

Original Article

Characterization of combinatorial histone modifications on lineage-affiliated genes during hematopoietic stem cell myeloid commitment

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Hematopoietic stem cells (HSCs) are multipotent stem cells capable of self-renewal and multilineage differentiation. Mechanisms regulating the maintenance of HSCs' multipotency and differentiation are still unclear. In this study, we observed the role of combinatorial histone modification pattern in the maintenance of HSCs' pluripotency and differentiation. HSCs (CD34⁺CD38^{low}) were collected from human umbilical cord blood and induced to differentiate to committed cells *in vitro*. The histone modifications on lineage-specific transcription factors/genes in multipotent HSCs and differentiated progenies, including megakaryocytes, granulocytes, and erythrocytes, were analyzed by chromatin immunoprecipitation-quantitative polymerase chain reaction. Our results showed that a certain level of acH4 and acH3 together with high level of H3K4me2, low level of H3K4me3, and a certain level of H3K9me3 and H3K27me3 were present in lineage-specific genes in CD34⁺CD38^{low} HSCs. As CD34⁺CD38^{low} cells differentiated into granulocytes, erythroid cells, and megakaryocytes, the modification levels of acH3, acH4, and H3K4me2 on lineage-specific genes remained the same or elevated, while H3K4me3 level was increased greatly. At the same time, H3K9me3 and H3K27me3 modification levels became lower. In non-lineage-specific genes, the acH3 and acH4 levels were decreased, and H3K4me3 level remained at low level, while H3K9me3 and H3K27me3 levels were increased drastically. Our data suggest that combinatorial histone modification patterns have implicated function in maintaining the multipotency of HSCs and keeping the accuracy of gene expression program during HSC differentiation.

Keywords histone modification; hematopoietic stem cells; myeloid commitment; lineage-affiliated genes

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Introduction

Hematopoietic stem cells (HSCs) are multipotent cells that have the potential to differentiate into all cells of erythromyeloid and lymphoid lineages, as well as to maintain their numbers by means of controlled self-renewal [1,2]. Previous studies showed the co-expression of lymphoid and myeloid genes in HSCs and early hematopoietic progenitors at low level of transcription. The process, known as multilineage gene priming, maintains the multipotency of HSCs [3–6]. And HSC differentiation requires the coordinated regulation of multiple gene expression programs, leading to the activation of lineage-specific genes and repression of lineage-inappropriate genes [7]. Although several cell-intrinsic and microenvironmental factors can regulate these processes, the mechanism behind them has yet to be fully elucidated.

Recent studies suggested that epigenetic-based mechanisms, especially histone modifications, play an important role in the self-renewal and differentiation of stem cells [8–16]. Histone modifications include acetylation, phosphorylation, methylation, and ubiquitinylation, etc. [17]. The hallmarks of active chromatin states are acetylation of histone H3 (acH3), acetylation of histone H4 (acH4), dimethylation of lysine 4 of histone H3 (H3K4me2), and trimethylation of lysine 4 of histone H3 (H3K4me3). Whereas trimethylation of histone H3 lysine 9 (H3K9me3) and trimethylation of histone H3 lysine 27 (H3K27me3) are hallmarks of repressive chromatin states. These modifications are essential for the formation and maintenance of active or repressive chromatin states. Previous studies have reported that epigenetic modifications serve as an important mechanism to control HSC multipotency [8,11,12,18]. Progression from HSCs to differentiated progenies involves the coordinated regulation of multiple gene expression programs leading to the activation or repression of lineage-specific genes. In this aspect, histone modifications

which are crucial in chromatin structure formation determine ‘open’ or ‘close’ state for the expression of relevant genes. To date, little is known about the characterization of combinatorial histone modifications during HSC differentiation towards myeloid lineages (including granulocytic, erythroid, and megakaryocytic cells).

In this study, we investigated the characteristics of combinatorial histone modification pattern of a series of lymphoid- and myeloid-affiliated genes in HSCs and myeloid committed cells. We showed that lineage-affiliated genes are associated with a combination of histone modifications, including a certain level of histone acetylation, high level of H3K4me2, low level of H3K4me3, and a certain level of repressive histone modification in HSCs. These histone modification patterns ensure the characteristics of chromatin conformation which keeps HSCs in multipotent state. During lineage commitment, active histone modifications, especially H3K4me3, were further added at the lineage-affiliated genes, whereas the active histone marks were replaced by high level of repressive histone modifications (H3K9me3 and H3K27me3) at the lineage-inappropriate genes. Changes of histone modifications maintain the lineage-specific expression or repression, and ensure the accuracy of lineage commitment. These results highlight the role of combinatorial histone modification pattern in the maintenance of multilineage potential in HSCs and the accuracy of lineage commitment.

Materials and Methods

Cell isolation

Human umbilical cord blood samples were collected from normal full-term deliveries, after obtaining informed consent of the mothers according to the approved institutional guidelines of XiangYa Hospital (Changsha, China). After Ficoll density gradient separation (Lymphocytes Separation Medium; Sigma, St Louis, USA), CD34⁺ cells were collected by magnetic cell sorting using the immunomagnetic positive selection system (Miltenyi Biotec, Bergisch Gladbach, Germany). CD34⁺ cells (purity >90%) were further sorted to obtain CD34⁺CD38^{low} cells, which comprise up to 10% of the CD34⁺ cell population. CD34⁺CD38^{low} cells were routinely isolated from pools of 5–10 cord blood samples by negative selection using immunomagnetic sorting. The obtained CD34⁺CD38^{low} cells were rich in HSCs and multipotent progenitors and therefore considered as CD34⁺CD38^{low} HSCs.

In vitro differentiation

Freshly isolated CD34⁺CD38^{low} HSCs were differentiated towards the granulocyte, erythroid, and megakaryocyte lineages *in vitro*, induced by a combination of different cytokines. Differentiation was monitored by fluorescence-activated cell sorter analysis based on phenotypic changes of the expression of cell surface markers including CD15, CD235a, and CD41a.

Induction condition for granulocyte lineage is: 10% fetal calf serum (FCS) (Gibco, New York, USA), 100 ng/ml stem cell factor (SCF) (Peprotech, Rocky Hill, USA), 50 ng/ml interleukin-3 (IL-3) (Peprotech), and 100 ng/ml granulocyte colony-stimulating factor (Peprotech). Induction condition for erythroid lineage is: 30% FCS, 100 ng/ml SCF, 50 ng/ml IL-3, and 100 ng/ml erythropoietin (Peprotech). Induction condition for megakaryocyte lineage is: 10% FCS, 100 ng/ml SCF, 50 ng/ml IL-3, and 100 ng/ml thrombopoietin (Peprotech). The cells were cultured for 14 days at 37°C and 5% CO₂, cytokines were added every other day, and culture media were changed every 3 days [19,20].

Fluorescence flow cytometry analysis of the surface markers

Cells were washed with phosphate-buffered saline (PBS), and 10 µl of CD34-FITC, CD38-PE, CD15-FITC, CD235a-PE, or CD41a-FITC antibodies (BD, New Jersey, USA) was added to each tube containing 10⁵ cells. After being mixed and incubated at room temperature for 15 min in the dark, cells were washed twice with PBS and analyzed by flow cytometry (BD). IgG-FITC and IgG-PE were used as isotype control.

RNA purification and quantitative real-time polymerase chain reaction

Total RNA from the cultured cells was isolated with Trizol reagent according to the manufacturer’s recommended protocol. cDNA was synthesized using 1 µg of RNA with avian myeloblastosis virus-reverse transcriptase enzyme and oligo(dT)15 primer according to the manufacturer’s protocol. Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted using SYBR green PCR master mix reagent (TaKaRa, Otsu, Japan) and detected by an ABI 7900HT fast real-time PCR system (Applied Biosystems, Foster City, USA). Primer sequences were designed by using Primer 5 and listed in **Supplementary Table S1**. Fold expression relative to β-actin was calculated by using 2^{-ΔΔCt} method.

Chromatin immunoprecipitation-qPCR assays

Chromatin immunoprecipitation (ChIP)-qPCR was performed as previously described [21]. Purified CD34⁺CD38^{low} HSCs were cross-linked with 0.1% formaldehyde in RPMI medium containing 10% FCS for 10 min at 37°C. Then cells were washed with 10 volumes of RPMI medium. Cells (1.4 × 10⁶) were then lysed in 400 µl lysis buffer containing 1% sodium dodecyl sulfate (SDS), 10 mM EDTA, and 50 mM Tris-HCl, pH 8.0, and were sonicated with a Branson Sonifier (Cole-Parmer, Illinois, USA) in 1.5 ml Eppendorf tubes. ChIP experiments were performed on solubilized chromatin in 10-fold diluted ChIP buffer (Upstate Biotechnology, Charlottesville, USA) using 2 × 10⁵ cells per histone modification. All immunoprecipitation and washing steps were performed in Eppendorf tubes at 4°C.

Chromatin was pre-cleared by incubating with 60 ml salmon sperm DNA/protein A agarose beads for 1 h and then incubated overnight with antibodies against acH4, acH3, H3K4me2, H3K4me3, H3K9me3, and H3K27me3 (all from Upstate Biotechnology). Immunocomplexes were collected by incubating with 60 ml salmon sperm DNA/protein A agarose beads, and then washed with 1 ml of low-salt immune complex wash buffer, high salt immune complex wash buffer, LiCl immune complex wash buffer, and TE buffer. The modified histone/DNA complexes were eluted with 200 μ l of 0.1 M NaHCO₃ containing 1% SDS. After reversal of cross-link, DNA was purified by phenol/chloroform extraction. An aliquot of the sonicated chromatin was treated identically for use as input. ChIP experiments for the six kinds of histone modifications were performed on the same chromatin sample. Chromatin of CD34⁺CD38^{low} HSCs isolated from ~30 cord blood samples was pooled for each complete experiment.

qPCR was performed to determine the relative enrichment of gene segments in ChIP compared with input DNA. Reactions were performed in triplicate using SYBR Green PCR master mix reagent and detected by the ABI 7900 Sequence Detection System (Applied Biosystems). Percent of immunoprecipitation was calculated by the average value of the corresponding input normalized by dilution factor. To compare histone modifications in different cell types, acH3, acH4, and H3K4me2/3 modification levels were routinely normalized to β 2-microglobulin (*B2m*), as it is a ubiquitously expressed gene; for H3K9me3 and H3K27me3 modification levels, non-hematopoietic related *THP* gene was used as internal reference. PCR primers were designed within the promoter regions or known regulatory sequences (Supplementary Table S2).

Results

Isolation and differentiation of CD34⁺CD38^{low} HSCs

CD34⁺CD38^{low} HSCs were isolated from human umbilical cord blood. The purity of CD34⁺CD38^{low} cells reached

91.25% (Supplementary Fig. S1). Differentiations of CD34⁺CD38^{low} HSCs towards CD15⁺ (granulocytic) cells, CD235a⁺ (erythroid) cells, and CD41a⁺ (megakaryocytic) cells were induced through a combination of different cytokines. After induction, the positive cells were detected by fluorescence-activated cell sorting. The CD41a positive cells are 86.52%, CD15 positive cells are 91.49%, and CD235a positive cells are 95.55% (Supplementary Fig. S2). In the meantime, lineage-specific gene expression profile was detected by qRT-PCR. *PU.1* and *MPO* expressions were increased during HSC differentiation into granulocytes, likewise *EPOR* expression was increased during differentiation into erythroid cells, and *CD41a* expression was increased during differentiation into megakaryocytes (Supplementary Fig. S3).

Lineage-affiliated genes in CD34⁺CD38^{low} HSCs are associated with a certain level of acH3 and acH4, high level of H3K4me2, and low level of H3K4me3

We investigated the pattern of active histone modifications by ChIP experiments in CD34⁺CD38^{low} HSCs. In multipotent CD34⁺CD38^{low} HSCs, many genes were associated with a certain level of acH3 and acH4 modifications (Fig. 1). The level of acH4 on some lineage-affiliated genes was even similar to that of *B2m*, such as *MPO*, *CD11b*, *PAX5*, and *CD79a*. In general, the level of acH3 modification was similar to that of acH4 modification at those genes in CD34⁺CD38^{low} HSCs. At the same time, we observed the pattern of H3K4me2 and H3K4me3 modifications by ChIP experiments in CD34⁺CD38^{low} HSCs. As shown in Fig. 1, a high H3K4me2 modification level was found in these gene loci, but H3K4me3 level was very low or undetectable. These results indicated that some active histone modifications such as histone acetylation and H3K4me2 at these lymphoid- and myeloid-affiliated genes occur before transcription and lineage commitment, suggesting that the histone modification is involved in the maintenance of activation potential rather than gene expression.

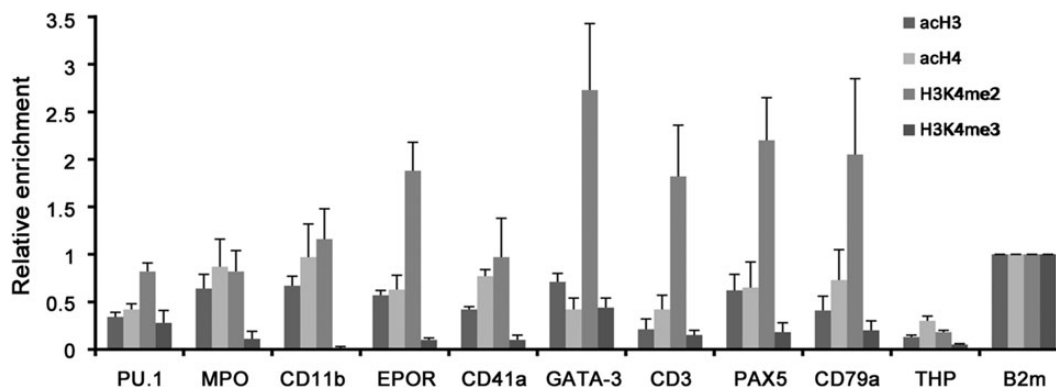


Figure 1. Active histone modifications of lineage-specific genes in CD34⁺CD38^{low} cell population, analyzed by ChIP-qPCR. Histogram shows the enrichment values (bound/input) normalized to *B2m* control. Error bars represent the mean \pm SD of three independent ChIP experiments.

Lineage-affiliated genes in CD34⁺CD38^{low} HSCs are associated with repressive histone modifications to some extent

We further examined the presence of the repressive modifications (H3K9me3 and H3K27me3) at these lymphoid- and myeloid-affiliated genes in CD34⁺CD38^{low} HSCs (Fig. 2). Most of the genes analyzed also had a certain level of H3K9me3 or H3K27me3 modification in CD34⁺CD38^{low} HSCs. Particularly, lymphoid-affiliated genes have a similar modification level of H3K9me3 and H3K27me3 to that of non-hematopoietic-related *THP* gene, higher than that of myeloid-affiliated genes (Fig. 2). Interestingly, H3K9me3 modification is more popular than H3K27me3 at the promoter of lineage genes in CD34⁺CD38^{low} HSCs. Together, these results showed the coexistence of active and repressive histone modifications at most myeloid- and lymphoid-affiliated genes in CD34⁺CD38^{low} HSCs, supporting the idea that bivalent modification keeps lineage-specific genes in a 'poised' state, thus maintaining the multipotent state of stem cells. In CD34⁺CD38^{low} HSCs, the level of repressive histone modifications of *PU.1* was much lower than that of other genes, indicating that *PU.1* possesses a more open chromatin structure in HSCs.

Dynamic changes of histone modifications during differentiation of CD34⁺CD38^{low} HSCs towards granulocytic, erythroid, and megakaryocytic cells

We then analyzed the dynamic changes of histone modifications from CD34⁺CD38^{low} HSCs to committed cells. After differentiation into granulocytic cells, the levels of acH3, acH4, and H3K4me2 on granulocytic-affiliated genes (*PU.1*, *MPO*, and *CD11b*) were increased or remained the same (Fig. 3A–C), while H3K4me3 level was increased significantly (Fig. 3D). At the same time, the repressive histone modifications were decreased to a lower level (Fig. 4). In granulocytic cells, for erythroid/megakaryocytic-affiliated (*EPOR* and *CD41a*) and lymphoid-affiliated genes (*GATA-3*, *CD3*, *PAX5*, and *CD79a*), it was shown that the levels of acH3, acH4, and H3K4me2 were decreased,

the level of H3K4me3 remained low, while the repressive histone modifications (H3K9me3 and H3K27me3) were drastically elevated.

When CD34⁺CD38^{low} cells differentiated towards erythroid or megakaryocytic cells, changes of histone modifications of the erythroid- or megakaryocytic-affiliated genes and other genes showed similar patterns (Figs. 3 and 4). After differentiation into erythroid cells, levels of acH3, acH4, and H3K4me2 in *EPOR* gene was increased or remained the same, H3K4me3 level was increased significantly, and repressive histone modifications (H3K9me3 and H3K27me3) became lower. When HSCs differentiated into megakaryocytes, the histone modifications at *CD41a* gene showed the same changes as *EPOR* gene in erythrocytes (Figs. 3 and 4). In erythroid/megakaryocytic cells, granulocytic-affiliated genes (*PU.1*, *MPO*, and *CD11b*) and lymphoid-affiliated genes (*GATA-3*, *CD3*, *PAX5*, and *CD79a*) showed the same pattern with erythroid/megakaryocytic-affiliated and lymphoid-affiliated genes in granulocytic cells. Observation in myeloid committed cells showed that lymphoid-affiliated genes have more repressive histone modifications than myeloid-affiliated genes (Fig. 4). The above results indicate that during lineage commitment of HSCs, lineage-specific genes have higher level of active histone modifications, especially H3K4me3, and lower repressive histone modifications. In contrast, other non-lineage-specific genes have higher level of repressive histone modifications and lower level of active histone modifications. Together, those changes in the level of histone modifications result in accurate control of lineage commitment, and keep the committed cells to a more restricted differentiation capacity.

Discussion

In this study, we investigated the histone modification pattern in primary CD34⁺CD38^{low} HSCs and myeloid lineage-committed cells. We analyzed six histone modifications specific for active or repressive chromatin state in a panel of lymphoid- and myeloid-affiliated genes. Although

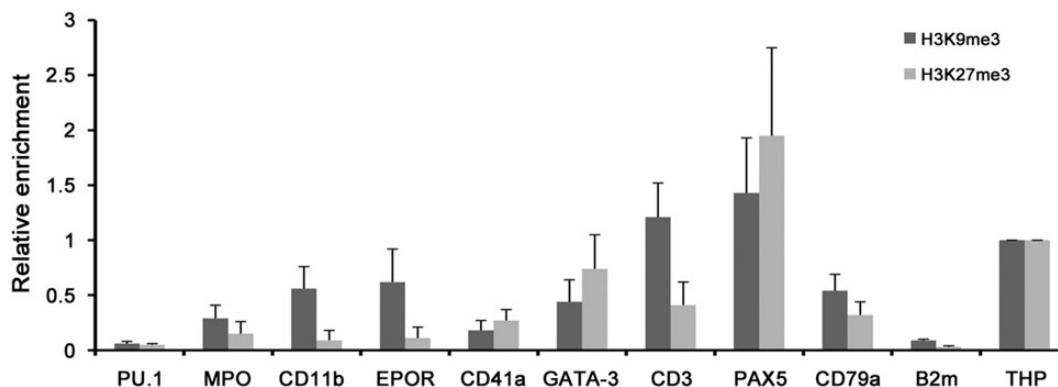


Figure 2. Repressive histone modifications of lineage-specific genes in CD34⁺CD38^{low} cell population, analyzed by ChIP-qPCR Histogram shows the enrichment values (bound/input) normalized to *THP* control. Error bars represent the mean \pm SD of three independent ChIP experiments.

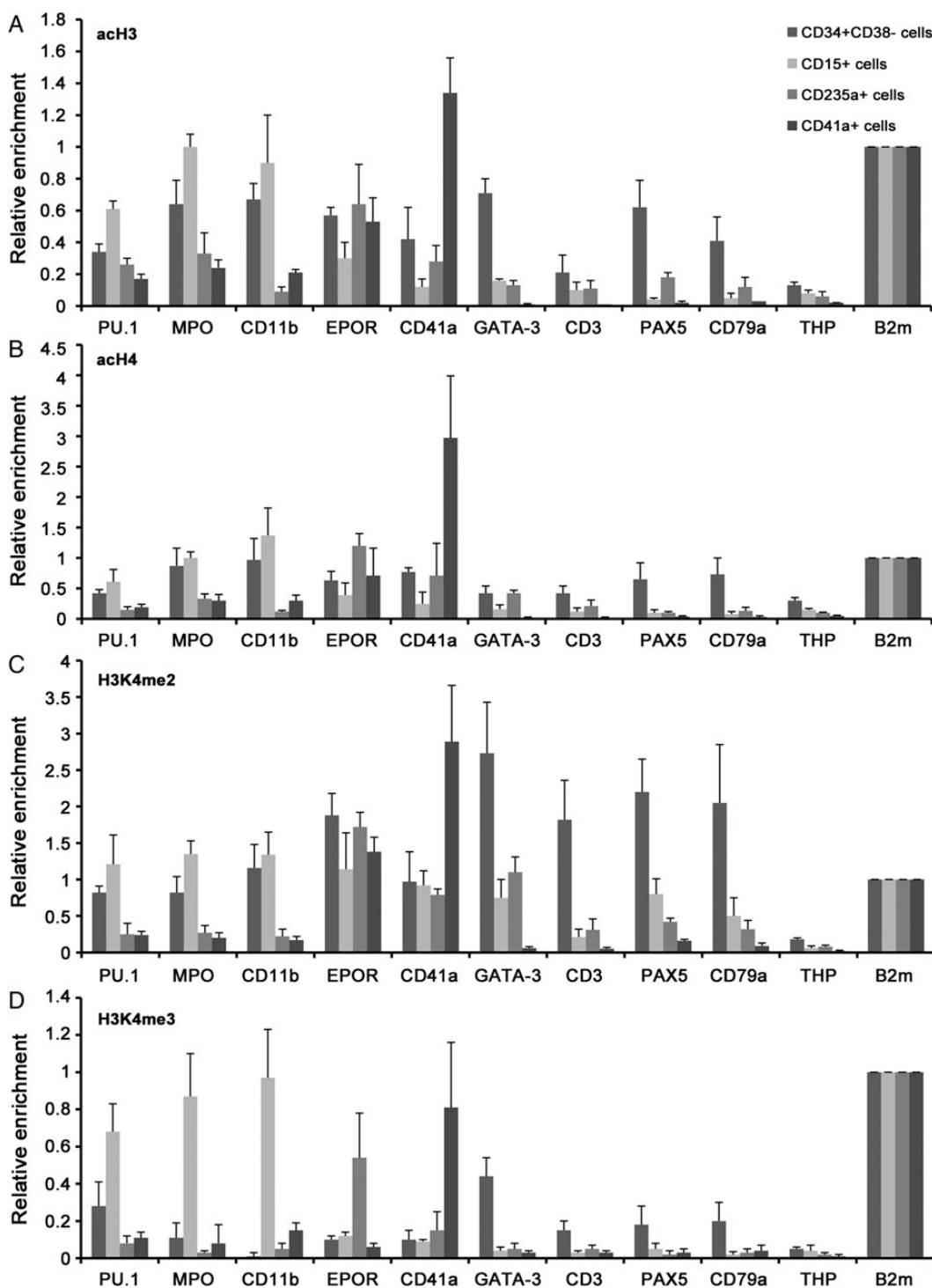


Figure 3. Changes of active histone modifications of lineage-affiliated genes in different cell types Histogram shows the enrichment values (bound/input) normalized to *B2m* control. Error bars represent the mean \pm SD of three independent ChIP experiments.

the results presented here were not based on a genome-wide analysis, clear trends were observed.

First, in multipotent cord blood CD34⁺CD38^{low} HSCs, coexisting of the active histone modifications (acH3, acH4, and H3K4me2) and repressive modifications (H3K9me3 and H3K27me3) was found at the promoter of most lineage-affiliated genes, and H3K4me3 modification level

was low. Previous studies showed that histone acetylation is closely related to gene activation [17]. Here, our results suggested that histone acetylation is associated with open/accessible chromatin conformation in addition to gene activation. Maës *et al.* [22] observed similar phenomenon through analysis of a panel of lymphoid-affiliated genes in HSCs. Although H3K4me2 was believed to be a mark of

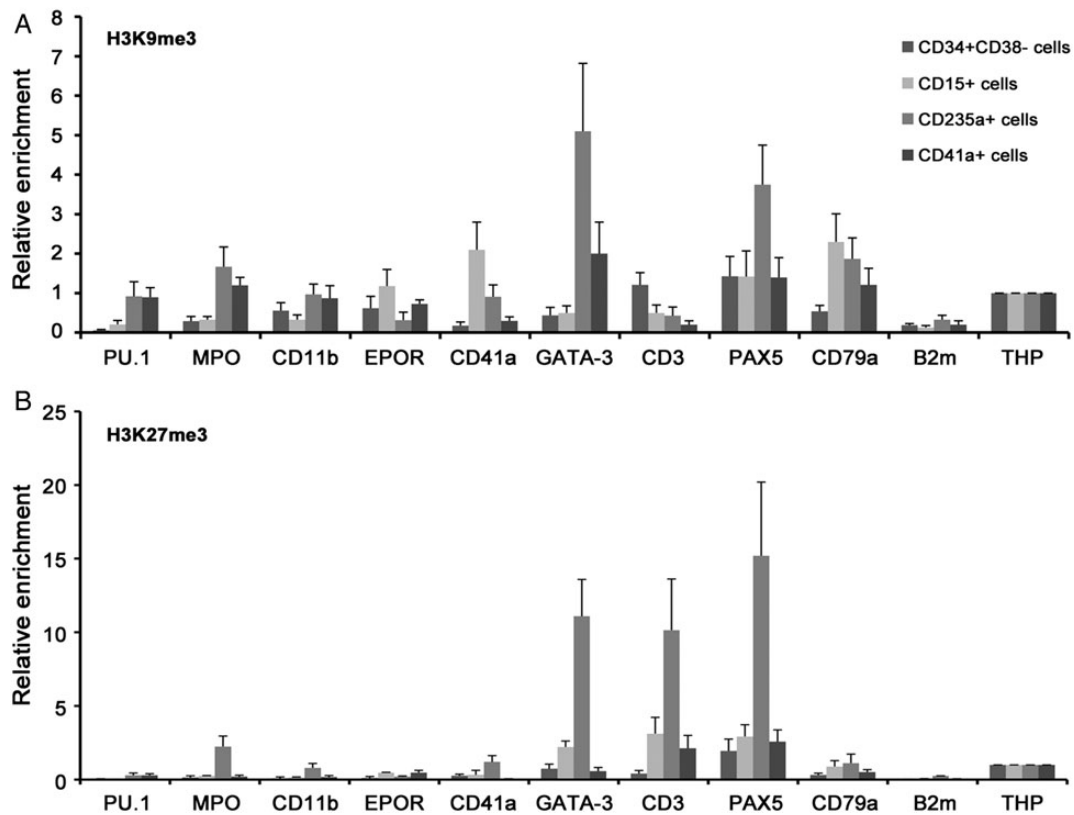


Figure 4. Changes of repressive histone modifications of lineage-affiliated genes in different cell types Histogram shows the enrichment values (bound/input) normalized to *THP* control. Error bars represent the mean \pm SD of three independent ChIP experiments.

activation, our results showed that H3K4me2 is not associated with gene expression. H3K4me2 modification enrichment at regulatory regions of differentiation genes prior to their activation suggests that H3K4me2 is involved in the maintenance of activation potential required for differentiation. This result is consistent with other reports. By comparing the histone modification characteristics between HSCs and ESCs, Orford *et al.* [11] found that a multipotential hematopoietic cell line maintains a population of genes in a unique epigenetic state, defined by the presence of dimethylation, but not trimethylation of histone H3 (H3K4me2⁺/H3K4me3⁻). Attema *et al.* [18] also reported this phenomenon in murine HSCs. In murine HSCs, H3K4me2 was enriched at lineage-affiliated genes, which ensured that these genes are ‘primed’ for expression when differentiation starts, although these genes were expressed at low levels or not at all in murine HSCs. Others also reported that monomethylations such as H3K4me1, H3K9me1, and H3K27me1 are associated with differentiation genes prior to their activation [12,13]. These pieces of evidence suggested a more complex chromatin-modifying system involved in the maintenance of the genes ready for expression. Maës *et al.* [22] reported that they did not detect the repressive H3K9me3 and H3K27me3 marks on lymphoid-affiliated genes. In our experiment, we observed that these lineage-specific genes have some

repressive modifications in CD34⁺CD38^{low} HSCs. The level of these repressive modifications in CD34⁺CD38^{low} HSCs is significantly lower than that in committed myeloid cells. Weishaupt *et al.* [8] observed the repressive histone modifications on lineage-specific genes in mHSCs using high throughput ChIP-Seq technology, which is consistent with our results. So in CD34⁺CD38^{low} HSCs, coexistence of the active histone modifications (acH3, acH4, and H3K4me2) and repressive modifications at the promoter of most lineage-affiliated genes ensures the chromatin in an open/access state and makes sure lineage-specific genes be ‘poised’ for transcription. These results supported the multilineage priming hypothesis that low-level transcription of lineage-specific genes expresses in HSCs/HPCs. And in CD34⁺CD38^{low} HSCs, low level of H3K4me3 modification and a certain level of repressive modifications (H3K9me3 and H3K27me3) in the lineage-affiliated genes ensure these lineage-specific genes in low expression state in HSCs, further maintaining the multipotency of HSCs.

Secondly, differentiation of multipotent hematopoietic progenitors towards granulocytic, erythroid, and megakaryocytic cells leads to significantly elevated H3K4me3 and lower H3K9me3/H3K27me3 modifications in lineage-affiliated genes, while losing histone H3 and H4 acetylation, and addition of repressive H3K9me3 or H3K27me3 mark at

lineage-inappropriate genes. Our results confirmed that H3K4me3 is generally associated with gene expression. During HSC differentiation, lineage-affiliated genes have higher levels of H3K4me3 modification, consistent with higher expression of these genes. H3K27me3 and H3K9me3 were generally correlated with gene silencing. In our experiment, we observed this phenomenon. Interestingly, at the promoter of lineage-affiliated genes, H3K9me3 modification is more popular than H3K27me3. Previous studies have shown that α -globin had a significant H3K27me3 modification level in non-erythroid cells, but β -globin was different [23]. These results indicated that H3K9me3 and H3K27me3 may have different roles in gene silencing. In addition, DNA methylation also plays an important role in maintaining non-lineage-specific genes [24]. Recently, it was reported that changes in gene subnuclear location relative to pericentromeric heterochromatin appear to be determined by whether the gene will be permanently silenced or activated [25]. Our results show that combinatorial histone modification pattern at these regulatory regions ensures inheritance of epigenetic states to maintain lineage-specific expression or repression during the process of lineage commitment. In addition, differentiation of HSCs into terminal mature cells requires silencing of multipotency genes in HSCs. Kerényi *et al.* [26] reported that Lsd1, a histone demethylase, is important in silencing hematopoietic stem and progenitor cell genes during HSC differentiation. In this study, HOXA9, a multipotency transcription factor in HSCs, was analyzed after HSC differentiation. It was found that repressive histone modification marks were enriched in the promoter region of this gene and the active histone modifications were decreased, rendering its expression silencing activity (data not shown).

In summary, combinatorial histone modification patterns have been implicated in the maintenance of the multipotency of HSCs and the accuracy of gene expression program during HSC differentiation.

Supplementary Data

Supplementary data are available at *ABBS* online.

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