Histone demethylase LSD1-mediated repression of GATA-2 is critical for erythroid differentiation

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Introduction

GATA-1 and GATA-2 are two important transcription factors that regulate hematopoietic lineage commitment and differentiation. While GATA-1 is mainly expressed in differentiated hematopoietic lineages, such as erythroid, megakaryocytic, eosinophilic, and mast cells, GATA-2 is mainly expressed in hematopoietic stem and progenitor cells. The dynamic expression of GATA-1 and GATA-2 is critical for erythroid differentiation, and this is termed as “GATA factor switching”. Transcription of GATA-2 can be initiated from two different first exons, ie, the proximal first exon (1G promoter) and the distal first exon (1S promoter). While the 1G promoter and 1S promoter are mainly located in hematopoietic and progenitor tissues. At the early stage of erythroid differentiation, GATA-2 binds to five GATA motifs in the GATA-2 locus (~77 kb, ~3.9 kb, ~2.8 kb, ~1.8 kb, and +9.5 kb from the 1S promoter) and promotes its own expression. At the late stage of erythroid differentiation, GATA-1 replaces GATA-2 and binds to these GATA motifs in the GATA-2 locus, which inhibits...
\textit{GATA-2}} expression.\textsuperscript{7,8} Thus, \textit{GATA-2} expression is regulated by both \textit{GATA-2} and \textit{GATA-1} during erythroid differentiation. In addition, \textit{GATA-2} transcription can be regulated by several other transcription factors, including V-\textit{ets} ETS1,\textsuperscript{14} BMP4,\textsuperscript{15} NOTCH1,\textsuperscript{16,17} PU.1,\textsuperscript{18} and EVI1,\textsuperscript{19,20} and cytokines, such as IL-1 and TNF\textsubscript{\alpha}.\textsuperscript{21}

Histone modifications, including acetylation, phospho-
rylation, methylation, and ubiquitination, play important roles in altering chromatin structure and regulating gene expression.\textsuperscript{22–24} Lysine methylation is an important histone modification that marks either negative or positive expression of genes, depending on the position of the lysine and the number of methyl moieties.\textsuperscript{25,26} For instance, genes that are repressed are generally associated with methylations at lysine 9 or 27 of histone H3 (H3K9 and H3K27), whereas actively transcribed genes are marked by methylations at the H3K4, H3K36, and H3K79 residues.\textsuperscript{27} Epigenetic changes are reversible, and chromatin modifiers are often proteins with enzymatic activities. Lysine methylation is regulated by the opposite activities of lysine methyltransferases (KMTs) and lysine demethylases (KDMs).\textsuperscript{28–33} KDMs consist of the FAD-dependent lysine-specific demethylases, LSD1 and LSD2,\textsuperscript{34–36} as well as the Jumonji C domain-containing histone demethylases.\textsuperscript{29} LSD1 is the first identified histone demethylase and forms a core complex with corepressors, including HDAC1/2 and CoREST, and represses the transcription of target genes.\textsuperscript{37} LSD1 is crucial for a variety of cellular processes, such as embryonic stem (ES) cell pluripotency, cellular proliferation, and differentiation.\textsuperscript{38–42} In addition, abnormal expression of LSD1 in hematopoietic progenitors perturbs the development of several hematopoietic lineages, suggesting that LSD1 is essential in hematopoietic development.\textsuperscript{43} We previously demonstrated that LSD1 cooperates with TAL1 to regulate the transcription of the \textit{P4.2} gene.\textsuperscript{44} In this study, we report that LSD1 associates with TAL1 to regulate the expression of the \textit{GATA-2} gene during erythroid differentiation.

\textbf{Materials and methods}

\textbf{Plasmids and antibodies}

Plasmids pSUPERretro vector and pSUPERretro-shLSD1 were gifts from Dr Suming Huang at the University of Florida. Anti-LSD1 and anti-TAL1 antibodies (ab17721, ab138276) were purchased from Abcam plc (Cambridge, UK).

\textbf{Cell culture, transfection, and short hairpin (sh)RNA-mediated knockdown}

Murine erythroleukemia (MEL) cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) and induced to differentiation with dimethyl sulfoxide (DMSO) as previously described.\textsuperscript{45} Mouse ES cells were cultured in DMEM and induced to differentiation with erythropoietin (EPO) as previously described.\textsuperscript{46} Retroviral packaging and infection were performed as previously described.\textsuperscript{35} To generate stable LSD1-knockdown cell lines, 5×10\textsuperscript{5} exponentially growing MEL or ES cells were cultured with virus particles in the presence of Polybrene\textsuperscript{®} (4 μg/mL) for 24–48 hours followed by puromycin selection (1 μg/mL) for 2 weeks.

\textbf{Chromatin immunoprecipitation (ChIP) and Double ChIP}

Real-time polymerase chain reaction (RT-PCR)-based quantitative ChIP analysis and Double ChIP were performed as previously described.\textsuperscript{47} The RT-PCR primer sequences have been listed in Table S1.

\textbf{RNA isolation and quantitative RT-PCR}

Total RNA was isolated using the SV Total RNA Isolation System kit (Promega Corporation, Fitchburg, WI, USA), and reverse transcription was performed according to the manufacturer’s instructions (Invitrogen; Life Technologies Corp, Carlsbad CA, USA). SYBR Green quantitative polymerase chain reaction (PCR) was performed as previously described.\textsuperscript{48} The RT-PCR primer sequences have been listed in Table S2.

\textbf{Western blot analyses}

For the western blot analysis, whole-cell extracts were prepared by boiling cells for 10 minutes in sodium dodecyl sulfate (SDS) sample buffer (50 mM tris(hydroxymethyl) aminomethane [Tris] [pH 6.8], 100 mM dithiothreitol [DTT], 2% SDS, 0.1% bromophenol blue, 10% glycerol) and were resolved using SDS–polyacrylamide gel electrophoresis (PAGE). Primary antibodies were detected using horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies and visualized using enhanced chemiluminescence detection (ECL) (ECL reagents were from Thermo Fisher Scientific Inc, Waltham, MA, USA). Western blot analysis of β-actin (Thermo Fisher Scientific Inc) was used as a loading control.

\textbf{Differentiation and proliferation assays}

For the differentiation assay, MEL cells were treated with DMSO to induce differentiation and stained with benzidine blue (for hemoglobin), and benzidine blue–positive cells were quantified at day 3 and 5 of DMSO treatment. The proliferation of cells was determined using alamarBlue® assays (Invitrogen; Life Technologies Corp). Briefly, 1×10\textsuperscript{5} cells were seeded in a 96-well plate with 100 μL of DMEM and cultured for 10 days.
Next, 10 μL (1/10 volume) of alamarBlue reagent was directly added to the cells in culture medium and incubated for 4 hours at 37°C. The absorbance at optical density (OD) 570 nm and OD 600 nm were monitored, and the relative proliferation was calculated according to the manufacturer’s instructions.

**Statistical analysis**

The statistical analysis for data was performed employing the SPSS software package 19.0 (IBM Corporation, Armonk, NY, USA). Student t-test and one way analysis of variance (ANOVA) were employed for two groups and multiple group comparison. Data were presented as mean ± standard deviation (SD). \( P < 0.05 \) represented a statistically significant difference.

**Results**

**Knockdown of LSD1 inhibits the differentiation of MEL cells**

To investigate the function of LSD1 in erythroid differentiation, we generated stable LSD1-knockdown clones from MEL and ES cells using shRNA-mediated gene silencing (Figure 1A and B). The LSD1-knockdown cells were induced to differentiate and were stained with benzidine to detect hemoglobin production. In our experiments, we performed at least three technical replicates with the one optimal MEL cell clone and one optimal ES cell clone. Compared with control cells, the LSD1-knockdown cells showed a significantly lower percentage of benzidine-positive cells \( (P < 0.05) \) (Figure 1C), demonstrating that knockdown of LSD1 inhibited the differentiation of MEL cells into hemoglobin-producing cells. In addition to the inhibition of erythroid differentiation, knockdown of LSD1 also significantly inhibited MEL cell proliferation \( (P < 0.05) \) (Figure 1D) and resulted in increased cell death \( (P < 0.05) \) (Figure 1D and E) compared with the control cells.

Furthermore, we also detected the effects of LSD1-knockdown in ES cells. Our results also indicated that LSD1-knockdown inhibits EPO-induced ES cell differentiation to erythroid cell lineage (with the same changes in the MEL cells [data not shown]).

**Knockdown of LSD1 results in the decreased expression of GATA-1 and increased expression of GATA-2 in differentiated cells**

Because \( GATA-1 \) and \( GATA-2 \) are two master transcription factors in hematopoietic differentiation, we examined the
expression of GATA-1 and GATA-2 in MEL and EPO-induced differentiated ES cells. While normal MEL and ES cells showed low levels of GATA-2 and high levels of GATA-1 expression upon induced differentiation, knock-down of LSD1 resulted in an increased expression of GATA-2 and decreased expression of GATA-1 (Figure 2A and B). More importantly, while GATA-1 was the dominant form of the GATA switch in differentiated MEL and ES cells, GATA-2 became the dominant form of the GATA switch in LSD1-knockdown cells, demonstrating an essential role of LSD1 in regulating the expression of GATA switch factors (Figure 2C). Consistent with the decreased expression of the GATA-1 gene in LSD1-knockdown cells, LSD1-knockdown cells showed decreased expression of β-major, a well-established erythroid differentiation marker (P<0.05). Meanwhile, the western blot analysis results indicated that the LSD knockdown (both in MEL and ES cells) also significantly increased the GATA-2 protein levels and decreased the GATA-1 levels compared with the blank vectors (both in MEL and ES cells) (all \( P<0.05 \)) (Figure S1).

Furthermore, we also detected the GATA-2 or GATA-1 levels in the undifferentiated MEL and ES cells. Our preliminary results indicated that there were no differences in GATA-2 or GATA-1 levels in the MEL and ES cells before induction of differentiation (\( P>0.05 \)) (Figure S2).

**LSD1 colocalizes with TAL1 at the 1s promoter of the GATA-2 locus**

Within the GATA-2 locus, multiple sites have been suggested to be involved in the regulation of GATA-2 expression (Figure 3A).\(^9,10\) To study the molecular mechanism underlying LSD1 regulation of GATA-2 expression during erythroid differentiation, we examined the enrichment of LSD1 at

![Figure 2](image-url)  
**Figure 2** Knockdown of LSD1 results in decreased expression of GATA-1 and increased expression of GATA-2 in differentiated cells.  
**Notes:** (A) MEL cells were treated with DMSO for 5 days to induce differentiation, and the expression of GATA-1, GATA-2, and β-major mRNA was measured by reverse-transcription quantitative PCR from the differentiated cells. (B) ES cells were treated with EPO for 9 days to induce differentiation. Expression of GATA-1, GATA-2, and β-major mRNA was measured by reverse-transcription quantitative PCR from the differentiated cells. (C) The relative expressions of GATA-1, GATA-2, and β-major in MEL and ES cells from (A) and (B) were quantified (n=3) for the “relative mRNA level”, which was analyzed as the GATA-2 or GATA-1 level normalized to β-actin gene. *\( P<0.05 \) represents the GATA-2, GATA-1, or β-major mRNA transcription in the LSD1-knockdown group (MEL or ES cells) compared with the control vector group (MEL or ES cells). This experiment was repeated at least for three times.  
**Abbreviations:** DMSO, dimethyl sulfoxide; EPO, erythropoietin; ES, embryonic stem; LSD1-kd, LSD1-knockdown; MEL, murine erythroleukemia; mRNA, messenger RNA; PCR, polymerase chain reaction; Vector, vector control.
Figure 3 LSD1 colocalizes with TAL1 at the 1S promoter of the GATA-2 locus.

Notes: (A) Schematic representation of the GATA-2 locus. Cross-linked chromatin from MEL cells was precipitated using either (B) an anti-LSD1 or (C) anti-TAL1 antibody, and analyzed by PCR with primers specific to the indicated sites of the GATA-2 locus. (D) Cross-linked chromatin from MEL cells treated or untreated with DMSO was immunoprecipitated with an anti-TAL1 antibody and subjected to subsequent immunoprecipitation using an anti-LSD1 antibody. The precipitated DNA was analyzed by PCR with primers specific to the indicated sites in the GATA-2 locus. The “relative fold enrichment” refers to the value of LSD1 normalized to the IgG control. This experiment was repeated at least for three times.

Abbreviations: ChIP, chromatin immunoprecipitation; DMSO, dimethyl sulfoxide; Ig, immunoglobulin; MEL, murine erythroleukemia; PCR, polymerase chain reaction.

different regulatory sites in the GATA-2 locus using ChIP assay and found that LSD1 was mainly recruited to the 1S promoter of the GATA-2 locus in MEL cells (Figure 3B). Because LSD1 does not have a DNA-binding domain, we hypothesized that LSD1 is recruited by sequence-specific transcription factors to the GATA-2 locus. It has been reported that TAL1 recruits LSD1 to regulate gene transcription in normal and malignant hematopoiesis. Thus, we reasoned that TAL1 might also recruit LSD1 to the 1S promoter of the GATA-2 locus during erythroid differentiation. To test this hypothesis, we performed ChIP assays using the TAL1 antibody and found that TAL1 bound predominantly to the 1S promoter in the GATA-2 locus (Figure 3C). To further test whether LSD1 colocalizes with TAL1 at the 1S promoter, we performed TAL1 and LSD1 sequential ChIP assays. Expectedly, LSD1 and TAL1 co-occupied the 1S promoter, suggesting that TAL1 recruited LSD1 to the GATA-2 1S promoter (Figure 3D).

LSD1-knockdown increases H3K4me2 at the GATA-2 locus

LSD1 is an important histone demethylase. Our results showed that the enrichment of LSD1 was increased at the 1S promoter in the GATA-2 locus (Figure 3D), suggesting that LSD1 might decrease H3K4me2 at the 1S promoter via its lysine demethylases activity. Thus, we investigated the alteration of histone methylation levels at the GATA-2 locus during erythroid differentiation. The result of ChIP assays
demonstrated that H3K4me2 was significantly decreased at −2.8 kb, 1S promoter, and 1G promoter in the GATA-2 locus in DMSO-treated MEL cells on day 3 compared with that on day 0 (P<0.05) (Figure 4A). The decreased H3K4me2 at the GATA-2 locus, particularly at the 1S promoter, was correlated with the decreased expression of GATA-2 during erythroid cell differentiation, suggesting that LSD1 might inhibit the expression of GATA-2 via histone modification.

To confirm the role of LSD1 in the regulation of H3K4me2 at the GATA-2 locus during erythroid differentiation, we performed ChIP analysis for H3K4me2 enrichment at the GATA-2 locus in LSD1-knockdown MEL cells. H3K4me2 levels were significantly increased in the LSD1-knockdown cells compared with the control vector cells (P<0.05) (Figure 4B), demonstrating that LSD1 is required for the decreased H3K4me2 at the GATA-2 locus during erythroid differentiation.

**Discussion**

The dynamic expression of GATA-1 and GATA-2 is crucial for erythroid differentiation. Our results, together with previous findings, led us to propose a novel epigenetic mechanism for the regulation of GATA-2 expression. During erythroid differentiation, LSD1 is recruited to the GATA-2

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**Figure 4** LSD1-knockdown causes increased H3K4me2 at the GATA-2 locus.

**Notes:** (A) MEL cells were treated with DMSO for the indicated number of days. Cross-linked chromatin DNA was immunoprecipitated with the anti-H3K4me2 antibody and analyzed by PCR with primers specific to the indicated sites in the GATA-2 locus. (B) Cross-linked chromatin DNA from MEL cells with or without LSD1-knockdown was immunoprecipitated with an anti-H3K4me2 antibody and analyzed by PCR with primers specific to the indicated sites in the GATA-2 locus. # P<0.05 represents the relative fold enrichment on day 3 compared with that on day 0. * P<0.05 represents the relative fold enrichment in LSD1-knockdown cells compared with the blank vector cells. This experiment was repeated at least for three times.

**Abbreviations:** DMSO, dimethyl sulfoxide; LSD1-kd, LSD1-knockdown; MEL, murine erythroleukemia; PCR, polymerase chain reaction.

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**Figure 4:** LSD1-knockdown causes increased H3K4me2 at the GATA-2 locus. Notes: (A) MEL cells were treated with DMSO for the indicated number of days. Cross-linked chromatin DNA was immunoprecipitated with the anti-H3K4me2 antibody and analyzed by PCR with primers specific to the indicated sites in the GATA-2 locus. (B) Cross-linked chromatin DNA from MEL cells with or without LSD1-knockdown was immunoprecipitated with an anti-H3K4me2 antibody and analyzed by PCR with primers specific to the indicated sites in the GATA-2 locus. # P<0.05 represents the relative fold enrichment on day 3 compared with that on day 0. * P<0.05 represents the relative fold enrichment in LSD1-knockdown cells compared with the blank vector cells. This experiment was repeated at least for three times.
locus by TAL1. The demethylation activity of LSD1 results in decreased H3K4me2, which subsequently reduces the transcription of GATA-2. Thus, LSD1 is an essential epigenetic regulator of GATA-2 expression.

While this work highlights the significance of LSD1-mediated epigenetic silencing of GATA-2 expression, previous work has demonstrated that GATA-1 also suppresses GATA-2 expression. Genome-wide studies have demonstrated that TAL1 and GATA-1 extensively co-occupy genomic sites in erythroid cells. Thus, it is likely that LSD1 collaborates with TAL1 and GATA-1 to regulate gene expression during erythroid differentiation. Moreover, previous studies have already confirmed the interaction between LSD1 and TAL1. However, despite repeated efforts, our coimmunoprecipitation results do not show such an interaction between GATA-1 and LSD1 (data not shown), suggesting that LSD1 and GATA-1 regulate GATA-2 expression via different protein complexes.

In addition to increased expression of GATA-2, LSD1-knockdown cells also exhibited decreased expression of GATA-1 during erythroid differentiation. However, the precise mechanisms underlying LSD1-mediated increased expression of GATA-1 is still unknown. It remains to be determined whether and how LSD1 cooperates with TAL1 or GATA-2 to regulate GATA-1 expression.

LSD1 is a component of several histone deacetylase complexes, though it silences genes by functioning as a histone demethylase. Alternative splicing results in multiple transcript variants. The histone modification mediated by LSD1 always plays an important role in some diseases, such as leukocytopenia and some cancers. Adamo et al reported that LSD1 regulates the balance between self-renewal and differentiation in human ES cells. Therefore, we speculated that the LSD1 may become a clinical therapeutic target for leukocytopenia. GATA-2 is expressed in hematopoietic progenitors, including early erythroid cells, mast cells, and megakaryocytes, and also in nonhematopoietic ES cells. In chicken erythroid progenitors, forced expression of GATA-2 promotes proliferation at the expense of differentiation. Multiple mutations on the GATA-2 gene have been recently implicated as the cause of primary immunodeficiency in patients with MonoMAC syndrome, and cases of dendritic cell, monocyte, B and natural killer (NK) lymphoid deficiency, and leukocytopenia. Also, GATA-2 has recently been implicated in non–small cell lung cancer. Targeting processes that occur downstream of GATA-2 signaling, with clinically approved drugs, had a significant effect in mouse models of the disease. All of these studies showed that the GATA-2 may be a therapeutic target for some kinds of diseases, especially for cancers and leukocytopenia. However, no effective and available compounds targeting GATA-2 have been discovered. Our study proved, for the first time, that LSD1 could regulate the GATA-2-mediated erythroid differentiation, which would provide the potential clue for new anticancer drugs.

In conclusion, our data revealed that LSD1 mediates erythroid differentiation via epigenetic modification of the GATA-2 locus.

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Disclosure
The authors report no conflicts of interest in this work.

References


Supplementary materials

Table S1 Primer sequences of GATA-1, GATA-2, and β-major

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences</th>
</tr>
</thead>
</table>
| GATA-1 | Forward: ATGGATTTTCTGCGTCAGGGGC  
Reverse: TCAAAGACTCTGCGGCGACGCGC |
| GATA-2 | Forward: GGGCGGGCATGAGGATGG  
Reverse: CCAATCCATGAGCGGCC |
| β-major | Forward: TGGAGGCTGCAGGGCTCTG  
Reverse: ACCAGCAAGGACGAGAAGG |
| β-actin | Forward: GGGTGATTCTCCCCGCTCGG  
Reverse: CCAATGCTGTTAACGTCGG |

Table S2 Primer sequences of the GATA-2 locus

<table>
<thead>
<tr>
<th>GATA-2 locus</th>
<th>Primer sequences</th>
</tr>
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</table>
| −9.3 | Forward: GAGATGAGCTAATCCCGCTGTGA  
Reverse: AAGGGCTGATTATTCCCAAGGCC |
| −4.2 | Forward: GAAATTGCCTGCGCCGTCCAT  
Reverse: GACGCGTGTTCTTGGT |
| −45.8 | Forward: GAAATAGCAGGGATCATCAGG  
Reverse: ATTCGGGCCTAGGGCC |
| −2.8 | Forward: GCCCTGTCAACACCCCGATCCT  
Reverse: TTGGTCCCAGGCGAGGATAAT |
| IS | Forward: CCCCTCGAAATGATGTCGAA  
Reverse: TCTGCGGTGTCCTCCGTTTC |
| IG | Forward: AGATAACCCAGAAGTGACACGTG  
Reverse: GCATACCCCTGACCC |
| Exon 3 | Forward: GCAAACTAAGGCAGCGATTCC  
Reverse: CAGAAACAGCTATGACC |
| Intr. 4 | Forward: GACCGAGACTACACCCCATCCC  
Reverse: GACCGAGACATACCCCACCATC |
| 3′UTR | Forward: GACATGCCCTAGATATAACACAATGTAC  
Reverse: CATTCCTTATATTACCTAAACCCC |

Figure S1 Knockdown of LSD1 results in decreased expression of GATA-1 protein and increased expression of GATA-2 protein in differentiated cells.

Notes: (A) MEL cells were treated with DMSO for 5 days to induce differentiation, and the expression of GATA-1, GATA-2, and β-major protein was measured, by western blot, from the differentiated cells. (B) ES cells were treated with EPO for 9 days to induce differentiation. Expression of GATA-1, GATA-2, and β-major protein was measured by western blots from the differentiated cells. (C) The relative expressions of GATA-1, GATA-2, and β-major protein in MEL and ES cells from (A) and (B) were quantified (n=3) for the “relative protein level”, which was analyzed as the GATA-2 or GATA-1 level normalized to β-actin protein. *P<0.05 represents the GATA-2 or GATA-1 or β-major protein expression in the LSD1-kd group (MEL or ES cells) compared with the control vector group (MEL or ES cells). This experiment was repeated at least for three times.

Abbreviations: DMSO, dimethyl sulfoxide; EPO, erythropoietin; ES, embryonic stem; LSD1-kd, LSD1-knockdown; MEL, murine erythroleukemia; Vector, vector control.
Figure S2 Knockdown of LSD1 did not affect the expression of GATA-1 or GATA-2 in undifferentiated cells.

Notes: (A) The expression of GATA-1, GATA-2, and β-major mRNA was measured by reverse-transcription quantitative PCR in undifferentiated MEL cells. (B) The expression of GATA-1, GATA-2, and β-major mRNA was measured by reverse-transcription quantitative PCR in undifferentiated ES cells. (C) The relative expressions of GATA-1, GATA-2, and β-major in MEL and ES cells from (A) and (B) were quantified (n=3) for the "relative mRNA level", which was analyzed as the GATA-2 or GATA-1 level normalized to β-actin gene. This experiment was repeated at least for three times.

Abbreviations: ES, embryonic stem; LSD1-kd, LSD1-knockdown; MEL, murine erythroleukemia; mRNA, messenger RNA; PCR, polymerase chain reaction; Vector, vector control.