Hydroxymethylcytosine and demethylation of the \(\gamma\)-globin gene promoter during erythroid differentiation

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**Abbreviations:** 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; HbF, fetal hemoglobin; DNMT, DNA methyltransferase; BM, bone marrow; FL, fetal liver; LSD1, lysine specific demethylase 1; TC, tranylcypromine

The mechanism responsible for developmental stage-specific regulation of \(\gamma\)-globin gene expression involves DNA methylation. Previous results have shown that the \(\gamma\)-globin promoter is nearly fully demethylated during fetal liver erythroid differentiation and partially demethylated during adult bone marrow erythroid differentiation. The hypothesis that 5-hydroxymethylcytosine (5hmC), a known intermediate in DNA demethylation pathways, is involved in demethylation of the \(\gamma\)-globin gene promoter during erythroid differentiation was investigated by analyzing levels of 5-methylcytosine (5mC) and 5hmC at a CCGG site within the \(5'\) \(\gamma\)-globin gene promoter region in FACS-purified cells from baboon bone marrow and fetal liver enriched for different stages of erythroid differentiation. Our results show that 5mC and 5hmC levels at the \(\gamma\)-globin promoter are dynamically modulated during erythroid differentiation with peak levels of 5hmC preceding and/or coinciding with demethylation. The Tet2 and Tet3 dioxygenases that catalyze formation of 5hmC are expressed during early stages of erythroid differentiation and Tet3 expression increases as differentiation proceeds. In baboon CD34\textsuperscript{+} bone marrow-derived erythroid progenitor cell cultures, \(\gamma\)-globin expression was positively correlated with 5hmC and negatively correlated with 5mC at the \(\gamma\)-globin promoter. Supplementation of culture media with Vitamin C, a cofactor of the Tet dioxygenases, reduced \(\gamma\)-globin promoter DNA methylation and increased \(\gamma\)-globin expression when added alone and in an additive manner in combination with either DNA methyltransferase or LSD1 inhibitors. These results strongly support the hypothesis that the Tet-mediated 5hmC pathway is involved in developmental stage-specific regulation of \(\gamma\)-globin expression by mediating demethylation of the \(\gamma\)-globin promoter.

Introduction

The human \(\beta\)-globin gene complex spans approximately 70 kb on the long arm of chromosome 11 and includes 5 distinct genes, \(\alpha\)-, \(G\gamma\)-, \(A\gamma\)-, \(\delta\)-, and \(\beta\)-, that are activated sequentially in a highly regulated manner during development. Increased understanding of the mechanism(s) that regulate developmental expression of this cohort of genes, particularly the duplicated \(\gamma\)-globin genes, is of critical importance in the development of new therapeutic interventions for sickle cell disease and \(\beta\)-thalassemia because increased levels of fetal hemoglobin (HbF; \(\alpha_2\gamma_2\)) inhibit polymerization of deoxygogenated sickle hemoglobin and are associated with decreased risk of pain crises and death in patients with sickle cell disease.\textsuperscript{1,2} Current evidence suggests that the mechanism of developmental globin gene regulation involves the targeting of repressive epigenetic modifications to critical regulatory elements by recruitment of corepressor complexes.\textsuperscript{3-5} A mechanistic role for DNA methylation in developmental stage-specific repression of the \(\gamma\)-globin gene has long been supported by experimental studies. Previous analysis of the DNA methylation status of the \(\gamma\)-globin promoter by Southern blot following digestion of DNA with methylation sensitive restriction enzymes established a strong negative correlation between the
level of DNA methylation of the γ-globin promoter and γ-globin expression during development. Studies in the experimental non-human primate baboon model showed that pharmacological inhibitors of DNA methyltransferase (DNMT) induced high levels of HbF. Subsequent clinical trials in patients with sickle cell disease and β-thalassemia confirmed that administration of DNMT inhibitors increased HbF to therapeutic levels in patients with sickle cell disease and β-thalassemia. Phylogenetic footprinting studies showing that CpG residues within the 5' γ-globin promoter region were acquired during the evolutionary transition from prosimians to simian primates that coincided with recruitment of the γ-globin gene to fetal stage-specific expression suggested that DNA methylation was critically involved in developmental regulation of γ-globin expression. More recent studies have shown that the DRED and Bcl11A-Nurd co-repressor complexes, both containing the DNMT1 protein, repress the γ-globin gene in adult erythroid cells. Inhibition of the histone demethylase LSD1, another component of these co-repressor complexes, using the pharmacological agent tranylcypromine increased γ-globin expression in cultured human erythroid progenitors and human β-globin YAC mice, thus demonstrating that other epigenetic modifications, in addition to DNA methylation, are involved in γ-globin repression.

The mechanism responsible for the dramatic differences in DNA methylation of the γ-globin promoter between fetal and adult erythroid cells is unknown. Previous studies from our laboratory analyzing changes in DNA methylation of the γ-globin promoter in FACS-purified cells of adult baboon bone marrow (BM) and early gestational age fetal liver (FL) enriched for different stages of erythroid differentiation showed that the γ-globin gene promoter was demethylated in a progressive manner during erythroid differentiation in FL and to a lesser extent in adult BM. The loss of DNA methylation observed during differentiation of the murine erythroleukemia cell line, during erythroid differentiation in FL and to a lesser extent in adult BM, supports a functional role for 5hmC in erythroid differentiation. Mechanistic pathways responsible for DNA demethylation have been described that involve covalent modification of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC), 5-formylcytosine, and 5-carboxylcytosine by activity of the Tet oxygenases and its subsequent demethylation by active mechanisms involving enzymatic base excision repair pathways and passive DNA replication-dependent mechanisms. The role of Tet genes and 5hmC in normal and malignant hematopoiesis has been the subject of a number of recent reviews. Dynamic changes in hydroxymethylcytosine levels, both globally and at gene-specific regulatory regions, including the γ-globin promoter, during differentiation of cultured human erythroid progenitors and in FACS-purified subpopulations of erythroid cells from baboon BM cells have recently been described. Disruption of erythroid differentiation and 5hmC patterning in patient-derived Tet2 mutant CD34+ early progenitor/stem cells supports a functional role for 5hmC in erythroid differentiation. A recent report shows that active demethylation of the avian adult α (D)-globin promoter occurs during differentiation of the avian HD3 erythroblast cell. The timing of this event coincides with incorporation of 5hmC at the promoter and is consistent with a mechanistic role for hydroxymethylation and demethylation in globin gene switching in chickens.

The hypothesis that 5hmC mediates demethylation of the γ-globin promoter during erythroid differentiation was investigated by analyzing levels of 5hmC, 5mC, and unmodified cytosine (Cyt) at the −54 CCGG site within the 5' γ-globin promoter region in FACS-purified subpopulation enriched for different stages of erythroid differentiation from adult baboon BM and early gestational age baboon FL derived from timed matings. Experiments have been performed to determine the relationship between γ-globin promoter 5mC and 5hmC and γ-globin expression in cultured baboon BM erythroid progenitor cells grown in conditions resulting in either high or low levels of γ-globin expression. Because recent reports have shown that Vitamin C stimulates DNA demethylation by acting as a Tet cofactor to increase Tet-mediated conversion of 5mC to 5hmC, the effect of Vitamin C, alone and in combination with known pharmacological inducers of HbF, on DNA methylation of the γ-globin promoter and γ-globin expression, has also been investigated. Our results strongly support a role for the Tet-mediated 5hmC pathway in regulation of γ-globin expression through demethylation of the γ-globin promoter.

Results

Analysis of γ-globin promoter 5hmC during adult BM erythroid differentiation

Subpopulations of adult baboon BM cells were isolated using a combination of immunomagnetic column separation and FACS. Baboons were phlebotomized for 4 days immediately prior to the day of BM aspiration to increase the number of erythroid precursors and CFUe in the marrow in order to maximize cell numbers for purification and analysis. Previous data from our laboratory has shown that increases in HbF to 5–10% of total hemoglobin are associated with phlebotomy in baboons. A subpopulation consisting of terminal erythroid precursors, reticulocytes, and erythrocytes was isolated by immunomagnetic column separation using an antibody specific for baboon RBCs. The negative flow-through cells were then separated by FACS using antibodies to CD117 and CD36, an early erythroid marker, to obtain subpopulations consisting of CD117+CD36−, CD117+CD36+, and CD117-CD36+ cells (Figs. A and B). Colony assays showed that the terminal erythroid precursors and CD117-CD36+ subpopulations did not contain colony-forming cells. The CD117+CD36+ subpopulation was enriched in erythroid colony-forming cells (15.9 ± 9.3%), CFUe and small BFUe, and was devoid of non-erythroid colony forming cells, while large BFUe, mixed, and granulocytic colonies were found in the CD117+CD36− subpopulation. DNA isolated from these subpopulations was used to determine the levels of 5hmC, 5mC, and Cyt at a CCGG MspI site located at −54 within the 5' γ-globin promoter region by Epimark assays. Levels of 5hmC in the terminal erythroid precursor cells (2.34 ±
0.82%) were enriched >12-fold compared to peripheral blood WBC (0.18 ± 0.19%; \( P < 0.0001 \)) demonstrating that 5hmC levels associated with the \( \gamma \)-globin promoter are significantly higher than in non-erythroid lineage cells (peripheral blood WBC). The highest levels of 5hmC were observed in the CFUe-enriched CD117\(^+\)CD36\(^-\) population (7.15 ± 1.34%), while significantly reduced levels were observed in the CD117\(^+\)CD36\(^-\) subpopulation (2.87 ± 1.85%; \( P < 0.01 \)), the CD117\(^-\)CD36\(^+\) subpopulation (2.34 ± 0.82; \( P < 0.001 \); Fig. 1C). Thus the level of \( \gamma \)-globin promoter 5hmC was approximately 2.5-fold higher in the CD117\(^+\)CD36\(^-\) fraction enriched in CFUe than in the CD117\(^+\)CD36\(^-\) subpopulation, 3-fold higher than in the terminal erythroid subpopulation, and 36-fold higher than in peripheral WBC. Similar levels of 5mC at the −54 site were observed in peripheral blood WBC (83.46 ± 6.30%) and the CD117\(^+\)CD36\(^-\) subpopulation (83.40 ± 5.77%), which were significantly higher (\( P < 0.01 \)) than levels observed in the CD117\(^+\)CD36\(^+\) (66.31 ± 7.03%), CD117\(^+\)CD36\(^-\) (63.77 ± 9.65%) and the terminal erythroid precursor subpopulations (63.70 ± 7.67%; Fig. 1C). In contrast, the level of unmodified Cyt at the −54 position was similar in the terminal erythroid precursors (33.23 ± 7.22%) and the CD117\(^+\)CD36\(^+\) subpopulation (33.17 ± 8.4%); the level in the terminal erythroid precursors was significantly higher compared to both the CD117\(^+\)CD36\(^-\) (10.44 ± 5.44%; \( P < 0.02 \)) and the CD117\(^+\)CD36\(^+\) (23.31 ± 7.33; \( P < 0.03 \); Fig. 1C) subpopulations. These results show that demethylation of the −54 site occurs as erythroid differentiation proceeds from the CD117\(^+\)CD36\(^+\) CFUe-enriched subpopulation to the terminal erythroid precursors. Reduction in the level of 5hmC with no difference in the level of 5mC between the CD117\(^+\)CD36\(^+\) CFUe-enriched population and the terminal erythroid precursors suggests that the limited demethylation of the −54 site during erythroid differentiation of adult baboon BM cells proceeds through a 5hmC intermediate.

Figure 1. (A) FACS profile of baboon BM cells separated by PE-labeled CD117 and FITC-labeled CD36. (B) Wright’s stained cytospin preparations showing morphology of FACS-sorted CD117\(^+\)CD36\(^-\), CD117\(^+\)CD36\(^+\), and CD117\(^-\)CD36\(^+\) and immunomagnetic column purified bRBC (terminal erythroid precursor) cell subpopulations. (C) Analysis of 5hmC, 5mC, and Cyt present at the \( \gamma \)-globin promoter −54 CCGG site in purified subpopulations of baboon BM cells. CD36\(^-\)CD117\(^-\) (checkerboard), CD117\(^+\)CD36\(^-\) (black bars), CD117\(^+\)CD36\(^+\) (diagonal bars), terminal erythroid subpopulation (white bars) (D) Analysis of 5hmC, 5mC, and Cyt present at the \( \gamma \)-globin promoter −54 CCGG site in purified subpopulations of baboon fetal liver cells. CD36\(^-\)CD117\(^-\) (checkerboard), CD117\(^+\)CD36\(^-\) (black bars), CD117\(^+\)CD36\(^+\) (diagonal bars), terminal erythroid subpopulation (white bars).
Analysis of globin mRNA levels within these subpopulations showed that levels of \(\gamma\) and \(\beta\)-globin transcripts were 1000-fold higher in the terminal erythroid precursors compared to more primitive CD117+/CD36− subpopulation and 50-fold higher compared to the CFUe-enriched CD117+/CD36+ subpopulation (Fig. S1A). The proportion of \(\gamma\)-globin mRNA relative to \(\beta\)-globin mRNA did not differ significantly between these subpopulations (Fig. S1B). Levels of globin transcripts were 20 fold higher in the CD117+/CD36+ subpopulation compared to the more primitive CD117+/CD36− subpopulation, showing that a significant increase in globin gene transcription coincides with increased levels of 5hmC at the \(\gamma\)-globin promoter during erythroid differentiation.

Bisulfite sequence analysis was performed to compare the DNA methylation at 5 CpG sites within the 5′ \(\gamma\)-globin promoter region in DNA samples isolated from the CD117+/CD36+ subpopulation (\(n=4\)) and the terminal erythroid precursor subpopulation (\(n=4\)) to determine to what extent demethylation of the −54 site during erythroid differentiation reflected more widespread changes in the level of DNA methylation at other \(\gamma\)-globin promoter CpG sites. Analysis of 5 CpG sites (−54, −51, +5, +16, +48) showed that the level of DNA methylation in the CD117+/CD36+ subpopulation (87.7 ± 5.7%) was significantly higher (\(P < 0.05\)) than in the terminal erythroid precursors (73.9 ± 8.1%; Fig. S2A). Comparison of CpG methylation at the −54, +5, and +48 sites showed that the level at the −54 CpG site (73.0 ± 8.1%) was lower than the +5 site (90.5; \(P < 0.06\)) and the +48 site (98%; \(P < 0.02\)) in the CD117+/CD36+ subpopulation (Fig. S2B). Due to polymorphisms that eliminate the CpG residues at −51 and +16 these sites were not included in our analysis. This pattern persisted in terminal erythroid precursors as DNA methylation of the −54 site (55 ± 12%) was lower than the +5 site (91 ± 6.6%; \(P < 0.01\)) and also the +48 site (76.3 ± 14.4%; \(P < 0.07\); Fig. S2B).

Analysis of \(\gamma\)-globin promoter 5hmC during fetal liver erythroid differentiation

Analysis of changes in 5hmC, 5mC, and Cyt at the −54 site was also performed in subpopulations enriched for different stages of erythroid differentiation purified from early gestational age baboon FL with high levels of HbF expression (>90%) in contrast to adults that express predominantly HbA. FL cells were purified from 2 early gestational age (54 d, 62 d) baboon fetuses obtained through timed matings. FL cells were separated in an identical manner as the adult-derived BM cells by a combination of immunomagnetic column selection of the terminal erythroid precursors followed by FACs-purification of the CD117+/CD36−, CD117+/CD36+, and CD117-CD36+ populations (Fig. S3). High levels of 5hmC were observed in the most primitive CD117+/CD36− subpopulation and were greatly reduced in the CD117+/CD36+ subpopulation (Fig. 1D). Rapid demethylation was observed as the level of 5mC in the CD117+/CD36− subpopulation was reduced to <5% in the CD117+/CD36+ cells while the level of Cyt in the CD117+/CD36− subpopulation increased to >95% in CD117+/CD36+ subpopulation (Fig. 1D). Thus, demethylation of the −54 site occurs to a greater extent and progresses with greater rapidity at an earlier stage of erythroid differentiation in FL compared to adult BM.

Relationship between \(\gamma\)-globin promoter 5hmC, 5mC and \(\gamma\)-globin expression

The relationship between the level of \(\gamma\)-globin expression and \(\gamma\)-globin promoter 5hmC, was investigated by comparing levels of 5hmC, 5mC, and Cyt at the \(\gamma\)-globin promoter −54 CCGG site in adult baboon CD34+ BM-derived erythroid cells expressing high levels of \(\gamma\)-globin when grown in liquid culture to those expressing low levels of \(\gamma\)-globin when grown in a stromal cell derived co-culture system. Expression of \(\gamma\)-globin in cultured cells was measured by HPLC analysis of globin chains (\(\gamma/\gamma+\beta\)) on d14. Analysis of paired cultures (liquid vs. co-culture) derived from BM aspirates from 6 different individual baboons showed that globin synthesis (\(\gamma/\gamma+\beta\)) was significantly greater (mean difference = 0.44 \(\gamma/\gamma+\beta\); \(P < 0.001\); Fig. 2A) in cells cultured in liquid media compared to the stromal cell line co-culture system. Levels of 5hmC in d14 purified erythroid cells were significantly higher in liquid cultures compared to stromal cell line co-cultures (mean difference 4.93%; \(P < 0.005\); Fig. 2B) while levels of 5mC were significantly lower (mean difference −26.0%; \(P < 0.01\); Fig. 2C). Cyt levels were higher in cells from liquid cultures compared to stromal cell co-cultures (mean difference = 16.5%; \(P < 0.001\); Fig. 2D). Combined analysis of data from all cultures showed that \(\gamma\)-globin expression was negatively correlated with 5mC (\(\rho = 0.854\); \(P < 0.001\); Fig. S4A) and positively correlated with both Cyt (\(\rho = 0.758\); \(P < 0.01\); Fig. S4B) and 5hmC levels (\(\rho = 0.729\); \(P < 0.01\); Fig. S4C).

Effect of Vitamin C

Experiments were performed to test the effect of Vitamin C, a cofactor of the Tet dioxygenases, on \(\gamma\)-globin expression and \(\gamma\)-globin promoter DNA methylation. CD34+ BM cells were isolated from 4 different baboons and cultured in both liquid media and stromal cell line co-cultures for 14d in the presence and absence of Vitamin C (100 \(\mu\)M). HPLC analysis showed that \(\gamma\)-globin expression was significantly higher in cultures treated with Vitamin C compared to untreated controls (mean difference 0.12 \(\gamma/\gamma+\beta\); \(P < 0.01\); Figs. 3A, D). A representative HPLC chromatogram of globin chain expression shows the increased levels of \(\gamma\)-globin chain expression in liquid and stromal cell co-cultures treated with Vitamin C compared to the untreated controls (Fig. 3A). Epimark assays showed that levels of 5mC at the −54 CCGG site of the \(\gamma\)-globin promoter were significantly lower in Vitamin C-treated cultures (mean difference −29.0%; \(P < 0.001\); Fig. 3B) compared to untreated controls, while Cyt levels were higher (mean difference 31.9%; \(P < 0.001\); Fig. 3C). Bisulfite sequence analysis showed that DNA methylation at 5 CpG sites within the 5′ \(\gamma\)-globin promoter region was significantly lower in cultures supplemented with Vitamin C (45.1 ± 20.3%) compared to untreated controls (74.4 ± 10.3%; \(P < 0.01\); Fig. 4). Site-specific analysis showed that reduced 5mC was observed at all 3 non-polymorphic CpG sites (−54, +16, +48) in Vitamin C-treated cultures compared to untreated controls (mean difference 26.0%; \(P < 0.01\); Fig. S4C).
Combinatorial effects of vitamin C with decitabine, hydroxyurea, or tranylcypromine

Experiments were then performed to test the effect of Vitamin C in combination with the known HbF-inducing drugs decitabine, hydroxyurea, and tranylcypromine on γ-globin expression in cultured baboon erythroid progenitors. Triplicate stromal cell line co-cultures of CD34+ baboon BM cells grown in the presence and absence of Vitamin C were treated on d7 with decitabine (1 × 10⁻⁷ M), hydroxyurea (100 μM) or tranylcypromine (5 μM). Results showed that γ-globin expression on d14 was significantly higher in cultures treated with Vitamin C (0.32 ± 0.02 γ/γ+β), decitabine (0.33 ± 0.02 γ/γ+β), hydroxyurea (0.33 ± 0.08 γ/γ+β), and tranylcypromine (0.33 ± 0.02 γ/γ+β) compared to untreated controls (0.18 γ/γ+β; P < 0.001; Fig. 5A). Expression of γ-globin was increased in cultures treated with a combination of Vitamin C with either decitabine (0.49 ± 0.02 γ/γ+β) or tranylcypromine (TC) (0.54 ± 0.02 γ/γ+β) to higher levels than any of these agents alone (P < 0.001; Fig. 5A, B). A representative HPLC chromatogram shows (Fig. S5B) increased γ-globin chain expression in a culture treated with the combination of decitabine and Vitamin C compared to cultures treated with either agent alone and the untreated control. In contrast, in cultures treated with the combination of Vitamin C and hydroxyurea, γ-globin expression (0.33 ± 0.04 γ/γ+β) was similar to cultures treated with hydroxyurea or Vitamin C alone. The effect of DNA methylation of 5 CpG sites within the 5′ γ-globin promoter was assessed by bisulfite sequence analysis of pooled DNA isolated from terminal erythroid precursors purified on d14 by immunomagnetic column purification from pooled triplicate cultures (Fig. 5C). The level of DNA methylation of 5 CpG sites within the 5′ γ-globin promoter region was reduced in cultures treated with Vitamin C (52.6%) and decitabine (73.1%) but not with TC (85.1%) compared to the untreated control (87.0%). The modest effect on DNA methylation levels in the decitabine-treated sample likely reflects the low dose (1 × 10⁻⁷ M) used. The level of DNA methylation in cells treated with a combination of Vitamin C and decitabine (52.6%) or TC and decitabine (55.7%) was similar to that in cultures with Vitamin C alone (52.8%).

**Tet expression during erythroid differentiation**

Expression of the 3 Tet genes, Tet1, Tet2, and Tet3 was analyzed by RT-PCR. Primers that distinguished the Tet2A and Tet2B alternatively spliced transcripts that encode 2 different isoforms of Tet2 that differ at the C-terminal end encoding most of the catalytic domain were included in the analysis. Tet gene expression was determined relative to GAPDH in RNA isolated from purified CD117⁺CD36⁻ (n = 3), CD117⁺CD36⁺ (n = 4), CD117⁻CD36⁻ (n = 3), the terminal erythroid precursors from BM (n = 4), and fetal liver (n = 1), CD34⁺ BM-derived d14 cultures (n = 3), and human CD34⁺ cord blood-derived d14 FACS purified glycoporphin A⁺ cells (n = 3; Fig. 6). Expression of Tet1 was not detected in these samples. Within the CD117⁺CD36⁺-subpopulation the relative expression of Tet2A was 2.8-fold higher than Tet3 (P < 0.05). Within the CD117⁺CD36⁺-subpopulation Tet2A expression (0.002 ± 0.001) appeared lower than Tet3 (0.008 ± 0.005) but the difference was not significant. Within the CD117⁻CD36⁺-subpopulation, Tet3 expression (0.015 ± 0.001) was 3-fold higher (P < 0.001) than Tet2A (0.005 ± 0.001), while in the terminal BM erythroid precursors subpopulation Tet3 expression (0.025 ± 0.008) was 6.7-fold higher (P < 0.01) than Tet2A (0.004 ±
0.001) in BM terminal erythroid precursors and 10-fold higher in-terminal erythroid cells from d14 CD34+ BM-derived erythroid progenitor cultures. Levels of Tet3 (0.030 ± 0.015) were nearly tenfold (P < 0.05) higher than Tet2A (0.0031 ± 0.0026) in terminal erythroid cells isolated from d14 CD34+ BM-derived erythroid progenitor cultures, and higher levels of Tet3 relative to Tet2A were also observed in FL terminal erythroid precursors and glyA+ cells from CD34+ human cord blood-derived cultures. Expression of the Tet2B alternatively spliced transcript lacking the C-terminal region encoding the catalytic domain appeared to be increased in-terminal erythroid cells to levels equivalent to Tet3 although this was not observed in cells isolated from d14 CD34+ BM-derived erythroid progenitor cultures. This data demonstrates that expression of both Tet2 and Tet3 is modulated during erythroid differentiation.

Discussion

Analysis of FACS-purified cells from baboon BM and FL showed elevated levels 5hmC at the −54 CpG site within the γ-globin gene promoter at stages of differentiation immediately preceding and/or coinciding with the timing of demethylation of this site. Bisulfite sequence analysis showed reduced levels of DNA methylation at the −54 site compared to CpG residues at +5 and +48 in the adult BM CD117+CD36− subpopulation enriched in CFUe suggesting preferential demethylation of this site during erythroid differentiation. Preferential loss of methylation at this site during differentiation of human erythroid progenitors in vitro was previously reported. The extent of demethylation is more rapid and markedly more complete in FL compared to BM. In FL cells, the highest levels of 5hmC are observed within the CD117+CD36− primitive cell subpopulation and the demethylation of the γ-globin promoter is essentially complete by transition to the CD117+CD36− stage. In adult BM, the highest levels of 5hmC are observed in the CD117+CD36− population that is enriched in erythroid colony-forming cells although levels of 5hmC within the most primitive erythroid progenitors within the CD117+CD36− subpopulation may be underestimated due the presence of non-erythroid granulocytic...
progenitors in this population. Previous results have shown that differences in 5hmC at the \(\gamma\)-globin promoter within these subpopulations reflect differences in genomic 5hmC and therefore are part of global changes in DNA methylation occurring in the early stages of erythroid differentiation. Due to the nature of the assay used in our current studies, 5hmC data is limited to a single site. Although it would likely be more informative to have more sequence information across larger genomic regions, such methods for large-scale analysis are not optimized for the rare cell types utilized in our studies. Analysis of Tet gene expression clearly shows that both Tet2 and Tet3 transcripts are readily detectable in all erythroid subpopulations isolated from both primary BM and fetal liver and also from cultured CD34+ erythroid progenitors. Expression of Tet2 decreases while Tet3 increases as erythroid differentiation proceeds. High levels of Tet3 were observed in all subpopulations of terminal erythroid precursors, including FACS-purified gly A+ cells from cultured human cord blood CD34+ cells. A role for Tet3 in maintenance and terminal differentiation of neural progenitor cells has recently been demonstrated. Analysis of hematopoiesis in Tet3 conditional knockout mice showed that 5hmC levels were decreased in the absence of any significant effect on numbers of terminally differentiated cells. Further analysis of these mice showed Tet3 deficiency augmented the repopulation capacity of primitive haematopoietic stem cells and it has been suggested that Tet2 and Tet3 may be functionally redundant. Interestingly, \textit{Xenopus} Tet3, shown to regulate the expression of a key set of developmental genes important in eye and neural development is targeted to specific sequences through the binding activity of a CXXC domain in the N terminus that exhibits strong in vitro binding to the CmCGG sequence (homologous to the \(\gamma\)-globin promoter –54 site). Although our data showing that Tet3 expression and \(\gamma\)-globin promoter 5hmC levels during erythroid differentiation are inversely related...
would appear to preclude a functional role for Tet3 in catalyzing the formation of 5hmC at the γ-globin promoter, it is probable that repressive chromatin modifications established during erythroid differentiation block access of the γ-globin promoter to modification by action of the Tet proteins and that hydroxymethylation/demethylation is restricted to a specific stage of erythroid differentiation. Future experiments will be performed to test the roles of Tet2 and Tet3 in hydroxymethylation/demethylation of the γ-globin promoter.

Our in vitro culture results comparing 5hmC and 5mC levels at the γ-globin promoter with levels of γ-globin expression show that higher levels of 5hmC and lower levels of 5mC are observed in the terminally differentiated erythrocyte precursors derived from liquid cultures that express increased levels of γ-globin. Addition of Vitamin C, a cofactor of the Tet dioxygenases, clearly decreased 5mC levels and increased γ-globin expression, consistent with a role for the Tet dioxygenase in demethylation of the γ-globin gene promoter. The negative correlation between the difference in 5mC levels between Vitamin C and untreated controls with the level of 5mC (Fig. S7) suggests that the final methylation state may be influenced by multiple factors that affect the efficiency of demethylation of the 5hmC site and may be variable between individuals. Recent studies have demonstrated that Vitamin C stimulation of Tet activity increases DNA demethylation30–34 and the effect of Vitamin C on γ-globin promoter DNA methylation are clearly consistent with these studies. However, Vitamin C is also a cofactor for other Fe (II) 2-oxoglutarate dependent dioxygenase including the histone demethylases of the Jumonji family that are capable of removing repressive methylation/demethylation of lys9 of Histone H3.34 A recent study showed Vitamin C is also a cofactor for other Fe (II) 2-oxoglutarate dependent dioxygenase including the histone demethylases of the Jumonji family that are capable of removing repressive methylation from lys9 of Histone H3.34 A recent study showed Vitamin C enhanced neural differentiation in the developing midbrain in a Tet1 and Jmjd3-dependent manner.39 Thus, a role for additional other Fe (II) 2-oxoglutarate-dependent enzymes in mediating increased γ-globin expression cannot be excluded. Notably, we have observed that Vitamin C increases γ-globin expression in an additive manner when combined with 2 drugs, the DNMT inhibitor decitabine and the LSD1 inhibitor tranylcypromine, that target enzymes that establish and maintain repressive epigenetic modifications at the γ-globin promoter that repress γ-globin expression. It is possible that the failure of the combination of Vitamin C and hydroxycurea to elicit additive effects on γ-globin expression could be due to inhibition of DNA synthesis by hydroxycurea. Previous studies have shown that DNA demethylation in differentiating mouse fetal liver erythroblasts requires DNA synthesis20 and hydroxycurea blocks the ability of 5-azacytidine to increase HbF in baboons.8

A high frequency of Vitamin C deficiency has been observed in patients with sickle cell disease.46 Interestingly, one study reported a small but significant increase in HbF following Vitamin C supplementation in children with sickle cell disease.41 The low levels of Vitamin C in patients could limit the HbF response to these drugs, one of which (decitabine) is currently in clinical trials. Thus, these results could have important clinical implications for drug therapies targeting epigenetic modifications designed to increase HbF in patients with sickle cell disease who exhibit a high frequency of Vitamin C deficiency.

Materials and Methods

Baboons

Adult baboons (P. anubis) were housed at the University of Illinois Biologic Resources Laboratory. Baboons were phlebotomized to increase erythroid cell numbers in the bone marrow by daily removal of 17% of the blood volume for 4 consecutive days. Aspiration of bone marrow (30–40 ml) from the hips or shoulders was performed on the fifth day.

Livers were obtained from baboon fetuses (n = 2) produced through timed matings. Fetuses were delivered by Caesarian section and euthanized by injection with euthobarb prior to removal of tissue.

All procedures were approved by the IACUC of the University of Illinois at Chicago.

Purification of subpopulations of erythroid cells from baboon BM and fetal liver

Low-density mononuclear cells were isolated from BM aspirates by centrifugation (1500 RPM; 15 min) in pre-formed 70% Percoll gradients. The mononuclear cell population was incubated with a baboon-specific anti-red blood cell antibody (BD Bioscience, #51299) for 15 min at 4°C. Cells were washed 3 times by centrifugation (1500 RPM; 10 min) in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin and 2 mM EDTA followed by incubation (20 min; 4°C) in: immunomagnetic anti-mouse IgG microbeads (Miltenyi #130-047-101) according to the instructions of the manufacturer. Cells were washed an additional 3 times in PBS followed by separation of terminal erythroid precursors (anti-RBC positive cells) using LS columns (Miltenyi #130-042-401). The anti-RBC negative population was suspended in Iscove’s media containing 10% fetal bovine serum and incubated in T75 tissue culture flasks from 45–60 min at 37°C in a CO2 incubator to allow adherent cells to attach. The nonadherent cells were removed and incubated 30–60 min at 4°C with fluorescein-labeled anti-CD36 (Beckman Coulter #IM0766U, clone FA6.152) and phycoerythrin-labeled CD117 (Biolegend #313240, clone 104D2) and appropriate isotype controls. Following incubation, cells were washed in PBS containing 0.5% bovine serum albumin. Cell purification of CD117+CD36−, CD117+CD36+, and CD117-CD36− subpopulations was performed using a Beckman Coulter Mo-Flo at the UIC Research Resources Center Flow Cytometry Service.

Epimark site-specific quantitative analysis of 5hmC, 5mC, and Cyt

The levels of 5hmC, 5mC and Cyt at the −54 CCGG site 5′ to the γ-globin gene were quantitated by Epimark assays.42,43 DNA was purified from cell pellets using QIAamp DNA blood Mini kits (Qiagen #51104). In a 1.5-ml reaction tube, 2–5 μg of genomic DNA was added with 80 μM UDP-Glucose and 1X NEBuffer4 to a total volume of 200 μl. The reaction was divided into 2 tubes. Twenty units of T4 β-glucosyltransferase (T4-BGT) was added to one tube and nuclease-free water to the other (control). Both tubes were incubated at 37°C overnight. Each tube was then equally aliquoted into 3 separate tubes. The
addition of the enzymes into each tube was as follows: Tube 1 (T4-BGT): 100 unitsMspI; Tube 2 (T4-BGT): 50 units HpaII; Tube 3 (T4-BGT) no enzyme; Tube 4 (control): 100 units MspI; Tube 5 (control): 50 units HpaII; and Tube 6 (control): no enzyme. The tubes were incubated at 37°C overnight. Proteinase K was added (1 μl; 20 mg/ml) and reactions were incubated at 40°C for 30 min. Proteinase K was inactivated at 95°C for 10 min. DNA was then analyzed by real-time PCR using primers specified below that targeted the γ-globin promoter and spanned the −54 CCGG site. The copy number of each reaction was calculated from a standard curve derived from cloned amplicons. Copy numbers of all reactions were normalized by the copy number of Tube 6 (control). Normalized copy numbers were used to calculate 5mC, 5mC and Cyt values. Primers: γ 5hmc F-5'TCTTGGCCCTTGACCAATAGCC and γ 5hmc R-5'AGCCTTGTCCCTCCTGTGTA.

CD34+ erythroid progenitor cultures

CD34+ cells were purified from baboon BM aspirates by immunomagnetic column purification using the anti-CD34 monoclonal antibody (clone 12.8; Dr. Ira Bernstein, Fred Hutchinson Cancer Research Center) and rat anti-mouse IgM microbeads (Miltenyi #130-047-302). For liquid cultures, cells were grown in Iscove’s media containing 30% fetal bovine serum, 3 U/ml erythropoietin, 200 ng/ml stem cell factor, and 1 microbead (Miltenyi #130-047-302). For liquid cultures, cells were cultured in media with and without Vitamin C (100 μM, L-ascorbic acid, Fisher #A61-25), a dose that showed maximum effect in dose-response experiments. A complete change of the media was performed every 2-3 days for both Vitamin C-supplemented and control cultures to maintain Vitamin C levels.

HPLC analysis of globin chains

Analysis of globin chain expression was performed by HPLC. Washed cell pellets (5 × 10^6 cells) were suspended in 5mM EDTA and lysed by 5 freeze-thaw cycles in a dry ice-methanol bath. Lysates were clarified using 0.2 μM cellulose acetate filters (Nalgene #171-0020) and globin chains separated with a LiCart 250-4 LiChrospher 100 RP-8 column (EMD Chemicals) column using a gradient of acetonitrile-methanol and a Spectra HPLC system (ThermoFinnegan). Globin chain peaks were quantitated by absorbance at 215.".

Bisulfite sequence analysis

For bisulfite sequence analysis, DNA was purified from washed cell pellets using QIAamp Blood DNA Minikits. Bisulfite modification was performed using Epitect Bisulfite kits (Qiagen #59104) according to the instructions of the manufacturer. Amplification of bisulfite modified DNA was performed with using the BG2 5’- AATAACCTTTATCCTTCTCTATTAAA- TACC and BG5 5’- GGTGGTTAGTTTGGTTTGGAT- TAATAAG primers. 46 Amplicons from 3 separate PCR reactions were pooled and the amplicons cloned using Topo TA cloning kits (Life Technologies #K4575-01SC). DNA was isolated from individual clones and sequence analysis performed at the University of Illinois DNA Sequence Laboratory.

RNA Analysis

For analysis of Tet1, Tet2, and Tet3 expression, RNA was isolated using the RNeasy Minikit (Qiagen #74104) and treated with RNase-free DNase I (Ambion #AM1906) according to the manufacturer’s instructions. Purified RNA was used for the synthesis of cDNA using RevertAid First Strand cDNA Synthesis Kits (Thermo Scientific #K1622). Real time PCR was performed using SYBR green reagent. Primers were: Hum Tet1F-5’CCGAATCAACCGGGAAGATA, Hum Tet1 R- 5’CCTGGAGATGCTCCTTTCA; Bab Tet1 F- 5’CGAGTCAAGCGGAAGAGTAA; Bab Tet1 R- 5’AGATGCGCTCCTTTCAC; Tet2 F- 5’AATTTATGTGATACACCTGTCACACTC; Tet2A R- 5’CCGGCTCTCAGTAGGTGATAAAAG; Tet2B R-5’GATAAACGCCATGTGCTCTCAG TACA; Tet3 F- 5’GCACCTCGGGAGAATCG; Tet3 R- 5’GGAGTACACGCTGTTGATGC. As indicated, human Tet1 primers were used for Tet1 analysis of human samples but were modified for the analysis of baboon samples due to sequence differences; human primers were used for Tet2 and Tet3 amplifications. Control amplifications of cDNA reactions incubated without reverse transcriptase were performed for all reactions. PCR reactions yielded single bands in gel electrophoresis. Amplicons were cloned and sequenced to ensure amplification of the correct sequences in all cases. Standard curves were constructed by amplification of the cloned amplicons. The level of expression measured was relative to GAPDH.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website.

References

1. Platt OS, Thorton BD, Brambilla DJ, Milner PF, Rosse WF, Vichinsky E, Kinney TR. Pain in sickle cell disease. Rates and risk factors. N Engl J Med 1991; 325:11-6; PMID:1710777; http://dx.doi.org/10.1056/NEJM199107043250103

2. Platt OS, Brambilla DJ, Rosse WF, Castro O, Stenberg MH, Klug PP. Mortality in sickle cell disease. Life expectancy and risk factors for early death. N Engl J
15. Cui S, Kolodziej KE, Obara N, Amaral-Psarris A, Tagle DA, Koop BF, Goodman M, Slightom JL, Hess
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14. Yao H, Trowbridge JJ, Mandel G, Orkin SH. Corepressors that mediate 
imprinted expression by BCL11A. Proc Natl Acad Sci USA 2013; 110:6158-23; http://dx.doi.org/10.1073/pnas.1303976110

13. Shl I, Cui S, Engel JD, Tanabe O. Lyso- specific demethylase 1 is a therapeutic target for fetal hemoglo- bin induction. Nat Med 2013; 19:291-4; PMID:23461702; http://dx.doi.org/10.1038/nn.3101

12. Singh M, Lavelle D, Vaitkus K, Mahmud N, Hanke- wych M, DeSimone J. The g-globin promoter promotes widespread demethylation. J Biol Chem 2011; 286:3666-70; PMID:21699873; http://dx.doi.org/10.1002/jbc.ZK06

11. Christman JK, Weich N, Schoenbrun B, Schneiderman N, Acar G. Hypomethylation of DNA during differen- tiation of Friend erythroleukemia cells. J Cell Biol 1988; 106:802-3; PMID:6931107; http://dx.doi.org/10.1083/jcb.106.3.802

10. Sheehan JR, Pop R, Bock C, Boyle P, Meisler A, Soccolovsky M. Global DNA demethylation during mouse erythropoiesis in vivo. Science 2011; 331:799- 802; PMID:22007567; http://dx.doi.org/10.1126/science.1207306

9. Yu Y, Mo Y, Blumberger D, Bhattarcharya S, Liu H, Sun- darave J, Giricz O, Wontakal S, Cartier J, Caces B, et al. High resolution methylation analysis reveals wide- spread functional hypomethylation during adult erythropoiesis. J Biol Chem 2013; 288:8805-14; PMID:23833603; http://dx.doi.org/10.1074/jbc.M112.423376

8. Menon EL, Mariani CJ, Zullow H, Jeff-Eke M, Litwin E, J. 2-deoxy 5-azacytidine and fetal hemoglobin induc- tion in baboon fetal liver and adult bone marrow. Exp Hematol 2007; 35:48-55; PMID:17198873; http://dx.doi.org/10.1016/j.exphem.2006.09.001

7. Shi L, Cui S, Engel JD, Tanabe O. Lyso-specific demethylase 1 is a therapeutic target for fetal hemoglo- bin induction. Nat Med 2013; 19:291-4; PMID:23461702; http://dx.doi.org/10.1038/nn.3101

6. DeSimone J, Keller P, Hall L, Zwiars D. 5-azacytidine increases gamma-globin synthesis increases fetal hemo- globin levels in baboons. Exp Hematol 1982; 10:415-20; PMID:6931107; http://dx.doi.org/10.1016/0387-5703(82)90086-0

5. Miyata F, Giampappalo A, Care A, Migliaccio G, Calan- drini M, Russo G, Pagliardi G, Maseri N, Guarrera P, Tanabe O. Nuclear receptors TR2 and TR4 recruit multiple epigenetic transcriptional corepressors that mediate 
imprinted expression by BCL11A. Proc Natl Acad Sci USA 2013; 110:6158-23; http://dx.doi.org/10.1073/pnas.1303976110

4. Senior EA, Court BL, Young WL. G Ascorbate induces ten eleven translocation (Tet) methylcytosine deoxynucleoside-mediated generation of 5-hydroxymethyl- cytosine. 2013; 288:13660-74; PMID:23548903

3. Yin R, Mao SQ, Zhao R, Choung S, Zick V, Zhao C, Zhang D, Huang H, Gao J, Li Z, et al. Ascorbic acid enhances Tet-mediated 5-methylcytosine oxidation and promotes DNA demethylation in mammals. J Am Chem Soc 2013; 135:10396-403; PMID:23768288; http://dx.doi.org/10.1021/ja4028346

2. Bessler L, Gacasova S, Chen YH, Hoffman R, DeSimone J. 2-deoxy 5-azacytidine and fetal hemoglobin induc- tion in baboon fetal liver and adult bone marrow. Exp Hematol 2007; 35:48-55; PMID:17198873; http://dx.doi.org/10.1016/j.exphem.2006.09.001

1. Cui S, Kolodziej KE, Obara N, Amaral-Psarris A, Tagle DA, Koop BF, Goodman M, Slightom JL, Hess
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fetal hemoglobin expression in adult erythroid cells. Transl Res 2013; 165:115-25; PMID:24800417

5. Trowbridge JJ, Mandel G, Orkin SH. Corepressors that mediate 
imprinted expression by BCL11A. Proc Natl Acad Sci USA 2013; 110:6158-23; http://dx.doi.org/10.1073/pnas.1303976110
45. Leone L, Monteleone M, Gabutti V, Amoine C. Reversed phase high performance liquid chromatography of human hemoglobin chains. J Chromatogr 1985; 321:407-19; http://dx.doi.org/10.1016/S0021-9673(01)90459-5
46. Lavelle D, Vairkus K, Hankewych M, Singh M, DeSimone J. Developmental changes in DNA methylation and covalent histone modifications of chromatin associated with the ε-, γ-, and β-globin gene promoters in *Papio anubis*. Blood cells Mol Dis 2006; 36:269-78; PMID:16527500; http://dx.doi.org/10.1016/j.bcmd.2006.01.004
47. Moran-Crusio K, Reavie L, Shih A, Abdel-Wahab O, Ndiaye-Lobey D, Lobey C, Figuerosa MN, Vasantha Kumar A, Patel J, Zhao X, et al Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. Cancer Cell 2011; 20:11-24; PMID:21723200; http://dx.doi.org/10.1016/j.ccr.2011.06.001
48. Langemeijer SM, Kuiper RP, Berends M, Knops R, Adanyan MG, Massop MG, Stevens-Linders E, van Hoogen P, van Kessel AG, Raymakers RA, et al Acquired mutations in TET2 are common in myeloproliferative syndromes. Nat Genet 2009; 41:838-42; PMID:19483684; http://dx.doi.org/10.1038/ng.391