PU.1 Activation Relieves GATA-1–Mediated Repression of Cebpa and Cbfb during Leukemia Differentiation

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Abstract

Hematopoietic transcription factors GATA-1 and PU.1 bind each other on DNA to block transcriptional programs of undesired lineage during hematopoietic commitment. Murine erythroleukemia (MEL) cells that coexpress GATA-1 and PU.1 are blocked at the blast stage but respond to molecular removal (downregulation) of PU.1 or addition (upregulation) of GATA-1 by inducing terminal erythroid differentiation. To test whether GATA-1 blocks PU.1 in MEL cells, we have conditionally activated a transgenic PU.1 protein fused with the estrogen receptor ligand-binding domain (PUER), resulting in activation of a myeloid transcriptional program. Gene expression arrays identified components of the PU.1-dependent transcription program negatively regulated by GATA-1 in MEL cells, including CCAAT/enhancer binding protein α (Cebpa) and core-binding factor, β subunit (Cbfb), which encode two key hematopoietic transcription factors. Inhibition of GATA-1 by small interfering RNA resulted in derepression of PU.1 target genes. Chromatin immunoprecipitation and reporter assays identified PU.1 motif sequences near Cebpa and Cbfb that are co-occupied by PU.1 and GATA-1 in the leukemic blasts. Significant derepression of Cebpa and Cbfb is achieved in MEL cells by either activation of PU.1 or knockdown of GATA-1. Furthermore, transcriptional regulation of these loci by manipulating the levels of PU.1 and GATA-1 involves quantitative increases in a transcriptionally active chromatin mark: acetylation of histone H3K9. Collectively, we show that either activation of PU.1 or inhibition of GATA-1 efficiently reverses the transcriptional block imposed by GATA-1 and leads to the activation of a myeloid transcriptional program directed by PU.1.

Introduction

During hematopoiesis, precise levels of specific transcription factors regulate lineage determination, and changes in their levels block or divert this process (1–3). PU.1 (Sfp1, Spi-1) and GATA-1 are two lineage-specific transcription factors that play key roles in determining the fate of multipotent progenitors (4). PU.1 is an Ets family member that dose-dependently guides the development and differentiation of granulocyte-macrophage and common lymphoid progenitors by interacting with lineage-specific cofactors on DNA (5). Differentiation into myeloid precursors also involves CCAAT/enhancer binding protein α (Cebpa) and core-binding factor, β subunit (Cbfb), which cooperate with PU.1 in the further specification and maturation of cells (6, 7). PU.1 levels below a certain threshold (∼20%) cause a block of hematopoietic differentiation accompanied with accelerated proliferation (8, 9). Mutations of PU.1 and some of its target genes, including Cebpa and Cbfb, are associated with a differentiation block in human acute leukemias (10). GATA-1 is a zinc finger transcription factor regulating erythro-megakaryopoiesis by sequence-specific targeting as well as cooperating with lineage-specific cofactors in chromatin, such as nuclear factor [erythroid-derived 2 (Nfe2)] and Friend of GATA-1 (Zfp1); refs. 11, 12). Mutations affecting either the length of GATA-1 protein or its interactions with Zfp1 are also associated with acute leukemias (13–15). Murine erythroleukemia (MEL) cells are acute leukemia blasts blocked from further erythroid differentiation by deregulated expression of PU.1 (16). Removal of PU.1 (17) or addition of GATA-1 (18) causes erythroid differentiation of MEL cells, the incompleteness of which, however, suggests an involvement of additional factors. In MEL cells, PU.1 physically interacts with GATA-1 (19–21) and silences the transcription of its target genes by creating a repressive chromatin structure (22, 23) that forms when PU.1 binding on GATA-1 involves...
deacetylation of histone H3 lysine 9 (H3K9) and its trimethyla-
tion (22, 23). Ectopic expression of PU.1 also blocks chemically
induced erythroid differentiation of MEL cells (24). Previous
studies showed that the Ets domain of PU.1 interacts with and
mediates the repression of GATA-1 (19, 21) without altering
dNA binding (22, 23). Conversely, the C finger of GATA-1
can interact with and mediate the repression of PU.1 (20, 21).

We asked whether GATA-1 blocks PU.1 in MEL cells, and
whether by increasing the PU.1/GATA-1 level ratio we could
induce PU.1 target genes and drive non-erythroid differentia-
tion. Our data show that, indeed, activated PUER induces
non-erythroid differentiation of MEL proerythroblasts into
cell cycle–arrested non–erythroid-like cells. Using gene ex-
pression arrays, we have identified a specific set of genes
regulated positively by PU.1 and inhibited by GATA-1 that
contains two key hematopoietic transcription factors, Cebpa
and Cbfb, both required for proper myeloid development. Mu-
tations in CEBPA and CBFB in human acute leukemias block
this process at the blast stage (10, 25-29). Our data, supported
by chromatin immunoprecipitation (ChIP) and reporter analy-
ses, indicate that Cebpa and CBFB are repressed by the inhibi-
tory activity of GATA-1 on PU.1 in MEL cells, and that
increase in PU.1 levels could reverse this repression and lead
to MEL cell differentiation.

**FIGURE 1.** Conditional activation of PUER and GER in MEL cells restarts myeloid and erythroid program, respectively, and inhibits cell proliferation. MELPUER (A) and MELGER (C) cells were cultured in the presence or absence of 10^{-7} mol/L of 17β-estradiol (E) for the indicated time periods. Total RNA was purified and subjected to quantitative reverse transcription-PCR as described in Materials and Methods. Y-axis, mRNA expression of indicated
genes relative to housekeeping gene Gapdh. B. Aliquots of MELPUER cells stimulated for the indicated time periods were immunostained using antibodies
against Itgam, Ptprc, and Ly6g and control isotypic antibodies, as described in Materials and Methods. Y-axis, percentage of immunostained cells by flow
cytometry analysis. Bars, SE calculated for two independent experiments. Proliferation of MELPUER (D) and MELGER (E) cells unstimulated or stimulated by
17β-estradiol for 96 h. Y-axis, maximum of cell number (%); X-axis, time (days).
PUER Activation Results in Non-Erythroid Differentiation of MEL Cells

Previous work from our laboratory showed that activation of ectopic GATA-1-estrogen receptor (GER) induces GATA-1 target genes in MEL cells by a mechanism overriding the repressive block imposed by PU.1 on DNA (23). To determine whether PU.1, apart from its repressive function on GATA-1, could also activate its target genes directly on DNA in MEL cells, we first tested stable transfectants containing PU.1 cDNA driven by strong EF1α promoter (24) and observed mRNA upregulation of known PU.1 target genes, Itgam, Il7r, and Rag1 (data not shown). Second, we used MEL cells stably transfected with vector encoding inducible form of PU.1 fused to the ligand-binding domain of the estrogen receptor (PUER; ref. 18), in which the control MEL cells contained stable GER transgene (18).

Quantitative reverse transcription-PCR analysis confirmed our initial observation of activation of PU.1 target genes (Itgam, Cd14, Mpo, Cebpα, and Cbfβ) in MELPUER cells stimulated for 4 and 16 hours by 17β-estradiol (Fig. 1A). Stimulation of MELGER cells with 17β-estradiol at identical time points resulted in rapid upregulation of GATA-1 target genes: Nfe2, Zfp41 (Fig. 1C), Hba-a1, Hbb-b1, and Klf1 (Supplementary Fig. S1). Induced MELGER cells overtly hemoglobinized between 72 and 120 hours, whereas MELPUER cells remained pale in the floating cell fraction. Both MELPUER and MELGER cells significantly inhibited their proliferation rates following 48 hours of induction (Fig. 1D and E). Flow cytometry analysis of MELPUER cells revealed significant induction of myeloid surface markers Itgam (CD11b), Ptpc (CD45), and Ly6g (Gr-1) at indicated time points after 17β-estradiol treatment (Fig. 1B). These surface markers were not induced in MELGER cells stimulated for the same periods of time (data not shown). The MEL cells lacking a transgene, either untreated or treated with 17β-estradiol, grew exponentially with a similar doubling time (18). 17β-Estradiol did not affect PU.1 or GATA-1 expression levels in MEL cells indicating that, indeed, either the activated Puer or the activated GER transgene mediates the specific effects (18).

The presence of specific PU.1-dependent transcriptional responses and of protein markers associated with non-erythroid differentiation of MEL cells led us to examine the morphology of the cells following PUER induction (Fig. 2). Parental MEL cells (clone DS19) represent a heterogeneous cell population (diameter, 15-25 μm) of proerythroblasts (26%) and partially differentiated basophilic erythroblasts (74%), detected by May-Grünwald-Giemsa staining. A similar appearance of these respective populations was found in unstimulated MELPUER (23% and 77%, respectively) and MELGER cells (53% and 47%). As expected, erythroid differentiation induced within 72 hours in MELGER cells by 17β-estradiol induction resulted in a significant shift of the cell proportions toward the basophilic erythroblast stage (4% and 96%) which was also associated with the lack of observable mitoses. In turn, the cell line with 17β-estradiol–activated PUER (MELPUER) for 72 hours displayed a significant decrease in proerythroblasts (only 8%), little or no change in proportion of basophilic erythroblasts (64%), and lack of mitoses. Importantly, we also observed a new population of atypical cells (28%) with irregularly shaped nuclei and with mildly basophilic and azurophilic granular cytoplasm (see Fig. 2 and Supplementary Fig. S2 for summary and details; dashed arrows). These atypical cells stained positive for α-naphthyl butyrate esterase, and unlike MEL and MELGER cells, they poorly destained, retaining diffuse granular positivity after the addition of sodium fluoride (data not shown).

FIGURE 2. Distinct cell types are induced by PUER and GER transgenes from MEL cell line. MEL (A), MELPUER (B), and MELGER (C) cells were cultured in the presence of 10−7 mol/L of 17β-estradiol for 72 h, fixed, cyto-spinned, and stained using May-Grünwald-Giemsa. Microscopy (Olympus BX-51 apparatus, E-410 Camera) was done under x1,000 magnification according to the manufacturer’s recommendations. Black arrow, proerythroblasts; dashed arrows, basophilic erythroblasts; empty arrows, atypical cells. The table displays the distribution of these cell types in MEL, MELPUER, MELGER induced with 17β-estradiol for 3 d, MELGER, and MELGER cells induced with 17β-estradiol for 3 d.

<table>
<thead>
<tr>
<th></th>
<th>MEL</th>
<th>MELPUER</th>
<th>MELPUER +E</th>
<th>MELGER</th>
<th>MELGER +E</th>
</tr>
</thead>
<tbody>
<tr>
<td>proerythroblasts</td>
<td>26</td>
<td>23</td>
<td>8</td>
<td>53</td>
<td>96</td>
</tr>
<tr>
<td>basophilic erythroblast</td>
<td>74</td>
<td>77</td>
<td>64</td>
<td>47</td>
<td>96</td>
</tr>
<tr>
<td>atypical</td>
<td></td>
<td>-</td>
<td>28</td>
<td></td>
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*Stopka et al., unpublished observation.*
shown). This observation indicates that these cells display similarities with monocytes.

Induced PUER Activates Its Endogenous Target Genes and Represses GATA-1 Targets in MEL Cells

As shown above, MEL cells can be differentiated by conditional GER and PUER activation into two distinct cell cycle–arrested populations within 96 hours. The mRNA and protein analyses revealed the induction of respective erythroid and non-erythroid expression programs. To identify complete profiles of PUER and GER activation in MEL cells, we used oligonucleotide expression arrays in biologically replicated profiling of MEL\textsuperscript{GER} or MEL\textsuperscript{PUER} cells stimulated with 10\textsuperscript{−7} mol/L of 17β-estradiol for the following time periods: 0, 2, 4, 8, 12, 16, 20, and 24 hours. The expression profiling data analysis identified 3,109 significant genes positively regulated by PU.1 and negatively regulated by GATA-1 (Supplementary Fig. S3A; Fig. 3A, left) and 4,292 significant genes positively regulated by GATA-1 and negatively regulated by PU.1 (Supplementary Fig. S3B; Fig. 3A, right). Subgroups of these genes encode critical lineage-specific regulatory molecules: transcription factors that are repressed in MEL cells and rapidly induced in MEL\textsuperscript{PUER} cells, but not in MEL\textsuperscript{GER} cells, including Cebpa, Cbfb, and also its established partner Runx1 (Fig. 3B; ref. 30). Runx1 cooperates with Cbfb on DNA and its expression is required for normal myelopoiesis, whereas its mutations predispose to leukemia (31-33). We also observed specific PU.1-dependent upregulation of Meis1, a known heterodimeric partner of Hoxa9, which is involved in myelopoiesis and also in leukemias harboring translocations with the mixed lineage leukemia gene (34, 35). PUER, but not GER, upregulated inhibitor of DNA binding 2, a known modulator of PU.1 and GATA-1 activities via interaction with transcription factor PU.1 (36); PUER also induced Ets1, another Ets family protein. We have also identified a subgroup of genes regulated by PUER, but not by GER, which belong to previously characterized differentiation-associated hematopoietic markers (Fig. 3B, bottom). Examples include markers of monocytes (Cd14 and Itgam), granulocytes (Mpo and Mmp9), and lymphocytes (Il7r and Thy1). An expanded set of the hematopoietic candidate targets of PU.1 and GATA-1 that are mutually inversely regulated is shown in Supplementary Fig. S3C to G. In addition, expression analysis also indicates that PU.1 and GATA-1 regulate two distinct sets of cell cycle genes involved in transcription factor–induced cell cycle arrest (Supplementary Fig. S3C-G). As shown in Fig. 3B, the majority of GATA-1 targets are already expressed in MEL cells, whereas the PU.1 targets are generally not expressed in MEL cells, indicating that the transcriptional program of PU.1 is markedly more inhibited than that of GATA-1, a fact also supported by the phenotypic appearance of MEL proerythroblasts (see Fig. 2; Supplementary Fig. S2).

Activated PUER Induces Active Chromatin Structure Near Cebpa and Cbfb Genes, whereas Activated GER Inhibits This Effect

Our data indicated that PU.1 activates a large set of genes in MEL cells and inhibits a set of GATA-1 target genes. In contrast, activated GER induced genes inhibited by PUER (Fig. 3B). Among these genes were transcription factors...
previously associated with normal and leukemia cell differentiation. We have focused on key hematopoietic transcription factors Cbfβ and Cebpa to determine the mechanism by which they are targeted and regulated by PU.1 and GATA-1. First, we measured the co-occupancy of PU.1 and GATA-1 by quantitative ChIP (qChIP) assay that covered 1-kb intervals of the selected genes (ranging from 10 kb upstream to 10 kb downstream relative to the transcription start site). Our data show co-occupancy of PU.1 and GATA-1 near their PU.1 binding sites at both Cbfβ (Fig. 4A) near the PU.1 binding site at +1531 kb downstream of the transcription start site and Cebpa (Fig. 4B) near the PU.1 binding site at −2977 kb upstream of the transcription start site (see Tables 1 and 2; Supplementary Fig. S4E and F). Co-occupancy of PU.1 and GATA-1 near
Table 1. GATA-1 and PU.1 Binding Sites Near Zfpm1, Nfe2, Cbfb, and Cebpa Genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amplicon (+/− bp Relative to TSS)</th>
<th>Binding Site (+/− bp Relative to TSS)</th>
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<tbody>
<tr>
<td>Zfpm1</td>
<td>3537 to 3630</td>
<td>+3579, AGATAA</td>
</tr>
<tr>
<td>Nfe2</td>
<td>−2223 to −2111</td>
<td>−1589, AGATAA</td>
</tr>
<tr>
<td></td>
<td>−1502 to −1390</td>
<td>−1532, AGATAG</td>
</tr>
<tr>
<td></td>
<td>−1084 to −966</td>
<td>−1525, AGATAA</td>
</tr>
<tr>
<td></td>
<td>−475 to −359</td>
<td>−1052, AGATAG</td>
</tr>
<tr>
<td>Cbfb</td>
<td>22 to 194</td>
<td>−804, AGATAG</td>
</tr>
<tr>
<td>Cebpa</td>
<td>1812 to 1913</td>
<td>+1531, GAGGAAGCT</td>
</tr>
<tr>
<td></td>
<td>−3260 to −3170</td>
<td>−2978, GAGGAGAG</td>
</tr>
</tbody>
</table>

NOTE: Numbers indicate their relative position to the transcription start site.

these early response gene targets, Cbfb and Cebpa, indicated that PU.1 and GATA-1 might regulate the expression of these target genes directly by a mechanism that involves regulatory changes in chromatin structure. This notion is also supported by studies showing that GATA-1 does not block binding of PU.1 to its DNA binding site (20, 21). To test this hypothesis, we have determined the levels of histone H3K9 acetylation near the candidate genes as this dynamic modification of chromatin was previously correlated with mutual antagonism of GATA-1 and PU.1 and with its disruption (23). As shown in Fig. 4A and B, PUER stimulation resulted in an increase of H3K9 acetylation near Cbfb and Cebpa genes (up to 2-fold and 3- to 4-fold, respectively) in comparison with GER unstimulated MEL cells (data not shown). The direction of the H3K9 acetylation pattern correlated with the expression levels of the genes, and the chromatin change was not observed in other portions of the selected genes, indicating that the regulatory regions responded specifically to the activated (PUER and GER) transgenes.

We then asked whether these PU.1 binding/response regions were functional in reporter assays. The PU.1 binding sites (see Materials and Methods) were subcloned into pGL3 reporter vector (see Table 2) encoding luciferase gene (Promega), transfected into MELPUER cells, and stimulated with 17β-estradiol for 48 hours. As shown in Fig. 4A and B (right), activated PUER in MEL cells stimulates the luciferase activity of these vectors, indicating that they respond to PU.1. The reporters were transfected into MELPUER (Fig. 4C) and MELGER (Fig. 4D) and stimulated for 72 hours, followed by analysis of luciferase activity (data not shown) and by transfected qChIP (details in Materials and Methods) using antibodies to PU.1 and GATA-1. The data indicate that PU.1 binds and transcriptionally stimulates the specific PU.1 DNA regions downstream of PU.1 binding sites Cbfb (+1531) and Cebpa (+2978) cloned in reporter plasmids. The data also indicate that GATA-1 could occupy these regions in the presence of PU.1. However, induction of MELPUER results in the depletion of GATA-1 from its association with PU.1 on DNA (Fig. 4E and F, right) followed by derepression of luciferase expression (data not shown).

Next, we used synthetic trimeric and pentameric PU.1 binding sites cloned into luciferase vectors (PU3x and PU5x) that were previously used to show PU.1 activation as well as its repression by GATA-1 in reporter assays (21). Supplementary Fig. S4A to D shows that the reporter vectors PU3x and PU5x are stimulated by PU.1 factor cotransfected into HeLa cells (Supplementary Fig. S4A-D) or induced as PUER in MEL cells by 17β-estradiol (Supplementary Fig. S4B). Supplementary Fig. S4C and D show that GATA-1 can inhibit PU.1 on its binding site in PU3x or PU5x and override the effect of transcription factor c-Jun, a known coactivator of PU.1 on DNA (20).

Activated GER Induces Active Chromatin Structure Near Zfpm1 and Nfe2 Genes, whereas Activated PUER Inhibits This Effect

The GATA-1 binding region near Nfe2 gene contains five putative GATA-1 binding sites (−1589, −1532, −1525, −1052, and −804 bp relative to the transcription start site; see Tables 1 and 2; Supplementary Fig. S5E). This GATA-1 binding region was previously shown to be negatively regulated by PU.1 or GATA-1 on DNA, which resulted in significant depletion of acetylated H3K9 (23). We used this region as a positive control (Fig. 5B) and tested whether another putative GATA-1 target gene, Zfpm1, was directly repressed by PUER and directly activated by GERA. A qChIP assay done at a relatively large portion of the Zfpm1 gene revealed significant co-occupancy of GATA-1 and PU.1 (Fig. 5A) near the GATA-1 binding site at the 3′ portion of Zfpm1 gene (+3579; see Tables 1 and 2; Supplementary Fig. S5D). Furthermore, induction of MELPUER cells resulted in marked deacetylation near Zfpm1 and Nfe2 genes. An inverse pattern was observed at Nfe2 and Zfpm1 genes after the induction of GER (Fig. 5A and B). Subcloned GATA-1 binding sites were then tested using reporter assays in both MELGER and HeLa cells, and these experiments revealed that they are indeed induced by GATA-1 and that its effect was blocked dose-dependently by PU.1 (see Fig. 5, right; Tables 1 and 2). As controls, we used luciferase reporter constructs

Table 2. Oligonucleotide Sequences of the Cloned Regions of Mouse Zfpm1, Nfe2, Cbfb, and Cebpa Genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligo Sequence</th>
<th>Relative Position from TSS</th>
</tr>
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<tbody>
<tr>
<td>Zfpm1</td>
<td>CTCTTTGAGAAATAGATCGTGAGATAAGCTCAGGCGAGTCTACAGGAGGCG</td>
<td>+3557 to +3606</td>
</tr>
<tr>
<td>Nfe2</td>
<td>TTGATATCTCTGATATCTCTGTTCAGTACGAGTTAGAGGAGAGGAGA</td>
<td>Three segments: −1583 to −1559, −1537 to −1515, and −1055 to −1042</td>
</tr>
<tr>
<td></td>
<td>−1052, AGATAG</td>
<td>−828 to −818, −807 to −796, −687 to −676, −428 to −417, and −327 to −316</td>
</tr>
<tr>
<td>Cbfb</td>
<td>TGAAATATAGGACTATCGCTTCCTGCTCCCTCCCCCTCGTATACCT</td>
<td>−1509 to +1559</td>
</tr>
<tr>
<td>Cebpa</td>
<td>CTCCTGTACCGGACCTCCCTGCCGAGGAAGGAGGGGTTGGAGGAAAGGAAAGCTTGG</td>
<td>−2999 to −2949</td>
</tr>
</tbody>
</table>

NOTE: Numbers indicate their relative position to the transcription start site.
containing the chicken α-globin promoter fragment αD3 and its mutant αD4 (see Supplementary Fig. S5A-C). Significant co-occupancy of GATA-1 and PU.1 near GATA-1 binding sites near Zfpm1 and Nfe2 was determined in unstimulated MEL<sub>G4E</sub> cells. Levels of H3K9 hyperacetylation in these cells in the presence of 10<sup>−7</sup> mol/L of 17β-estradiol for 24 h (third graphs) was determined relative to acetylation determined in stimulated MEL<sub>PUER</sub> cells under the same conditions. Primary binding sites of GATA-1 are functional in reporter assays. A. Right, HeLa cells were transfected with reporter plasmid [Zfpm1, 0.25 μg (R)] and cDNA constructs [pXM-GATA-1, 0.125 μg (G) and pXM-PU.1, 0.125 μg (P); 0.375 μg (3P); 1.125 μg (9P)]. B. Right, MEL<sub>GER</sub> cells were lipofected with Nfe2 reporter plasmid (1.7 μg) and either unstimulated (−E) or further stimulated with 17β-estradiol after 24 h (+E) followed by measurement of luciferase activity at 72 h (for details, see Materials and Methods). C. MEL<sub>PUER</sub> cells were either stimulated with 17β-estradiol (+E) or treated with PU.1-inhibiting siRNAs (si) for 48 h, and GATA-1 and PU.1 occupancy was detected by qChIP near Nfe2 (0 kb) and Zfpm1 (+3.5 kb) genes. All ChIP bars in C indicate the fold change (Y-axis) of DNA fragment in specific immunoprecipitates above the immunoprecipitates using control antirabbit IgG antibody. Bars, SE of at least two independent experiments.

Inhibition of GATA-1 Results in Non-Erythroid Differentiation of MEL Cells and Does Not Affect the Binding of PU.1 to Cebpa and Cbfb

Our data indicate that the transcription program of PU.1 is inhibited by GATA-1 on DNA, and that on activation of PU.1 by using PUER transgenes, we achieved efficient non-erythroid differentiation. To test whether GATA-1 is responsible for the inhibition of PU.1, we used siRNA oligonucleotides and inhibited GATA-1 expression and measured selected PU.1 target gene expression after 48 hours. As shown in Fig. 6A, inhibition of GATA-1 levels below 5% in MEL cells resulted in efficient derepression of the PU.1 target genes. Derepression of Chib and Cebpa genes seems to be more efficient after GATA-1 siRNA-mediated knockdown compared with stimulation of MEL<sub>PUER</sub> cells by 17β-estradiol, supporting our observation of GATA-1-mediated repression of PU.1 target genes in MEL cells. In addition, derepression of Itgam and Cdl4 genes after GATA-1 inhibition was achieved with slightly decreased efficiency compared with stimulation of PUER, indicating that full activation...
of Itgam and Cd14 might require additional PU.1 cofactors (such as Cebpa and Cbfb) at later time points. Conversely, PU.1-specific siRNA treatment resulted in the accumulation of mRNAs from GATA-1 target genes, Zfpm1 and Nfe2 (Fig. 6B). Our data indicate that GATA-1 is required in MEL cells to hold the repressive state of PU.1 target genes (see also Fig. 3), that both mechanisms of PU.1 repressing GATA-1 and of GATA-1 repressing PU.1 are functional in MEL cells, and that leukemia differentiation could be achieved by manipulating either of these two factors.

To test the mechanism by which PUER overcomes GATA-1-mediated repression on DNA, the occupancy of PU.1 near Cebpa and Cbfb genes was tested in either stimulated MELPUER cells (48 hours) or unstimulated MELPUER cells with knockdown of GATA-1. The qChIP technique using antibodies to PU.1 and GATA-1 was carried out as described in Materials and Methods. Activation of PUER (see Fig. 4E, +E on X-axis) or inhibition of GATA-1 by siRNA resulted in loss of GATA-1 occupancy at the PU.1 binding sites near Cebpa and Cbfb genes. Manipulation of GATA-1 or PU.1 did not result in a loss of PU.1 occupancy at these sites. These data strongly suggest that PUER overrides GATA-1-mediated repression of Cebpa and Cbfb by binding to DNA and that GATA-1 is not able to further associate with these regions. In addition, siRNA knockdown-mediated depletion of GATA-1 and its decreased occupancy near Cebpa and Cbfb genes results in transcriptional activation of Cebpa and Cbfb genes.

Discussion

PU.1 and GATA-1 are key transcription factors controlling the myelo-erythroid and myelo-lymphoid development of hematopoietic multipotential progenitors (4). PU.1 re-expression in PU.1−/− multipotential progenitors is sufficient to repress the program of GATA-1 by recruiting a repressive protein complex on its target genes (23). The next step of PU.1-dependent differentiation involves interactions with the PU.1-cooperating transcription factors Cebpa, Nab2, Gfi1, and Egr2 to progress further into the granulocyte-macrophage progenitor stage (5). In contrast, induction of erythroid differentiation in multipotential progenitors is followed by downregulation of PU.1 (37), a process that is likely prevented from completion in MEL cells by sustained low expression of PU.1 (16).

Our data support the hypothesis that PU.1-mediated block of GATA-1 in MEL cells resembles the block of erythroid differentiation that mediates PU.1 during early myeloid commitment of multipotential progenitors (23). The data show that MEL cells retain their multipotentiality and responsiveness to PU.1 and GATA-1 (see Fig. 3; Supplementary Fig. S4), and that PU.1 activity at the lineage commitment crossroads could redirect the erythroid commitment decision and drive the nonerythroid (monocyte-like and mixed) differentiation and cell cycle arrest of MEL cells. The manipulation of transcription factors PU.1 or GATA-1 that are directly involved in commitment decisions thus harbors a therapeutic potential for treating human leukemias. Our other data show that retroviral PU.1 rescue of several cellular systems of human acute leukemias that retained PU.1 and GATA-1 coexpression (NB4, K562 cell lines) or lacking their expression (U937) successfully induced differentiation and cell cycle arrest concomitant with the re-expression of multiple PU.1 target genes.8 These data are supported by experiments reported in other human leukemia cell systems (38, 39).

We report a strategy of using MEL cells stimulated with mutually antagonizing transcription factors PU.1 or GATA-1 that
enabled us to identify mutually opposite gene expression programs regulating leukemia differentiation in a lineage-specific manner. These distinct programs are regulated at the chromatin DNA level, and whether and how these programs are propagated is determined by molecular interaction of GATA-1 and PU.1 at specific DNA-binding sites distributed near genes encoding transcription factors required for lineage specification such as Nfe2, Zfp1m1, Cebpα, and Cbfb. This is in agreement with the studies indicating the critical and indispensable roles of Cebpα, Cbfb, Nfe2, and Zfp1m1 genes for normal hematopoietic lineage specification (25-28). Furthermore, Cebpα and Cbfb are often mutated (10, 25-29) or epigenetically dysregulated (40) in human acute leukemias. Our data collectively indicate that leukemia differentiation in blocked leukemic MEL blasts is decided by a stoichiometric balance between PU.1 and GATA-1, the individual levels of which are sufficient to initiate specific changes of chromatin structure within their target genes that retained flexible responsiveness. The mechanisms of PU.1 and GATA-1, in a leukemic state and upon leukemia differentiation, involve the following putative steps: in myeloid genes such as Cebpα, PU.1 binds directly to DNA but is repressed by GATA-1 that binds directly to PU.1 molecules on DNA. Activation of PUER and stable levels of GATA-1 create excess of available PU.1, which is not paired by available GATA-1 on DNA, thus allowing gene activation. Similarly, in erythroid genes such as Nfe2, GATA-1 is bound to DNA but is repressed by PU.1 that binds to this GATA-1 molecule. Activation of GER creates an excess of available GATA-1, which is not paired on DNA by available PU.1, also allowing gene activation. Our mechanistic study implicates that transcription factor manipulation, such as inhibition of GATA-1 or activation of PU.1 in erythroleukemias, may represent an efficient tool of inducing leukemic blast differentiation.

Materials and Methods

Cell Cultures

MEL and HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics as described previously (22-24). The PUER and GER conditional activation in MEL cells containing these transgenes (MELPUER and MELGER) was induced by 10^{-7} mol/L of 17β-estradiol.

Extraction of Total RNA

Total cellular RNA was purified with modified TRIzol reagent (Invitrogen) involving enhanced precipitation by adding 1 vol of isopropanol to the extracted aqueous phase, precipitating at −20°C overnight, and centrifuging the RNA for 30 min at 14,000 rcf at 4°C. The concentration, purity, and integrity of total RNA were determined by NanoDrop ND-1000 and Agilent 2100 Bioanalyzer.

Microarray mRNA Profiling and Data Analysis

mRNA profiling in MELPUER and MELGER cells in a biologically duplicate experiment was done using Affymetrix Mouse Genome arrays MG-430A 2.0 containing 22,694 probes, following the one-cycle labeling protocol and standardized array processing procedures recommended by Affymetrix. The raw data (CEL files) were normalized using robust multi-chip average algorithm in GeneSpring GX software and filtered in the TM4 suite using significance analysis of microarrays with the false discovery rate set to 1%. The microarray data are deposited at the European Molecular Biology Laboratory’s European Bioinformatics Institute (ArrayExpress accession: E-MTAB-125).

ChiPs for qChiP

Chromatin from 3 × 10^7 cells from MELPUER and MELGER cells expressing PU.1-estrogen receptor or GATA-1-estrogen receptor (PUER and GER) fusion protein (in the absence or presence of 10^{-7} mol/L 17β-estradiol for 24 h) was cross-linked with 1% formaldehyde for 10 min at room temperature. Subsequently, cells were lysed by a set of lysis buffers to isolate the nuclei from cells that were resuspended in 2 mL of low-salt buffer and sonicated (45% intensity, 500 cycles of 2 s, in an ice-ethanol cooling bath) with a Branson Sonic Dismembrator model 500 equipped with a microtip to yield 200 to 400 bp DNA fragments (22, 23). ChiP was done using antibodies against PU.1 (T21, Santa Cruz), GATA-1 (N6, Santa Cruz), and acetyl-histone H3 (Lys9; Upstate); normal rabbit IgG (Calbiochem) was used as a control nonspecific antibody. DNA extracted from the immunoprecipitates was used as the template for SYBR Green–based quantitative real-time PCR reactions as described below.

Transfected ChiP

Transient transfections into MELPUER and MELGER cells (10^6/mL) were carried out with the Lipofectamine 2000 reagent (Invitrogen) and the cells were simultaneously treated with 10^{-7} mol/L of 17β-estradiol. After 48 h, chromatin from ~10^6 cells was immunoprecipitated as described above.

Quantitative Real-time PCR

The quantity of specific DNA fragments in immunoprecipitates was determined by quantitative real-time PCR reactions. We used 7900 HT SDS PCR cycler with a 396-well configuration (Applied Biosystems). For the qChiP assays, 0.5 to 2 ng of immunoprecipitated DNA were amplified in each 1× SYBR Green Master Mix reaction (Applied Biosystems). All quantitative real-time PCR amplifications were done in 8-μL reaction volumes and consisted of 40 cycles of 10 s at 95°C, 20 s at 60°C, and 30 s at 72°C or in the presence of TaqMan probe (Roche) with the recommended protocol of 40 cycles of 15 s at 95°C and 1 min at 60°C. Fluorescence was read at both the annealing and polymerization steps. For each individual primer pair, a standard curve was generated using serial dilutions of the input DNA. The dissociation curve was determined for each PCR reaction to ensure the production of a single and specific product. Fluorescence was read in the exponential amplification phase (in case of SYBR Green reaction mix), and the raw data were expressed as CT values. Using a standard curve equation for each PCR primer pair, the CT values were transformed into DNA copy numbers. The copy number of a specific DNA fragment in each immunoprecipitate was compared with the copy number of that fragment in 1/100 dilution of the DNA obtained from the input sample used for immunoprecipitation (1% input DNA), and the “percentage of the input” was calculated. Percentage of input was also determined for each DNA fragment in immunoprecipitates using appropriate control antibodies (background), and these values were subtracted from the values
obtained with the specific antibodies (details are also given in refs. 22, 23).

Immunoblotting
MEL\textsuperscript{PUER} and MEL\textsuperscript{GER} cells (3 × 10\(^6\)) were cultured in medium containing 10\(^{-7}\) mol/L of 17\(\beta\)-estradiol for 3 d and lysed for immunoblotting analysis using radioimmunoprecipitation assay buffer supplemented with proteinase inhibitors. Denatured lysates (20 µg protein per lane) were resolved on 10% PAGE. The gels were stained with Coomassie dye for load control and wet-blotted onto polyvinylidene difluoride membranes. Blots were blocked in 7.5% nonfat milk-PBS-Tween 20. Primary antibodies were used at 1:500 dilution [anti-PU.1 (sc-352), anti–GATA-1 (sc-265; Santa Cruz), and anti–estrogen receptor \(\alpha\) (ab31949, Abcam)]. After washing with PBS-Tween 20, the membranes were stained with secondary horseradish peroxidase–labeled antibodies, followed by luminescence detection on X-ray films.

Transient Transfections and Reporter Assays
The putative GATA-1 binding sites in murine Zpml (+3579, AGATAA) and Nfe2 (−1588 and −1524 AGATAA; −1531, −1051 and −803 AGATAG) genes and the PU.1 binding sites in murine Cbfb (+1531, GAGGAACT) and Cebpa (−2977, GAGGAAGT) genes and their deletion mutants were subcloned into the pGL3-basic vector (Promega). Transient transfections into MEL\textsuperscript{PUER}, MEL\textsuperscript{GER}, and HeLa cells were carried out with the Lipofectamine 2000 reagent (Invitrogen). Firefly luciferase activity was measured ∼4 h after transfection using the Steady-Glo Luciferase Assay System (Promega) and shown as fold activation over background. Individual transfection experiments were done in duplicate and the results are reported as mean firefly fold activation ± SD.

Flow Cytometry
MEL\textsuperscript{PUER} and MEL\textsuperscript{GER} cells (1 × 10\(^5\)) induced with 17\(\beta\)-estradiol for the indicated time points were incubated with 2 µL of phycoerythrin-conjugated mouse monoclonal CD45 antibody (clone 30-F11, PharMingen), biotin-conjugated monoclonal CD11b antibody (clone M1/70, PharMingen), or biotin-conjugated monoclonal Gr-1 antibody (clone RB6-8C5, PharMingen). Biotinylated CD11b and Gr-1 antibodies were visualized with 2 µL of streptavidin-phycoerythrin (PharMingen). Flow cytometry analyses were done on an Aria cell sorter (BD).

SiRNA Inhibition
MEL\textsuperscript{PUER} cells were cultured in DMEM supplemented with 10% fetal bovine serum. SiRNA (Ambion) complementary to GATA-1 or negative control siRNA (Ambion) was transfected using the Lipofectamine 2000 reagent (Invitrogen). After 48 h, cells were harvested and total RNAs were isolated and analyzed by quantitative real-time PCR as described above. Custom siRNA (Qiagen) complementary to PU.1 (5′-ggaggugcugag-gagaa-3′) with 3′ dTdT overhang.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
27. Stopka T, Amanatullah DF, Papetti M, Skoulthci AI. PU.1 inhibits the erythroid program by binding to GATA-1 on DNA and creating a repressive chromatin structure. EMBO J 2005;24:3712–23.
PU.1 Activation Relieves GATA-1–Mediated Repression of *Cebpa* and *Cbfb* during Leukemia Differentiation

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