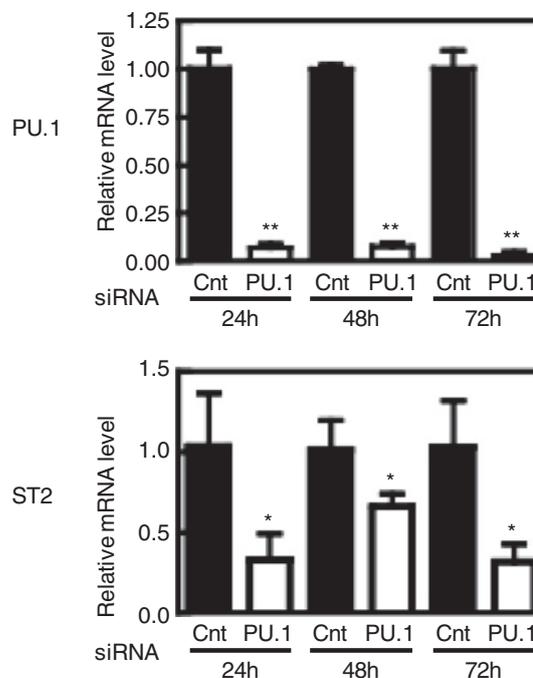
**Fig. 3** PU.1 and GATA2 cooperatively transactivates the human *ST2* promoter. Relative luciferase activity is displayed as the ratio of luciferase activity versus that in pGL4-Basic and mock transfectant. Data represent means  $\pm$  SD of triplicate samples. The total amount of co-expression plasmid was adjusted to 6  $\mu$ g as follows: m, 6  $\mu$ g mock vector (pCR3.1); P, 3  $\mu$ g pCR-PU.1 and 3  $\mu$ g pCR3.1; P + G, 3  $\mu$ g pCR-PU.1 and 3  $\mu$ g pCR-GATA2. ns, not significant. \* $p$  < 0.05.

### EFFECT OF PU.1 siRNA ON ST2 EXPRESSION

To examine the involvement of PU.1 in *ST2* expression, the expression of PU.1 was knocked down by siRNA. By the detection of red fluorescent oligonucleotides, which were introduced by electroporation using a Neon system, the transfection efficiency in KU812 was confirmed to be over 98% (data not shown). When PU.1 siRNA was introduced in KU812 under the same conditions, PU.1 mRNA level was reduced by 90% at 24 h after transfection and the reduction was continued until at least 72 h (Fig. 4, top). In these conditions, the amount of *ST2* mRNA was significantly lower than that in control siRNA transfectants (Fig. 4, bottom). These results indicate that PU.1 participates in expression of *ST2*.

### DISCUSSION

IL33 receptor is encoded by the human *IL1RL1/ST2* gene that possesses two promoters, the distal promoter for hematopoietic lineages and the proximal promoter for non-hematopoietic lineages.<sup>9,10</sup> Among hematopoietic lineages known as the target of IL33, mast cells and basophils play important roles in IL33-mediated Th2-type immune responses. In previous studies regarding the transcriptional regulation mechanism of the mouse *ST2* promoter, two GATA-motifs in the distal promoter were identified to be critical *cis*-elements, which were recognized with GATA1 in mouse mast cells<sup>10</sup> and with GATA3 in mouse T cells.<sup>11</sup> However, when nucleotide sequences of the *ST2* promoters were compared between human and mouse, transcription start site of the human *ST2* gene was shown to be located approximately 60 bp upstream compared with that of mouse and the sequence around a GATA-motif at 5'-



**Fig. 4** Effect of PU.1 knockdown on *ST2* expression. The mRNA expression levels of PU.1 (top) and *ST2* (bottom) in PU.1 siRNA transfectants (open bar) are shown as the ratio of mRNA levels vs. that in control siRNA transfectants (closed bar). Data represent means  $\pm$  SD of six transfectants, each of which was analyzed by real-time PCR of triplicate samples. \* $p$  < 0.05; \*\* $p$  < 0.01 in two-tailed unpaired Student's *t* test.

site was highly conserved containing a typical Ets-motif, but another GATA-motif was absent (Fig. 1A). In the present study, the involvement of this Ets-motif in promoter function was examined, because we have previously found that the Ets-motif close to the GATA-motif exhibited mast cell/basophil-specific enhancing activity mediated by PU.1.<sup>12</sup>

A mutant promoter carrying nucleotide replacement in the Ets-motif at -39/-36 showed significantly reduced transcriptional activity compared with the wild-type *ST2* promoter in LAD2 and KU812 (Fig. 1). Among several bands in EMSA using nuclear extract from KU812, a major band was identified to represent a complex of probe and PU.1 (Fig. 2). In addition, exogenous expression of PU.1 transactivated the *ST2* promoter (Fig. 3). These results suggested that an Ets-motif at -39/-36 is critical for the *ST2* promoter and that PU.1 is one of the candidates transactivating the *ST2* promoter via this Ets-motif. Finally, PU.1 knock-down by siRNA resulted in suppression of *ST2* expression in KU812 (Fig. 4). From these results, it is concluded that PU.1 transactivates the human *ST2* promoter via binding to an Ets-motif at -39/-36 in these lineages.

Although we have tried to identify other transcription factor(s) that bind to a critical element at -39/-36 in EMSA using Abs against various transcription factors, no additional specific disappearance of bands has been observed so far. Further experiments to identify the transcription factor(s) that bind to the element at -39/-36 will be required to reveal the mechanism of *ST2* expression in mast cells and basophils. Regardless, we demonstrated that a *cis*-element at -39/-36 is critical for *ST2* promoter activity and that PU.1 is involved in *ST2* expression in the present study.

We have previously reported that PU.1 is involved in transcription of the human *FcεRI α* gene.<sup>12</sup> Recently, we introduced PU.1 siRNA into LAD2 to confirm our previous findings by using the up-to-date experimental technologies, and we found that PU.1 knock down reduced the cell expression of *FcεRI* (Inage, E., unpublished data). Therefore, PU.1 knock down may exhibit anti-allergic effects by suppressing multiple pathways in mast cells/basophils including IL33- and IgE-mediated activation signaling.

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