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Establishment of a Screening System to Identify Novel GATA-2 Transcriptional Regulators

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Hematopoietic stem cells can self-renew and differentiate into all blood cell types. The transcription factor GATA-2 is expressed in hematopoietic stem and progenitor cells and is essential for cell proliferation and differentiation. Heterozygous germline *GATA2* mutations induce GATA-2 deficiency syndrome, characterized by monocytopenia, a predisposition to myelodysplasia and acute myeloid leukemia, and a profoundly reduced dendritic cell (DC) population, which is associated with increased susceptibility to viral infections. Because patients with GATA-2 deficiency syndrome could retain a wild-type copy of GATA-2, boosting residual wild-type GATA-2 activity may represent a novel therapeutic strategy for the disease. Here, we sought to establish a screening system to identify GATA-2 activators using human U937 monocytic cells as a potential model of the DC progenitor. Enforced GATA-2 expression in U937 cells induces CD205 expression, a marker of DC differentiation, indicating U937 cells as a surrogate of human primary DC progenitors. Transient luciferase reporter assays in U937 cells reveals a high promoter activity of the −0.5 kb *GATA-2* hematopoietic-specific promoter (1S promoter) fused with two tandemly connected *GATA-2* +9.9 kb intronic enhancers. We thus established U937-derived cell lines stably expressing tandem +9.9 kb/−0.5 kb 1S-luciferase. Importantly, forced GATA-1 expression, a repressor for GATA-2 expression, in the stable clones caused significant decreases in the luciferase activities. In conclusion, our system represents a potential tool for identifying novel regulators of GATA-2, thereby contributing to the development of novel therapeutic approaches.

Keywords: dendritic cells; GATA-1; GATA-2; GATA-2 deficiency syndrome; U937
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Introduction

Differentiation of hematopoietic stem cells (HSCs) into specific progenitor cells, and ultimately into diverse blood cell types, is controlled by transcription factors represented by the GATA family of zinc finger DNA-binding proteins (Orkin and Zon 2008; Bresnick et al. 2010; Fujiwara et al. 2017). GATA-1, GATA-2, and GATA-3 are hematopoietic GATA factors, given their important roles in this process (Orkin and Zon 2008; Bresnick et al. 2010; Fujiwara et al. 2017). Among them, GATA-2 is required for the maintenance and expansion of HSCs, multipotent progenitors, or both, during early hematopoiesis (Tsai et al. 1994, 1997; Ezoe et al. 2002; Rodrigues et al. 2005).

The mechanisms underlying *GATA-2* transcription have been extensively analyzed. Two independent first exons of the *GATA-2* gene, the 1S and 1G, were identified in mice and humans (Minegishi et al. 1998; Pan et al. 2000). Transcripts involving the 1G promoter are commonly found in tissues expressing GATA-2, whereas 1S transcripts play an important role in hematopoietic cells (Minegishi et al. 1998; Pan et al. 2000). During erythroid differentiation, GATA-2 levels concomitantly decrease with increased GATA-1 levels (Bresnick et al. 2010). Based on a murine model of erythroid differentiation, GATA-1 represses *GATA-2* transcription by displacing GATA-2 from sites at −77, −3.9, −2.8, −1.8, and +9.5 kb relative to the 1S promoter, each of which is considered as "GATA switch site" (Bresnick et al. 2010; Moriguchi et al. 2014). Among these enhancers, deletion of the +9.5 site involving GATA-binding motif leads to delayed embryonic lethality compared with global *Gata2* knockout (Johnson et al. 2012). Thus, the 1S promoter and +9.5 kb enhancer regions could be considered important regulatory components for hematopoietic *GATA-2* expression.

Heterozygous *GATA-2* germline mutations, inherited

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and *de novo*, cause three overlapping clinical entities characterized by a predisposition to myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) as follows: (i) familial MDS/AML, ii) Emberger syndrome, characterized by lymphedema and predisposition to MDS/AML, and iii) an immunodeficiency that is characterized by monocytopenia and *Mycobacterium avium* complex (MonoMAC) or by dendritic cells (DCs), monocyte, B- and natural killer (NK)-lymphoid deficiency (DCML) (Dickinson et al. 2011; Hsu et al. 2011; Ostergaard et al. 2011). These conditions are collectively termed GATA-2 deficiency syndrome. Approximately 100 different *GATA-2* mutations seem to share certain characteristics, such as reduced or abrogated GATA-2 transcriptional activity (Collin et al. 2015). Some missense mutations could also exhibit dominant-negative effects; mutated GATA-2 protein overexpression inhibits chromatin occupancy of wild-type GATA-2 (Fujiwara et al. 2014). Approximately two-thirds of all cases have mutations in either N- or C-terminal zinc finger domains within GATA-2 protein (Fig. 1A). Although familial MDS/AML was specifically caused by *GATA-2* missense mutation within the C-terminal zinc finger domain, mutations observed in MonoMAC or Emberger syndrome include whole gene deletion, frameshift mutation, missense mutation, and regulatory mutation involving GATA-2 intronic enhancer region (Johnson et al. 2012; Collin et al. 2015). Moreover, in humans, a heterozygous mutation of the intronic enhancer at +9.9 kb, which corresponds to +9.5 kb in mice, occurs in patients with GATA-2 deficiency (Johnson et al. 2012). In patients with GATA-2 deficiency syndrome, monocytes, B cells, NK cells, and DC populations are profoundly diminished or undetectable, whereas neutrophil, macrophage, and T cell populations remain unaltered (Dickinson et al. 2011; Hsu et al. 2011; Ostergaard et al. 2011). DCs play crucial roles in the immune system (Banchereau and Steinman 1998), and as their numbers are profoundly decreased in GATA-2 deficiency syndrome; however, boosting GATA-2 activity may represent a novel therapeutic strategy for protecting severe life-threatening infections in patients with GATA-2 deficiency syndrome.

Here, we have established a screening system to identify a novel activator of GATA-2 during DC differentiation.

Materials and Methods

Cell culture

Human erythroleukemia cell lines, YN-1 (Endo et al. 1993) and K562 (Dorfman et al. 1992); human T cell leukemia cell line, Jurkat; human pre-B cell leukemia cell line, Nalm6; and human monocytoid cell line, U937 were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Biowest, Miami, FL, USA) and 1% penicillin/streptomycin (Sigma-Aldrich, St Louis, MO, USA). U937 cells stably expressing pGL4.20 (*GATA-2* +9.9/1S; described below) were cultured in RPMI-1640 containing 10% FBS, 1% penicillin/streptomycin, and 1 μg/ml puromycin (Sigma-Aldrich). K562 and U937 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and the Cell Resource Center for Biomedical Research at Tohoku University (http://www2.idac.tohoku.ac.jp/dep/ccr/), respectively. The human embryonic kidney cell line, HEK293T and retrovirus packaging cells (PLAT-F and PLAT-GP) were maintained in Dulbecco modified Eagle medium containing 10% FBS (Biowest) and 1% penicillin/streptomycin (Sigma-Aldrich) (Kamata et al. 2014).

Generation of an anti-GATA-2 antiserum

Three synthetic peptides, FLGGPASSFTPKQRSKARSC, HKMNGGNRPLIKPKRRLSA, and SLSFGHPHPSSMVTAMG, corresponding to amino acid positions 270-289, 323-341, and 464-480, respectively, within human GATA-2 (GenBank No: NP_001139133), were conjugated to keyhole limpet hemocyanin using an m-maleimidobenzoyl-N-hydroxysuccinimide ester method (Sigma-Aldrich, Ishikari, Hokkaido, Japan) (Fig. 1A). Each peptide (400 μg) was injected into a rabbit on day 0, and 200 μg of the peptide was subsequently injected on days 14, 28, and 42. Each anti-GATA-2 antiserum was prepared on day 56.

Quantitative chromatin immunoprecipitation analysis

Real-time-PCR-based quantitative chromatin immunoprecipitation (ChIP) analysis was performed as described (Fujiwara et al. 2009). K562 cells or GATA-1-overexpressing U937 cells were cross-linked with 1% formaldehyde for 10 min at room temperature (2×10^6 cells/IP condition). The nuclear lysate was sonicated to reduce DNA length by using a Branson sonifier. The protein-DNA complexes were immunoprecipitated using a specific antibody (1.5 vol% of antiserum, or 6 μg of purified IgG) and collected with Protein A Sepharose (Sigma-Aldrich). Immunoprecipitated DNA fragments were quantified using real-time polymerase chain reaction (PCR) to amplify regions of 75-150 bp overlapping with the appropriate motif. The products were measured with SYBR Green fluorescence in 20-μl reactions, and the amount of products was determined relative to a standard curve generated from titration of input chromatin. The post-amplification dissociation curves showed that the primer pairs generated single products. The primer sequences are shown in Table 1.

Plasmids and gene transfer

Primers linked to restriction enzyme sites were used to amplify the *GATA-2* genomic region to be included in the plasmids (Table 1) and were cloned into pGL3 (Luc) and pGL4.20 (Luc2/puro) vectors (Promega, Madison, WI, USA) as previously described (Saito et al. 2015). *Renilla* vector pGL4.74 was purchased from Promega. The GATA-2 +9.9/1S pGL4.20 plasmid was used to transfect U937 cells using an Amaxa Nucleofector (Nucleofector solution C, Nucleofector program W-001; Lonza Group). Subsequently, the cells were cultured in a medium containing 1 μg/ml puromycin (Sigma-Aldrich) to select transduced cells. To identify integration of the pGL4.20 luciferase vector, genomic DNA was extracted from each clone using the DNeasy Blood and Tissue Kit (QIAGEN).

To overexpress GATA-1 and GATA-2, each cDNA was cloned into the pBABE-puro vector (Addgene Plasmid 1764; Addgene Cambridge, MA, USA) (Fujiwara et al. 2014) or MSCV-IRES-GFP vector (Kamata et al. 2014). Retroviral overexpression was conducted as previously described (Fujiwara et al. 2014; Kamata et al. 2014). In brief, the retroviral vectors encoding human GATA-1 or GATA-2, and the env (envelope glycoprotein) gene from the vesicular stomatitis virus (VSV-G) were co-transfected into the retroviral pack-

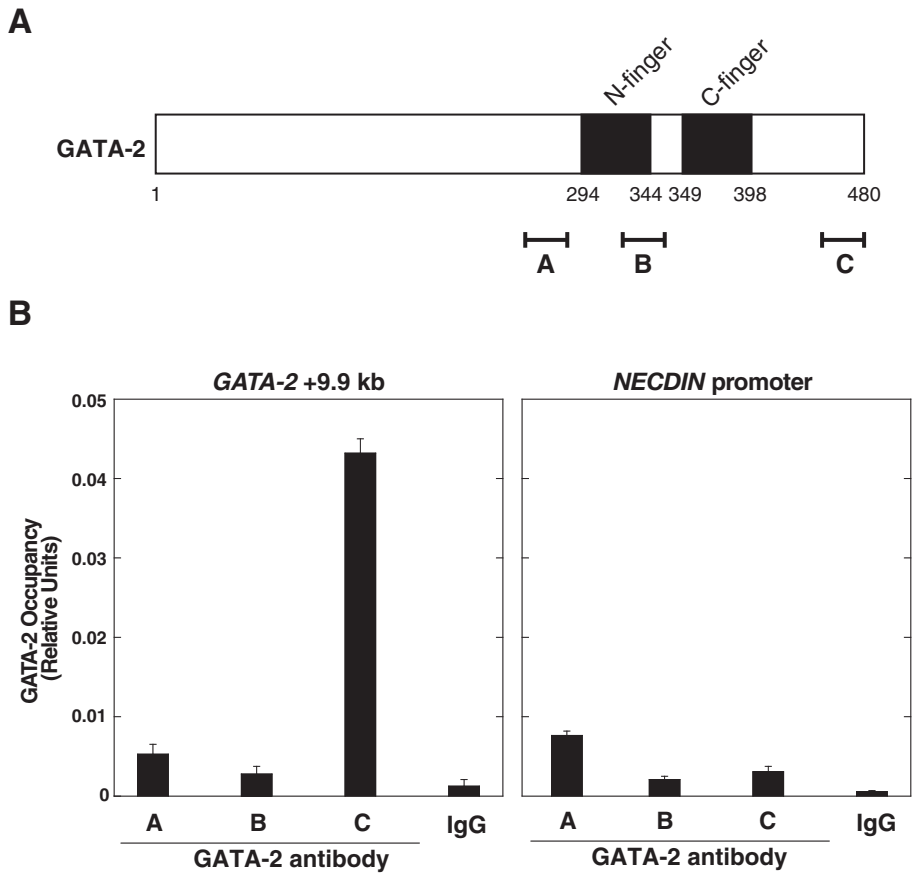

Fig. 1. Generation of an anti-GATA-2 antiserum.
(A) Three different GATA-2 peptides (A, B, and C) were used to immunize rabbits. (B) Quantitative ChIP analysis to detect endogenous GATA-2 occupancy in parental K562 cells, expressing abundant amounts of GATA-2 (Fujiwara et al. 2009). The *NECDIN* promoter served as a negative control (mean ± SD, n = 3).

aging cell lines (PLAT-GP or PLAT-F) with FuGene HD (Promega). The viral supernatant was used for infection 72 h after transection. After spin infection into the U937 cells at 3400 rpm for 2 h, GFP-positive cells were sorted by BD FACSAria II flow cytometers (BD Biosciences, Franklin Lakes, NJ, USA) for the selection of transduced cells. For transient GATA-2 overexpression in HEK293T cells, pBABE-puro vector encoding human *GATA-2* mRNA was used to transfect FuGene HD (Promega).

Promoter activity assay

To evaluate *GATA-2* transcriptional activity, aliquots of U937 cells were transfected with 1 μg of the *GATA-2* promoter construct and 100 ng of the pGL4.74 [*hRluc*/TK] vector (Promega) with FuGene HD (Promega). The cells were harvested 24 h after plasmid transfection, and Firefly and *Renilla* luciferase activity levels in the cell extracts were determined using a Dual-Luciferase Reporter Assay System (Promega). For the analysis of U937 clones stably expressing luciferase, 1×10^6 cells were counted and harvested, and each Firefly luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega).

Real-time quantitative reverse transcription (RT)-PCR

Real-time quantitative RT-PCR was performed using the SYBR Green master mix (Qiagen, Venlo, The Netherlands) based on a previously described protocol (Fujiwara et al. 2009). To obtain plasmids for standards for use in quantitative RT-PCR, an amplified cDNA fragment of the gene of interest was cloned into the pGEM™-T Easy Vector (Promega, Madison, WI, USA). The primer sequences are listed in Table 1.

Western blotting analysis

Whole-cell lysates were prepared by boiling 1×10^7 cells/ml in sodium dodecyl sulfate (SDS) buffer. Samples (5 μl) were resolved using SDS–polyacrylamide gel electrophoresis and analyzed using specific antibodies (Fujiwara et al. 2009). We used 10% polyacrylamide gel to detect GATA-1, GATA-2, and alpha-tubulin.

Reagents

A specific alpha-tubulin antibody was purchased from Calbiochem (Darmstadt, Germany). Rabbit control IgG was purchased from Abcam (Cambridge, MA, USA).

Statistical analysis

Statistical significance was assessed using a two-sided Student *t* test.

Table 1. Oligonucleotide primers.

Primers	Direction	Sequence (5'→ 3')
RT-PCR		
28S	Forward	TGGGTTTTAAGCAGGAGGTG
	Reverse	CCAGCTCACGTTCCCTATTA
GATA2	Forward	CAAGGCTCGTTCCTGTTCA
	Reverse	GCCCATTCATCTTGTGGTAGA
GATA1	Forward	GGCCTCTATCACAAGATGAATG
	Reverse	ACTGAGTACCTGCCCGTTTACT
CD205	Forward	GCTCTTCTGGTTCTTCGATCTC
	Reverse	CCTCAGTTTCATCACAGTCGTC
CD83	Forward	GAAACCTAAGTGGCAAGGTGAT
	Reverse	AGAAAATAACCAGAGCCAGCAG
PCR		
0.5kb/EE/Luc2	Forward	ATAAGGAAACTTCGTGTATCTGT
	Reverse	AATGGGAAGTCACGAAGGTG
ChIP		
NECDIN promoter	Forward	GAAGAGCTCCTGGACGCAGA
	Reverse	TGCAAAGTTAGGGTCGCTCAG
GATA2 +9.9kb	Forward	GACATCTGCAGCCTGAAGATAAG
	Reverse	CATTATTTGCAGAGTGGAGGGTATTAG
Cloning		
+9.9kb Enhancer insert	Forward	ATAGGTACCGAGCTCTACTGTGTATTT
	Reverse	CAGCTGTGGACGCGTTATTTGCAGAGT
0.5kb Promoter insert	Forward	GAGGATGTCCACGCGCCACAGCTGTGT
	Reverse	TAGGATAGATCTGGCGGCAGGCAATAGACAG
1.0kb Promoter insert	Forward	CACAATGCTAGCGTCACCACCAGATTTGGAGAAAG
	Reverse	TAGGATAGATCTGGCGGCAGGCAATAGACAG
1.8kb Promoter insert	Forward	TACACTGCTAGCTATGCTGTTAAAGACAGATGGACATGCA
	Reverse	TAGGATAGATCTGGCGGCAGGCAATAGACAG
3.8kb Promoter insert	Forward	CTTACGCGTGCTAGCCCCACGCCGGGCACACCGGGGC
	Reverse	CGCAGATCTCGAGCCCCGACGGGGCCCTGCTAGGATG
4.5kb Promoter insert	Forward	GCTAGCCTCGAGGATCCGAGACCCCGCCGCCGCCAGG
	Reverse	GAGGCCAGATCTTGATCGACGGGGCCCTGCTAGGATG
GATA1 pMX IRES GFP	Forward	TTTTTGGATCCCAGAGGCTCCATGGAGTTCCCTG
	Reverse	TTTTTGAATTCCTCTGTGCCCTCATGAGCTGAGC
Sequence		
RV3	Forward	GACGATAGTCATGCCCCGCG

Results and Discussion

Generation of an anti-GATA-2 antiserum

We first generated rabbit anti-GATA-2 antiserum. The synthetic peptides (A, B, C), corresponding to amino acid positions 270-289, 323-341, and 464-480 of human GATA-2, respectively, were used (Fig. 1A). We subsequently performed quantitative ChIP analysis to assess GATA-2 occupancy at the *GATA-2* intronic enhancer at +9.9 kb (corresponding to +9.5 kb in mice), which plays a crucial role in endogenous *GATA-2* expression (Johnson et al. 2012). The *NECDIN* gene promoter, which does not contain a GATA-binding motif and was not bound by GATA-2 (Fujiwara et al. 2009), served as a negative control. GATA-2 chromatin occupancy was detected using GATA-2 antiserum C, which was used for further analyses (Fig. 1B). The specificity of antiserum C was also confirmed with the western blotting analysis of control and GATA-2-silenced K562 cells (data not shown).

GATA-2 overexpression in U937 cells induces expression of a DC marker

GATA-2 plays an important role in the differentiation of DCs (Onodera et al. 2016). Therefore, we evaluated the phenotypic changes by the restoration of GATA-2 expression in DC precursors. We first confirmed that endogenous expression levels of GATA-2 and GATA-1 mRNAs were quite low in the U937 cells (Fig. 2A, B). Subsequently, GATA-2 was overexpressed in human U937 monocytic cells transfected with the MSCV-IRES-GFP retroviral vector, which was confirmed using western blotting with the anti-GATA-2 antiserum C (Fig. 2C). We focused on the expression changes of DC-related genes, such as *CD205* and *CD83* (Schwede et al. 2014). The expression of CD205 was significantly increased, and that of CD83 was marginally increased ($p = 0.09$) (Fig. 2D). However, we did not detect obvious morphological changes indicating differentiation toward DCs (data not shown).

The molecular mechanism by which GATA-2 induces *CD205* expression is unknown. For example, genome-wide analysis of GATA-2 chromatin occupancy based on the coupling of next-generation DNA-sequencing technology with ChIP-seq demonstrates that a significant GATA-2 peak is not detected in the *CD205* locus (Fujiwara et al. 2009), presumably excluding the possibility of direct transcriptional regulation of the *CD205* gene by GATA-2. We have recently demonstrated that GATA-2 has an important role in cell-fate specification toward the myeloid versus T-lymphocyte lineage by regulating lineage-specific transcription factors in DC progenitors, thereby contributing to DC differentiation (Onodera et al. 2016). In support of our observations, a previous study suggested that moderate GATA-2 overexpression might induce myeloid expansion after the granulocyte monocyte progenitor stage, while blocking lymphoid differentiation (Nandakumar et al. 2015). Thus, we speculate that GATA-2-mediated activation of signals to induce myeloid differentiation might indirectly activate *CD205* expression in the U937 cells (Fig. 2D).

Taken together, identification of GATA-2 upstream mechanisms in U937 cells might be a feasible approach to treat GATA-2 deficiency syndrome.

GATA-2 1S promoter fused to tandem +9.9 kb enhancers induces high promoter activity

To discover novel regulators of *GATA-2* expression, we sought to establish a screening system. For this purpose, we constructed a luciferase plasmid containing GATA-2 regulatory elements. Subsequently, the plasmid was used to transfect U937 cells to establish a stable clone. The established screening system will be applicable for screening libraries comprising cDNA, interfering RNAs (RNAi), or small molecules (Fig. 3). In this study, we focused on the results regarding the construction of a reporter vector and establishment of a stable clone expressing luciferase, with several validation experiments by overexpressing potential GATA-2 upstream regulators (as described below).

First, we performed a transient luciferase promoter assay to determine the optimal configuration for siRNA screening. *GATA-2* transcription involves two different first exons, distal (1S) and proximal (1G) promoters; the former is particularly important in hematopoietic cells (Minegishi et al. 1998; Pan et al. 2000; Bresnick et al. 2010) (Fig. 4A). Thus, we selected the 1S promoter as a potential promising tool to identify novel upstream factors of GATA-2 in hematopoietic cells. The −0.5-kb 1S promoter exhibited modest promoter activity, and extension from −0.5 to −4.6 kb from the transcription start site of the 1S promoter did not significantly affect its activity (Fig. 4B). This may be partially explained by the repressive effect of CCAAT/enhancer-binding protein, alpha (CEBPA) through its binding sites at −1.2 and −2.4 kb (Fig. 4B) (Cortes-Lavaud et al. 2012) or the presence of other unrecognized repressive elements at this position. In contrast, the addition of a +9.9 kb *GATA-2* enhancer to these promoters resulted in higher luciferase activity, although the changes were relatively modest for −3.5 and −4.6 kb (Fig. 4B).

A direct sequence connection between +9.9 kb and the 1S promoter reflecting a physiological promoter–enhancer interaction *in vivo* has not been established. For example, Wozniak et al. (2007) analyzed the murine +9.5/1S construct and demonstrated that the +9.5/1S construct drives high luciferase activity, and the disruption of the GATA-binding element within +9.5 kb eliminated its enhancer activity. Moreover, when the +9.9 kb site was linked in tandem to the −0.5 kb 1S promoter, the promoter activity was more strongly induced than the single +9.9 kb fused with the 1S promoter and the 1S promoter lacking the +9.9 kb site (Fig. 4C), implying that the enhancer would be functional. Therefore, we selected two tandemly connected *GATA-2* +9.9 kb intronic enhancers fused to the 1S pro-

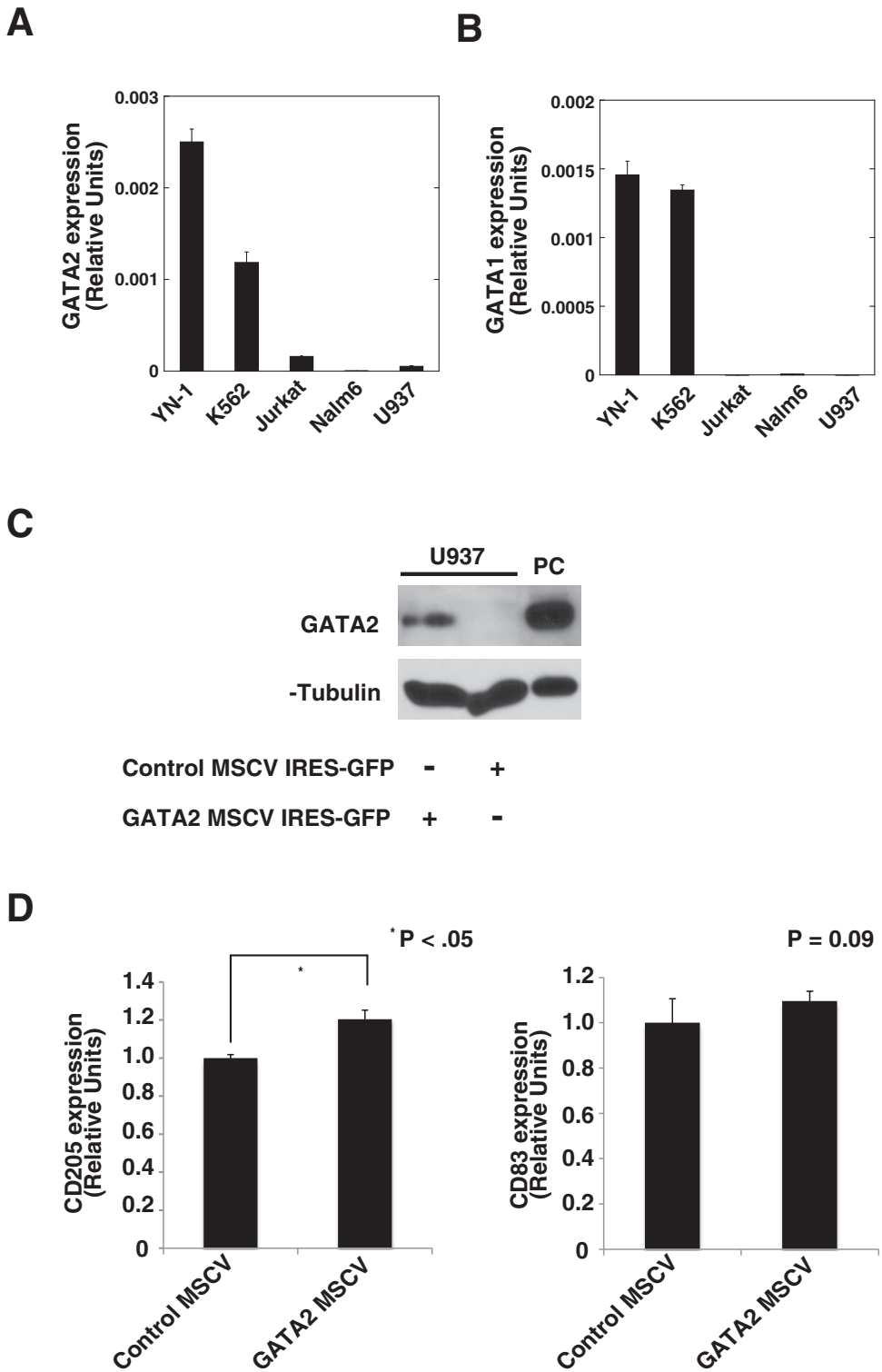

Fig. 2. Effects of *GATA-2* overexpression in monocytic U937 cells.
(A, B) Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed to measure the relative expression levels of *GATA-2* mRNA (A) and *GATA-1* mRNA (B) in five hematopoietic cell lines (mean ± SD, n = 3). 28S ribosomal RNA was used as a control. (C) Anti-GATA-2 western blotting analysis of whole-cell extracts from U937 cells infected with GATA2-MSCV-IRES-GFP or the empty vector. Alpha-tubulin was used as a loading control. For detecting GATA-2, anti-GATA-2 antiserum C was used (Fig. 1). PC, positive control, which was derived from a whole- cell extract of HEK293T cells transiently overexpressing the pBABE-puro vector encoding human *GATA-2* mRNA. (D) Quantitative RT-PCR analysis of *CD205* and *CD83* expression (mean ± SD, n = 3). 28S ribosomal RNA was used as a control. *P < 0.05.

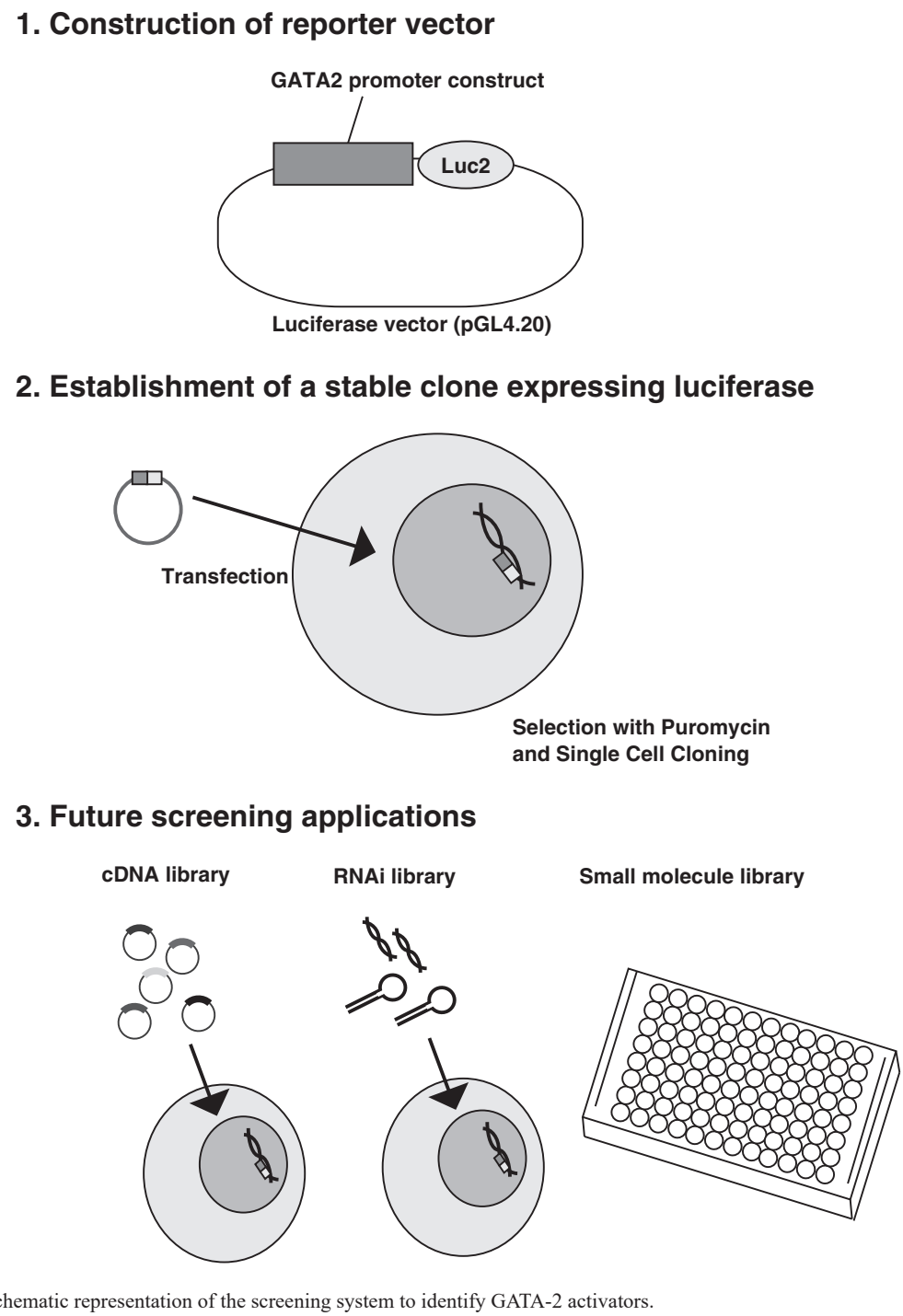

Fig. 3. Schematic representation of the screening system to identify GATA-2 activators.
To discover novel regulators of *GATA-2* expression, we sought to establish a screening system. First, we constructed a luciferase plasmid containing GATA-2 regulatory elements. Subsequently, the plasmid was used to transfect U937 cells to establish a stable clone. The established screening system will be applicable for screening libraries comprising cDNA, interfering RNAs (RNAi), or small molecules.

moter luciferase construct for screening.

Establishment of U937 cell clones that stably express luciferase activity under the control of tandem +9.9 kb enhancers fused to the 1S promoter

The luciferase plasmid was introduced into the parent U937 cells, which were subsequently selected with puromycin to ensure stable expression. To evaluate the integration of the genomic region of the luciferase plasmid, we designed a primer to cover the GATA-2 regulatory region and luciferase sequences (Fig. 5A), which could not be amplified in the parental U937 cells or in the integration-negative clones. After transfection of the luciferase vector containing the tandem +9.9 kb enhancer sites fused to the

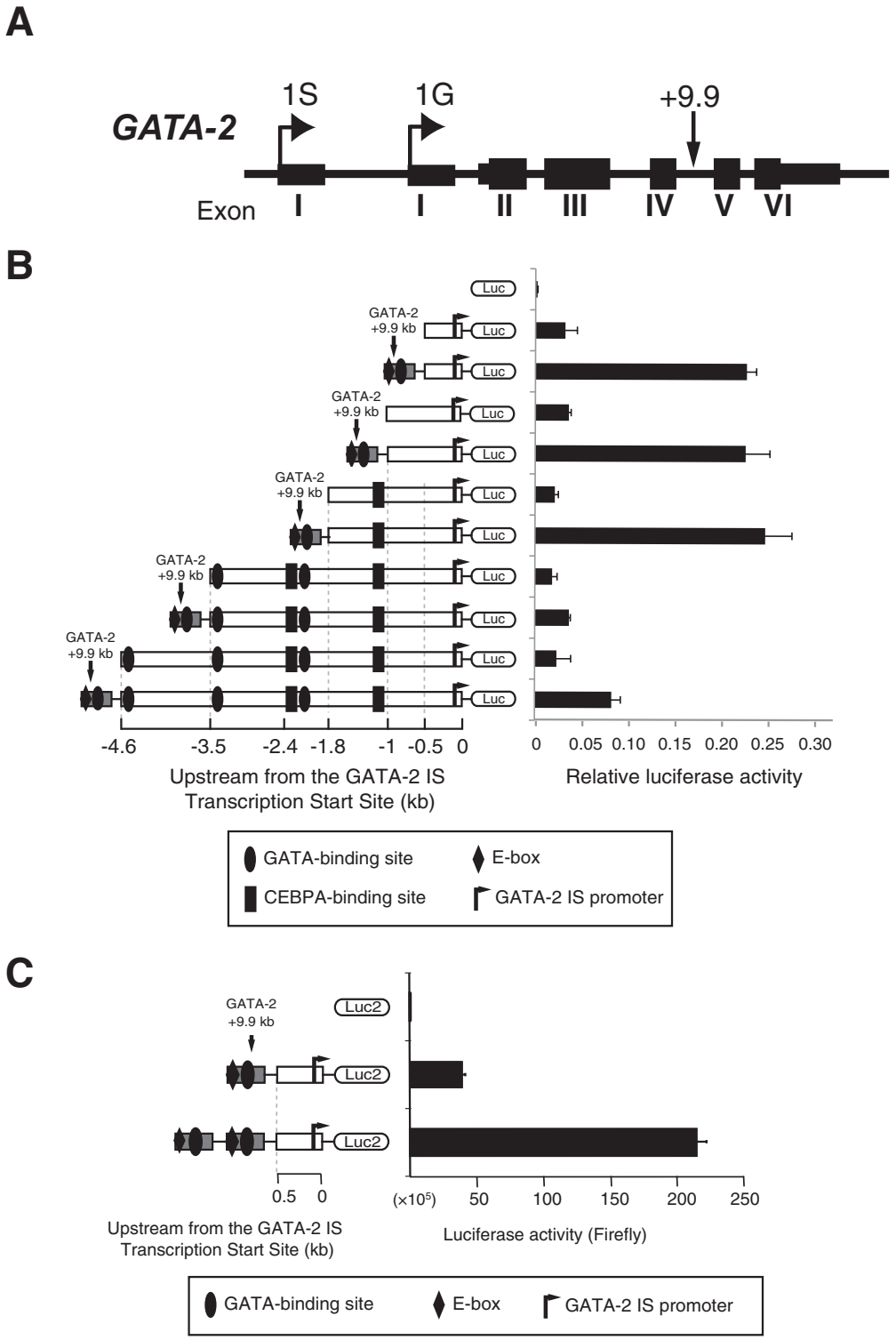

Fig. 4. Luciferase assay of GATA-2 regulatory elements in U937 cells.
(A) Structure of the human *GATA-2* gene. The human *GATA-2* gene is transcribed from either the 1S or 1G promoter, and the +9.9 kb intronic enhancer region is indicated. (B) U937 cells were transiently cotransfected with pGL3.0, with or without the upstream 1S promoter sequence, +9.9 kb enhancer site, or both, and *Renilla* (pRL). Putative *GATA*-binding sites, CCAAT/enhancer-binding protein, alpha (CEBPA)-binding sites, and E-box are indicated. The results are presented as ratio of luciferase activities (mean values of Firefly/*Renilla* ± SD, n = 3). (C) U937 cells were transfected with pGL4.20 (empty, GATA-2 −0.5 kb 1S/+9.9 kb enhancer, or GATA-2 −0.5 kb 1S/tandem +9.9 kb enhancers), selected using puromycin, and the luciferase activities were subsequently evaluated (mean values of Firefly ± SD, n = 3). For the luciferase assay, 1×10^6 cells were used for each analysis.

−0.5 kb 1S promoter, the individual clones (#12, #14, #15, #16, #17, #18, #20, #22, #23, and #24) were subsequently selected based on a serial dilution with a medium containing puromycin. Fig. 5B shows the detection of strong luciferase activities in clones #12, #14, #16, #17, #18, #20, #22, and #24. Concomitantly, the integration of the *GATA-2* regulatory region and luciferase sequences was confirmed in these clones (Fig. 5C).

Based on the established clones containing the *GATA-2* +9.9 kb enhancer/1S −0.5 kb, we tested whether the luciferase activity of these clones was increased or decreased by introducing established GATA-2 upstream regulators. Before the increase in GATA-1 expression occurs during erythroid differentiation, GATA-2 is expressed in HSCs, and GATA-1 directly represses *GATA-2* transcription at the *GATA-2* locus (the "GATA switch") (Bresnick et al. 2010). We demonstrated that GATA-1 overexpression in the U937 clones containing the *GATA-2* +9.9 kb enhancer/1S −0.5 kb (Fig. 6A) and found that the luciferase activities of three of four clones significantly decreased (Fig. 6B). Although we confirmed that GATA-1 directly occupied GATA-2 +9.9 kb element based on GATA-1 overexpressed U937 cells (Fig. 6C), we were unable to confirm if GATA-1 overexpression repressed endogenous *GATA-2* expression, due to an extremely low endogenous *GATA-2* expression level in the U937 cells (Fig. 2A).

Furthermore, because GATA-2 has been reported to positively autoregulate its own expression in HSCs (Bresnick et al. 2010), we tested if GATA-2 overexpression increases the luciferase activity of the U937 clones. Unexpectedly, GATA-2 overexpression decreased the luciferase activity (Fig. 7). In this context, the myelo-lymphoid transcription factor PU.1 negatively regulates the GATA-2 expression (Walsh et al. 2002), and PU.1 is abundantly expressed in U937 cells (Yeamans et al. 2007). We therefore speculate that GATA-2 overexpression might nega-

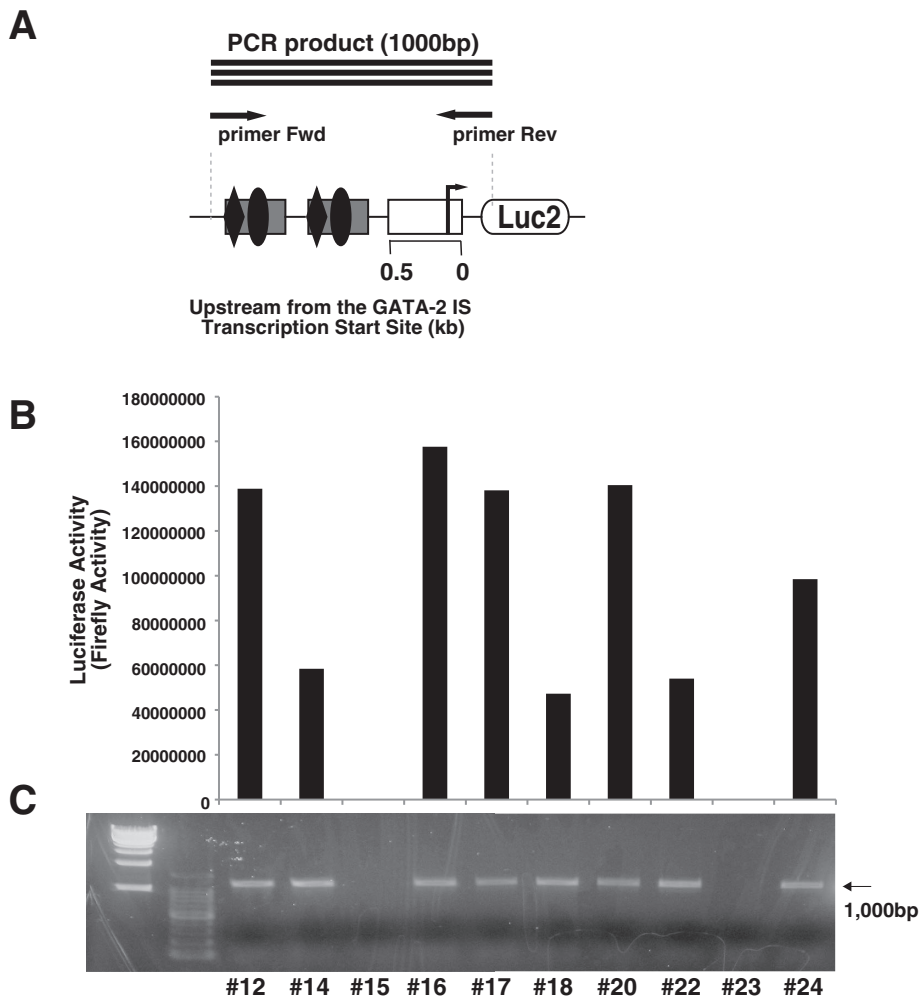

Fig. 5. Establishment of U937-derived cell lines stably expressing two tandemly connected +9.9 kb enhancers/−0.5 kb 1S promoter luciferase.
(A) Primer design to detect integrated DNA of the plasmid containing the GATA-2 regulatory region and luciferase sequences. When the plasmid with tandem GATA-2 +9.9 kb enhancers/−0.5 kb 1S promoter was used to establish a stable clone, an approximately 1000 bp band was predicted based on the PCR analysis of the positive clones. (B, C) Luciferase activity (B) and PCR analysis (C) of each clone (mean values of Firefly ± SD, n = 3). For the luciferase assay, 1×10^6 cells were used for each analysis.

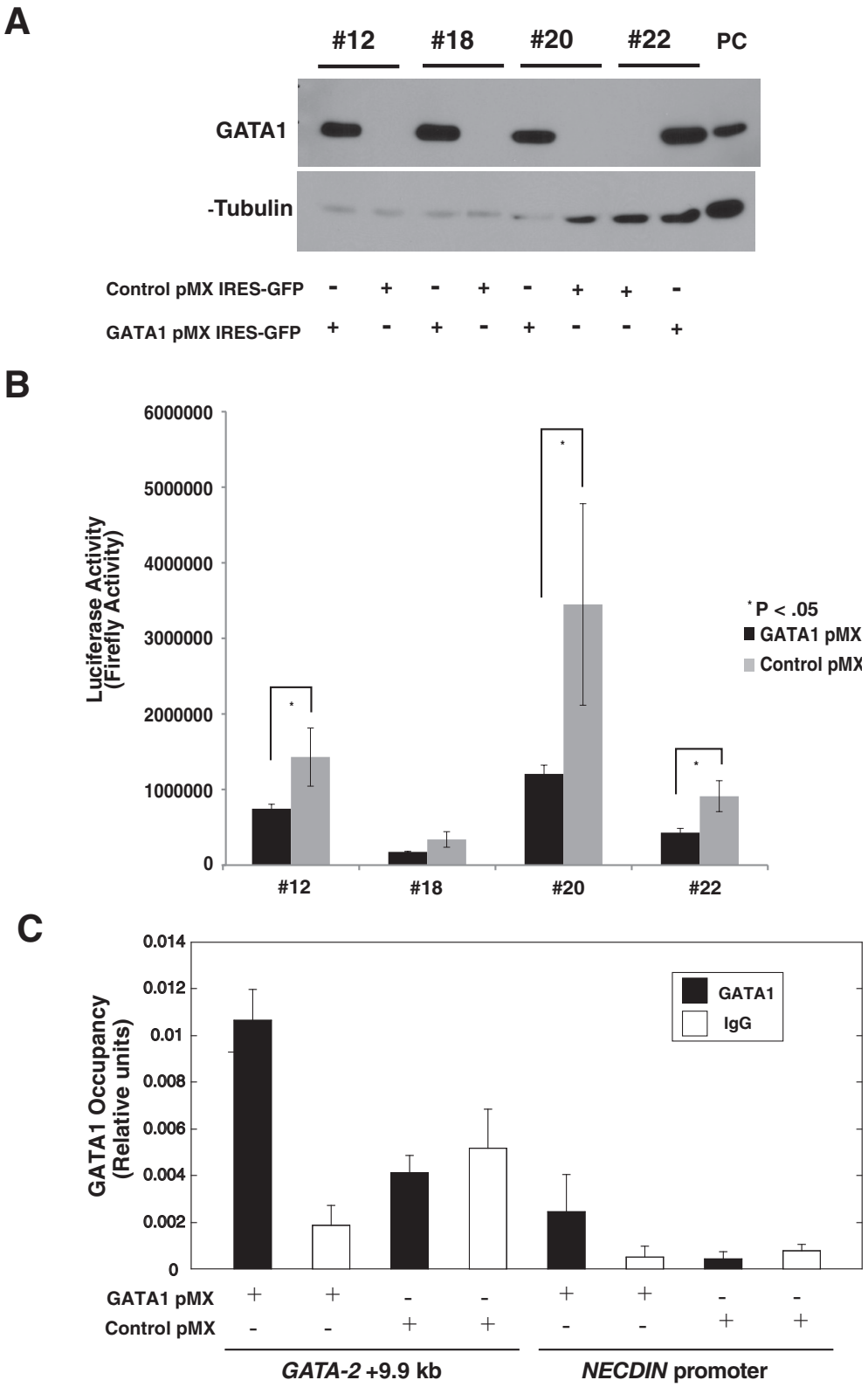

Fig. 6. GATA-1 overexpression represses GATA-2 luciferase activity in U937-derived cell lines.
(A) Western blot analysis to detect exogenous GATA-1 expression in U937 cell clones capable of stably expressing tandem +9.9 kb/−0.5 kb 1S promoter luciferase. Clones, #12, #18, #20, and #22, were used. PC, positive control, which was derived from a whole-cell extract of K562 cells, which expresses abundant amounts of GATA-1 (Fujiwara et al. 2009). (B) Luciferase analysis of GATA-1 overexpression (mean values of Firefly ± SD, n = 3). For the luciferase assay, 1×10^6 cells were used for each analysis. *P < 0.05. (C) Quantitative ChIP analysis to detect GATA-1 occupancy at *GATA-2* +9.9kb enhancer in U937 cells, infected with GATA-1 pMX or control pMX vector. The *NECDIN* promoter served as a negative control (mean ± SD, n = 3).

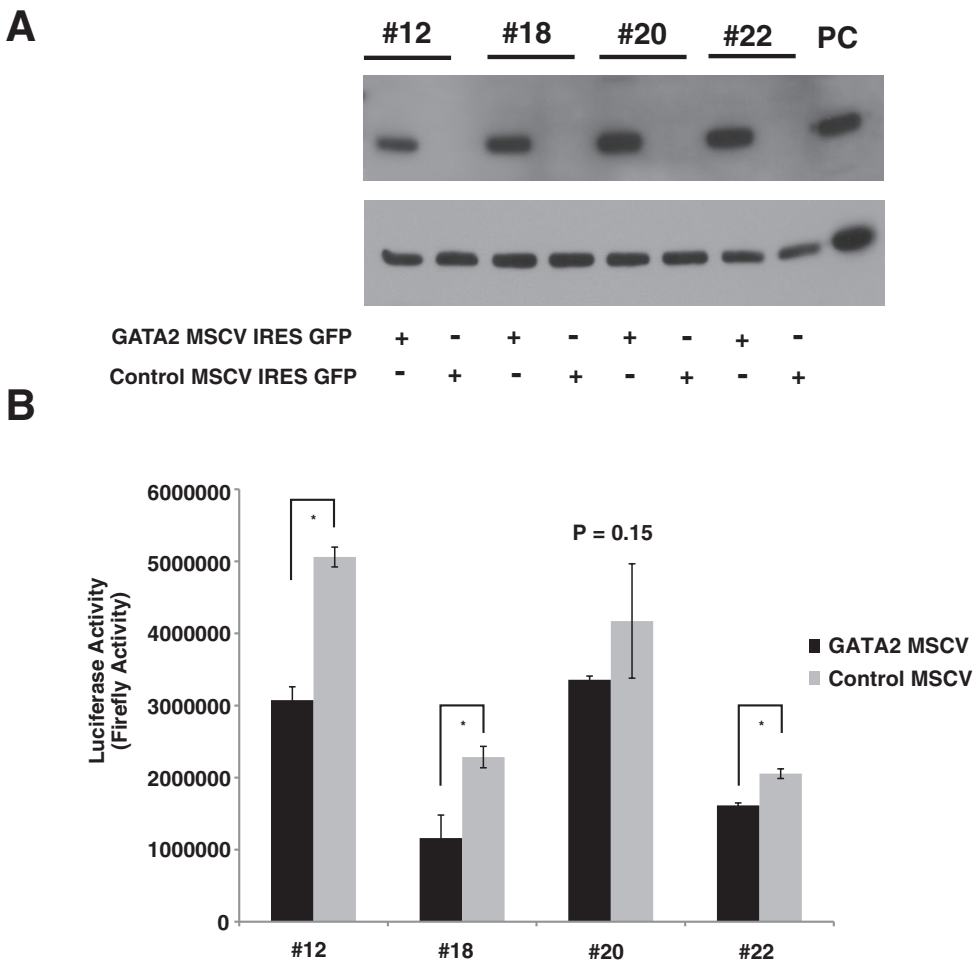

Fig. 7. GATA-2 overexpression represses GATA-2 luciferase activity in U937-derived cell lines.
(A) Western blot analysis to detect exogenous GATA-2 expression in U937-derived clones capable of stably expressing tandem +9.9 kb/−0.5 kb 1S promoter luciferase. Clones, #12, #18, #20, and #22, were used. PC, positive control, which was derived from a whole-cell extract of K562 cells, which expresses abundant amounts of GATA-2 (Fujiwara et al. 2009). (B) Luciferase analysis of GATA-2 overexpression (mean values of Firefly ± SD, n = 3). For the luciferase assay, 1×10^6 cells were used for each analysis. *$P < 0.05$.

tively affect endogenous *GATA-2* expression by interacting with PU.1 at *GATA-2* +9.9 kb intronic enhancer. Further analyses would be required to reveal the molecular mechanisms of context-dependent GATA-2 activities on its locus.

To the best of our knowledge, GATA-1-mediated direct repression of *GATA-2* in monocytic cells has not been demonstrated. During erythroid differentiation, GATA-1 requires friend of GATA-1 (FOG1) to repress endogenous GATA-2 by interacting with the nucleosome remodeling and deacetylation (NuRD) complex (Bresnick et al. 2010). Although FOG1 expression has not been detected in monocytic cells, other mechanisms, such as histone demethylase LSD1-mediated epigenetic repression (Guo et al. 2015), might contribute to GATA-1-mediated repression of *GATA-2*. Further analysis will be required to determine the regulatory mechanism of GATA-2 in the context of committed myeloid cells. Nevertheless, we conclude that our system can be applied to a variety of screening systems (Fig. 3).

Because all patients with GATA-2 deficiency syndrome seem to retain a wild-type copy of GATA-2 in all cells, boosting residual wild-type GATA-2 activity may represent a novel therapeutic strategy for the disease. However, the approach could also induce mutated GATA-2 activity, which might potentially act as a dominant-negative effect, which would require further investigations. Nevertheless, our system potentially represents a powerful tool to identify the regulatory mechanisms of GATA-2 and may lead to the development of novel therapeutic approaches.

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Conflict of Interest

The authors declare no conflict of interest.

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