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

Keiichi Ohashi,1 Tohru Fujiwara,1 Koichi Onodera,1 Yo Saito,1 Satoshi Ichikawa,1 Masahiro Kobayashi,1 Yoko Okitsu,1 Noriko Fukuhara,1 Yasushi Onishi1 and Hideo Harigae1

1Department of Hematology and Rheumatology, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan

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

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 (Tsi 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 (Minigishi 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 (Minigishi 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...
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 improve protective immunity against the 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, Namal; and human monocyteoid 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/crcr), 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, FLGGPASSFPQKRSKARSC, HKMNGGQRPLKPKRRLAS, and SLSFGHPPSSMVATMG, 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-maleimido-dobenzoyl-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-
aging cell lines (PLAT-GP or PLAT-F) with FuGene HD (Promega). The viral supernatant was used for infection 72 h after transfection. After spin infection into the U937 cells at 3400 rpm for 2 h, GFP-positive cells were sorted by BD FACS Aria 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 × 10⁶ 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 × 10⁷ 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.
### Primers Direction Sequence (5’ → 3’)

| Primers       | Direction | Sequence (5’ → 3’)     |
|---------------|-----------|------------------------|
| **RT-PCR**    |           |                        |
| 28S           | Forward   | TGGGTTTTAAGCAGGAGGTG   |
|               | Reverse   | CCAGCTACGTTCCCTATTA    |
| GATA2         | Forward   | CAAGGCTCGTCTGTTCCTCA   |
|               | Reverse   | GCCCATTCATCTTTGTGGTGA  |
| GATA1         | Forward   | GGGCTCTATCAACAAGATGAT  |
|               | Reverse   | ACTGAGTACCTGCCCGTTTACT |
| CD205         | Forward   | GCTCTTCTGGTTCTCGATTC   |
|               | Reverse   | CCTCAGTTCTCATACAGTGCT  |
| CD83          | Forward   | GAAACCTAAGTGCAAGGTAT   |
|               | Reverse   | AGAAAATAACCAGAGCCAGAG |
| **PCR**       |           |                        |
| 0.5kb/EE/Luc2 | Forward   | ATAAGGAAACTTCGTGTATCT  |
|               | Reverse   | AATGGGAAGTCAAGGAAGTG   |
| **ChIP**      |           |                        |
| NECDIN promoter | Forward | GAAGAGCTCCTGGAGGCAGA  |
|               | Reverse   | TGCAAAAGTCTGGGCTGCTCAG |
| GATA2 +9.9kb  | Forward   | GACATCTGCAAGCTAGAAGAT  |
|               | Reverse   | CATTATAGCAGATGGAGGTATT |
| **Cloning**   |           |                        |
| +9.9kb Enhancer insert | Forward | ATAGGTACCGAGCTCTACTGTTTAGT |
|               | Reverse   | CAGCTGGGACGCGTTATTTGCAGA  |
| 0.5kb Promoter insert | Forward | GAGGATGTCACCGCCACAGCTGTGT |
|               | Reverse   | TAGGATAGATCTGCGGCGAGGCAGAGAGAAG |
| 1.0kb Promoter insert | Forward | CACAATGCTAGCGTCACCCAGATTTGGGAGAAG |
|               | Reverse   | TAGGATAGATCTGGCCGAGGCAATAGACAG |
| 1.8kb Promoter insert | Forward | TACACTGCTAGCTAGTGGTTGAAAGACAGATGAGCATGCA |
|               | Reverse   | TAGGATAGATCTGCGAGCGCTAGAAGAGAAG |
| 3.8kb Promoter insert | Forward | TACACTGCTAGCTAGTGGTTGAAAGACAGATGAGCATGCA |
|               | Reverse   | TAGGATAGATCTGCGAGCGCTAGAAGAGAAG |
| 4.5kb Promoter insert | Forward | GCTAGGCTCGAGGATCCGACCCCGCAGCGCGCGCCAG |
|               | Reverse   | GAGGGCGACTCTGGAGCGGCGCGGCGCTAGAAGAGAAG |
| GATA1 pMX IRES GFP | Forward | TTTTTAGTCAGAGGCTCTAGGTATGACAG |
|               | Reverse   | TTTTTGAATTCCTCTGTGCCCCCTATGATGAGGCG |
| **Sequence**  |           |                        |
| RV3           | Forward   | GACGATAGTCCTGGGGAGGCG  |
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 over-expressing 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 with 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.
1. Construction of reporter vector

GATA2 promoter construct

Luciferase vector (pGL4.20)

2. Establishment of a stable clone expressing luciferase

Transfection

Selection with Puromycin and Single Cell Cloning

3. Future screening applications

cDNA library RNAi library Small molecule library

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.

Promoter 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 IS 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 × 10⁶ 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 (Yeaman et al. 2007). We therefore speculate that GATA-2 overexpression might nega-

![Fig. 5](image-url)
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 × 10⁶ cells were used for each analysis.  *P < 0.05.  (C) Quantitative ChIP analysis to detect GATA-1 occupancy at GATA-2 +9.9 kb 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).
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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