RESEARCH PAPER

Epigenetics 10:5, 397–407; May 2015; Published with license by Taylor & Francis Group, LLC

Hydroxymethylcytosine and demethylation of the γ-*globin* gene promoter during erythroid differentiation

Maria Armila Ruiz[1,2], Angela Rivers[1,3], Vinzon Ibanez[1,2], Kestis Vaitkus[1,2], Nadim Mahmud[2], Joseph DeSimone[1,2], and Donald Lavelle[1,2,*]

[1]Jesse Brown VA Medical Center; Chicago, IL USA; [2]Department of Medicine; University of Illinois at Chicago; Chicago, IL USA; [3]Department of Pediatrics; University of Illinois at Chicago; Chicago, IL USA

Keywords: baboon, decitabine, DNA methylation, erythropoiesis, globin gene switching, hydroxymethylcytosine, tranylcypromine, vitamin C, γ-*globin* gene

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 γ-*globin* gene expression involves DNA methylation. Previous results have shown that the γ-*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 γ-*globin* gene promoter during erythroid differentiation was investigated by analyzing levels of 5-methylcytosine (5mC) and 5hmC at a CCGG site within the 5′ γ-*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 γ-*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+ bone marrow-derived erythroid progenitor cell cultures, γ-*globin* expression was positively correlated with 5hmC and negatively correlated with 5mC at the γ-*globin* promoter. Supplementation of culture media with Vitamin C, a cofactor of the Tet dioxygenases, reduced γ-*globin* promoter DNA methylation and increased γ-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 γ-globin expression by mediating demethylation of the γ-*globin* promoter.

Introduction

The human β-*globin* gene complex spans approximately 70 kb on the long arm of chromosome 11 and includes 5 distinct genes, ε-, Gγ-, Aγ-, δ-, and β-, 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 γ-*globin* genes, is of critical importance in the development of new therapeutic interventions for sickle cell disease and β-thalassemia because increased levels of fetal hemoglobin (HbF; $\alpha_2\gamma_2$) inhibit polymerization of deoxygenated sickle hemoglobin and are associated with decreased risk of pain crises and death in patients with sickle cell disease.[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 co-repressor complexes.[3-5]

A mechanistic role for DNA methylation in developmental stage-specific repression of the γ-*globin* gene has long been supported by experimental studies. Previous analysis of the DNA methylation status of the γ-*globin* promoter by Southern blot following digestion of DNA with methylation sensitive restriction enzymes established a strong negative correlation between the

*Correspondence to: Donald Lavelle; Email: dlavelle@uic.edu
Submitted: 01/22/2015; Revised: 03/30/2015; Accepted: 04/05/2015
http://dx.doi.org/10.1080/15592294.2015.1039220

level of DNA methylation of the γ-*globin* promoter and γ-globin expression during development.[6,7] Studies in the experimental non-human primate baboon model showed that pharmacological inhibitors of DNA methyltransferase (DNMT) induced high levels of HbF.[8] 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.[9-13] 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.[14] 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.[15,16] 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,[17] 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.[18] The loss of DNA methylation observed during differentiation of the murine erythroleukemia cell line,[19] during differentiation of mouse fetal liver erythroblasts *in vivo*[20] and during erythroid differentiation of cultured human CD34+ cells[21] suggests that modulation of DNA demethylation plays a critical role 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.[22] The role of Tet genes and 5hmC in normal and malignant hematopoiesis has been the subject of a number of recent reviews.[23-26] 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.[27] 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.[28] 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.[29]

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[30-34] 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. 1A 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 *Msp*I 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 ±

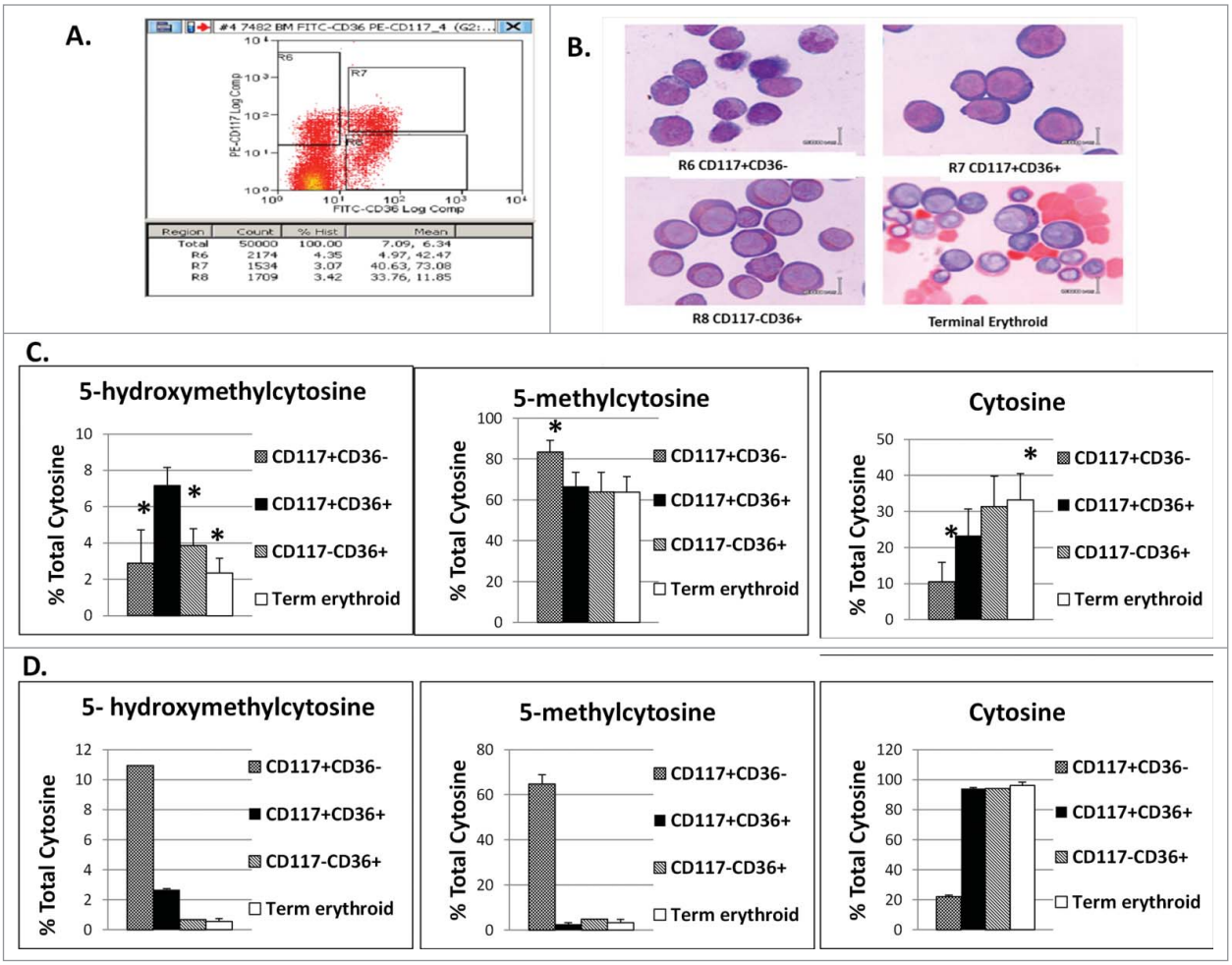

Figure 1. (**A**) FACS profile of baboon BM cells separated by PE-labeled CD117 and FITC-labeled CD36. (**B**) Wright's stained cytospin preparations showing morphology of FACS-sorted CD117+CD36−, CD117+CD36+, and CD117-CD36+ and immunomagnetic column purified bRBC+ (terminal erythroid precursor) cell subpopulations. (**C**) Analysis of 5hmC, 5mC, and Cyt present at the γ-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 γ-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).

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 γ-*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 (3.85 ± 0.93; $P < 0.01$), and terminal erythroid precursors (2.34 ± 0.82; $P < 0.001$; **Fig. 1C**). Thus the level of γ-*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 eythroid 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 (31.37 ± 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.

Analysis of globin mRNA levels within these subpopulations showed that levels of γ- and β-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 γ-globin mRNA relative to β-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 γ-*globin* promoter during erythroid differentiation.

Bisulfite sequence analysis was performed to compare the DNA methylation at 5 CpG sites within the 5′ γ-*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 γ-*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 ($P < 0.05$) the 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 γ-*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 γ-*globin* promoter 5hmC, 5mC and γ-*globin* expression

The relationship between the level of γ-globin expression and γ-*globin* promoter 5hmC, was investigated by comparing levels of 5hmC, 5mC, and Cyt at the γ-*globin* promoter −54 CCGG site in adult baboon CD34+ BM-derived erythroid cells expressing high levels of γ-globin when grown in liquid culture to those expressing low levels of γ-globin when grown in a stromal cell derived co-culture system. Expression of γ-globin in cultured cells was measured by HPLC analysis of globin chains (γ/γ+β) on d14. Analysis of paired cultures (liquid vs. co-culture) derived from BM aspirates from 6 different individual baboons showed that globin synthesis (γ/γ+β) was significantly greater (mean difference = 0.44 γ/γ+β; $P < 0.001$; **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 γ-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 γ-globin expression and γ-*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 μM). HPLC analysis showed that γ-globin expression was significantly higher in cultures treated with Vitamin C compared to untreated controls (mean difference 0.12 γ/γ+β; $P < 0.01$; Figs. **3A, D**). A representative HPLC chromatogram of globin chain expression shows the increased levels of γ-globin chain expression in liquid and stromal cell co-cultures treated with Vitamin C compared to the untreated controls (**Fig. S5A**). Epimark assays showed that levels of 5mC at the −54 CCGG site of the γ-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′ γ-*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 (**Fig. S2C**). Levels of 5mC at the −54 site were negatively correlated ($\rho = 0.732$; $P < 0.001$) and Cyt levels were positively correlated ($\rho = 0.633$; $P < 0.01$) with γ-globin expression (**Fig. S6**). No significant difference in 5hmC levels was observed between Vitamin C-treated and untreated control cultures. A positive correlation was observed between the difference in 5hmC levels in Vitamin C and untreated control cultures and the 5mC levels in both Vitamin C treated cultures ($r^2 = 0.63$) and the untreated control cultures ($r^2 = 0.57$; **Fig. S7**).

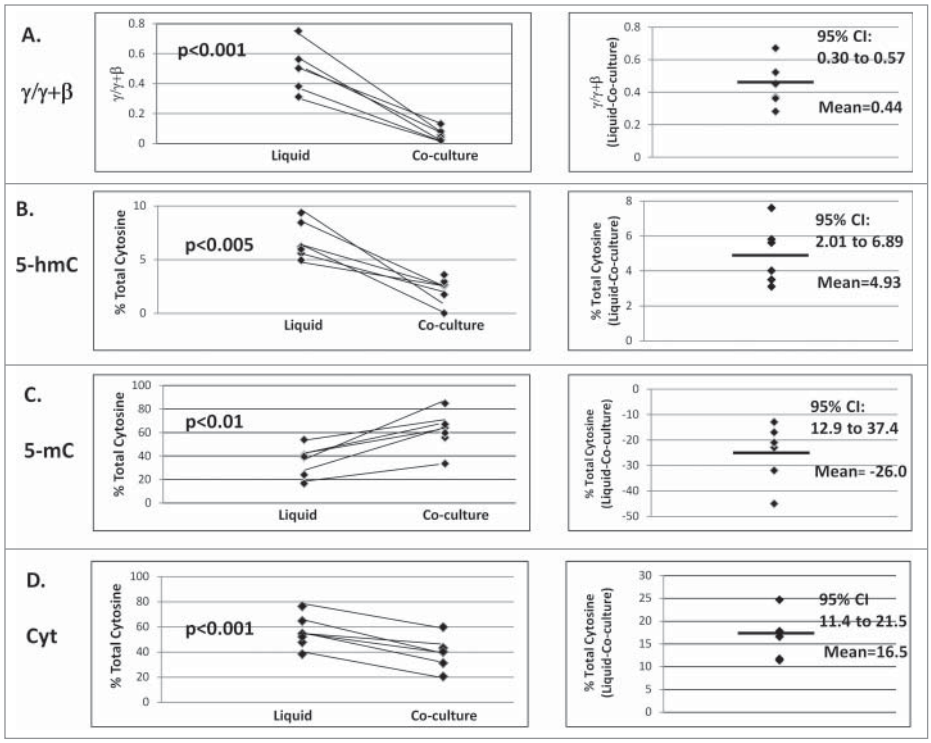

Figure 2. Comparison of (**A**) γ-globin expression (γ/γ+β), (**B**) 5hmC, (**C**) 5mC, and (**D**) Cyt at the γ-globin promoter −54 CCGG site between CD34+ baboon BM erythroid progenitor cells grown in liquid cultures and co-cultures with the AFT024 stromal cell line.

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**) shows 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 glycophorin 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 ±

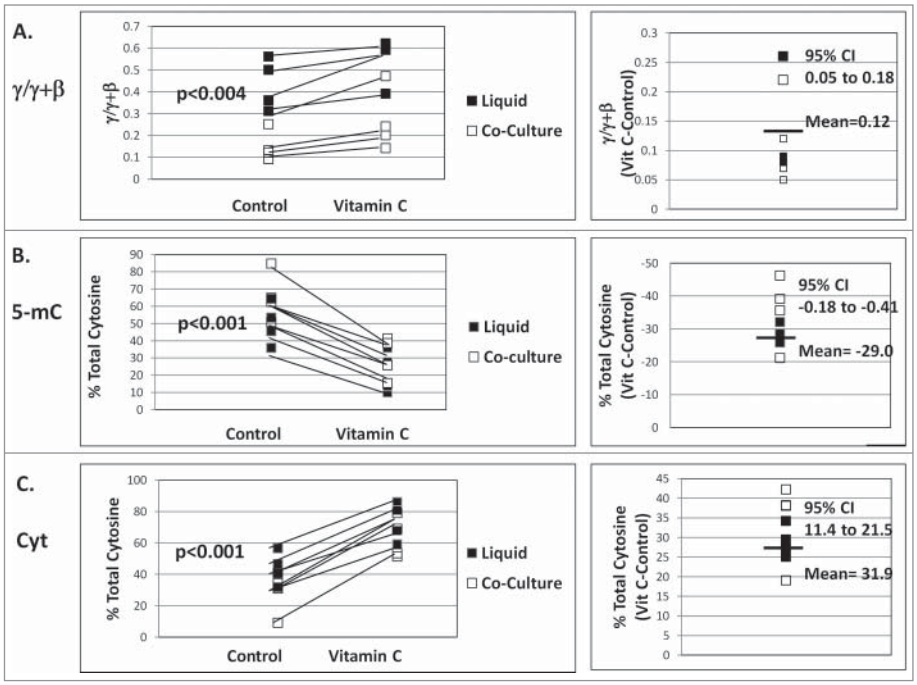

Figure 3. Effect of Vitamin C on (**A**) γ-globin expression (γ/γ+β), (**B**) 5mC, and (**C**) Cyt, at the γ-*globin* promoter −54 CCGG site in baboon BM erythroid progenitors grown in liquid and stromal cell line co-cultures.

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.

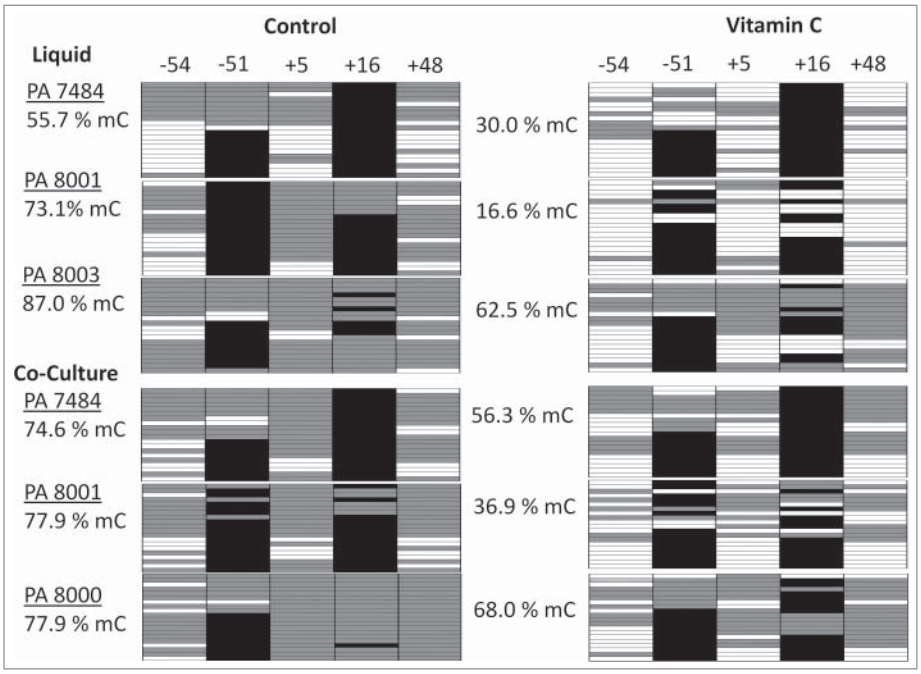

Figure 4. Bisulfite sequence analysis showing the effect of Vitamin C on DNA methylation of 5 CpG sites located in the 5′ γ-*globin* promoter region. Methylated CpG (gray boxes), unmethylated CpG (white boxes), polymorphic sites that do not contain CpG (black boxes).

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.[35] 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 γ-*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.[27] 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.[36] 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.[37] 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.[25] Interestingly, *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 γ-*globin* promoter −54 site).[38] Although our data showing that Tet3 expression and γ-*globin* promoter 5hmC levels during erythroid differentiation are inversely related

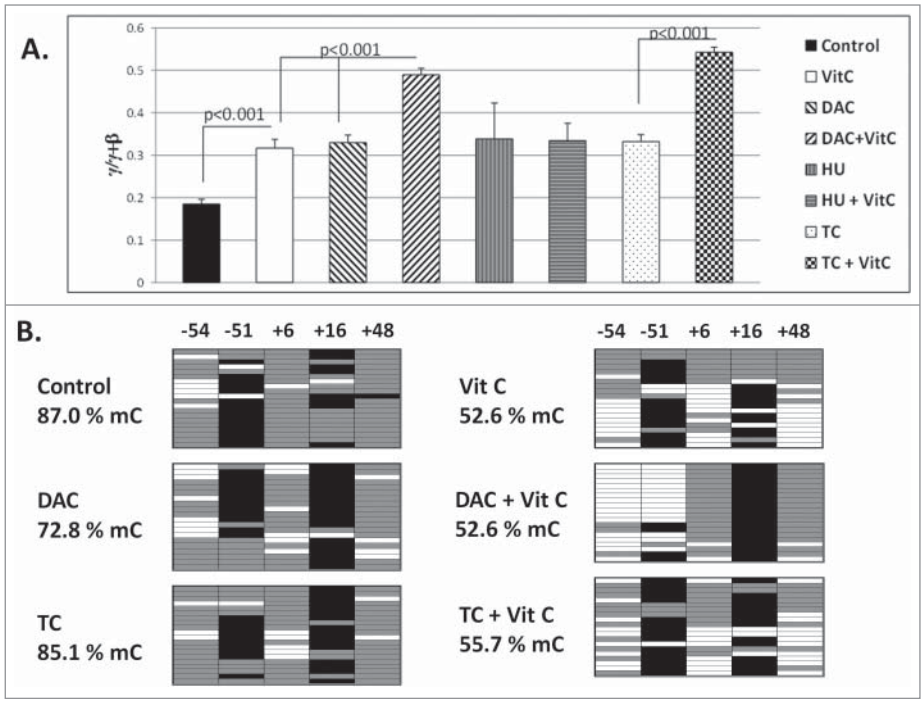

Figure 5. (**A**) Effect of decitabine, TC, and HU alone and in combination with Vitamin C on γ-globin expression. (**B**) Bisulfite sequence analysis showing effect of decitabine, TCP alone, and in combination with Vitamin C on DNA methylation of 5 CpG sites within the 5′ γ-*globin* promoter region. Methylated CpG (gray boxes), unmethylated CpG (white boxes), and polymorphic sites that do not contain CpG (black boxes) are shown.

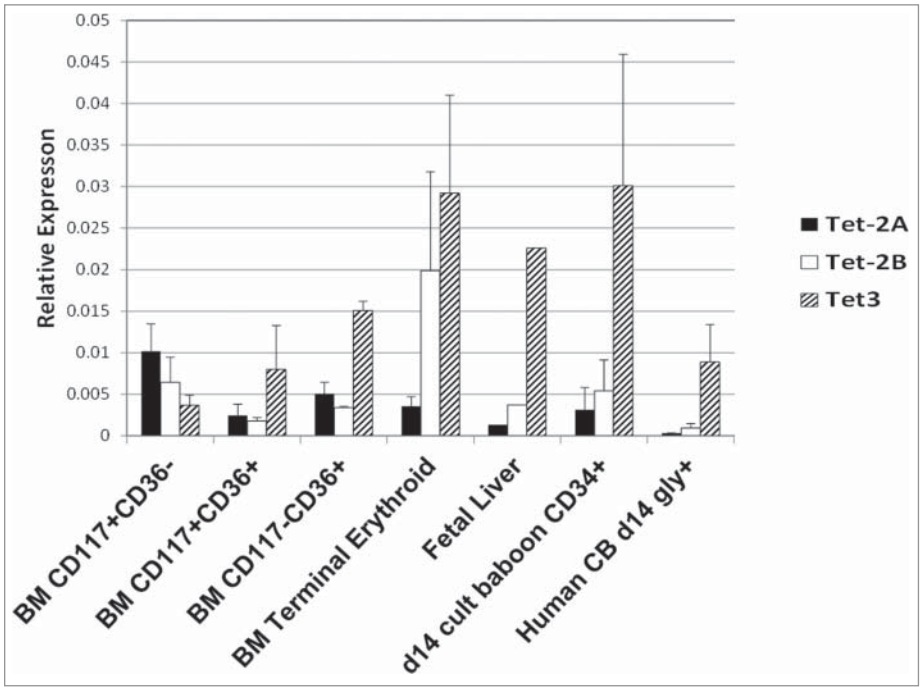

Figure 6. RT-PCR analysis of Tet2A, Tet2B, and Tet3 expression. Expression was measured relative to GADH for each based on standard curves derived from each amplicon.

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 erythroid 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 5hmC 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 demethylation[30-34] 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 from lys9 of Histone H3.[34] A recent study showed Vitamin C enhanced neural differentiation in the developing midbrain in a Tet1 and Jmjd3-dependendent 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 hydroxyurea to elicit additive effects on γ-globin expression could be due to inhibition of DNA synthesis by hydroxyurea. Previous studies have shown that DNA demethylation in differentiating mouse fetal liver erythroblasts requires DNA synthesis[20] and hydroxyurea 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.[40] 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, #551299) 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 Minikits (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 units MspI; Tube 2 (T4-BGT); 50 units *Hpa*II; Tube 3 (T4-BGT) no enzyme; Tube 4 (control): 100 units *Msp*I; Tube 5 (control): 50 units *Hpa*II; 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 5hmC, 5mC and Cyt values. Primers: γ 5hmc F- 5′TCTTGCCTTGACCAATAGCC and γ 5hmc R- 5′AGCCTTGTCCTCCTCTGTGA.

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 1×10^{-6} M dexamethasone. On days 1–3 culture media was also supplemented with IL-3 (10 ng/ml). For co-cultures, $2–3 \times 10^5$ CD34+ cells were seeded onto monolayers of the ATF024 murine fetal liver stromal cell line (ATCC) and grown in the same media as for liquid cultures as previously described.[44] In experiments designed to test the effect of Vitamin C, 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.

HLPC analysis of globin chains

Analysis of globin chain expression was performed by HPLC.[45] 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 215λ.

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′- AATAACCTTATCCTCCTCTATAAAA-TAACC and BG5 5′- GGTTGGTTAGTTTTGTTTTGAT-TAATAG 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[47,48] were: Hum Tet1F- 5′CCGAATCAAGCGGAAGAATA, Hum Tet1 R- 5′C CTGGAGATGCCTCTTTCAC; Bab Tet1 F- 5′CGAGT CAAGCGGAAGAGTAA; Bab Tet 1R- 5′AGATGCCTCTTT CACTGGATG; Tet2 F- 5′AATTTATTGGAT ACACCTGT-CAAGACTC; Tet2A R- 5′ACCTGCTCCTAGATGG GTA-TAAAAAG; Tet2B R-5′GATAAACGCCATGTGTCTCAG TACA; Tet3 F- 5′GCACTCCGGAGAAGATCAAG; Tet3 R-5′GGAGTACACGCTGTTCATGC. 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.

Funding

This work was supported by NIH 1R01HL114561-01 and 1U01HL117658 funds.

Supplemental Material

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

References

1. Platt OS, Thorington 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, Steinberg MH, Klug PP. Mortality in sickle cell disease. Life expectancy and risk factors for early death. N Engl J

Med 1994; 330:1639-44; PMID:7993409; http://dx.doi.org/10.1056/NEJM199406093302303
3. Sankaran VG, Xu J, Orkin SH. Advances in the understanding of haemoglobin switching. Br J Haemotol 2010; 149:181-94; http://dx.doi.org/10.1111/j.1365-2141.2010.08105.x
4. Ginder GD. Epigenetic regulation of fetal globin gene expression in adult erythroid cells. Transl Res 2015; 165:115-25; PMID:24880147
5. Suzuki M, Yamamoto M, Engel JD. Fetal globin gene repressors as drug targets for molecular therapies to treat the hemoglobinopathies. Mol Cell Biol 2014; 34:3560-9; PMID:25022757; http://dx.doi.org/10.1128/MCB.00714-14
6. Van der Ploeg LH, Flavell RA. DNA methylation in the human gamma delta beta-globin locus in erythroid and nonerythroid tissues. Cell 1980; 1:947-58; http://dx.doi.org/10.1016/0092-8674(80)90086-0
7. Mavilio F, Giampaolo A, Care A, Migliaccio G, Calandrini M, Russo G, Pagliardi GL, Mastroberardino G, Marinucci M, Peschle C. Molecular mechanisms of human hemoglobin switching: Selective undermethylation and expression of globin genes in embryonic, fetal and adult erythroblasts. Proc Natl Acad Sci USA 1983; 80:6907-11; PMID:6316333; http://dx.doi.org/10.1073/pnas.80.22.6907
8. DeSimone J, Heller P, Hall L, Zwiers D. 5-azacytidine increases gamma-globin synthesis increases fetal hemoglobin in anemic baboons. Blood 1982; 67:4428-31.
9. Ley TJ, DeSimone J, Noguchi CT, Turner PH, Schechter AN, Heller P, Nienhuis AW. 5-azacytidine increases gamma-globin synthesis and reduces the proportion of dense cells in patients with sickle cell anemia. Blood 1983; 62:370-80; PMID:6191799
10. Ley TJ, DeSimone J, Anagnou NP, Keller NP, Humphries RK, Turner PH, Young NS, Keller P, Nienhuis AW. 5-azacytidine selectively increases gamma-globin synthesis in a patient with beta+ thalassemia N Engl J Med 1982; 307:1469-1475; PMID:6183586; http://dx.doi.org/10.1056/NEJM198212093072401
11. Koshy M, Dorn L, Bressler L, Molokie R, Lavelle D, Talischy N, Hoffman R, van Overveld W, DeSimone J. 2-deoxy 5-azacytidine and fetal hemoglobin induction in sickle cell anemia. Blood 2000; 96:2379-384; PMID:11001887
12. DeSimone J, Koshy M, Dorn L, Lavelle D, Bressler L, Molokie R. Talischy N. Maintenance of elevated fetal hemoglobin levels by decitabine during dose interval treatment of sickle cell anemia. Blood 2002; 99:3905-8; PMID:12010787; http://dx.doi.org/10.1182/blood.V99.11.3905
13. Saunthararajah Y, Hillery CA, Molokie R, Dorn L, Bressler L, Gacasova S, Chen YH, Hoffman R, DeSimone J. Effects of 5-aza-2′-deoxycytidine on fetal hemoglobin levels, red cell adhesion, and hematopoietic differentiation in patients with sickle cell disease. Blood 2003;; 102:3865-70; PMID:12907443; http://dx.doi.org/10.1182/blood-2003-05-1738
14. Tagle DA, Koop BF, Goodman M, Slightom JL, Hess DL, Jones RT. Embryonic epsilon and gamma globin genes of a prosimian primate (Galago crassicaudatus). Nucleotide and amino acid sequences, developmental regulation, and phylogenetic footprints. J Mol Biol, 1988; 203:43-455; http://dx.doi.org/10.1016/0022-2836(88)90011-3
15. Cui S, Kolodziej KE, Obara N, Amaral-Psarris A, Demmers J, Shi L, Engel D, Grosveld F, Strouboulis J, Tanabe O. Nuclear receptors TR2 and TR4 recruit multiple epigenetic transcriptional corepressors that associate specifically with the embryonic b-type globin promoters in differentiated adult erythroid cells. Mol Cell Biol 2011; 31:3298-311; PMID:21670149; http://dx.doi.org/10.1128/MCB.05310-11
16. Xu J, Bauer DE, Kerenyi MA, Vo TD, Hou S, Hsu YJ, Yao H, Trowbridge JJ, Mandel G, Orkin SH. Corepressor-dependent silencing of fetal hemoglobin expression by BCL11A. Proc Natl Acad USA 2013; 110:6518-23; http://dx.doi.org/10.1073/pnas.1303976110
17. Shi L, Cui S, Engel JD, Tanabe O. Lysine-specific demethylase 1 is a therapeutic target for fetal hemoglobin induction. Nat Med 2013; 19:291-4; PMID:23416702; http://dx.doi.org/10.1038/nm.3101
18. Singh M, Lavelle D, Vaitkus K, Mahmud N, Hankewych M, DeSimone J. The g-globin gene promoter progressively demethylates as the hematopoietic stem progenitor cells differentiate along the erythroid lineage 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
19. Christman JK, Weich N, Schoenbrun B, Schneiderman N, Acs G. Hypomethylation of DNA during differentiation of Friend erythroleukemia cells. J Cell Biol 1980; 86:366-70; PMID:6931107; http://dx.doi.org/10.1083/jcb.86.2.366
20. Shearstone JR, Pop R, Bock C, Boyle P, Meissner A, Socolovsky M. Global DNA demethylation during mouse erythropoiesis in vivo. Science 2011; 334:799-802; PMID:22076376; http://dx.doi.org/10.1126/science.1207306
21. Yu Y, Mo Y, Ebenezer D, Bhattacharyya S, Liu H, Sundaravel S, Giricz O, Wontakal S, Cartier J, Caces B, et al, High resolution methylome analysis reveals widespread functional hypomethylation during adult human erythropoiesis. J Biol Chem 2013; 288:8805-14; PMID:23306203; http://dx.doi.org/10.1074/jbc.M112.423756
22. ShenL, Song CX, He C, Zhang Y. Mechanism and function of oxidative reversal of DNA and RNA methylation. Annu Rev Biochem 2014; 83:585-614; PMID:24905787; http://dx.doi.org/10.1146/annurev-biochem-060713-035513
23. Madzo J, Vasanthakumar A, Godley LA. Perturbations of 5-hydroxymethylcytosine patterning in hematologic malignancies. Semin Hematol 2013; 50: 61-9; PMID:23507484; http://dx.doi.org/10.1053/j.seminhematol.2013.01.004
24. Jeong M, Goodell MA. New answers to old questions from genome-wide maps of DNA methylation in hematopoietic cells. Exp Hematol 2014; 42:609-17; PMID:24993071; http://dx.doi.org/10.1016/j.exphem.2014.04.008
25. Ko M, An J, Pastor WA, Koralov SB, Rajewsky K, Rao A. TET proteins and 5-methylcytosine oxidation in hematological cancers Immunol Rev 2015; 263:6-21; PMID:25510268; http://dx.doi.org/10.1111/imr.12239
26. Moen EL, Mariani CJ, Zullow H, Jeff-Eke M, Litwin E, Nikitas JN, Godley LA. New themes in the biological functions of 5-methylcytosine and 5-hydroxymethylcytosine. Immunol Rev 2015; 263:36-49; PMID:25510270; http://dx.doi.org/10.1111/imr.12242
27. Madzo J, Liu H, Rodriguez A, Vasanthakumar A, Sundaravel S, Caces DB, Looney TJ, Zhang L, Lepore JB, Macrae T, et al Hydoxymethylation at gene regulatory regions directs stem/early progenitor cell commitment during erythropoiesis. Cell Rep 2014; 6:231-44; PMID:24373966; http://dx.doi.org/10.1016/j.celrep.2013.11.044
28. Pronier E, Almire C, Mokrani H, Vasanthakumar A, Simon A, da Costa reis Monte Mor B, Masse' A, Le Couedic JP, Pendino F, Carbonne B, et al Inhibition of TET2-mediated conversion of 5-methylcytosine to 5-hydroxymethylcytosine disturbs erythroid and granulomonocytic differentiation of human hematopoietic progenitors. Blood 2011; 118:2551-5; PMID:21734233; http://dx.doi.org/10.1182/blood-2010-12-324707
29. Valdes-Quezada C, Arriaga-Canon C, Fonseca-Guzman Y, Guerrero G, Recillas-Targa F. CTCF demarcates chicken embryonic a-globin gene autonoumous silencing and contributes to adult stage-specific gene expression. Epigenetics 2013;; 8:827-38; PMID:23880533; http://dx.doi.org/10.4161/epi.25472
30. Chung TL, Brena RM, Kolle G, Grimmond SM, Berman BP, Laird PW, Pera MF, Wolvetang EJ. Vitamin C promotes widespread yet specific DNA demethylation of the epigenome in human embryonic stem cells. 2010; 28:1848-55; PMID:20687155
31. Minor EA, Court BL, Young JL, Wang G. Ascorbate induces ten eleven translocation (Tet) methylcytosine dioxygenase-mediated generation of 5-hydroxymethylcytosine. 2013; 288:13669-74; PMID:23548903
32. Yin R, Mao SQ, Zhao B, Chong Z, Yang Y, 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:23768208; http://dx.doi.org/10.1021/ja4028346
33. Blascjke K, Ebata KT, Karimi MM, Zepeda-Maryinez JA, Goyal P, Mahapatra S, Tam A, Laird DJ, Hirst M, Rao A et al Vitamin C induces Tet-dependent DNA demethylation and a blastocyst-like state in ES cells. Nature 2013; 500:222-226; PMID:23812591; http://dx.doi.org/10.1038/nature12362
34. Monfort A, Wutz A. Breathing-in epigenetic change with vitamin C. EMBO Rep 2013; 14:337-46; PMID:23492828; http://dx.doi.org/10.1038/embor.2013.29
35. Mabaera R, Richardson CA, Johnson K, Hsu M, Fiering S, Lowrey CH. Developmental- and differentiation-specific patterns of human gamma- and beta-globin promoter DNA methylation. Blood 2008; 110:1343-52; http://dx.doi.org/10.1182/blood-2007-01-068635
36. Plongthongkum N, Diep DH, Zhang K. advances in the profiling of DNA modifications: cytosine methylation and beyond. Nat Rev Genet 2014; 15:647-661; PMID:25159599; http://dx.doi.org/10.1038/nrg3772
37. Li T, Yang D, Li J, Yang J, Le W. Critical role of Tet3 in neural progenitor cell maintenance and terminal differentiation. Mol Neurobiol 2015; 51:142-54; PMID24838624
38. Xu Y, Xu C, Kato A, Temple W, Abreu JG, Bian C, Hu Y, Hu D, Zhao B, Cerovina T et al Tet3 CXXC domain and dioxygenase activity cooperatively regulate key genes for Xenopus eye and neural development. Cell 2012; 151:1200-13; PMID:23217707; http://dx.doi.org/10.1016/j.cell.2012.11.014
39. He X, Kim M, Kim S, Yi S, Rhee Y, Kim T, Lee E, Park C, Dixit S, Harrison FE, Lee S. Vitamin C facilitates dopamine neuron differentiation in fetal midbrain through Tet1- and Jmjd3-depdendent epigenetic control manner. Stem Cells 2015; 33:1320-32; PMID 25535150
40. Chiu D, Vichinsky E, Ho SL, Liu T, Lubin BH. Vitamin C deficiency in patients with sickle cell anemia. Am J Pediatr Hematol Oncol 1990; 12:262-7; PMID:2240472; http://dx.doi.org/10.1097/00043426-199023000-00003
41. Jaja Sl, Ikotun AR, Gbenebitse S, Temiye EO. Blood pressure, hematologic and erythrocyte fragility changes in children suffering from sickle cell anemia following ascorbic acid. J Trop Pediatr 2002; 48:366-70; PMID:12521281
42. Kinney SM, Chin HG, Vaisila R, Bitinaite J, Esteve P-O, Feng S, Stroud H, Jacobsen SE, Pradhan S. Tissue-specific distribution and dynamic changes of 5-hydroxymethylcytosine in mammalian genomes. J Biol Che 2011; 286:24685-93; http://dx.doi.org/10.1074/jbc.M110.217083
43. Davis T, Vaisivila R. High sensitivity 5-hydroxymethylcytosine detection in Balb/C brain tissue. J Vis Exp 2011; 48:pii 2661; PMID:NOT_FOUND
44. Lavelle D, Vaitkus K, Ruiz MA, Ibanez V, Kouznetsova T, Saunthararajah Y, Mahmud N, DeSimone J. Effect of AGM and fetal liver-derived stromal cell lines on globin expression in adult baboon (*P. anubis*) bone marrow-derived erythroid progenitors. Plos One 2012; 7:e36846; PMID:22693559; http://dx.doi.org/10.1371/journal.pone.0036846

45. Leone L, Monteleone M, Gabutti V, Amoine C. Reversed phase high performance liquid chromatography of human hemoglobin chains. J Chromatog 1985; 321:407-19; http://dx.doi.org/10.1016/S0021-9673(01)90459-5
46. Lavelle D, Vaitkus K, Hankewych M, Singh M, DeSimone J. Developmental changes in DNA methylation and covalent histone modifications of chromatin associated with the e-, g-, and b-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, Ndiave-Lobry D, Lobry C, Figueroa MN, Vasanthakumar 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, Aslanyan MG, Massop MG, Stevens-Linders E, van Hoogen P, van Kessel AG, Raymakers RA, et al Acquired mutations in TET2 are common in myelodysplastic syndromes. Nat Genet 2009; 41:838-42; PMID:19483684; http://dx.doi.org/10.1038/ng.391