Histone Lysine-specific Demethylase 1 (LSD1) Protein Is Involved in Sal-like Protein 4 (SALL4)-mediated Transcriptional Repression in Hematopoietic Stem Cells*

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Background: SALL4 plays important roles in regulating the growth of hematopoietic progenitor cells.

Results: SALL4 dynamically recruits histone demethylase LSD1 to specific target genes. LSD1 negatively regulates SALL4-mediated gene expression by affecting local chromatin structure.

Conclusion: Multiple epigenetic modifiers cooperatively modulate SALL4-mediated gene repression.

Significance: This report provides a novel mechanism by which stem cell gene SALL4 controls hematopoietic progenitor cell properties.

The stem cell protein SALL4 plays a critical role in hematopoiesis by regulating the cell fate. In primitive hematopoietic precursors, it activates or represses important genes via recruitment of various epigenetic factors such as DNA methyltransferases, and histone deacetylases. Here, we demonstrate that LSD1, a histone lysine demethylase, also participates in the trans-repressive effects of SALL4. Based on luciferase assays, the amine oxidase domain of LSD1 is important in suppressing SALL4-mediated reporter transcription. In freshly isolated adult mouse bone marrows, both SALL4 and LSD1 proteins are preferentially expressed in undifferentiated progenitor cells and co-localize in the nuclei. Further sequential chromatin immunoprecipitation assay confirmed that these two factors share the same binding sites at the promoter regions of important hematopoietic regulatory genes including EBF1, GATA1, and TNF. In addition, studies from both gain- and loss-of-function models revealed that SALL4 dynamically controls the binding levels of LSD1, which is accompanied by a reversely changed histone 3 dimethylated lysine 4 at the same promoter regions. Finally, shRNA-mediated knockdown of LSD1 in hematopoietic precursor cells resulted in altered SALL4 downstream gene expression and increased cellular activity. Thus, our data revealed that histone demethylase LSD1 may negatively regulate SALL4-mediated transcription, and the dynamic regulation of SALL4-associated epigenetic factors cooperatively modulates early hematopoietic progenitor proliferation.

SALL4 is a zinc-finger transcription factor and is essential for human embryonic development (1–3). We and others have earlier reported that SALL4 plays important roles in maintaining the properties of embryonic stem cells by interacting with Oct4 and Nanog (4–7). Moreover, SALL4 is one of the few genes that are also involved in tissue stem cell self-renewal and multipotency maintenance (8–10). In isolated mouse bone marrow (BM) Lin⁻/Sca1⁺/ckit⁺ (LSK) cells, forced expression of SALL4 dramatically activates multiple hematopoietic stem and progenitor cell (HSPC) regulatory genes including HoxB and Notch factors and leads to a rapid ex vivo HSPC expansion, as well as increased cell repopulating abilities in vivo (10, 11). More strikingly, by using the SALL4 transduction methodology, the HSPCs obtained from human peripheral blood are capable of rapid and efficient ex vivo expansion by >10,000-fold in the presence of appropriate cytokines (12). These findings provide a novel avenue for achieving clinically significant expansion of human HSPCs. We have sought to examine the potential transcriptional and/or epigenetic mechanisms underlining the observed SALL4 effects on BM progenitor cells. To this end, we and others have reported that SALL4 can silence lineage differentiation genes and modulate cell proliferation by recruiting epigenetic regulators, including DNA methyltransferases (DNMT1, 3A, 3B, 3L) and histone deacetylases (HDAC1 and HDAC2), to target genes (13–15). In addition, SALL4-mediated activation of Bmi-1, another important hematopoietic stem cell gene, involves methylation of lysine 4 of histone H3.

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§ The abbreviations used are: BM, bone marrow; CFU, colony-forming unit; BFU-E, burst-forming unit-erythroid; co-IP, co-immunoprecipitation; DNMT, DNA methyltransferase; H3K4, lysine 4 of histone 3; HDAC, histone deacetylase; HSPC, hematopoietic stem and progenitor cell; LSD1, lysine-specific demethylase 1; NuRD, nucleosome remodeling and histone deacetylase; PCPA, trans-2-phenylcyclopentylamine hydrochloride; qPCR, quantitative PCR; qRT-PCR, quantitative RT-PCR.

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(H3K4) at its promoter region (16). Based on recent discoveries, there seem to be various ways of cross-talking between histone modifiers and DNA methyltransferases. For example, the histone lysine-specific demethylase 1 (LSD1) has been shown not only to act as component of the nucleosome remodeling and histone deacetylase (NuRD) complex which is necessary for embryonic stem cell differentiation, it also directly interacts with DNMT1 and affects global DNA methylation status (17). LSD1 is the first of several protein lysine demethylases discovered. It specifically removes histone H3K4me2 to H3K4me1 or H3K4me0 and functions as a transcriptional corepressor (18, 19). Interestingly, LSD1 also appears to act as a central regulator for HSPC proliferation and differentiation. In a conditional gene knockdown model, LSD1 reduction was found to expand BM progenitor numbers by enhancing their proliferative behavior (20). Additionally, knockout of LSD1 resulted in depression of stem and progenitor cell gene signatures along with impaired granulocytic and erythroid maturation (21). In the current study, we aim to identify whether LSD1 also acts as an enzymatic partner for SALL4 and how they may function together and coordinately regulate BM progenitor cell proliferation.

EXPERIMENTAL PROCEDURES

Mice—C57BL/6J mice at 8–12 weeks of age were obtained from the Jackson Laboratory (Bar Harbor, ME). The SALL4flox/flox mice were described previously (3). All animal experiments were preapproved by the State University of New York, Stony Brook Institutional Animal Care and Use Committee.

Plasmids, Cell Culture, Transfections, and Luciferase Assays—The LSD1- and LSD1ΔC-encoded plasmids were described in Ref. 22. The LSD1 CDNA was cloned into the EcoRI/XhoI site of entry vector pENTR3C (Invitrogen). A Gateway reaction in the attL and attR sites was carried out to subclone the cDNA into the lentiviral expression vector pDEST-CMVFG12 (11). Plasmids expressing SALL4 isoforms and shortened mutants were described elsewhere (13). The pEBFI-Luc reporter was kindly provided by Dr. Rudolf Grosschedl (23). Transfection of plasmids into cultured cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendation. Luciferase assays were performed as described previously (13). PCPA was purchased from Sigma. When used for luciferase assays, drugs were added at 12 h, 20 h following transfection, and cells were collected after another 4 h.

Isolation of Murine Hematopoietic Stem/Progenitor Cells—Mouse bone marrow Lin−/Sca1− cells were purified from 4–8-week-old wild-type or gene-targeted C57BL/6J mice by using either the EasySep Mouse Hematopoietic Progenitor Cell Enrichment kit (STEMCELL Technologies) or as described previously (11).

Immunofluorescence Staining—Cells were fixed with 3% paraformaldehyde, permeabilized with 0.1% saponin, and then blocked with 20% goat serum at room temperature for 1 h. The treated cells were incubated with primary antibodies for 30 min and washed with PBS, followed by staining with a secondary antibody at room temperature for another 30 min. For double staining, the cells were blocked with murine IgG and then further stained with mouse Alexa Fluor 488 or rabbit Alexa Fluor 594. For immunofluorescent analysis, the cells were examined with an Evos fluorescent microscope, and images were captured and analyzed.

Retrovirus Production and LSD1 Knockdown—Five short hairpin RNA-expressing plasmids, one control (pRS, no. 12), and four LSD1-specific (no. 47, 48, 49, and 50; all from Origene), were transfected into 293T packaging cells (Origene) using Lipofectamine 2000. Shed virus was harvested 48 h after transfection, and control or stable LSD1 knockdown clones were obtained under puromycin (1.5 μg/ml) selection after 5 days. Sequences were as follows: no. 47, cttatacaatctggttaacaagagat; no. 48, cacacccggattctctgtaagaagagc; no. 49, cagtgccgagctggctacaagc; and no. 50, acacgctatgctctgctcaccaggat.

Flow Cytometry—LSD1 knockdown and control cells were centrifuged at 300 × g for 7 min and washed once with Robo buffer, then incubated with primary mAb FITC-Sca1 (BioLegend), PE-cKit (BioLegend), and biotinylated lineage antibody mixture (Miltenyi Biotec) at 4 °C for 20 min. After washes, some cells were labeled further with strepavidin-PE/Cy5 (BioLegend) at 4 °C for 20 min and then analyzed on FACScalibur flow cytometer (Stony Brook University flow cytometry facility).

Adenoviral and Lentiviral Transduction and qRT-PCR Assays—Detailed transduction procedures for mouse bone marrow Lin−/Sca1− cells are described in Ref. 11. For SALL4 knockout, GFP or Cre recombinase adenovirus (Vector Biolabs) at a multiplicity of infection of 100 was added to the cells at 37 °C. The cells were infected overnight for 12–15 h and then recovered in culture medium. Total RNA was extracted by TRIzol (Invitrogen), and qRT-PCR was performed as described previously (11).

Co-immunoprecipitation and Western Blotting—Protein interaction assays were conducted as described (13). LSD1 antibody was purchased from Cell Signaling.

LSD1 Demethylase Activity Assay—These experiments were carried out using the EpiQuik LSD1 Activity/Inhibition Assay kit. Briefly, HEK293 cells were transfected with wild-type SALL4 or different SALL4-HA mutants. Nuclear proteins were extracted from cells using the EpiQuik Nuclear Extraction kit (Epigentek). The LSD1 activity were fluorometrically quantified by SpectraMax M3 at 530–590 nm.

Chromatin Immunoprecipitation (ChIP) and RE-ChIP—For regular ChIP, BM HPSCs were cross-linked with 1.0% formaldehyde (Sigma) at room temperature for 10 min, lysed, and sonicated. Chromatin was isolated by using Chromatin Extraction kit (Epigentek). ChIP assays were conducted by One-Step ChIP kit (Epigentek) with specific antibodies against FLAG (Sigma), SALL4 (Novus Biologicals), LSD1 (Cell Signaling). All ChIP enrichment results were evaluated by qPCR relative to input DNA. For RE-ChIP, we used the kit from Active Motif. Briefly, SALL4-HA and FLAG-LSD1 co-transfected HEK293 cells were cross-linked with 1.0% formaldehyde and collected in lysus buffer. The anti-HA and anti-FLAG antibodies were sequentially used in first and second ChIP. IgG was used as negative control. For PCR detection, primers were designed based on ChIP-chip probe information provided by NimbleGen.
Colony-forming Unit (CFU) Assay of BM Cells—LSD1 shRNA or pRS-transduced BM cells were prepared at 10 x the final concentration as required. The BFU-E, CFU-GM and CFU-G, and CFU-M colonies were counted under the microscope 9 days after the cells were plated in MethoCult® medium (Stem Cell Technologies) or Methylcellulose Complete Media (R&D Systems). A colony with >50 cells was counted as a positive colony.

Statistical Analysis—A Student’s nonpaired t test was used to determine the statistical significance, where indicated.

RESULTS

LSD1 Is Highly Expressed in Mouse HSPCs and Interacts Directly with SALL4—To determine the roles of LSD1 in HSPC proliferation and differentiation, the expression of LSD1 in mouse BM Lin-Sca1 cells was first examined. Similar to the expression profile of SALL4, immunofluorescence analysis using an LSD1-specific antibody revealed that LSD1 is extensively expressed in the nuclei of HSPCs (Fig. 1A). Moreover, a large portion of SALL4 co-localized with LSD1 in the nuclei of these cell populations. By contrast, in the differentiated lineage-positive cells, expression of both genes is substantially reduced, and their proteins were hardly seen under the microscope. Consistent with these microscopic findings, further qRT-PCR and Western blot analysis confirmed that both SALL4 and LSD1 are preferentially expressed in BM undifferentiated Lin^-/Sca1^+ HSPC populations (Fig. 1, B and C).

Based on their shared cellular distribution pattern and previous findings that either SALL4 or LSD1 actively interacts with epigenetic modifiers DNMT1, HDAC1, and HDAC2 (13, 17, 24), we then performed a co-immunoprecipitation (co-IP) experiment to verify their potential physical interactions. Earlier we have generated wild-type SALL4 as well as a series of deletion mutant plasmids which are linked with a HA tag. In this study we transfected each of these plasmids into HEK293 cells. Co-IP assays were then performed after 36 h. Interest-
ingly, as demonstrated in Fig. 2, A and B, except for the shortened N-terminal 174-amino acid fragment, the wild-type SALL4 isoforms and other deletion mutants all successfully immunoprecipitated LSD1. It then seems hard to predict the exact interacting site between SALL4 and LSD1. In our previous study, however, we have shown that the N-terminal 174-amino acid sequence may act as a “common” site for interactions of SALL4 with HDAC1 and different DNMT proteins (13). Thus, LSD1 may associate with SALL4 via different domain(s) as these epigenetic co-factors do. To further demonstrate the observed protein interactions and verify their associated functions, we then conducted LSD1-specific enzyme activity assays. Our data showed that in agreement with the co-IP findings, SALL4 isoforms and the deletion mutants which exhibited LSD1 protein interaction purified significantly increased enzyme activity from HEK293 cells nuclear lysates (Fig. 2C). It is notable that among the analyzed SALL4 variants, the Δ2 fragment, which bears the first C2H2 double zinc-finger motif, purified the highest LSD1 activity (up to ~50-fold). Combined with above co-IP data, this may reflect that the Δ2 fragment contains the essential sequence for SALL4-LSD1 binding. Further, the wild-type SALL4 isoforms may possess inhibitory sequence(s) for LSD1 activity.

LSD1 is involved in SALL4-mediated transcriptional regulation via its histone demethylase activity—The above results led us to examine possible involvement of LSD1 in SALL4-mediated transcriptional regulation. We initially performed a series of luciferase reporter assay using a pEBF1-Luc reporter plasmid. EBF1 has been identified as one of SALL4 downstream target genes in BM progenitor cells (see below). Our data showed that forced expression of SALL4 activated pEBF1-Luc reporter activity in a dose-dependent fashion (Fig. 3A). However, when the cells were co-transfected with LSD1-encoded plasmid, the SALL4-mediated EBF1 promoter activation was largely blocked. In contrast, deletion of the amine oxidase domain, which is essential for the histone demethylase activity of LSD1, resulted in a complete loss of repression of SALL4-directed transcription (Fig. 3B), indicating that LSD1 represses SALL4-mediated transcriptional activity via its histone demethylase activity. This notion was further supported by results from an additional luciferase reporter assay. In the presence of PCPA, a nonselective monoamine oxidase inhibitor and an LSD1 enzymatic inhibitor, overexpression of LSD1 failed to block SALL4-mediated activation of EBF1 promoter activity.
(Fig. 3B). On an interesting note, PCPA alone leads to a further activation of SALL4-mediated *EBF1* promoter activity to ~5.5-fold, suggesting that endogenous LSD1 still plays a repressive role, or other unknown regulatory mechanisms are involved in SALL4-mediated transcriptional regulation.

**SALL4 Dynamically Recruits LSD1 to Hematopoietic Regulatory Genes and Modulates Their Expression**—Because SALL4 directly interacts with LSD1 and recruits high LSD1 enzymatic activity, we next performed sequential chromatin immunoprecipitation coupled with PCR detection (Re-ChIP-PCR) to examine whether SALL4 and LSD1 proteins may share the same binding site(s) at SALL4 target gene promoters. HEK293 cells were co-transfected with SALL4-HA- and FLAG-LSD1-encoded plasmids, and anti-HA and anti-FLAG antibodies or IgG were sequentially used in first and second ChIP. We started from the following candidate genes: *EBF1*, *GATA1*, *CEBPA*, and *TNF*, as our earlier ChIP-chip assays from both HEK293 cell (Fig. 4A), and isolated human CD34+ HSPCs (data not shown) reveal that SALL4 binds to the promoter regions of these genes. More importantly, all of these genes are key regulators for normal BM progenitor proliferation and differentiation. Specifically, *EBF1* drives early B cell lineage specification and commitment (25), and *GATA1* is important in erythroid development by regulating the switch of fetal hemoglobin to adult hemoglobin (26) whereas *TNF* induces apoptosis in hematopoietic as well as other cell types via stepwise activation of caspase family proteases (27). Based on the probe sequence adopted for our ChIP-chip assays, we designed PCR primers which specifically amplify the SALL4-binding sites for each gene. As shown in Fig. 4B, chromatin samples in which the first antibody used was anti-HA and the second antibody was anti-FLAG show good amplification of *EBF1* DNA after the second ChIP. By contrast, chromatin samples in which IgG was used as the second antibody show little amplification of tested *EBF1* sequence, indicating that SALL4 and LSD1 co-localize at the same region of the *EBF1* promoter. This result is further confirmed by a separate Re-ChIP assay in which anti-FLAG used as the first antibody and anti-HA as the second antibody (Fig. 4B). Similarly, using the same method, we demonstrate that SALL4 and LSD1 co-occupy the same regions of *GATA1*, *CEBPA*, and *TNF* promoters (Fig. 4B).

To prove that SALL4 does dynamically recruit LSD1 to its target genes, we next conducted regular ChIP in BM progenitor cells which were obtained from either gain- or loss-of-function models. Lin−/Sca1− cells were isolated from BMs of either wild-type BL6 or homozygous *Sall4*^flax/flax^ mice and then cultured in dishes. The wild-type cells were infected with either GFP (control) or SALL4-expressing lentivirus, whereas the gene-targeted cells were treated with control or Cre-adenovirus. We compared ChIP data from each of these groups by using anti-SALL4, anti-LSD1 and anti-dimeH3K4 antibodies at day 4 of treatment. As expected, upon forced expression of SALL4 in wild-type cells, the *EBF1* promoter-bound SALL4 was markedly increased, which was followed by an also remarkable increase of *EBF1*-binding LSD1 (approximately 8.5-fold). In contrast, the dimerH3K4 level was dramatically decreased at the same region (Fig. 5A). In accordance with these overexpression studies, Cre-induced deletion of *SALL4* in gene-targeted BM progenitors resulted in a remarkable decrease of SALL4-bound and LSD1-bound *EBF1*, which was accompanied by a ~750-fold increase of dimerH3K4 (Fig. 5A). Thus, data from both models confirmed that SALL4 dynamically recruits LSD1, which is followed by reduced histone H3K4 dimethylation at downstream genes. Similarly, the SALL4-mediated induction or reduction of bound LSD1 in above treated models was also observed in the promoter regions of *TNF* and *GATA1* genes, which was consistently accompanied by a reversely affected dimerH3K4 level at the same sites (Fig. 5, B and C). Note that in either SALL4 overexpression or gene knockout samples, we failed to monitor altered SALL4 binding levels at the tested *CEBPA* promoter region or relevant changes of bound LSD1 or dimerH3K4 (data not shown). This may suggest that in BM progenitors, the tested *CEBPA* sequence is not a functional regulating site for SALL4, or the SALL4-LSD1 complex only selectively controls specific targets during cell proliferation. The latter may be supported by further ChIP assays on two other HPSC regulatory genes, *HOX9A* and *MEIS1*. Both were previ-
ously identified as downstream targets and activated by SALL4 (28, 29), but we did not detect meaningful co-binding of LSD1 at the tested SALL4-bound sites in these studies (data not shown).

We next examined the transcription levels of the above tested genes. Corresponding to the promoter binding status of SALL4 and LSD1, qRT-PCR assay showed that overexpression of SALL4 largely represses the mRNA levels of EBF1, GATA1, and TNF, whereas deletion of SALL4 caused a general up-regulation of these gene transcripts (Fig. 5D). Activation of HOX9A and MEIS1 mRNA levels were included here as a system control. Regarding the repression of EBF1 by SALL4, it may appear contradictory to the luciferase assay result, in which the H3K4me2 level was reversely affected compared with LSD1. A and C, SALL4 modulated promoter binding of LSD1 and relevant H3K4me2 level were also tested in GATA1 (B) and TNF (C) genes. D, the relative mRNA levels of SALL4 targets upon overexpression and knockout of SALL4 were determined by qRT-PCR assays. Data are represented as means ± S.D. from at least two independent experiments. Error bars, S.D. *, p < 0.05; **, p < 0.01.

**Knockdown of LSD1 in BM HSPCs Leads to Altered SALL4 Downstream Gene Expression and Increased Cellular Activity**—LSD1 is a putative transcriptional repressor. We further tested whether/how LSD1 expression levels may affect SALL4 downstream gene regulation and subsequent cell growth. In a pilot experiment, we used RNA interference strategies to reduce LSD1 expression. Four short hairpin (shRNA) retroviral constructs that target different regions of LSD1 mRNA, termed no. 47, no. 48, no. 49, and no. 50, were tested against the pRS control (no.12) in BM HSPCs by Western blotting. Among them, the no. 48 construct was more effective in suppressing LSD1 expression and was used in all further experiments (Fig. 6a and data not shown). The BM Lin^−/Sca1^− cells were first infected with shRNA-expressing retrovirus and then grown under puromycin selection for 5–6 days, until mock-infected cells were dead. Compared with pRS control, LSD1 knockdown resulted in a ~1.3-fold increase of total alive cell numbers starting from day 5, as judged by counting the trypan blue-excluding cells in a
hemocytometer. Another LSD1 knockdown experiment by using no. 47, also produced similar results (Fig. 6A). To further rule out potential off-target effects of the LSD1 knockdown, we performed RNA interference (RNAi) rescue experiments by expressing the human LSD1 cDNA (hLSD1). Although highly homologous (≈98%) in amino acid sequence, the mouse and human LSD1 mRNAs do differ at 6 of the 29 no. 48 shRNA target nucleotides, which enables us to test its RNAi specificity. We have carried out RNAi assay in mouse NIH3T3 fibroblasts which has lower level of endogenous LSD1. As expected, high level ectopic hLSD1expression was successfully detected after lentiviral administration, whereas further shRNA treatment failed to decrease its expression levels by Western blotting (Fig. 6B). In BM Lin−/Sca1+ cells, transduction of hLSD1-expressing lentivirus since day 3 largely blocked the increased cell proliferation mediated by RNAi. These group data suggest that the growth stimulation induced by no. 48 is truly due to a specific depletion of LSD1 protein and not mediated by the accidental depletion of other proteins by a nonspecific effect.

Triggered by these findings, we conducted flow cytometry analysis and identified that in LSD1 reduction cells, their BM HSPC-specific cell surface markers are moderately increased (Sca1+/H11001 80.0%/H11006 1.4% versus pRS control 75.2%/H11006 1.9%, p<0.017; cKit+/H11001 56.4%/H11006 2.4% versus pRS control 53.6%/H11006 2.1%, p=0.112; Sca1+/cKit+ 56.2%/H11001 1.3% versus pRS control 51.1%/H11001 0.9%, p=0.042), whereas their overall mature lineage markers are slightly but significantly decreased (Lin−/H11001 78.7%/H11006 4.6% versus pRS control 82.1%/H11006 4.2%, p<0.05).

FIGURE 6. Knockdown of LSD1 in BM HSPCs leads to altered SALL4 downstream gene expression and increased cellular activity. A, BM HSPCs were transduced with pRS, no. 47 or no. 48 retrovirus. After 2 days, 1.5 µg/ml puromycin was added and maintained for the indicated days. Viable cell numbers were determined by the average of cells in three wells for each group, and data are represented as mean ± S.D. (error bars). *, p < 0.05 versus pRS control. Western blot validation for each shRNA treatment showed LSD1 expression levels at day 2. B, for RNAi rescue experiment, GFP control or hLSD1-expressing lentivirus was added to the no. 48-mediated LSD1 knockdown (KD) cells at day 3. Then cell growth was monitored as described above. *, p < 0.05 versus pRS and no.48 plus hLSD1. To validate the shRNA effect and target specificity, mouse NIH3T3 fibroblasts with or without hLSD1 treatment were transduced with pRS or no. 48 retrovirus. After 72 h, Western blotting was conducted to examine total LSD1 expression levels. C, the levels of Sca1, cKit, and Lineage markers were analyzed by flow cytometry in pRS control and LSD1 knockdown cells which were under puromycin selection for 4 days. D, pRS control and LSD1 knockdown cells were plated as 5000 cells/35-mm dish in Methylcellulose Complete Media. After 9 days the BFU-E, CFU-GM, CFU-M, and CFU-G colonies were counted under a microscope. E, the relative mRNA levels of the indicated genes in control and LSD1 knockdown cells were determined by qRT-PCR and quantified based on two independent experiments. Error bars denote S.D. *, p < 0.05.
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21.4% ± 0.7% versus pRS control 24.9% ± 1.1%, p = 0.006, Fig. 6C). In line with the cell proliferation and cellular phenotype assay findings, a methylcellulose-based CFU assay showed that LSD1 reduction cells retain the ability to generate various colonies including CFU-GM, CFU-G, CFU-M, and BFU-E since day 9. However, the total numbers of each type of colony are lower than that from the pRS control-infected cells (Fig. 6D). Thus, knockdown of LSD1 in BM HSPCs down-modulate myeloid differentiation versus proliferation from early primitive cells.

Based on the functional observations, we asked how the SALL4-bound genes may be affected by LSD1 reduction, as LSD1 is assumedly a co-repressor and involved in SALL4-mediated differentiation- and apoptosis-inducing gene repression. Our qRT-PCR assay showed that among the tested genes, the mRNA levels of LSD1, EBF1, GATA1, TNF, and CEBPA are all largely decreased (ranging from −2 to −18 log2). The SALL4 as well as HOXA9 and MEIS1 transcripts, however, were markedly increased (ranging from +2.5 to +12.5 log2, Fig. 6E). These data support the observed cell behavior of LSD1 knockdown HSPCs, but raise an immediate question: if LSD1 acts as a transcriptional co-repressor for SALL4, why did reduction of LSD1 not cause derepression of SALL4-silenced genes in these cells? To answer this, we checked several other SALL4 co-factors and evaluated their interplay on an overall level.

LSD1 and Other Epigenetic Modifiers Cooperatively Contribute to SALL4 Repressive Effects—In our previous work, we proposed that SALL4 and various epigenetic repressors (including DNMT1, DNMT3A, DNMT3B, DNMT3L, MBD2, and HDAC1, HDAC2 and most likely others) form a large complex which cooperatively regulates downstream genes (13). As LSD1 also directly interacts with SALL4 (although not via the same site as do the above listed factors) and affects its downstream gene histone methylation status, we considered the possibility that LSD1 and these epigenetic repressors may competitively interact with SALL4 during transcriptional regulation. Thus, in the absence of ample LSD1 proteins (e.g. after shRNA-induced knockdown), SALL4 may compensatively recruit more other epigenetic modifiers to downstream genes and repress their expression. To examine this possibility, BM Lin−/Sca1+ cells were infected with shRNA-expressing retrovirus and then placed under puromycin selection for 4 days. We then performed a ChIP assay using anti-SALL4, anti-LSD1, anti-DNMT1, and anti-DNMT3L antibodies to detect the gene binding levels of each of these factors. As shown in Fig. 7, A–C, and as expected, at the promoter regions of EBF1, TNF, and GATA1, binding of LSD1 was consistently decreased. However, there was variously increased binding of SALL4, DNMT1, or DNMT3L at the same sites. We also examined the binding status of these factors at the CEBPA promoter, which did not exhibit significant changes of bound SALL4 or LSD1 in previous experiments. Consistently, none of these factors showed differed binding levels at the same area after LSD1 knockdown (Fig. 7D). These data, at least in part, suggest that LSD1 and other epigenetic modifiers cooperatively contribute to SALL4 regulatory effects during normal BM progenitor proliferation.

DISCUSSION

LSD1 is a lysine-specific histone demethylase that suppresses gene expression by converting dimethylated H3K4 to mono- and unmethylated H3K4 (18). LSD1 has been implicated in hematopoietic development and differentiation by its ability to activate or repress gene expression. It associates with hematopoietic specific repressors Gfi-1, Gfi-1b, and affects erythroid, megakaryocytic, and granulocytic differentiations (31). It is also shown to contribute to the activity of the Blimp-1-dependent repression complex during plasma cell differentiation (32). By interacting with TAL1, the TAL1-LSD1-HDAC1 complex appears to control the onset of erythroid differentiation programs (22). Two recent in vivo studies further addressed that LSD1 plays a critical role in inhibiting HSPC gene expression programs and restraining progenitor proliferation, and LSD1 deficiency resulted in severe granulocytic and erythroid differentiation (20, 21). We report that reduction of LSD1 stimulated the HSPC subpopulation from cultured bone marrows cells and preserved their primitive cell phenotypes (Fig. 6), reinforcing that LSD1 may act as a key controller for HSPC proliferation and differentiation. One function of LSD1 in HSPCs may be to...
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...contribute not only to activity of the SALL4-dependent repression network, but also to influence SALL4 expression directly, as SALL4 mRNA levels were substantially increased after LSD1 knockdown (Fig. 6E). Additionally, increased enrichment of SALL4 after LSD1 knockdown at the SALL4-LSD1 co-binding sites of different downstream gene promoters also supported this notion (Fig. 7). Further studies maybe required to determine the underlying mechanisms of the negative regulatory effects of LSD1 on SALL4. Moreover, given the potent regulatory roles of LSD1 on early hematopoiesis, detailed characterization of the gene expression profiles regulated specifically by SALL4 and LSD1 is awaited.

As reported in our previous data (11), forced expression of SALL4 led to expansion of BM HSPCs, which is associated with dramatic up-regulation of HSFC-activating genes MEIS1 and HOX9A and down-regulation of apoptosis-inducing gene TNF and hematopoietic differentiation genes EBF1 and GATA1. Suppression of the latter factors was shown to be associated with an increased recruitment of LSD1 and subsequent demethylation of dimeH3K4, supporting its role as a co-repressor by maintaining locally repressive chromatin structure. It appears that SALL4 actively interacts with multiple epigenetic factors during transcriptional regulation, as we have shown earlier that SALL4 associates with a group of DNA methyltransferases during subsequent gene silencing. Competitive or compensative involvement of these epigenetic factors during specific SALL4 regulatory process may occur, because under LSD1 knockdown conditions, we monitored increased gene binding of DNMT1 and DNMT3L at SALL4 target genes. Here, it may be critical to consider that histone modification and DNA methylation cooperatively regulate chromatin structure and gene activity. For example, loss of LSD1 mediated by shRNA is found to suppress HDAC mRNA expressions in breast cancer cells (33). We have also verified the reduced mRNA levels of HDAC1 and HDAC2 after LSD1 knockdown in BM HSPCs. Further, their binding to SALL4-LSD1 co-occupied gene sites was also moderately decreased (data not shown). It is possible that LSD1-HDACs and DNMTs are in a separate complex and cooperatively mediate gene transcription. Indeed, we have examined the SALL4-bound protein levels of DNMT1 and DNMT3L after LSD1 knockdown, and the Western blotting assay demonstrated that both factors are moderately increased based on the band intensity against the loading control (Fig. 7E). Although LSD1 is shown to be essential for the stability of DNMT1 protein, the catalytic activity of DNMT1 is not impaired in LSD1 deletion cells (17). Increased binding of more DNMTs at SALL4 downstream genes may suggest possible hypermethylation status at their promoter regions, which in part contributes to the repressive effects of SALL4 on its downstream genes after LSD1 depletion. However, given the complex genetic network controlling early hematopoiesis, other LSD1-related but SALL4-independent regulatory machinery may also exist.

Nevertheless, in current study, we identified that in primitive hematopoietic precursors, LSD1 also acts as an important co-factor for the repressing activity of SALL4. We propose that SALL4 orchestrates recruitment of multiple epigenetic modifiers, e.g. DNMTs, components of the Mi-2/NuRD complex, and components of the LSD1-HDAC complex, which cooperatively regulate local chromatin structure and coordinate contribute to the repressing activity of SALL4. These findings provide further insights into the molecular mechanisms of the silencing of lineage differentiation genes by SALL4 during the process of early hematopoiesis.

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