Onset and inheritance of abnormal epigenetic regulation in hematopoietic cells

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Abnormal epigenetic regulation of gene expression contributes significantly to a variety of human pathologies including cancer. Deletion of hypersensitive site 2 (HS2) at the human β-globin locus control region can lead to abnormal epigenetic regulation of globin genes in transgenic mice. Here, two HS2-deleted transgenic mouse lines were used as model to demonstrate that heritable alteration of chromatin organization at the human β-globin locus in multipotent hematopoietic progenitors contributes to the abnormal expression of the β-globin gene in mature erythroid cells. This alteration is characterized by specific patterns of histone covalent modifications that are inherited during erythropoiesis and, moreover, is plastic because it can be reverted by transient treatment with the histone deacetylase inhibitor Trichostatin A. Altogether, our results indicate that aberrant epigenetic regulation can be detected and modified before tissue-specific gene transcription, a finding which may lead to novel strategies for the prevention of chromatin-related pathologies.

INTRODUCTION

Gene expression in differentiated cells of higher eukaryotes is strongly influenced by chromatin organization (1). Heterochromatin and euchromatin, respectively, preclude and permit gene expression. Variations in chromatin organization are frequently induced by ATP-dependent chromatin remodeling (2) and histone modifying complexes (3). Transcriptionally active and transcriptionally inactive chromatin are each characterized by particular combinations of histone covalent modifications (4). For instance, hyperacetylated histone H3 and H4 (5), as well as K4 di- and tri-methylated histone H3 (6–8), are generally enriched at transcriptionally active genes, whereas histone H3 K9 (9–12) and K27 methylation (13,14) are often associated with transcriptionally restrictive chromatin.

The expression of different developmental- and tissue-specific genes is preceded by their ‘potentiation’ in progenitor cells. The term potentiation, also known as priming (15,16), refers to the mechanism of opening chromatin domains in progenitor cells (15–20) in order to render genes accessible to transactivators and facilitate full transcriptional activation in definitive cells (19,21–23).

Little is known about potentiation of genes subjected to abnormal epigenetic regulation. In Drosophila, the decision leading to gene silencing can be set before transcriptional activation and memorized until transcriptional initiation (24,25).

In mammals, it is not known whether abnormal gene silencing in differentiated cells can be influenced by epigenetic events taking place in multipotent progenitor cells. However, different investigations made in mammalian systems have brought indirect evidence suggesting that after transcriptional activation, some events characterized by abnormal epigenetic regulation are completely or partially inherited (26–29), whereas others are not (30). Abnormal epigenetic regulation can result from genomic rearrangements that relocate a gene from euchromatin to heterochromatin, thereby causing its complete or partial silencing. These phenomena are termed ‘position effects’. Among the different position effects, position effect variegation (PEV) is characterized by the activity of a gene that is restricted to a subset of cells from a homogenous population. Similar to the inactivation of tumor suppressor genes in cancer, the silencing associated with PEV is randomly set and its occurrence is consistent with cell-to-cell differences in the ability of heterochromatin to invade a particular gene locus located within euchromatic regions (31).

Transgene expression is also influenced by PEV, and the human β (huβ)-globin locus has been used to model epigenetic regulation in mammals (27,32–34). This locus consists of five developmentally regulated genes (ε–ζγ–εγ–δ–β). High-level globin gene expression is mediated by their direct interactions with the β-globin locus control region (LCR) (35–37). The huβ-globin LCR is composed of five
DNaseI hypersensitive sites (HS1–5) and is a major determinant of chromatin organization at the β-globin locus (38).

In humans (39) and in transgenic mice (27,32), human β-globin loci with partial LCR are susceptible to position effects. Two mouse lines with a huβ-globin locus in which the LCR hypersensitive site 2 (HS2) is deleted (Δ2B and Δ2C) have been characterized (27). In these lines, the Δ2 locus is integrated into pericentric heterochromatin, and the huβ-like globin genes display a PEV pattern of expression; thus, transcription of the huβ-globin gene is only observed in a subset of adult erythroid cells (EryC). In Δ2B and Δ2C, PEV is associated with variations of chromatin organization in EryC and can be influenced by modifications in the concentration of chromatin-associated proteins and transcription factors (40).

We recently reported that in hematopoietic progenitor cells (HPC), the huβ-globin locus is characterized by potent chromatin organization including gene- and developmental-specific patterns of histone covalent modifications (18). We suggested that the huβ-like globin genes are independently potentiated before being transcribed in EryC. Here, we verified whether aberrant epigenetic regulation of globin gene expression results from disrupted potentiation in HPC. To this end, we studied epigenetic regulation of the huβ-globin gene in the PEV line Δ2B or Δ2C and in line 2 mice (ln2), which express the globin genes normally (41). We show that the decision to variegate is set early during hematopoiesis and is inherited, with some restrictions, until final erythroid differentiation. In line with this finding, we demonstrate that important chromatin abnormalities characterize the huβ-globin locus in Δ2B and Δ2C HPC. Additionally, we show that transient Trichostatin A (TSA) treatment of Δ2B HPC can modify PEV in erythroid-derived cells, whereas TSA treatment of EryC has no effect on huβ-globin gene expression. Altogether, our data indicate that abnormal epigenetic regulation in mammals can result from the disruption of gene potentiation in multipotent progenitor cells. Furthermore, this phenomenon is inherited during tissue differentiation and can be effectively modulated before the onset of full gene activity.

RESULTS

Aberrant expression of the huβ-globin gene in EryC correlates with the disruption of the locus chromatin organization in HPC

The huβ-globin locus in each of ln2, Δ2B and Δ2C is integrated into pericentric heterochromatin. However, ln2 mice express the huβ-gene normally (41), whereas Δ2 lines display variated gene expression as detected in 25% (Δ2B) or 4% (Δ2C) of transgenic EryC (27). We recently showed that the huβ-gene and -promoter are epigenetically marked by histone H3 acetylation/K4 dimethylation (H3ac/H3-meK4) in human and in ln2 bone marrow HPC (18). These histone covalent modifications appear to be important for the potentiation of the huβ-globin gene in bone marrow cells.

If the abnormal chromatin organization associated with PEV expression pattern results from disrupted epigenetic events occurring early during hematopoiesis, epigenetic marks that characterize the locus in ln2 HPC are likely to be altered in Δ2B and Δ2C HPC. Thus, chromatin organization and histone covalent modifications at the huβ-globin locus (Fig. 1A) were analyzed in HPC and EryC purified from ln2, Δ2B or Δ2C bone marrow. Cells selected for their c-Kit+/CD31high/Ly-6c− phenotype (HPC) and Ter119+ (EryC) were subjected to DNaseI sensitivity and chromatin immunoprecipitation (ChIP) analyses (18). DNaseI sensitivity and histone covalent modifications were evaluated by quantitative duplex PCR analysis. One primer set was specific for a region of the locus and another primer set was specific for genes transcriptionally inactive in hematopoietic cells, namely ZFP227 (ZFP, Figs 1–3 and 5) or amylase 2.1y (data not shown, but results were the same). PCR reactions were performed under conditions of linear amplification, and the relative enrichment of globin sequences was normalized to DNaseI non-treated samples or ChIP input material (18). DNaseI sensitivity at mouse β major promoter (βmaj) was analyzed in all lines and used as reference for the assay. In both HPC (Fig. 1B) and EryC (Fig. 1C), βmaj sensitivity is similar among transgenic lines, indicating that the DNaseI digestion patterns illustrated in Figure 1 are comparable in ln2, Δ2B and Δ2C samples. The HS3 region of the LCR was shown to be significantly more sensitive to DNaseI digestion in ln2 HPC than in PEV HPC (Fig. 1D). For all lines, HS3 sensitivity is higher in EryC than in HPC; nevertheless, HS3 accessibility is considerably lower in PEV EryC than in ln2 EryC (Fig. 1E). A similar trend is observed for the HS4 region (data not shown). The huβ-promoter manifests little sensitivity to DNaseI in ln2 HPC (18), and DNaseI accessibility is lost in PEV HPC (Fig. 1F). As observed in fetal liver EryC (40), huβ-promoter accessibility in bone marrow-derived EryC appears to be proportional to the number of EryC capable to transcribe the huβ-gene (ln2 > Δ2B > Δ2C, Fig. 1G). Thus in PEV lines, chromatin ‘activation’ at HS3 and HS4 is affected early during hematopoiesis, suggesting that incomplete chromatin remodeling of the locus in HPC provides the basis for less efficient transcriptional activity in EryC.

ChIP analyses revealed that in HPC derived from either ln2 or PEV lines, HS3 is not significantly acetylated at histone H3 (Fig. 2A). In contrast, HS3 is significantly acetylated at histone H4 and K4 dimethylated at histone H3, but only in ln2 HPC (Fig. 2B and C). Similar results were obtained when the LCR HS4 region was analyzed (data not shown). In EryC, differences between PEV lines and ln2 are even more remarkable, as HS3 in ln2 EryC displays a higher level of H3ac, H4ac and H3-meK4 than that in Δ2B or Δ2C EryC (Fig. 2A–C). The huβ-promoter is not significantly acetylated at histone H4 either in ln2 HPC or in PEV HPC (Fig. 2E, ratio: 1), whereas huβ-promoter H3ac and H3-meK4 are greater in HPC purified from ln2 than that from PEV lines (Fig. 2D and F). Similarly, the huβ-globin gene displays H3ac, H4ac and H3-meK4 exclusively in HPC purified from ln2 (Fig. 2G–I). When compared with ln2, Δ2B and Δ2C EryC display lower levels of H3-meK4 at the huβ-promoter (Fig. 2F) and of H3ac, H4ac and H3-meK4 at the huβ-gene (Fig. 2G–I). Taken together, these results indicate that variegated huβ-gene expression in Δ2B and Δ2C EryC correlates with an abnormal pattern of chromatin organization and histone covalent modifications at the LCR (HS3 and HS4), huβ-gene and -promoter in EryC, and, remarkably, in HPC.
A heritable ‘silencing’ mark in HPC is associated with PEV in Δ2B EryC

It has been suggested that histone methylation serves as static mark for a gene to be either expressed or repressed. In particular, K9 or K27 methylation (H3-meK9 or H3-meK27) has been linked to heterochromatic gene silencing (9–14). As mentioned earlier, HPC purified from PEV lines are affected by a loss of histone acetylation/K4 dimethylation at HS3 and huβ-gene and -promoter, which correlates with a more compacted chromatin organization and with gene inactivation in EryC. Because the PEV pattern of huβ-gene expression appears to be linked to the disruption of chromatin potentiation, we looked for possible H3-meK9/K27 of the huβ-globin locus in Δ2B HPC. ChIP assays were performed with antibodies recognizing predominantly H3-meK9, but which

Figure 1. DNasel sensitivity assay of the huβ-globin locus in ln2, Δ2B and Δ2C. (A) Map of the huβ-globin locus; genes are shown as black boxes and vertical arrows indicate location of LCR HS. Amplicons are indicated by gray boxes. An expanded view of the huβ-globin gene region illustrates in more detail the amplified regions; each segment represents 100 bp. (B–G) PCR-based DNasel analyses. Each point of the curves represents samples of comparable molecular weight (ranging from 12 to 0.3 kb). PCR products were quantified by Phosphorimager and plotted on graphs relative to input samples (DNasel untreated chromatin). Y-axis: relative amount of PCR products (globin/ZFP); X-axis: increasing DNasel concentration, ranging from 0 to 0.35 U. Standard errors of mean (SEM) are indicated by vertical lines and are the results of at least three independent experiments. P-values were obtained by unpaired Student’s t-test. ZFP; ZFP37; βmaj: β major promoter; Huβp: huβ-promoter; Huβg: huβ-gene; HPC: hematopoietic progenitor cells; EryC: erythroid cells.
may not distinguish between dimethylated and trimethylated K9 or methylated K9 from methylated K27 (42). This analysis revealed that in Δ2B HPC, HS3, the huβ-gene and -promoter, together with the region immediately upstream (huβ5), are 1.5–2.5-fold more H3-meK9/K27 than ZFP (Fig. 3A). Similar levels of H3-meK9/K27 are maintained in EryC (Fig. 3A). As expected, H3-meK9/K27 is not detected either at HS3 or along the huβ-region in ln2 HPC (data not shown).

Additionally, we examined incorporation of the linker histone H1 (43) at HS3 and huβ-region in Δ2B- or ln2-derived cells. Histone H1 is suggested to facilitate chromatin compaction (44). Although no H1 binding in ln2-derived cells could be detected (data not shown), we observed that H1 is enriched at the huβ-gene and -promoter in Δ2B HPC, and also at huβ5 and HS3 in Δ2B EryC (Fig. 3B). These findings suggest that in Δ2B HPC, H3-meK9/K27 and H1 binding affect the chain of epigenetic events leading to proper chromatin activation in EryC, directing normal gene expression only on a stochastic basis in a subpopulation of cells.

**The decision to variegate is set and inherited before full transcriptional activation**

The abnormal chromatin organization of the huβ-globin locus in Δ2B and Δ2C HPC suggests that PEV is the result of abnormal potentiation of the locus during hematopoiesis. To characterize onset and inheritance of the abnormal chromatin organization in multipotent HPC of PEV lines, we used clonal assays in methylcellulose that permit ex vivo recapitulation of hematopoietic differentiation. In effect, with appropriate culture conditions, individual hematopoietic progenitors, known as colony forming units (CFUs), develop into clones that are identified as colonies. CFU-GEMM (colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte) indicates a pluripotent hematopoietic progenitor, which is capable to differentiate into erythroid and myeloid cells. Instead, BFU-E (burst forming unit-erythroid) and CFU-E (colony forming unit-erythroid) indicate, respectively, an early- or late-erythroid committed progenitor. BFU-E and CFU-E colonies are constituted exclusively by EryC.

If PEV does not follow abnormal potentiation of the huβ-globin locus in HPC and its onset is linked to high-level transcription of the huβ-gene in EryC, all CFU-GEMM, BFU-E and CFU-E colonies derived from Δ2B HPC should display the same PEV pattern of huβ-gene expression, i.e., the same percentage of EryC per colony will express the huβ-gene (Fig. 4A). However, if in accord with our chromatin analyses PEV results from abnormal potentiation of the locus in a subset of HPC that is inherited during hematopoiesis, the huβ-gene will be either expressed or silenced in all EryC of each colony, giving rise to an ‘all-or-none’ pattern of gene expression.
of human (hu) D2B CFU-GEMM and BFU-E colonies. The inconsistency (17 out of 24 tested) and 30% of CFU-E (9 out of 31 tested) 90% of CFU-GEMM (17 out of 19 tested), 70% of BFU-E and as internal reference. Hu (45), the latter being used as control for reaction efficiency.

BFU-E colonies. As exemplified in Figure 4F, in some instances (colonies 5 and 6) or undetectable (colonies 7 and 8) levels of hu-gene expression were seen in human EryC. For this purpose, hu-gene expression was analyzed by semi-quantitative RT–PCR with primers amplifying hu and βmaj mRNAs (45), the latter being used as control for reaction efficiency and as internal reference. Hu-gene expression was seen in 90% of CFU-GEMM (17 out of 19 tested), 70% of BFU-E (17 out of 24 tested) and 30% of CFU-E (9 out of 31 tested) colonies (exemplified in Fig. 4D). Interestingly, hu-gene expression appears to vary significantly between individual D2B CFU-GEMM and BFU-E colonies. The inconsistency of hu-gene expression suggests that the percentage of expressing cells differs from one colony to another, i.e., the individual CFU-GEMM and BFU-E possess distinct patterns of PEV expression. To better evaluate variegation patterns, hu-gene expression in separate D2B BFU-E colonies was quantified by real-time RT–PCR using ln2 BFU-E colonies as reference and gapdh mRNA as internal control. We observed that in D2B BFU-E colonies, the hu-gene was expressed at high (colonies 1–3), intermediate (colony 4), very low (colonies 5 and 6) or undetectable (colonies 7 and 8) levels (Fig. 4E). The aim of the real-time PCR analysis was to verify whether hu-gene expression is identical among D2B BFU-E colonies (we note that ratios presented in Fig. 4E do not take into account transgene copy number; three copies in D2B and one copy in ln2). PEV inheritance was further examined by single-cell RT–PCR (discussed earlier) of the hu-gene expression on individual EryC isolated from D2B BFU-E colonies. As exemplified in Figure 4F, in some BFU-E, all EryC express the hu-gene (e and h); whereas in others the hu-gene is not expressed at all (a and g); finally, many BFU-E colonies (b, c, d, f, i and j) are constituted by a variable proportion of expressing, as well as non-expressing EryC. Immunofluorescence analysis of single D2B BFU-E colonies with anti-human hemoglobin A antibodies did corroborate the RT–PCR results (Supplementary Material, Fig. S1). Therefore, single-cell analyses confirm that the number of EryC expressing the hu-gene is not constant among D2B BFU-E colonies.

PEV variability and the absence of hu-gene expression in some CFU-GEMM and BFU-E colonies strongly suggest that (i) PEV results from abnormal potentiation of the locus in a subset of multipotent HPC prior to the CFU-GEMM stage of hematopoietic differentiation and (ii) in a restrictive chromatin context, the abnormal epigenetic marks that characterize the hu-globin locus in early HPC are inherited during hematopoiesis, but their transmission to progeny cells is not always stable. This ‘imperfect’ inheritance would lead to low-frequency PEV reversion during hematopoiesis (Fig. 4C).

Figure 3. Histone H3 K9 methylation and histone H1 distribution in the PEV line D2B. D2B HPC (gray bars) or EryC (black bars) were subjected to ChIP analysis with (A) anti-K9/K27 dimethylated histone H3 (H3-meK9) or (B) anti-histone H1 (H1) antibodies. The level of enrichment of globin regions relative to the control (ZFP) and input samples is represented by bars, with their corresponding SEM deviations. A value of 1 indicates that no enrichment is detected. Huβ5: huβ-promoter 5’ region; Huβp: huβ-promoter; Huβg: huβ-gene.

We previously showed that the treatment of ln2 EryC with the histone deacetylase inhibitor TSA results in histone H3 hyperacetylation at the huβ-promoter without significantly modifying gene expression levels (18). The reduced levels of H3ac/ H3-meK4 found at the huβ-globin locus in D2B and D2C HPC and EryC could, in principle, render these lines responsive to TSA. Because both PEV lines display similar defects in chromatin activation in HPC and EryC, the effect of TSA treatment was analyzed exclusively in D2B cells. TSA-treated D2B EryC were purified and subjected to ChIP analysis using a panel of antibodies specific to acetylated or methylated histone H3 or H4. In TSA-treated EryC, H3ac at HS3 and hu-globin promoter significantly increases (Fig. 5A, black bars), whereas no change in H4ac is detected (Fig. 5B, black bars). As expected, TSA does not affect H3-meK4 in EryC (Fig. 5C, black bars). Using real-time RT–PCR and mouse gapdh mRNA as control (46), we observed that the hu-gene expression is not induced when EryC are treated with TSA (Fig. 6A). These data collectively indicate that TSA treatment of D2B EryC induces histone H3ac at HS3 and huβ-promoter but is not sufficient to modify the huβ-globin gene expression in these cells. Similar results were previously observed in ln2 TSA-treated EryC (18).

We then asked whether TSA treatment of HPC could modify histone covalent modifications leading to variation of hu-gene expression in EryC. For this purpose, D2B bone marrow cells were seeded on methylcellulose and transiently treated with TSA. Indeed, TSA, whose efficacy in culture is limited to ~20 h (47), was provided only once at the beginning of the culture. BFU-E and CFU-GEMM colonies were used for ChIP or real-time RT–PCR analysis. TSA treatment of D2B HPC enhances H3ac at the LCR (HS3) and hu-gene in erythroid-derived cells (Fig. 5A, gray bars). H4ac increases at HS3 and hu-gene but not at the huβ-promoter (Fig. 5B, gray bars), whereas the level of H3-meK4 increases at HS3 and hu-gene, and -promoter (Fig. 5C, gray bars). Therefore,
transient TSA treatment of Δ2B HPC induces H3ac, H4ac and indirectly H3-meK4 at the LCR (HS3) and huβ-gene in erythroid-derived cells. Real-time RT–PCR (Fig. 6A) and S1 nuclease protection assay (Fig. 6B) revealed that EryC derived from TSA-treated Δ2B HPC transcribe the huβ-gene more efficiently than ethanol-treated or fresh bone marrow derived EryC, whereas the huβ-gene expression does not increase in EryC derived from TSA-treated ln2 HPC (Fig. 6A). This important increase of the huβ-gene expression in Δ2B appears to be the consequence of higher frequency of individual huβ-globin expressing EryC. Indeed, single-cell RT–PCR performed with EryC isolated from independent Δ2B BFU-E colonies derived from TSA-treated HPC (exemplified in Fig. 6C) revealed that upon TSA treatment, almost all EryC transcribe the huβ-gene (compare Fig. 4F, no TSA, with Fig. 6C, presence of TSA).

These results not only confirm that appropriately ‘poised’ chromatin in HPC is necessary for correct huβ-gene transcription in EryC, but also indicates that (i) disrupted chromatin organization at the huβ-globin locus in HPC precedes abnormal huβ-gene expression in EryC and (ii) transient TSA treatment of HPC, but not of EryC, induces a significant increase of the huβ-gene activity in Δ2B erythroid-derived cells.

DISCUSSION
Abnormal gene expression and chromatin organization
In this study, we have investigated the role played by chromatin in the establishment and inheritance of tissue- and developmental-specific epigenetic control of gene expression. We show that, compared with ln2, the abnormal expression pattern of the huβ-gene in Δ2B and Δ2C EryC correlates with inappropriate chromatin organization of the locus (LCR and huβ-region) in HPC and EryC, which is characterized by decreased chromatin accessibility, lack of histone acetylation/K4 dimethylation, and by increased levels of K9/K27 methylation and H1 binding. Because histone H3-meK4 can block the interaction of the histone deacetylase NuRD complex and prevent H3-meK9, which in turn inhibits H3ac

Figure 4. PEV onset and inheritance. (A–C) Schematic view of the expected outcomes by clonal assays in methylcellulose. (A) PEV is set on a stochastic base in EryC (EryC). EryC-containing colonies (CFU-GEMM, BFU-E and CFU-E colonies) display the same level of huβ-gene expression, that is, the same variegation pattern. (B) PEV is set, on a stochastic base, in HPC. All EryC within CFU-GEMM, BFU-E or CFU-E colonies that originate from ‘active’ HPC express the huβ-gene. Conversely, all EryC within CFU-GEMM, BFU-E or CFU-E colonies that originate from ‘inactive’ HPC do not express the huβ-gene. (C) PEV is set, on a stochastic base, in HPC but epigenetic marks are not perfectly transmitted to the progeny. Some CFU-GEMM, BFU-E or CFU-E colonies show ‘all-or-none’ pattern of expression (as in B), but other colonies display different levels of huβ-gene expression, e.g., different variegation patterns, which depend on the degree of PEV reversion. (D) Representative examples of RT–PCR on single methylcellulose colonies. Single Δ2B CFU-GEMM, BFU-E or CFU-E colonies were used for one-step RT–PCR analysis, with primers amplifying both mouse βmaj (upper band) and huβ (lower band) gene transcripts (45). Each lane represents the expression pattern of one individual colony. Neg: negative control. Circle graphs indicate the proportion of colonies expressing the huβ-globin gene (black sectors). (E) Real-time PCR on single Δ2B BFU-E colonies (1–8). Values indicate ratios of huβ/gapdh transcript levels in individual BFU-E colonies. (F) Representative examples of RT–PCR analyses on single-cell purified from independent Δ2B BFU-E colonies. In parentheses are the percentages of huβ-expressing EryC/total EryC. ‘N’ indicates negative controls and asterisks indicate reactions that did not result in any detectable PCR products.
(48), the lack of H3ac/H3-meK4 observed in PEV HPC could target the huβ-promoter for H3-meK9 or similar histone covalent modifications that provide a code for the establishment/maintenance of heterochromatin (49,50). Thus, in PEV HPC the assembly of an appropriate ‘activated’ chromatin, necessary for correct chromatin competence during hematopoiesis is clearly impeded. Accordingly, our results provide compelling evidence that the abnormal epigenetic regulation of erythroid-specific gene (globins) expression can result from epigenetic regulation variability in progenitor cells.

**Disruption of huβ-gene potentiation and epigenetic inheritance**

Abnormal epigenetic regulation associated with silencing or variegated gene expression is frequently caused by spreading of heterochromatin into euchromatic regions. This can be the consequence of genomic rearrangements that juxtapose heterochromatic and euchromatic regions. Alternatively, it can result from changes in local concentrations of heterochromatin proteins, as frequently observed in chromosomal translocations that induce fusion of epigenetic factors, e.g. AML1-ETO translocation. In both situations, transcription is stochastically abolished or prevented in a subset of cells, which then confer this new epigenetic state to their progeny (24,25,51). Transgenic studies have shed some light upon how PEV and chromatin organization influence gene expression in mammals (29,40,52). However, the events leading to the onset and inheritance of disrupted chromatin organization causing an abnormal epigenetic regulation, in particular during development, differentiation and before high-level transcription, are not well understood.
Different markers of hematopoietic lineages, including the huβ-globin genes, are potentiated (or primed) in multipotent HPC, as well as in hematopoietic stem cells (15,17,18). In addition, some lineage-specific markers are expressed at basal level in early hematopoietic progenitors, suggesting that potentiation/priming is a common phenomenon for many hematopoietic genes (53–55). Thus, we wondered whether abnormal epigenetic regulation could be the end result of a disrupted potentiation in multipotent progenitor cells. To address these issues, we studied chromatin organization and expression profile of the huβ-gene during hematopoiesis by clonal assays in methylcellulose. Our data strongly support a mechanism of PEV that follows abnormal chromatin organization of the huβ-globin locus in multipotent HPC, which appears to be composed of a mixed population of competent HPC (active) or not competent HPC (inactive) for future huβ-gene expression in mature EryC. Additionally, inconsistent huβ-gene expression among erythroid colonies indicates that in early progenitors, abnormal epigenetic control of globin locus activation is plastic, i.e., chromatin at huβ-region can switch from an active to an inactive organization and vice versa (reversion) conducting, respectively, to expression or abolition of the huβ-gene expression in EryC. Plasticity is common during hematopoietic lineage commitment, and changes in single regulatory factor can modify cell fate (56). It is not clear which regulatory factors could influence either the epigenetic inheritance or the plasticity of the huβ-globin locus during hematopoiesis. The fact that ‘non-expressing CFU-GEMM’ colonies are less frequent than ‘non-expressing BFU-E’ colonies suggests that, as observed in Drosophila (25), reversion of variegated expression pattern is facilitated by cell division. Moreover, the all-or-none pattern of expression of the huβ-gene in CFU-E colonies indicates a very low frequency of reversion, which might result from the low number of cellular divisions between CFU-E progenitors and EryC.

**MATERIALS AND METHODS**

**Cell sorting**

Mouse c-Kit+ /CD31high/Ly-6C− cells (HPC) and Ter119+ cells (EryC) were purified and characterized as previously described (18).

**TSA treatment of bone marrow cells**

TSA treatment of EryC was performed as previously described (18). For TSA treatment of HPC, mouse bone marrow was seeded on methylcellulose (M3434, StemCell Technologies); 20 nM final TSA or equal volume of ethanol (TSA solvent) was added exclusively at day 0. Colonies were harvested at day 10.

**DNaseI sensitivity and chromatin immunoprecipitation assays**

DNaseI sensitivity and ChIP assays were carried out as previously described (18,40). ChIP antibodies (Upstate Biotechnology) were raised against unmodified histone H1, acetylated histone H3 (K9, K14) or H4 (K5, K8, K12, K16) and dimethylated histone H3, either on K4 or on K9. Cross-linked chromatin was sonicated to obtain fragments of 400 bp average size.

**S1 nuclease protection assay**

RNA samples were prepared using Trizol (Life Technologies), and the assay was carried out essentially as previously described (41).

**Duplex PCR and RT–PCR analyses**

Quantitative PCR and semi-quantitative RT–PCR analyses were performed as previously described (18,40). For single-colony
RT–PCR, mouse bone marrow was seeded on methylcellulose (M3434, StemCell Technologies) and single colonies were collected after 3 days (CFU-E) or 10 days (BFU-E and CFU-GEMM) of culture. For single-cell RT–PCR, BFU-E colonies were washed once in PBS and single cells were deposited into 96-well plates using FACS Vantage (Becton Dickinson).

Real-time RT–PCR analysis

Total RNA was extracted by RNeasy MiniKit (Qiagen) and used for cDNA synthesis by Sensiscript RT kit (Qiagen). cDNA was used in real-time PCR (iCycler iQ™, Bio-Rad) with Qiagen QuantiTect probes specific for huß-globin cDNA (5′ primer: tctggtgcctttagtgatg; 3′ primer: tgcctggagcctgga; TET-labeled QuantiProbe: acctttgccacactga; TET-QuantiProbe: acctttgccacactga) or mouse gapdh cDNA (FAM-labeled QuantiTect gene expression assay, Qiagen, GapdRT). To avoid genomic DNA contamination, HußRT and GapdRT primers were designed to span intron–exon junctions. HußRT and GapdRT PCR reactions were independently run at least in triplicate. The following equation (61) was used to quantify huß relative to gapdh mRNA

\[
\text{Ratio} = \frac{(E_{\text{target}}) \Delta CP_{\text{sample}}}{(E_{\text{ref}}) \Delta CP_{\text{sample}}} 
\]

where \( E_{\text{target}} \) represents HußRT PCR efficiency; \( E_{\text{ref}} \), GapdRT PCR efficiency; CP, crossing point. For ΔB BFU-E PCR: ΔCP target, CP deviation of ln2–Δ2B of huß-gene transcript; ΔCP ref, CP deviation of ln2–Δ2B of gapdh transcript. For TSA-treated samples: ΔCP target, CP deviation of ethanol–TSA of huß-gene transcript; and ΔCP ref, CP deviation of ethanol–TSA of gapdh transcript.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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