Multiple ETS Family Proteins Regulate \textit{PF4} Gene Expression by Binding to the Same ETS Binding Site

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Abstract

In previous studies on the mechanism underlying megakaryocyte-specific gene expression, several ETS motifs were found in each megakaryocyte-specific gene promoter. Although these studies suggested that several ETS family proteins regulate megakaryocyte-specific gene expression, only a few ETS family proteins have been identified. Platelet factor 4 (PF4) is a megakaryocyte-specific gene and its promoter includes multiple ETS motifs. We had previously shown that ETS-1 binds to an ETS motif in the PF4 promoter. However, the functions of the other ETS motifs are still unclear. The goal of this study was to investigate a novel functional ETS motif in the PF4 promoter and identify proteins binding to the motif. In electrophoretic mobility shift assays and a chromatin immunoprecipitation assay, FLI-1, ELF-1, and GABP bound to the \(-51\) ETS site. Expression of FLI-1, ELF-1, and GABP activated the PF4 promoter in HepG2 cells. Mutation of the \(-51\) ETS site attenuated FLI-1-, ELF-1-, and GABP-mediated transactivation of the promoter. siRNA analysis demonstrated that FLI-1, ELF-1, and GABP regulate PF4 gene expression in HEL cells. Among these three proteins, only FLI-1 synergistically activated the promoter with GATA-1. In addition, only FLI-1 expression was increased during megakaryocytic differentiation. Finally, the importance of the \(-51\) ETS site for the activation of the PF4 promoter during physiological megakaryocytic differentiation was confirmed by a novel reporter gene assay using in vitro ES cell differentiation system. Together, these data suggest that FLI-1, ELF-1, and GABP regulate PF4 gene expression through the \(-51\) ETS site in megakaryocytes and implicate the differentiation stage-specific regulation of PF4 gene expression by multiple ETS factors.

Introduction

Megakaryocytic differentiation is accompanied by drastic morphological changes that are induced by endomitosis and proplatelet formation. To understand the molecular mechanism of megakaryocyte-specific gene regulation during this unique differentiation process, several megakaryocyte-specific gene promoters, including the promoters of platelet factor 4 (PF4), c-Mpl, Glycoprotein (GP) IIb, GPV, GPIX, GPVI, GPlb and platelet basic protein (PBP), have been studied (reviewed in [1]). Each of these promoters includes multiple GATA and ETS motifs.

GATA-1 is shown to bind to the GATA motifs in most megakaryocyte-specific gene promoters. GATA-1 is one of the zinc finger transcription factors; it recognizes the T/AGATA/G motif and promotes megakaryocytic and erythroid development. GATA-1 binds to GATA motifs in the megakaryocytic gene promoters and activates the gene expression (reviewed in [1]). On the other hand, multiple ETS family transcription factors, such as FLI-1 and PU.1, are known to bind to ETS motifs in each megakaryocyte-specific gene promoter. The members of the ETS family of transcription factors share an evolutionarily conserved DNA-binding domain of 85 amino acids with a winged-helix-turn-helix configuration [2]. ETS factors bind to GGAA/T core sequences. FLI-1 is known to be a positive regulator of megakaryocyte-specific gene expression. FLI-1 regulates several megakaryocyte-specific genes, including GPlib, GPVI, GPIX and c-Mpl. FLI-1 interacts with GATA-1 and enhances its binding to DNA [3]. Homozygous FLI-1 knockout is embryonic lethal in mice and shows severe dysmegakaryopoiesis. PU.1 is known to be a transcriptional activator of the GPIV/GP and PBP genes [4,5]. However, PU.1 is also reported to interact with GATA-1 and inhibit GATA-1 function through several possible mechanisms.
[6, 7, 8, 9, 10, 11]. This indicates that PU.1 may function as a negative regulator of megakaryocyte-specific gene expression.

To investigate the mechanism underlying megakaryocyte-specific gene expression, we have been studying the regulatory mechanism of the PF4 gene expression by using the rat PF4 promoter. We demonstrated that several transcription factors (e.g. GATA-1, ETS-1, MEIS1, PBX1/2, PREP1 and USF1/2) bind to the proximal promoter region and activate the PF4 gene expression [12, 13, 14, 15]. ETS-1 has been shown to activate the PF4 promoter through the −73 ETS site. However, the functions of several other ETS motifs in the PF4 promoter are still unknown. In the present study, we identified the −51 ETS site as a novel functional site in the promoter. The importance of the −51 ETS site for the activation of PF4 gene expression in physiological megakaryocytic differentiation was demonstrated by a novel ES cell differentiation system. Based on EMSA and coexpression assays, 3 ETS family proteins, FLI-1, ELF-1, and GABP were shown to bind to the −51 ETS site and regulate the PF4 promoter. Analysis of expression patterns of FLI-1, ELF-1, and GABP during megakaryocytic differentiation suggested the differentiation stage-specific function of ETS factors. Thus, we succeeded in identifying multiple ETS family proteins that regulate megakaryocyte-specific PF4 gene expression through the novel ETS site.

Materials and Methods
Preparation of plasmids and targeting vectors
The Rat PF4 promoter (PF4-luc) and human PF4 (hPF4-luc) reporter constructs were previously described [12, 14]. To generate the PF4 promoter deletion constructs (Del-500, Del-300, and Del-100), rat PF4 promoter upstream fragments were amplified by polymerase chain reaction (PCR) using 3 different primer sets (DNA sequences are shown in Table S1). The resulting fragments were phosphorylated by T4 DNA kinase and cloned into the Smal site of PGV-B (TOYO B-Net, Tokyo, Japan). All DNA sequences were verified by automated DNA sequencing.

To generate the two PF4 promoter mutants containing a single mutation in −73 or −51 ETS site (−73 G/A, −73 T/A, −51 G/A, and −51 T/A), an intermediate plasmid containing mutations in both sites (−73 G/A−51 G/A) was prepared by two-step PCR amplification. In the first step, the rat PF4 promoter fragment was amplified by PCR using PF4-luc as a template and primers for the first PCR. In the second step, PCR was performed using primers (the first PCR product and a new primer) and PF4-luc as a template. The resulting 0.2-kb promoter fragment containing the −73 G/A−51 T/A mutation was digested with KpnI and ligated with the 6.4-kb fragment of PF4(−73 G/A−51 T/A) KpnI digested PF4-luc. To generate the −73 G/A−51 T/A mutation, Smal I and the 0.7-kb fragment containing the −51 T/A mutation was ligated with the 5.9-kb fragment of Smal I digested PF4-luc. To generate the −73 G/A−51 T/A mutation, Smal I and the 0.7-kb fragment was ligated with the 5.9-kb fragment of Smal I digested −73 G/A−51 T/A. The DNA sequences of all the constructs were verified by automated DNA sequencing. All the primer sequences are shown in Table S1.

To generate FLI-1, PU.1, and GATA-1 expression vectors, cDNAs for FLI-1, PU.1, and GATA-1 were PCR amplified using HEL cDNA and gene-specific primers (shown in Table S1). The resulting PCR fragments were phosphorylated with T4 DNA kinase and cloned into the EcoRV site of pcdNA3 (Invitrogen, Carlsbad, CA). DNA sequences were verified by automated DNA sequencing. To generate the ELF-1 expression vector (pcDNA3-ELF1), pcCl-ELF1 (a generous gift from Peter Ottgen) was digested with XhoI and XbaI, and the resulting ELF-1 fragment was cloned into pcdNA3. Preparation of ETS-1 and GABP expression vectors was described previously [12, 16].

To generate the Hprt-targeting vector with the rat PF4 promoter (pMP8II-PF4-AcGFP), a 1.1-kb region of the PF4 promoter was amplified by PCR using PF4-luc as a template. The resulting promoter fragment was digested with XhoI and SacI, and cloned into the XhoI-SacI site of AcGFP-P1 (Takara, Siga, Japan) to generate PF4-AcGFP. The promoter sequence was verified by automated DNA sequencing. PF4-AcGFP was then digested with AflII and blunt-ended with T4 DNA polymerase. The resulting DNA was digested with MluI. A 2.1-kb fragment containing PF4 promoter coupled to GFP was purified and cloned between the MluI and PstI sites of pMP8II [17]. To generate the targeting vector with a mutation of the −51 ETS site (pMP8II-PF4−51 ETSmut)-AcGFP), a 170 bp fragments spanning the mutation was PCR amplified using the −51 ETSmut plasmid as a template. The resulting promoter fragment was digested with SacI and SacII, and cloned between the SacI and SacII sites of PF4-AcGFP. The promoter sequence in the obtained plasmid (PF4−51 ETSmut−AcGFP) was verified by automated DNA sequencing. PF4−51 ETSmut−AcGFP was then digested with AflII and blunt-ended with T4 DNA polymerase. The resulting DNA was digested with MluI and 2.1-kb fragment was cloned between the MluI and PstI sites of pMP8II.

Cell culture and transient transfection assays
HEL cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. HepG2 cells were maintained under the same conditions except that Dulbecco’s modified Eagle’s medium (DMEM) was used. In transient transfection assays with HepG2 cells, 0.5 µg of PF4-luc or hPF4-luc were transfected into 2×10⁵ cells with or without 0.5 µg of each expression vector, using the Lipofectamine 2000 reagent (GIBCO BRL, Gaithersburg, MD). To control for transfection efficiency, the cells were also transfected with 0.5 µg of pβ-actin-lacZ. In the assay with HEL cells, 7 µg each of the reporter plasmids and pβ-actin-lacZ were transfected into 1×10⁶ cells by electroporation as described previously [18]. Transfected cells were cultured for 48 hr and assayed for luciferase and β-galactosidase activity. Each assay was performed in duplicate more than three times.

Electrophoretic mobility shift assay (EMSA)
Nuclear extracts were prepared from HEL cells using Nuclear Extract Kit (Active Motif, Carlsbad, CA). In vitro translated ETS-1, PU.1, FLI-1, ELF-1, and GABP were prepared using the TNT Quick Coupled transcription/translation System (Promega, Madison, WI) and 1 µg of expression vectors. To generate EMSA probes, oligonucleotides were annealed, labeled with T4 polynucleotide kinase and [γ-P³²]ATP, and purified with G-50 micro columns (Amersham Pharmacia Biotech, Uppsala, Sweden). Binding reactions were carried out using 50 fmol of each probe, 3 to 5 µg nuclear extract or 2 to 4 µl of in vitro translated proteins in binding buffer (2 mM HEPES-NaOH [pH 7.9], 2% glycerol, 0.01 mM EDTA, 0.25 mM DTT, 5 mM KCl, 0.1 mg/ml bovine serum albumin, 5 ng/µl poly dI-dC) for 40 min at 4°C. For competition and supershift assays, competitor oligonucleotides or antibodies were pre-incubated with nuclear extract or in vitro translated proteins for 10 min prior to addition of the probe. Antibodies to ETS-1 (C-20), PU.1 (T-21), FLI-1 (C-19), ELF-1 (C-20), GABPβ1/2 (H-180), GABPβ1/2 (H-265), and Stat5 (C-17) used as a control antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Gel electrophoresis was carried...
Chromatin immunoprecipitation (ChIP) assay
ChIP assays were performed using a ChIP-IT Express kit (Active Motif, Carlsbad, CA). Briefly, crosslinked genomic DNA was prepared from HEL cells and sheared by sonication using the Digital dicer model 250 (Branson, Danbury, CT). Resulting DNA-protein complexes were immunoprecipitated using 3 µg of antibodies against FLI-1, ELF-1, GABPα, or control IgG. The precipitated DNA fragments were analyzed by real-time PCR using primers (sequences are shown in Table S1) to amplify the promoter region including the −51 ETS sites or GAPDH locus as a control.

RT-PCR using cDNA from human megakaryocytes or cell lines
Buffy coat peripheral blood (PB) cells were obtained from volunteer blood donors. PB was collected after written consent that was obtained from the volunteer blood donors. All the protocols were approved by the ethic committees in Japanese Red Cross Osaka Blood Center and Osaka University. AC133+ cells were isolated from PB mononuclear cells by a MACS AC133 Cell Isolation Kit (Miltenyi Biotech, Auburn, CA), as described previously [20]. Purified AC133+ cells were cultured in IMDM containing 20% human serum, 50 IU/ml penicillin, 50 µg/ml streptomycin, and 10 ng/ml thrombopoietin (TPO) (PeproTech Inc., Rocky Hill, NJ). The same amount of TPO was added to the medium every two days, and half of the medium was replaced with new medium after 6 days of incubation. Cells were incubated for 0, 4, 6, or 10 days and then processed for total RNA using IsoGEN (Nippon gene, Tokyo, Japan). Each cDNA was synthesized from 0.5 to 1 g of total RNA using ReveTra Ace (Toyobo, Osaka, Japan) and used for real-time RT-PCR analyses. Expression levels of PF4, FLI-1, ELF-1, and GABPα were normalized by a GAPDH expression level. siRNA target and primer sequences are shown in Table S1.

Results
PF4 promoter activity is mediated by the ETS transcription factors ETS-1, PU.1, and FLI-1
We have previously shown that ETS-1 mediates expression of PF4 [12]. Other ETS factors, including PU.1 and FLI-1, have been implicated in megakaryocyte-specific gene expression [3,4]. To determine the temporal expression of these genes, we employed a human megakaryocyte differentiation assay. Hematopoietic progenitor (AC133+) cells from human peripheral blood cells were differentiated into megakaryocytes by adding thrombopoietin (TPO). The cells were processed 0, 4, 6, and 10 days later for RNA isolation and assayed by real-time PCR for expression of PF4 and the relevant transcription factors (Figure 1). PF4 demonstrated a time-dependent increase in expression, indicating that AC133+ cells were differentiating into the megakaryocytic lineage. A similar pattern was observed for NF-E2p45, a transcription factor that has been shown to play an important role during the late stages of megakaryocytic differentiation (reviewed in [22]). The expressions of ETS-1, PU.1, and FLI-1 preceded that of PF4. Detectable expression of the megakaryocytic transcription factor, GATA-1, coincided with that of PF4. These results are consistent with a role for FLI-1 and PU.1 in regulating PF4 gene expression.

To determine whether PU.1 or FLI-1 mediate PF4 promoter activity, we employed co-expression assays using HepG2 cells. We chose these cells because it lacks detectable expression of ETS-1, PU.1, FLI-1, and GATA-1 (Figure 2A). Overexpression of ETS-1 or PU.1 resulted in a small induction of the rat PF4 promoter activity, while FLI-1 strongly activated the PF4 promoter (Figure 2B). Overexpression of GATA-1 with FLI-1 but not with ETS-1 or PU.1 resulted in synergistic activation of the PF4 promoter (Figure 2B; Figure S1). A similar pattern was observed with the human PF4 promoter (Figure 2C). These
results suggest a positive role for FLI-1 in regulating PF4 gene expression.

FLI-1 activates the PF4 promoter activity through the −51 ETS site

In order to identify the FLI-1 binding site that regulates PF4 promoter activity, we cotransfected HepG2 cells with progressively shortened 5′-deletion fragments of the PF4 promoter. As shown in Figure 3A, deletion constructs containing 500, 300, and 100 bp of the upstream promoter region (Del-500, Del-300, and Del-100, respectively) were all activated by FLI-1. These findings suggest that the FLI-1-responsive DNA element is contained within 100 bp of the transcriptional start site. A comparative analysis of this region revealed 2 highly conserved ETS consensus elements at

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**Figure 1. Expression of PF4 and transcription factors during megakaryocytic differentiation.** The hematopoietic progenitor (AC133+) cells from human peripheral blood were cultured with TPO and differentiated into megakaryocytes. After culturing the cells for 0, 4, 6, and 10 days, the expression of PF4, the transcription factors, and GAPDH (as an internal control) were analyzed by real-time RT-PCR. Data are represented as the mean ± SE.

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**Figure 2. FLI-1 and GATA-1 synergistically activate the PF4 promoter.** (A) Expression of ETS family proteins and GATA-1 in megakaryocytic HEL cells and non-megakaryocytic HepG2 cells. (B, C) Coexpression assays were performed in HepG2 cells with expression vectors for ETS-1, PU.1, FLI-1, and GATA-1 and with luciferase constructs including the rat (B) or human (C) PF4 promoter. All the coexpression assays were performed 3 times. Data are represented as the mean ± SE. * p<0.05 between Control and each expression vector.

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All the coexpression and siRNA assays were performed 3 times. Data are represented as the mean ± SE. *p < 0.05 between the wild type and the mutant promoter. (E) Control or FLI-1 siRNA was transfected into HEL cells. The expression levels of PF4 and FLI-1 mRNA in the transfected cells were measured by real-time RT-PCR. All the coexpression and siRNA assays were performed 3 times. Data are represented as the mean ± SE. *p < 0.05 between Control and FLI-1 siRNA. doi:10.1371/journal.pone.0024837.g003

ETS transcription factors bind to the −51 ETS site

To determine which ETS factors bind to the −51 ETS site in megakaryocytic cells, electromobility shift assay (EMSA) was performed using a probe spanning the −51 ETS site and nuclear extract from HEL cells. Four shifted bands were observed (Figure 3B). To determine whether 1 or both sites were responsible for mediating the positive effect of FLI-1 on PF4 promoter activity, we co-transfected HepG2 cells with a FLI-1 expression vector and a wild type or a mutant PF4 promoter-reporter plasmid containing a mutation in the −73 or −51 ETS sites (−73 ETSmut and −51 ETSmut, respectively). Mutation of the −51 ETS site resulted in a significant reduction in FLI-1-mediated promoter activation, while mutation of the −73 ETS had little effect (Figure 3B). Consistent with this result, the mutation of the −51 ETS site also resulted in a significant reduction in synergistic promoter activation mediated by FLI-1 and GATA-1, while the mutation of the −73 ETS had no effect (Figure 3C). A similar result was obtained with an assay using Del-100, with or without a mutation in the −73 or −51 ETS site (Figure S2). These results indicated that the −51 ETS site is a FLI-1 responsive element and important for synergistic promoter activation by FLI-1 and GATA-1. A mutation of the −51 ETS site resulted in a significant decrease of the promoter activity in the PF4-expressing megakaryocytic HEL cell (Figure 3D). To determine whether FLI-1 plays a role in mediating the expression of the endogenous PF4 gene, siRNA against FLI-1 was transfected into HEL cells. FLI-1 siRNA resulted in 67% and 56% reduction of the endogenous FLI-1 and PF4 mRNA expression, respectively (Figure 3E). Together, these data suggest that FLI-1 regulates PF4 gene expression through the −51 ETS site.

Figure 3. FLI-1 activates the PF4 promoter through the −51 ETS site. (A) Schematic representation of the rat PF4 promoter-reporter constructs including the 1.1-kb promoter (wild type) and the deleted promoters (Del-500, Del-300, and Del-100). ETS consensus motifs, including the GGAAGa core sequence are indicated by closed boxes. The coexpression assay was performed by transfecting these constructs and a FLI-1 expression vector into HepG2 cells. (B) Two conserved ETS motifs and a functional GATA motif (reported in [12]) in the 100-bp 5′-upstream promoter. The asterisk indicates the base conserved between 3 species. (C) The coexpression assay was performed in the HepG2 cells using expression vectors for FLI-1 and GATA-1, and PF4-luc with or without a mutation at the −73 or −51 ETS site. (D) The transient transfection assay performed using PF4-luc with or without a mutation at the −51 ETS site and the megakaryocytic HEL cells. *p < 0.05 between the wild type and the mutant promoter. (E) Control or FLI-1 siRNA was transfected into HEL cells. The expression levels of PF4 and FLI-1 mRNA in the transfected cells were measured by real-time RT-PCR. All the coexpression and siRNA assays were performed 3 times. Data are represented as the mean ± SE. *p < 0.05 between Control and FLI-1 siRNA. doi:10.1371/journal.pone.0024837.g003
and this multiple protein binding of 3 ETS family proteins was not observed with the −73 ETS site (Figure 4F; Figure S3). Consistent with the EMSA, a chromatin immunoprecipitation (ChIP) assay using HEL cells also demonstrated the bindings of FLI-1, ELF-1, and GABP to the −51 ETS site (Figure 4G).

Together, these findings suggest that FLI-1, ELF-1, and GABP bind to the −51 ETS site in the PF4 promoter.

ELF-1 and GABP regulate the PF4 gene expression through the −51 ETS site
To determine the role of ELF-1 and GABP in mediating PF4 promoter activity, coexpression assays were performed. Overexpression of ELF-1 or GABP (GABPα, β, and γ) resulted in strong activation of the wild-type promoter, but not the −51 ETS mutant promoter in HepG2 cells (Figure 5A). The effect of GABP on promoter activation was recapitulated by combined expression of either GABPα/β or GABPα/γ (Figure 5B). To determine whether ELF-1 and/or GABP functionally interact with GATA-1 to mediate promoter activity, co-transfection assays in HepG2 cells were performed. Unlike FLI-1, ELF-1 and GABP did not synergistically activate the PF4 promoter with GATA-1 (Figure 5C). These results demonstrated that ELF-1 and GABP activate the PF4 promoter through the −51 ETS site while these factors cannot synergistically activate the promoter with GATA-1.

To investigate whether ELF-1 and/or GABP regulate the endogenous PF4 gene expression in megakaryocytic cells, siRNA against ELF-1 or GABP were transfected into HEL cells. ELF-1 siRNA resulted in 62% and 40% reduction in ELF-1 and PF4 mRNA expression (Figure 5D). On the other hand, GABP siRNA resulted in 32% reduction of GABP and 66% increase of PF4 mRNA expression (Figure 5E). These results indicated that both ELF-1 and GABP regulate the PF4 gene expression.

Figure 4. FLI-1, ELF-1, and GABP bind to the −51 ETS site. (A) EMSA performed with the −51 ETS probe. Nuclear extracts from HEL cells were added to lanes 2–8. Competition assays were performed with wild type (lanes 3 and 4) or −51 ETSmut (lanes 5 and 6) competitors. The supershift assays were performed with the antibody against FLI-1 and control antibody (lanes 7 and 8). Four shifted bands were labeled I, II, IIIa and IIIb. Asterisk indicates the specific shifted band IIIa. (B) EMSA was performed with the in vitro translated FLI-1 and control protein (Vector) (lanes 3 and 6). The supershift assay was performed with the antibody against FLI-1 and control antibody (lanes 5 and 6). (C) The supershift assay was performed with antibodies against ELF-1 and GABP or control antibody (lanes 3–7). GABPβ/γ antibody recognizes both GABPβ and its splicing isoform GABPγ. (D) EMSA was performed with the in vitro translated ELF-1 and control protein (lanes 3–6). The supershift assay was performed with the antibody against ELF-1 and control antibody (lanes 5 and 6). (E) EMSA was performed with the in vitro translated GABP (GABPα, β, and γ) and control protein (lanes 3–13). The supershift assay was performed with the antibody against GABP and control antibody. Shifted bands derived from GABPα, GABPα/β and GABPβ/γ complexes are labeled α, α/β, and α/γ, respectively. (F) EMSA was performed with the −51 ETSmut probe and in vitro translated FLI-1, ELF-1, GABP, and control protein. (G) ChIP assay was performed with HEL cells by using antibodies against ETS factors or control antibody. Immunoprecipitated DNA was measured by real-time PCR with primers to amplify the human PF4 promoter region, including the −51 ETS site, or the GAPDH locus as a negative control region. A representative of 3 independent experiments is shown.

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Although the result of co-expression assay showed that both ELF-1 and GABP are positive regulators for the PF4 gene expression, siRNA knockdown of GABP led to increased expression of PF4 in HEL cells. We speculated that this controversial effect was observed because the decrease of GABP promoted FLI-1 binding to the 51 ETS sites. In fact, EMSA using recombinant proteins showed competitive binding of FLI-1, ELF-1, or GABP at the 51 ETS site (Figure S4). Thus, these data suggest that FLI-1, ELF-1, and GABP competitively bind to the 51 ETS sites and regulate PF4 gene expression.

Expression patterns of FLI-1, ELF-1, and GABP in human megakaryocytic differentiation

To investigate and compare the temporal expression patterns of the ETS factors FLI-1, ELF-1, and GABP during megakaryocytic differentiation, mRNA levels of ELF-1 and GABP were measured by real-time PCR (using the same cDNA prepared in Figure 1). As shown in Figure 1, both ELF-1 and GABP were expressed during megakaryocytic differentiation. However, unlike the expression pattern of FLI-1, the expression of ELF-1 and GABP had decreased by day 4. This finding suggests that while all 3 ETS factors may play a role in mediating PF4 expression, they likely function in temporally distinct ways.

The 51 ETS site is important for PF4 promoter activation in differentiating megakaryocytes

To confirm a role for the 51 ETS site in mediating the PF4 gene expression in megakaryocytes, the wild type or the 51 ETSmut PF4 promoter was coupled to GFP. A single copy of the resulting transgenic cassette was targeted to the Hprt locus in ES cells by homologous recombination (Figure S5A; Figure 6A). Two correctly targeted recombinant clones for each construct were differentiated into megakaryocytic lineage by culturing with OP9 stroma cells and TPO. At day 10, a number of differentiated cells were positive for acetylcholine esterase activity (Figure 6B upper panel), and 30% of the cells were positive for CD41 (data not shown). In addition, proplatelets were also observed (Figure 6B lower panel), suggesting differentiation into the megakaryocytic lineage. Megakaryocytes and proplatelets from cells carrying the wild type PF4 promoter had strong GFP expression, while those from 51 ETSmut showed significantly decreased GFP expression (Figure 6C; Figure S5B). FACS analysis demonstrated that the mutation of the 51 ETS site significantly decreased the number of GFP positive megakaryocytic cells (Figure 6D). These observations suggest that the 51 ETS site is important for the activation of the PF4 promoter during megakaryocytic differentiation.

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Discussion

Multiple ETS binding sites are often found in the megakaryocyte-specific gene promoters. However, it has not been clearly shown how multiple ETS binding sites regulate megakaryocyte-specific genes. The rat PF4 promoter contains more than 20 ETS core sequences in it. In this study, we identified a novel, functional ETS binding site (−51 ETS site) in the PF4 promoter. To evaluate the physiological function of the −51 ETS site for the PF4 promoter activity in megakaryocytes, we developed and used a novel reporter gene assay using homologously recombined mouse ES cells (Figure 6; Figure S5). It has been difficult to analyze the promoter activity in intact megakaryocytes because of the small number of megakaryocytes in vivo and the technical difficulty of...
transient transfection of plasmids into megakaryocytes and proplatelets. Our system enables the promoter activity in normal differentiating megakaryocytes and proplatelets to be evaluated. Using this system we evaluated the function of the −51 ETS site using 2 independent ES cell lines containing a wild type or mutant PF4 promoter coupled to the GFP gene. The insertion of a singly copy transgene into the Hprt locus allowed to compare the expression levels of the reporter gene between ES cell lines in the same genomic DNA context [24]. By differentiating these ES cell lines into megakaryocytic lineage, we succeeded in demonstrating that the −51 ETS site is important for the activation of PF4 gene expression in megakaryocytes and proplatelets. This reporter assay system should be a powerful tool for analyzing physiological promoter function in normal megakaryocytes and proplatelets.

Our data demonstrate that multiple ETS family proteins regulate the PF4 gene expression. EMSA and coexpression assays indicate that FLI-1, ELF-1, and GABP bind to the −51 ETS site and activate the PF4 promoter. In addition, the ChIP assay showed that FLI-1, ELF-1, and GABP bind to the −51 ETS site in vivo. ELF-1 was originally described as a regulator of T-cell genes (e.g., TLX1) [25]. To our knowledge, this is the first study to demonstrate a role for ELF-1 in mediating the megakaryocyte-specific gene expression. GABP consists of 2 subunits, GABPα and GABPβ (or its splicing isoform, GABPγ). GABPα contains the ETS DNA-binding domain, while GABPβ is required for nuclear translocation and transactivation. Mammalian GABP is ubiquitously expressed in all tissues and has been implicated in several critical cellular processes such as cellular differentiation, cell cycle, cell survival and mitochondrial respiration [23]. In addition to regulating the expression of housekeeping genes, GABP has been shown to regulate the expression of cell-type specific genes in several distinct lineages, including myeloid cells, lymphocytes, mast cells and endothelial cells [16,23]. A few reports indicate the importance of GABP in the regulation of megakaryocyte-specific genes (e.g., GPIb and cAlp) [26,27]. Comparing with FLI-1, ELF-1, and GABP, ETS-1 has a small effect on the PF4 gene expression in the co-expression assay using HepG2 cells, although we have previously demonstrated that ETS-1 activates the PF4 promoter [12]. This difference may be caused by the difference in the cell types used in the co-expression assay. ETS-1 is known to inhibit its DNA binding via the autoinhibitory domain, and its DNA binding is enhanced by interacting with other transcription factors, such as CBFβ [28]. We speculated that some interacting protein that is expressed in HEL cells but not HepG2 cells is needed for the strong promoter activation by ETS-1.

It has been reported that multiple transcription factors can bind to the same site and regulate gene expression. Recent genome-wide ChIP-seq analyses have also shown an overlap in the chromatin regions commonly bound by different members of the ETS family [29,30,31]. In some cases, a transcriptional activator competes with a suppressor for binding to the same site. This mechanism enables positive or negative regulation of promoter activity through the same site. However, in our reporter gene assays, individual overexpression of FLI-1, ELF-1, and GABP resulted in similar levels of PF4 promoter activity. This suggests that all these factors are transcriptional activators of the PF4 promoter. The question is: why is the PF4 promoter regulated by multiple transcriptional activators through the same site? In the coexpression assay, FLI-1 but not ELF-1 and GABP synergistically activated the PF4 promoter with GATA-1 (Figure 3C). One potential mechanism to explain this synergistic activation may be a direct interaction between FLI-1 and GATA-1 on the GATA-1 binding site, because FLI-1 expression enhanced promoter activation by GATA-1 without binding to the −51 ETS site (Figure 3C; Figure S2). Alternatively, GATA-1 may enhance the FLI-1 binding to the other ETS site, such as the −71 ETS site. In either case, this synergistic activation by FLI-1 and GATA-1 suggests distinct roles for the ETS family proteins in mediating gene expression by interacting with other transcription factors. The siRNA knockdown of GABPs slightly increased the PF4 gene expression although the overexpression of GABP itself activated the promoter activity in the co-expression assay [Figure S4]. This controversial observation also suggests the possibility that FLI-1, ELF-1, and GABP competitively bind to the −51 ETS sites, and that the relative expression levels of these 3 factors are important for the regulation of the PF4 gene expression. Our real-time PCR analysis indicated that the expression of FLI-1 increased during megakaryocytic differentiation, whereas the expression of ELF-1 and GABP decreased at day 4 and did not drastically change at days 6 and day 10 (Figure 1). This suggests the possibility that FLI-1 plays a role in the late stage of megakaryocytic differentiation, whereas ELF-1 and GABP are necessary during the early stage. Consistent with this hypothesis, the importance of the FLI-1 at the late stage of megakaryocytic differentiation and gene expression was demonstrated by inducible FLI-1 knock out and FLI-1 mutant mice [32,33]. Furthermore, Pang et al. reported that GABP regulates megakaryocyte-specific genes that are expressed at the early differentiation stage, while FLI-1 regulates genes expressed at the late stage [27]. In the regulation of the late megakaryocytic marker PF4 either GABP or ELF-1 may bind to the −51 ETS site and moderately activate the promoter at the early stage, whereas FLI-1 may displace them and strongly activate the promoter with GATA-1 at the late stage. Our EMSA data indicate the possibility that FLI-1 displaces ELF-1 and GABP from the −51 ETS site (Figure S4). Together, these results implicate the differentiation stage-specific regulation of PF4 gene expression by multiple ETS factors.

Inconsistent with our result, Pang’s group demonstrated that GABP synergistically activates the GPIb promoter with GATA-1 under the existence of FOG-1. This suggests the possibilities that FOG-1 is essential for synergistic activation of the PF4 promoter by GABP and GATA-1 and that synergistic activation by GABP and GATA-1 does not occur on the PF4 promoter. More detailed analyses about ETS family proteins and their functionally associated proteins will be important to understand megakaryocyte-specific and differentiation stage-specific PF4 gene expression.

**Supporting Information**

**Figure S1 Expression of transcription factors in transient transfection assay.** Expression levels of the transcription factors and GAPDH (as a control) in the transfected HepG2 cells were analyzed by western blotting. The asterisks indicate bands derived from FLI-1 between non-specific bands. (TIF)

**Figure S2 Activation of the PF4 promoter by FLI-1 through the −51 ETS site.** Coexpression assay was performed in HepG2 cells by using FLI-1 and GATA-1 expression vectors and PF4-luc with or without a mutation in the −73 or −51 ETS site. (TIF)

**Figure S3 Binding of FLI-1, ELF-1, or GABP to the −73 ETS site.** EMSA was performed with the −73 ETS or −51 ETS probe, and FLI-1, ELF-1, and GABP prepared by in vitro translation. The arrows indicate the shifted bands derived from FLI-1 (F), ELF-1 (E) and GABP (G). The DNA sequences of oligonucleotides for the −73 ETS probe are shown in Table S1. (TIF)
Figure S4 Competitive binding of FLI-1, and ELF-1 or GABP to the −51 ETS site. A) EMSA was performed with the −51 ETS probe, and in the protein mixture containing ELF-1, and a variety of amounts of FLI-1. The arrows indicate the shifted bands derived from ELF-1 (E) and FLI-1 (F). B) EMSA was performed with the −51 ETS probe, and the protein mixture containing GABPz and various amounts of FLI-1. The arrows indicate the shifted bands derived from GABPz (G) and FLI-1 (F).

Figure S5 Promoter activity of the wild type or mutant PF4 promoter during megakaryocytic differentiation. (A) The transgene was inserted into the Hprt locus by homologous recombination. (B) Two ES cell lines with transgenes containing the PF4 promoter with or without a mutation in the −51 ETS site were differentiated into megakaryocytic lineage. The GFP expression level was compared between the 2 cell lines from days 6 to 12.

Table S1 Sequences of the oligonucleotides.

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Author Contributions

Conceived and designed the experiments: YO HNobori MS MW MY TNakai YK AWakimura NF HNaruse AWatanabe DD. Performed the experiments: YO HNobori MS MW MY TNakai YK AWakimura NF HNaruse AWatanabe DY SF KY. Analyzed the data: YO HNobori MS MW MY TNakai YK AWakimura NF HNaruse AWatanabe DD. Contributed reagents/materials/analysis tools: KK TNakano WCA. Wrote the paper: YO HNobori MS MW MY TNakai WCA. GD

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