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Original Article

Antagonistic effects of PU.1 on Gfi-1B-induced erythroid colony formation in human cord blood cells

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Abstract

Gfi-1B is a hematopoietic transcription factor essential for growth and differentiation of the erythroid/megakaryocytic lineages, and PU.1 is a master regulator for myeloid development. Herein, we demonstrate that PU.1 interacted with Gfi-1B *in vivo* by immunoprecipitation assay. GST pull-down assays showed that the binding sites were located in the Ets domain of PU.1 and the zinc finger domain of Gfi-1B. Luciferase reporter assays revealed that PU.1 and Gfi-1B antagonized each other's transcriptional activity in a dose-dependent manner. The transduction of Gfi-1B alone in human cord blood progenitor cells strongly enhanced erythroid colony formation. However, the transduction of PU.1 along with Gfi-1B in the progenitors significantly inhibited erythroid colony formation. Co-expression of Gfi-1B with a mutant PU.1, which bound to Gfi-1B but not to GATA1, another erythroid master regulator, also inhibited Gfi-1B-induced erythroid colony formation. Our results suggest that the function of Gfi-1B in the growth and differentiation of erythroid cells is antagonized by the expression of PU.1.

Keywords: Transcription Factor, Protein-protein interaction, Antagonistic effects, Hematopoietic differentiation, Human cord blood

1. Introduction

Transcription factors promote hematopoietic differentiation into a certain lineage but sometimes inhibit differentiation into others through protein-protein interactions and the regulation of target genes [1-3]. For example, the functional antagonism between a myeloid/B cell-restricted transcription factor, namely PU.1, and an erythroid/megakaryocyte-restricted transcription factor, namely GATA-1, has been reported [4-8]. Overexpression of PU.1 in hematopoietic progenitors or erythroblastic cells inhibits erythroid differentiation but promotes myeloid differentiation [9-11]. Conversely, overexpression of GATA-1 in myeloid progenitor cells inhibits myeloid differentiation but promotes erythroid/megakaryocytic differentiation [12-14].

The *PU.1/Spi-1* gene is a target for proviral integration in Friend virus-induced mouse erythroleukemia (MEL) [15] and is a member of the Ets family of transcription factor genes [16, 17]. We have reported that overexpression of PU.1 inhibits erythroid differentiation of MEL cells [9, 11]. Moreover, PU.1 inhibits the function of GATA-1, a master regulator for erythroid differentiation [7, 18]. Thus, PU.1 contributes to the development of erythroleukemia by blocking their differentiation.

Gfi-1B is a hematopoietic cell-restricted zinc finger transcription factor that shares homology in the SNAG and zinc finger domains with Gfi-1 [19, 20]. Gfi-1B contributes to the growth and differentiation of erythroid cells. Overexpression of Gfi-1B in $CD34^+$ cells induced a dras-

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tic expansion of erythroblasts in an erythropoietin-independent manner [21], and Gfi-1B$^{-/-}$ embryonic stem cells failed to contribute erythroid and megakaryocytic lineages [22]. Furthermore, forced expression of Gfi-1B in M1 myeloid cells blocked IL-6-induced G1 arrest and differentiation of the cells [23].

Both PU.1 and Gfi-1B are expressed in hematopoietic stem cells and progenitors. PU.1 is up-regulated during differentiation into the myeloid lineage but is down-regulated during differentiation into the erythroid lineage, and Gfi-1B exhibits the opposite behavior [10, 17, 20]. Moreover, it has also been shown that the interaction of PU.1 with proteins, such as CREB-binding protein and GATA-1, regulates the function of PU.1 itself or proteins that bind to PU.1 [14, 18, 24-27]. On the other hand, Ets-1 protein, which like PU.1 belongs to the Ets family of transcription factors, binds to the Bax promoter through protein interaction with Gfi-1 and represses expression of the Bax gene [28].

Based on these previous findings and the observation that the expression patterns of PU.1 and Gfi-1B genes are opposite in pluripotent hematopoietic progenitor cells, in the present study, we examined whether PU.1 and Gfi-1B interact to antagonize each function.

2. Materials and Methods

2.1. Cells

293T and HeLa cells were maintained in Dulbecco's modified Eagle minimum essential medium (Nissui, Tokyo, Japan) supplemented with L-glutamine containing 10% fetal bovine serum (FBS) (BioWest, FL, USA). The 503 cell line, which was kindly provided by Professor Tenen GD (Harvard Institutes of Medicine, Harvard Medical School), is a cytokine-dependent cell line established from bone marrow cells in a PU.1 knockout mouse [29] and was maintained in RPMI-1640 medium supplemented with 10% FBS and 50 ng/mL each of IL-3 and SCF (Sigma, Mo, USA). The cells were cultured under 5% CO_2 at 37°C. Fresh human cord blood (hCB) cells were obtained from the Tokyo Cord Blood Bank (Tokyo, Japan). Mononuclear cells were separated by density gradient centrifugation. $CD34^+$ cells were immunomagnetically enriched using a magnetic-activated cell sorting CD34 progenitor kit (Miltenyi Biotech, Auburn, CA, USA). The purity of hCB $CD34^+$ cells was > 90%. $CD34^+CD38^-$ cells were isolated by fluorescence-activated cell sorting using a JSAN desktop cell sorter (Bay Bioscience, Kobe, Japan). This study was approved by the institutional ethics committee of Chiba University (approval ID: 196).

2.2. Construction of the expression vectors

An expression vector of PU.1 was constructed by inserting mouse full-length cDNA into the pEF or pCMV-FLAG expression vector. Gfi-1B, AML1/RUNX1, and GATA-1 expression vectors were constructed by inserting the full-length cDNAs into the pcDNA3-Flag vector (Stratagene, CA, USA). For the GST pull-down assay, we recombined full-length PU.1 or PU.1 deletion mutants that were previously reported with pGEX4T expression vectors (Amersham Bioscience) [24-27]. Similarly, for Gfi1-B, full-length cDNA and a previously reported Gfi1-B deletion mutant were introduced into pGEX4T expression vectors [21].

2.3. Transfection

293T cells or HeLa cells were transfected with each expression vector using lipofectamine-Plus (Invitrogen, CA, USA) [24, 25].

2.4. Cell lysate

Transfected cells were collected in a tube and resuspended in 300 µL of glycerol buffer (0.5% NP-40 [vol/vol], 150 mM NaCl, 10 mM Tris-HCl [pH 7.4], 25% glycerol [vol/vol], 0.2 mM PMSF, 2 mM sodium vanadate, 0.1 µg/mL aprotinin, 0.2 mM benzamine, 0.1 µg/mL pepstatin, and 1 mM DTT). The cell suspension was subjected to sonication with Bioruptor (Orion Ltd., Tokyo, Japan) in ice water to isolate the cell lysate. The cell lysates were used for the immunoprecipitation assay, GST pull-down assay, and western blot analysis.

2.5. Immunoprecipitation

Immunoprecipitation and immunoblotting were performed as previously described [24, 25] with minor modifications. Briefly, 500 µg of each cell lysate was immunoprecipitated with 1 mg of anti-PU.1 antibody (T21, Santa Cruz, CA, USA). Each sample of the immunoprecipitate was washed four times with glycerol buffer (0.5% NP-40 [vol/vol], 150 mM NaCl, 10 mM Tris-HCl [pH 7.4], and 25% glycerol [vol/vol]), separated using a 4%–12% Nu-PAGE MOPS system (Invitrogen, CA, USA), and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, MA, USA) using a semidry blotting system.

2.6. GST pull-down assay

Bacterial lysates were prepared after transfection using the pGEX4T expression vectors described above. First, 100 µL of GST-fusion protein was incubated with 20 µL of glutathione sepharose beads for 2 h and then mixed with cell lysates for 1 h at 4°C. The beads were centrifuged and washed with PBS four times. The bound proteins were eluted with 20 µL of 2x SDS sample buffer by boiling for 10 min. The eluted proteins were then subjected to western blot analysis, as previously described [24-27].

2.7. Western blot analysis

First, 50 µg of total proteins were separated in a 4% to 12% gradient polyacrylamide gel (Nu-PAGE, Novex) by SDS-PAGE and blotted on a PVDF membrane (Immobilon-P, Millipore, MA, USA). The membrane was blocked with PBS containing 0.1% Tween-20 and 10% skim milk for 60 min and then reacted with mouse anti-Flag antibody (M2, Stratagene, CA, USA) and rabbit anti-PU.1 antibody (T21, Santa Cruz, CA, USA) for 60 min. Furthermore, 1 µg of peroxidase-labeled anti-mouse Ig antibody or peroxidase-labeled anti-rabbit Ig antibody was used as the secondary antibody. Bound antibody was detected using an electrogenerated chemiluminescence system (Amersham).

2.8. Luciferase assay

Cell extracts for luciferase assays were prepared as previously described [24-27]. The reporter plasmids, pTK100-PU x3-Luc, pTK100-PUmut x3-Luc, pTK81-Luc, pTK81-Gfi x4-Luc, and M-CSFR promoter-Luc were constructed. Reporter and effector vectors were co-transfected into 293T cells or HeLa cells cultured in 24-well plates. The concentrations of PU.1 and Gfi-1B expression vectors in

the luciferase reporter assay are those used in previous studies [21, 24-27]. Cell lysates were harvested 48 h after transfection, and luciferase activity was assayed with 10 µL of cell lysates and 50 µL of pikka gene substrate (Nippongene) using an LB9501 luminometer (Berthold).

2.9. Production of retrovirus

The retroviral vector GCDNsam (pGCDNsam), with an LTR derived from MSCV, has intact splice donor and splice acceptor sequences for the generation of subgenomic mRNA. The murine Gfi-1B cDNA was subcloned into a site upstream of an IRES-EGFP construct in pGCsam. The murine wild-type PU.1 and a series of mutant PU.1 cDNAs followed by IRES-nerve growth factor receptor truncated in the cytoplasmic domain (tNGFR) were subcloned into pGCDNsam. To produce the recombinant retrovirus, plasmid DNA was transfected into Phoenix cells along with the MLV env expression plasmid and the gag and pol expression plasmid by $CaPO_4$ coprecipitation, and supernatant from the transfected cells was collected to infect cells.

2.10. Transduction of cells

Murine early myeloid precursor 503 and hCB CD34+ cells were infected in culture dishes containing virus supernatant and 5 µg/mL protamine sulfate (Sigma, St. Louis, MO). Cells were centrifuged at 1,000 × g for 30 min. After transduction, cells positive for EGFP and/or NGFR were selected by cell sorting using a FACS Vantage system (Becton Dickinson, San Jose, CA) and subjected to subsequent analyses. To detect the expression of tNGFR on the cell surface, cells were stained with mouse anti-EGFP antibody (Abcam, Cambridge, UK) and rat anti-human NGFR antibody (Chemicon, Temecula, CA), followed by PE-conjugated goat anti-rat immunoglobulin.

2.11. Flow cytometric analysis

Expression of cell surface antigens was analyzed using a FACS Vantage system. To detect cell surface antigens, cells were stained with APC-conjugated anti-human CD11b, a part of the Mac-1 antibody (PharMingen). Cells that became stained with propidium iodide were gated out as dead cells.

2.12. Proliferation assay

$hCD34^+$ cells transduced with various expression vectors were plated (1×10^4 cells/well) in a 96-well plate with StemSpan medium containing 100 ng/mL rhSCF and library compounds and cultured for 7 days. WST-8 reagent (Kishida Chemical, Osaka, Japan) was used for the measurement of cell proliferation.

2.13. Colony-forming cell assay.

$hCD34^+$ cells, which were cultured with NR-101 or rhTPO for 7 and 10 days, were plated in Methocult GF H4435 methylcellulose medium containing 50 ng/mL human SCF, 10 ng/mL human IL-3, 10 ng/mL human TPO, and 3 U/mL human EPO (StemCell Technologies). After 7 days of culture, the colonies were counted. Colonies derived from high proliferative potential colony-forming cells (colony diameter > 1 mm) were recovered, cytospun onto glass slides, and then subjected to May-Grüenwald Giemsa staining for morphological examination.

2.14. Statistical analysis

Values are represented as means ± standard deviation. The one-way analysis of variance (ANOVA) is used to determine whether there are any statistically significant differences between the means of three or more independent groups. For the luciferase assay, cell proliferation and blood cell colony count, the one-way ANOVA was used because the number of samples was greater than three. If the one-way ANOVA showed a significant difference, the Bonferroni post hoc test for multiple comparisons was conducted. $P < 0.05$ was considered statistically significant.

3. Results

3.1. *In vivo* interaction of PU.1 with Gfi-1B

To verify the physical association between PU.1 and Gfi-1B, we first examined the association by co-immunoprecipitation assay. The mixture of lysates from 293T cells transfected with PU.1 and FLAG-tagged Gfi-1B expression vectors was immunoprecipitated with anti-PU.1 antibody, followed by western blot analysis using anti-FLAG antibody. Cell lysate from 293T cells transfected with AML1/RUNX1 was used as a positive control, considering that AML1 is known to interact with PU.1 [30]. The antibody against PU.1 co-precipitated Gfi-1B and AML1 (Fig. 1), suggesting that PU.1 interacts with Gfi-1B. Moreover, the bands seen in all lanes of the assay are thought to be non-specific (NS) bands.

3.2. Determination of the binding site of PU.1 for Gfi-1B

We then examined the interaction between PU.1 and Gfi-1B in the GST pull-down assay. To determine the binding site of PU.1 for Gfi-1B, various deletion forms of GST-PU.1 were constructed, namely wild-type PU.1 (GST-PU.1 WT), and GST-PU.1ΔA (PU.1Δ74-100 a.a.), GST-PU.1ΔE (PU.1Δ171-243 a.a.), GST-PU.1 E (PU.1 165-256 a.a.), GST-PU.1 EΔβ3/β4 (PU.1 171-243 a.a.), and GST-PU.1 β3/β4 (PU.1 244-256 a.a.), as shown in Fig. 2A. Cell lysate from 293T cells transfected with an expression plasmid of FLAG-Gfi-1B was incubated with the various GST-fused PU.1 deletion mutants and then

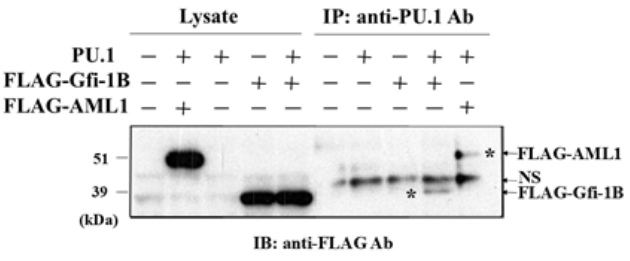

Fig. 1. Physical interaction between PU.1 and Gfi-1B. Co-immunoprecipitation of PU.1 along with Gfi-1B or AML1. The 293T nuclear lysates transfected with PU.1 and FLAG-tagged Gfi-1B or AML1 were incubated with anti-PU.1 antibody, and immunoprecipitates were separated in Nu-PAGE (Invitrogen, CA, USA). FLAG-tagged Gfi-1B and AML1 proteins (*) were detected by immunoblot analysis using anti-FLAG antibody (M2, Stratagene, CA, USA). Essentially, the same results were obtained from three independent experiments. The input was equivalent to 1% of the amount of nuclear lysates incubated. NS, non-specific.

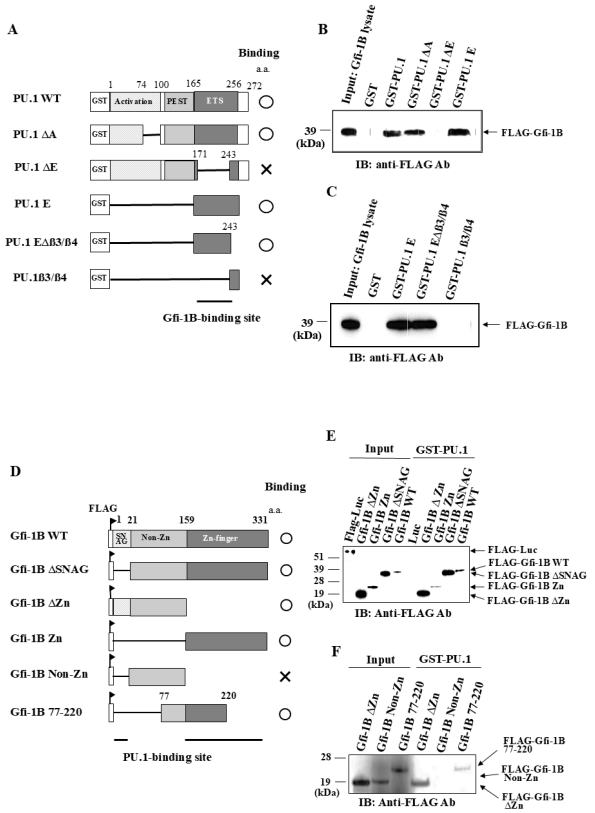

Fig. 2. Determination of the interaction sites between PU.1 and Gfi-1B. Schematic diagrams of the various deletion mutants of PU.1 fused to (A) GST and (D) FLAG-tagged Gfi-1B. Numbers correspond to the amino acid positions in (B-C) PU.1 and (E-F) Gfi-1B proteins. The GST pull-down assay was performed using a series of GST-PU.1 deletion mutants and 293T cell lysates transfected with a FLAG-tagged Gfi-1B WT expression vector. Immunoblot analysis was performed with anti-FLAG-tagged antibody (M2, Stratagene, CA, USA). Essentially, the same results were obtained from three independent experiments.

subjected to western blot analysis using anti-FLAG antibody. The results showed that FLAG-Gfi-1B was bound to GST-PU.1 WT, GST-PU.1ΔA, and GST-PU.1 E but not to GST-PU.1ΔE (Fig. 2B). It has been reported that GATA-1 and c-Jun exclusively interact with the β3/β4 region within the C-terminal Ets domain of PU.1 [14]. However, Gfi-1B barely interacted with GST-PU.1 β3/β4, whereas it interacted with GST-PU.1 EΔβ3/β4 (Fig. 2C). These results suggest that PU.1 directly interacts with Gfi-1B through its amino acids 171–243.

3.3. Determination of the binding site of Gfi-1B for PU.1

Next, we determined the binding site of Gfi-1B for PU.1. A schematic illustration of expression plasmids for the FLAG-tagged wild-type and mutant Gfi-1B is shown in Fig. 2D. Cell lysates from 293T cells transfected with the wild-type or each mutant Gfi-1B were incubated with GST-PU.1 WT and then subjected to western blot analysis using anti-FLAG antibody. As shown in Fig. 2E, Gfi-1B WT, Gfi-1BΔSNAG, and Gfi-1B Zn interacted with GST-PU.1. Gfi-1BΔZn also interacted with GST-PU.1 WT, whereas Gfi-1B 77-220 only weakly interacted. Moreover, Gfi-1B Non-Zn barely interacted with GST-PU.1 WT (Fig. 2F). These results suggest that Gfi-1B interacts with PU.1 mainly through its Zn finger domain but also through its SNAG domain.

3.4. Effect of PU.1 binding with Gfi-1B on PU.1-dependent transcription

The binding of Gfi-1B to PU.1 may affect PU.1-dependent transcriptional activation. To examine this possibility, a reporter assay was carried out in HeLa cells by transfection of the pTK100-PUx3-Luc reporter plasmid carrying 3 tandem repeats of PU.1 binding sites with or without expression vectors of PU.1 and/or Gfi-1B. The luciferase activity of pTK100-PUx3-Luc was enhanced approximately 25-fold by transfection of a PU.1 expression vector compared with an empty vector (Fig. 3A). The activity of pTK100-PUx3-Luc was inhibited by co-transfection of a Gfi-1B expression vector with a PU.1 expression vector in a dose-dependent manner, although transfection of Gfi-1B expression vector with the corresponding empty vector did not inhibit the activity. The activities of pTK100-PUmutx3-Luc and pTK100-Luc used as negative control reporter plasmids were not significantly changed, even by transfection of PU.1 and/or Gfi-1B expression vectors (data not shown). PU.1 regulates the expression of several myeloid-specific genes, including the M-CSF receptor (M-CSFR) gene [14]. Then, we examined the effect of Gfi-1B on the activity of M-CSFR promoter-Luc induced by PU.1. The promoter activity was enhanced approximately 9-fold by co-transfection with a PU.1 expression vector, but the activity was decreased by 3.5-fold when a Gfi-1B expression vector was introduced with a PU.1 expression vector (Fig. 3B). These results suggest that Gfi-1B inhibits PU.1-dependent M-CSFR promoter activity through protein-protein interactions.

3.5. Effect of the binding of Gfi-1B with PU.1 on Gfi-1B-dependent transcription

Then, we examined the effect of PU.1 on Gfi-1B-dependent transcription. The reporter assay was performed in 293T cells using the artificial reporter plasmid of pTK81-Gfix4-Luc carrying 4 tandem repeats of a Gfi-1B binding site with or without expression vectors of Gfi-1B and/or PU.1. The luciferase activity of pTK81-Gfix4-Luc was enhanced approximately 7.5-fold by transfection with a Gfi-1B expression vector compared with an empty vector (Fig. 3C). The activity of pTK81-Gfix4-Luc was inhibited by transfection of a PU.1 expression vector with a Gfi-1B expression vector in a dose-dependent manner, although transfection of a PU.1 expression vector with the corresponding empty vector did not inhibit the activity. The activity of pTK81-Luc used as a control reporter plasmid was not significantly changed even by transfection of Gfi-1B and/or PU.1 expression vectors (data not shown). These results suggest that PU.1 inhibits Gfi-1B-dependent promoter activity through protein-protein interactions.

3.6. Effect of negative cross-talk between PU.1 and Gfi-1B on myeloid differentiation

To analyze the functional interactions between the two transcription factors, we co-transduced both PU.1 and Gfi-1B expression vectors into the 503 cell line, a murine early myeloid progenitor line established from the spleen of a PU.1-knockout mouse.

Forced expression of PU.1 in 503 cells resulted in enhanced expression of Mac-1 (Fig. 4A), consistent with the

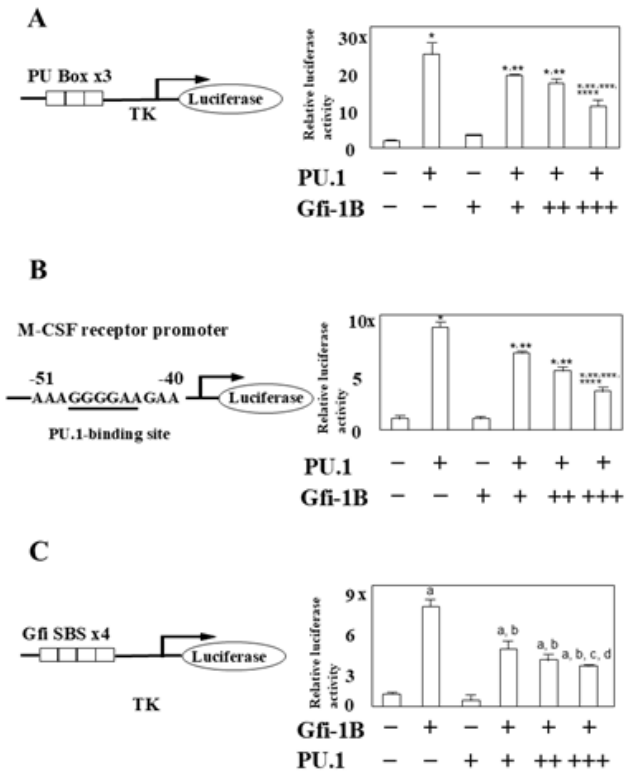

Fig. 3. Effects of PU.1 and Gfi-1B on transcriptional activities. Maps of the (A) pTK100-PUx3-Luc, (B) M-CSF receptor (M-CSFR) promoter-Luc and (C) pTK81-Gfix4-Luc reporter plasmid are shown (left). HeLa cells were transiently transfected with the indicated plasmids (0.2 μg), a PU.1 expression vector (0.4 μg), and increasing amounts (0.1, 0.2, and 0.4 μg) of a Gfi-1B expression vector. The data were expressed as ratios to the values of the empty vectors (set to 1). Values are represented as means ± standard deviation (n = 3). Multiple comparisons significance was determined using ANOVA followed by Bonferroni's post hoc test. *P < 0.05 vs. PU.1 (-) and Gfi-1B (-), **P < 0.05 vs. PU.1 (+) and Gfi-1B (-), ***P < 0.05 vs. PU.1 (+) and Gfi-1B (+), ****P < 0.05 vs. PU.1 (+) and Gfi-1B (++) (Fig. 3A, B). [a]P < 0.05 vs. PU.1 (-) and Gfi-1B (-), [b]P < 0.05 vs. PU.1 (-) and Gfi-1B (+), [c]P < 0.05 vs. PU.1 (+) and Gfi-1B (+), [d]P < 0.05 vs. PU.1 (++) and Gfi-1B (+) (Fig. 3C).

observation that PU.1 is a master regulator for macrophage development. The values of mean fluorescence intensity (MFI) were 906 in PU.1 transduced cells and 25 in cells transduced with the GFP control vector. Forced expression of Gfi-1B with PU.1 in 503 cells inhibited Mac-1 expression 2.3-fold compared with the expression induced by PU.1; the MFI was reduced from 906 to 396 in Gfi-1B co-transduced cells. However, this inhibition was weak and did not reach the levels required to induce Mac-1-negative cells, suggesting that the inhibitory effect of Gfi-1B on *in vivo* myeloid differentiation exists but is not strong.

3.7. Effect of negative cross-talk between PU.1 and Gfi-1B on erythroid differentiation

Forced expression of Gfi-1B in CD34$^+$ cells induced a drastic expansion of erythroblasts, even in the absence of erythropoietin [21], whereas overexpression of PU.1 in CD34$^+$ cells differentiated the cells into dendric cells [31]. We then co-transduced both PU.1 and Gfi-1B into CD34$^+$ human hematopoietic progenitors freshly isolated from cord blood. Compared with the effects of PU.1 and Gfi-1B expression on the differentiation of myeloid cells, their effects on the growth and differentiation of erythroid cells were more significant. As shown in Fig. 4B, double infection with PU.1 and Gfi-1B in CD34$^+$ cells markedly inhibited the Gfi-1B-induced expansion of erythroblasts. The cells exhibited growth inhibition and differentiated into dendric-like cells. Double infected cells exhibited a marked decrease in the numbers of glycophorin A-positive cells (Fig. 4C), suggesting that PU.1 inhibits Gfi-1B function *in vivo*.

3.8. Effect of an N-terminal deletion mutant of PU.1 on erythroid differentiation

To examine whether the binding sites of PU.1 for Gfi-1B and GATA-1 are the same, we constructed GST-PU.1ΔNΔβ3/β4, a deletion mutant lacking amino acids 1–70 at the N-terminus and the β3/β4 region of the Ets domain of PU.1. As shown in Fig. 5A, Gfi-1B was bound to GST-PU.1ΔNΔβ3/β4, GST-PU.1 WT, and GST-PU.1 EΔβ3/β4 in the GST pull-down assay. GATA-1 was barely bound to GST-PU.1ΔNΔβ3/β4 and GST-PU.1EΔβ3/β4, although it was strongly bound to GST-PU.1 WT and GST-PU.1 E (Fig. 5B). These results suggest that the binding site of PU.1 to Gfi-1B is different from that of GATA-1. Thus, the PU.1ΔNΔβ3/β4 mutant may inhibit Gfi-1B function without affecting GATA-1 function. Moreover,

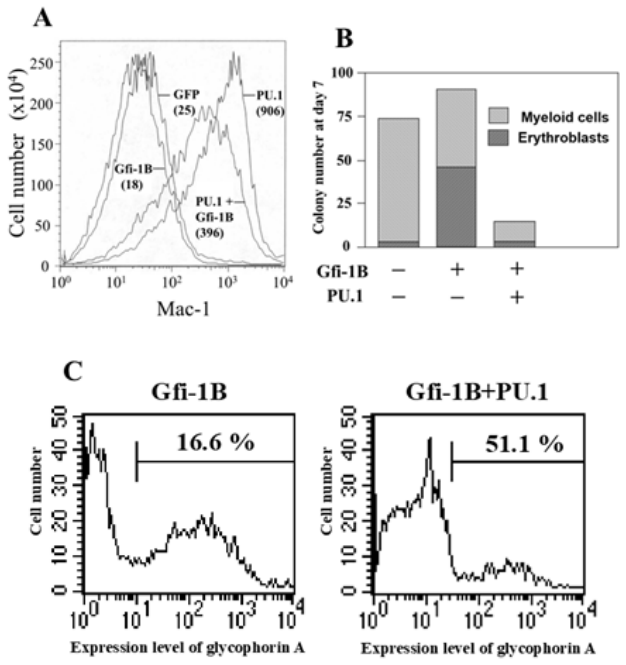

Fig. 4. Effect of cross-talk between PU.1 and Gfi-1B on hematopoietic differentiation. (A) Both PU.1 and Gfi-1B expression vectors were transduced into the myeloid progenitor 503 cell line from a PU.1-knockout mouse. A representative histogram of Mac-1 expression in the presence or absence of PU.1 and Gfi-1B is shown. Mean fluorescence intensity (MFI) is indicated in parentheses. (B) CD34$^+$ cells were sorted and cultured in micro-titer plates under myeloid culture conditions and incubated in the presence of SCF and GM-CSF. Cells were transduced with PU.1 and/or Gfi-1B expression vectors, as indicated. The numbers of myeloid and erythroid colonies were counted on day 7. (C) Flow cytometric analysis of CD34$^+$ cells, showing the expression of glycophorin A in CD34$^+$ cells in the presence of Gfi-1B with or without PU.1. Curves represent percentages of the cells stained with anti-glycophorin A antibody.

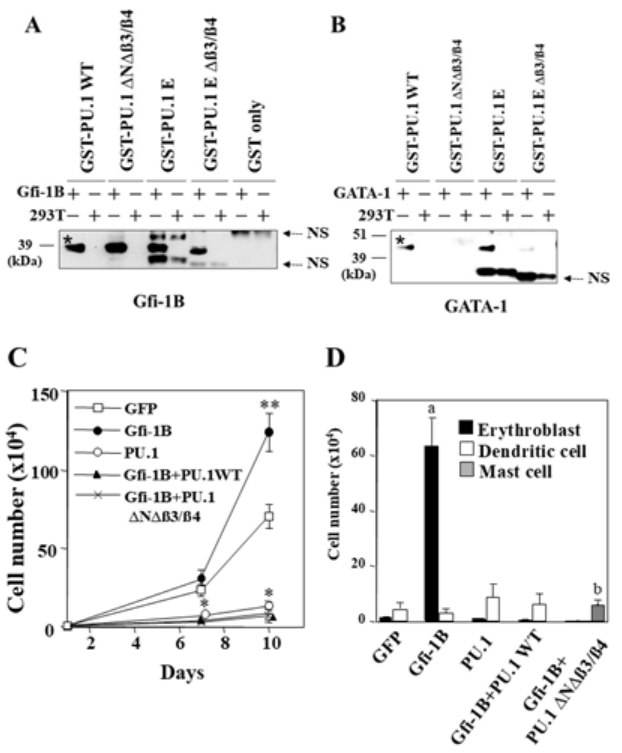

Fig. 5. Inhibition of Gfi-1B-induced erythropoiesis by co-expression of a PU.1 deletion mutant which did not bind to GATA-1. GST pull-down assays were performed using GST-PU.1 deletion mutants and cell lysates from 293T cells transfected with a (A) FLAG-tagged Gfi-1B WT or (B) FLAG-tagged GATA-1 WT expression vector. "293T" indicates control lysates from 293T cells without transfection. Immunoblot analyses were performed with anti-FLAG antibody (M2, Stratagene, CA, USA). Essentially, the same results were obtained from three independent experiments. NS, non-specific. (C-D) $CD34^+$ cells were transduced with Gfi-1B and/or PU.1 WT or PU.1ΔNΔβ3/β4 expression vectors, as indicated. The numbers of myeloid and erythroid colonies were counted on day 10. Values are represented as means ± standard deviation (n = 3). Multiple comparisons significance was determined using ANOVA followed by Bonferroni's post hoc test. *P < 0.05 vs. GFP-transfected cells (PU.1-, Gfi1-B + PU.1 WT- or Gfi1-B + PU.1 ΔNΔβ3/β4-transfected cells), **P < 0.05 vs. GFP-transfected cells (Gfi1-B-transfected cells) (Fig. 5C). ^{a}P < 0.05 vs. GFP-transfected erythroblast, ^{b}P < 0.05 vs. GFP-transfected mast cell (Fig. 5D).

the extra bands seen in Fig. 5A and 5B appear to be NS bands.

To clarify whether the binding of PU.1 with Gfi-1B is important for inhibiting erythroid differentiation *in vivo*, the effect of the mutant of PU.1ΔNΔβ3/β4 on cell growth and erythroid differentiation was then examined in transduction experiments. Growth of PU.1-transduced cells was markedly inhibited, whereas that of Gfi-1B-transduced cells was not. Co-transduction of Gfi-1B with PU.1 WT or PU.1ΔNΔβ3/β4 in 503 cells inhibited the cell growth (Fig. 5C). The colony assay revealed that transduction of a PU.1ΔNΔβ3/β4 expression vector with Gfi-1B WT in $CD34^+$ cells strongly inhibited erythroid expansion, similar to PU.1 WT (Fig. 5D), suggesting that inhibition of the growth and differentiation of erythroblasts by this mutant PU.1 is due to inhibition of Gfi-1B function but not due to inhibition of GATA-1 function. Hence, PU.1 probably inhibits the growth and differentiation of erythroid cells by inhibiting both GATA-1/GATA-2 and Gfi-1B.

Our results showed that PU.1 and Gfi-1B functionally interact with each other. Specifically, PU.1 strongly inhibits Gfi-1B-induced erythroid colony expansion and Gfi-1B weakly inhibits PU.1-induced myeloid differentiation.

4. Discussion

We showed that Gfi-1B was co-immunoprecipitated with PU.1. The GST pull-down assay revealed that PU.1 probably interacted with either the SNAG domain (amino acids 1–21) or the Zn finger domain (amino acids 159–331) of Gfi-1B through the Ets domain. It has been reported that Gfi-1B is required for the development of the erythroid and megakaryocytic lineages in mice [21, 22], whereas PU.1 is required for the development of the myeloid lineages [32, 33] and inhibits erythroid differentiation [9]. A reverse expression pattern of PU.1 and Gfi-1B in multipotent hematopoietic progenitors led us to speculate that PU.1 and Gfi-1B may function antagonistically and that the balance between these two transcription factors may be responsible for differentiation toward either myeloid or erythroid cells. This possibility was supported by the experimental results showing that Gfi-1B inhibited PU.1-mediated transactivation and that PU.1 inhibited Gfi-1B-dependent transcription in a dose-dependent manner. Several different mechanisms may be considered regarding how PU.1 and Gfi-1B inhibit each other's transcriptional activity. Our previous findings demonstrated that PU.1 can interact with HDAC1, MeCP2, and Dmnt3s [24-26], and Gfi-1B has a SNAG repression domain at the N-terminal region for binding co-repressors [19]. Mutual transrepression may be due to the recruitment of co-repressors on the promoters with PU.1- or Gfi-1B-binding sites (Fig. 6A). Alternatively, PU.1 binds to the DNA-binding domain of the Gfi-1B. As a result, physical binding of the Gfi-1B to DNA binding sites is thought to be suppressed, therefore, the transcriptional activity of the Gfi-1B is attenuated (Fig. 6B). In fact, a model has been proposed in which the PU.1 interacts with the DNA-binding domain of GATA-1 in the PU.1/GATA-1 complex, inhibiting GATA-1's DNA binding activity and thereby reducing GATA-1's transcriptional

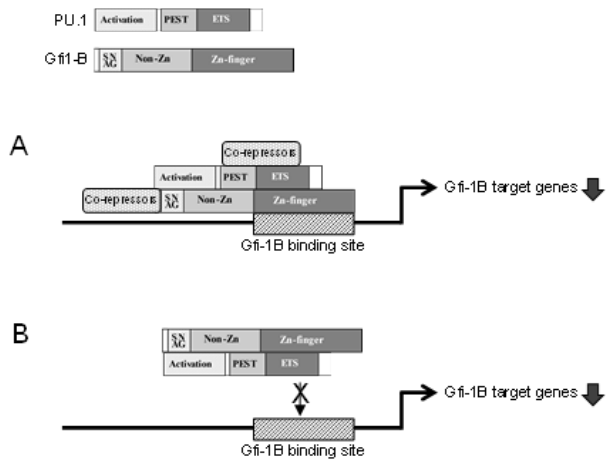

Fig. 6. Possible mechanisms of transcriptional repression of Gfi-1B by PU.1. (A) When Gfi-1B binds to PU.1, co-repressors are recruited to the Gfi-1B binding site, thus repressing the transcriptional activity of Gfi-1B. (B) When Gfi-1B binds to PU.1, the binding of Gfi-1B to its DNA-binding site is blocked and the transcriptional activity of Gfi-1B is repressed.

function [18]. A similar mechanism is also thought to be involved with PU.1 because Gfi-1B directly interacts with DNA binding domain of PU.1. However, there is a report that the DNA binding ability of PU.1 is not reduced, even though PU.1 and AML1-ETO, which is fusion protein generated in t(8;21) myeloid leukemia patients, bind to each other via their DNA binding domains [34], so further investigation is needed.

To elucidate the biological significance of the interaction between PU.1 and Gfi-1B, the effects of the interaction on myeloid and erythroid differentiation were examined in the 503 cell line. FACS analysis revealed that transduction of a PU.1 expression vector into 503 cells induced myeloid differentiation into macrophages that were monitored by Mac-1 expression. Transduction of a Gfi-1B expression vector with a PU.1 expression vector into 503 cells, however, did not strongly affect the PU.1-induced myeloid differentiation, although Mac-1 expression was reduced in the cells, consistent with the reduced M-CSFR promoter-Luc activity observed in Luc assays *in vitro*. The fact that myeloid differentiation was not markedly blocked by Gfi-1B expression despite the reduction of Mac-1 and M-CSFR expression indicates that the promoting effect of PU.1 on myeloid differentiation may overcome the inhibitory effect of Gfi-1B on myeloid differentiation.

In contrast to the effect of PU.1 and Gfi-1B interaction on myeloid differentiation, transduction of a PU.1 expression vector in hCB $CD34^+$ cells markedly inhibited Gfi-1B-mediated cell growth and colony formation of erythroblasts. In this case, the inhibitory effect of PU.1 on erythroid differentiation may overcome the promoting effect of Gfi-1B on the growth and differentiation of erythroid cells. It has been reported that PU.1 inhibits erythroid differentiation through direct interaction with GATA-1, an essential transcription factor for erythroid differentiation [14]. Moreover, the N-terminal region of PU.1 is necessary for inhibiting transcription of the erythroid-specific genes [4]. Thus, inhibition of erythroid differentiation by PU.1 is likely due to binding of the N-terminal region of PU.1 to GATA-1. In the present study, PU.1 lacked the N-terminal region and the β3/β4 region of the Ets domain (PU.1ΔNΔβ3/β4) did not interact with GATA-1 but was still bound to Gfi-1B in the GST pull-down assay. Furthermore, co-transduction of PU.1ΔNΔβ3/β4 and Gfi-1B expression vectors into $CD34^+$ cells strongly inhibited Gfi-1B-induced expansion of erythroid colonies, similar to transduction of PU.1 WT with Gfi-1B. Most of the transduced cells with PU.1ΔNΔβ3/4 stopped growing and appeared to be converted into mast cells, whereas transduced cells with PU.1 WT differentiated into dendric cells, consistent with a previous report [31].

Because PU.1 and Gfi-1B play an important role in blood cell differentiation, their deregulation can lead to the development of leukemia. Expression the Gfi-1B is negatively correlated with the prognosis of acute myeloid leukemia (AML) patients. For example, low-level or loss of Gfi-1B promotes AML development and negatively influences the prognosis of myelodysplastic syndrome (MDS)/AML patients [35]. On the other hand. Decreased expression of PU.1 is a cause of various hematological malignancies, such as AML [36, 37]. However, there are no clinical findings that suggest that the PU.1/Gfi-1B complex is involved in the development of leukemia in humans, and no studies have been conducted using animal models, so further investigation is needed.

5. Conclusions

Our results suggest that PU.1 inhibits the growth and differentiation of erythroblasts by inhibiting not only GATA-1 function but also Gfi-1B function in erythroid cells. Our results also showed that protein-protein interactions are critical for hematopoietic development and differentiation, as well as transcriptional regulation.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

NM, FKN, YN, and AI performed most of the experiments. TS, TY, and TO wrote the manuscript. TS, TY, AI, and TO contributed to the conceptualization of the study and interpretation of the results. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

The study using hCB cells was approved by the institutional ethics committee of Chiba University (approval ID: 196).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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