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siDNMT1 Increases γ -globin Expression in Chemical-Inducer-of-Dimerization (CID)-Dependent Mouse β YAC Bone Marrow Cells and in Baboon Erythroid Progenitor Cell Cultures

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Abstract

1) Objective—These studies were performed to test the hypothesis that DNMT1 is required for maintenance of DNA methylation and repression of the γ -globin gene in adult stage erythroid cells.

2) Methods—DNMT1 levels were reduced by nucleofection of siRNA targeting DNMT1 in chemical-inducer-of-dimerization (CID)-dependent multipotential mouse bone marrow (BM) cells containing the human β -globin gene locus in the context of a yeast artificial chromosome (β YAC) and in primary cultures of erythroid progenitor cells derived from CD34+ baboon BM cells. The effect of reduced DNMT1 levels on globin gene expression was measured by real time PCR and the effect on globin chain synthesis in primary erythroid progenitor cell cultures was determined by biosynthetic radiolabelling of globin chains followed by HPLC analysis. The effect on DNA methylation was determined by bisulfite sequence analysis.

3) Results—Reduced DNMT1 levels in cells treated with siDNMT1 were associated with increased expression of γ -globin mRNA, an increased $\gamma/\gamma+\beta$ chain ratio in cultured erythroid progenitors, and decreased DNA methylation of the γ -globin promoter. Similar effects were observed in cells treated with decitabine, a pharmacological inhibitor of DNA methyltransferase inhibitor.

4) Conclusion—DNMT1 is required to maintain DNA methylation of the γ -globin gene promoters and repress γ -globin gene expression in adult-stage erythroid cells.

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Conflict of Interest Disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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INTRODUCTION

A role for DNA methylation in the mechanism responsible for repression of the γ -globin gene in adults is strongly supported by multiple experimental studies (1). Expression of the γ -globin gene during the fetal period is correlated with DNA hypomethylation of its promoter sequence, while repression of this gene in the adult stage is associated with DNA methylation (2, 3, 4). Phylogenetic footprinting studies showed that recruitment of the γ -globin gene to fetal-stage specific expression occurred during the evolutionary transition from prosimian to simian primates and was coincident with the acquisition of 5 methylatable CpG residues within the 5' γ -globin gene promoter region suggesting that a new mechanism evolved utilizing methylation of these CpG residues to silence γ -globin expression in the adult stage (5). Studies in a transgenic mouse model allowing specific manipulation of the level of DNA methylation of the γ -globin gene promoter showed that DNA methylation repressed γ -globin expression (6). Other experiments showing reactivation of human γ -globin expression in β YAC MBD2^{-/-} mice strongly suggested both DNA methylation and the methyl binding domain protein MBD2 are required to maintain γ -globin repression in adult erythroid cells (7). Treatment with pharmacological inhibitors of DNA methyltransferase (DNMT) increased fetal hemoglobin (HbF) to high levels in experimental primates and patients with sickle cell disease and β -thalassemia. Reactivation of HbF correlated with DNA hypomethylation of the γ -globin gene promoter in these experiments (8-15). Increased expression of ϵ -globin also was observed in vivo following treatment with DNMT inhibitors (16).

At least three DNMTs are responsible for establishing and maintaining patterns of DNA methylation within mammalian cells (17, 18). The role of DNMT1 is primarily maintenance methylation while DNMT3A and 3B are most active in mediating de novo DNA methylation and these enzymes likely functionally interact to varying degrees. Experiments conducted in human erythroid progenitor cell cultures were consistent with a role for DNMT1 in maintenance of γ -globin promoter DNA methylation but did not demonstrate a role for either DNA methylation or DNMT1 in repression of γ -globin gene expression (19). Other recent experiments performed in the K562 cell line have suggested that DNMT3A is required for DNA methylation and repression of the γ -globin gene (20).

Increased HbF levels are clinically important in the treatment of sickle cell disease and β -thalassemia. Delineation of the role of individual DNMTs in the mechanism responsible for γ -globin repression would be important for the development of new therapies designed to increase HbF levels. Using RNAi, we performed experiments to investigate the role of DNMT1 in repression of the γ -globin gene. Our experiments were conducted in two experimental model systems. Initial experiments employed chemical-inducer-of-dimerization (CID) - dependent mouse BM cells harboring a human β -globin gene locus in the context of a yeast artificial chromosome (21). These cells were established from adult bone marrow of β YAC transgenic mice. The proliferation of these cells is driven by a thrombopoietin receptor derivative responsive to the CID AP20187 molecule. The cells express mouse α -, β^{maj} -, β^{min} -, and human β -globin, but not γ -globin transcripts. Because pharmacological DNMT inhibitors and γ -globin gene trans-activator proteins such as FKLF2 induce γ -globin expression in these cells, they are useful for the screening and

identification of transcription factors and pharmacological agents that increase γ -globin expression. This was recently demonstrated in a study identifying several artificial transcription factors capable of increasing γ -globin gene transcription (22). Experiments to test effects of DNMT1 knockdown were also performed in primary baboon erythroid progenitor cell cultures, a system well characterized in our laboratory (23). The baboon is a valuable animal model for studies on fetal globin gene regulation because expression of the γ -globin gene in the fetal stage is observed only in simian primates (5). Furthermore, studies in this model showing that pharmacological DNMT inhibitors increase HbF have been successfully translated into the clinic (8, 10-15). The arrangement of genes within the β -globin cluster and the pattern of developmentally-regulated expression of these genes is similar in baboon and man and therefore the mechanism(s) regulating γ -globin expression are also likely similar. Our results show that DNMT1 is involved in the maintenance of DNA methylation of the γ -globin gene promoter and repression of γ -globin expression in adult stage erythroid cells.

MATERIALS AND METHODS

Transfection of CID-dependent mouse β YAC BM cells

CID-dependent mouse β YAC BMCs were grown in Iscove's media containing 10% fetal bovine serum supplemented with nonessential amino acids, glutamine, 500ng/ml G418, and the CID molecule AP20187 (Ariad Pharmaceuticals, Cambridge, Ma.) as described (21). All transfections were carried out using the Nucleofector II device (Amaxa/Lonza, Basel, Switzerland). Optimum transfection conditions for the CID-dependent β YAC BM cells were determined using a Cell Line Optimization Nucleofector Kit (Amaxa/Lonza) as outlined in the product manual. In summary, a GFP plasmid (pMaxGFP) was transfected into the mouse BM cells using various pulse setting-nucleofector solution combinations. Flow cytometry readings were taken 24 hours after the pulse to assess the efficiency of transfection. In addition, cell viability was monitored using trypan blue staining. The highest viability count and fraction of cells expressing GFP was obtained using a combination of the Nucleofector solution V and pulse setting D-012 and thus was used for subsequent experiments.

Transfection of the CID-dependent mouse β YAC BM cells was carried out using the AMAXA Nucleofector Kit V. Cells were harvested by centrifugation at 100g for 10 minutes. Media was removed and the resulting cell pellet was then resuspended in a volume of the Nucleofector solution V to yield 5 million cells per 100 μ l of solution. For the siRNA experiments, 100 μ l of the cell solution containing 1 μ M siRNA or 1 μ M of the non-silencing negative control was transferred to the electroporation cuvettes. Cells were pulsed using the D-012 setting. The transfected cells were transferred to T25 flasks containing 5ml of prewarmed media. Controls containing no nucleic acid (no DNA, pulsed) were also set up. Transfection efficiency was determined by pulsing the cells with 2.5 μ g of GFP plasmid and evaluating the incorporation of the plasmid by flow cytometry 24 hours after initial pulse. Cells were then subjected to another round of transfection 24 hours after the initial pulse. Performing transfections on two successive days was critical to observe effects on globin gene expression. Samples for RNA extraction were obtained 24 and 72 hours after the final

pulse. Samples for DNA extraction and protein analysis were obtained 72 hours after final pulse.

Baboon BM CD34+ erythroid progenitor cell cultures

Erythroid progenitor cell cultures were derived from baboon CD34+ BM cells. Mononuclear cells were purified from baboon (*P. anubis*) BM aspirates (30-40ml) using Percoll sedimentation and further purification of CD34+ cells was performed by immunomagnetic bead chromatography (Miltenyi, Auburn, Ca.) using the 12.8 monoclonal antibody (gift of Dr. Rob Andrews). CD34+ cells were cultured in Iscove's media containing 30% fetal bovine serum (FBS, 2u/ml erythropoietin (epo), 200ng/ml stem cell factor (SCF) and 1μM dexamethasone.

Transfections of cultured baboon erythroid progenitor cells were carried out using the AMAXA Mouse T cell Nucleofector Kit. Transfections were performed in complete culture media supplemented with component A only. Transfections of siRNA were performed on two successive days (d7-8) of culture to analyze effects on globin gene expression. Cultured cells were harvested on day 7 and 8 by centrifugation at 100g for 10 minutes. The culture media was removed and the cell pellet was resuspended in a volume of the Mouse T cell Nucleofector solution to yield 5-9 million cells per 100μl of solution. The resulting solution was mixed with 1μM siRNA or 1μM of the nonsilencing negative control siRNA, transferred to the electroporation cuvettes, and pulsed using the Z-001 setting. Using 0.5ml of prewarmed transfection media, the electroporated cells were transferred to T25 flasks containing 5mL of prewarmed transfection media. Efficiency of transfection was assessed by transfecting the cells with 2.5ug of the GFP plasmid and flow cytometry performed 24 hours after initial pulse. Cells were then subjected to another round of transfection 24 hours after the initial pulse. Samples for RNA extraction were taken at 24 and 72 hours after the second pulse and samples for DNA extraction and biosynthetic radiolabelling of globin chains were obtained 72 hours after the second pulse.

siRNA

siRNA used in these experiments are listed in Supplemental Data and were purchased from Ambion (Austin, Tx.) and Dharmacon (Lafayette, Co.).

RNA purification and analysis

RNA was purified using the RNeasy Mini Kit (QIAGEN) according to manufacturer's instructions. RNA was treated with DNase I (Ambion) and used to prepare cDNA using kits (Fermentas, Glen Burnie, Md.). The primer-probe sets used for real time PCR analysis of human ε-, γ-, and β-globin and mouse α-globin in the CID-dependent mouse BM cells were obtained from Applied Biosystems (Foster City, Ca.) and are listed in Supplemental Data. Custom designed primer-probe combinations from Applied Biosystems were used for the analysis of ε- γ- β- and α-globin gene expression in baboon CD34⁺ cells. The sequences of these are also listed in Supplemental Data. Absolute numbers of globin transcripts were determined by extrapolation from standard curves prepared from the cloned amplicons or cDNA. Results were expressed as a ratio of total β-like (ε+γ+β)-globin mRNA. Statistical

significance was assessed using a two-tailed T test for either paired or unpaired observations.

Bisulfite sequence analysis

Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, Ca.) according to manufacturer's instructions. Bisulfite conversion was carried out using the EpiTect Bisulfite kit (Qiagen). PCR amplification of the bisulfite converted DNA was carried out as described (9). The PCR products were cloned in the pCR4-TOPO vector using a TOPO-TA cloning kit (Invitrogen, Carlsbad, Ca.). Clones were selected on LB plates containing ampicillin (50µg/mL) and plasmid DNA isolated from individual clones was sequenced using the ABI PRISM 3100 Genetic Analyzer at the University of Illinois at Chicago DNA Services Facility. Five CpG sites within the γ -globin gene promoter region (-54, -51, +5, +16, and +48) and three CpG sites within the ϵ -globin gene promoter (were analyzed for the presence or absence of cytosine methylation.

Western Blot Analysis

Whole cell extracts of the CID-dependent mouse β YAC BM cells were used for the western blot analysis. Protein purification, SDS PAGE and Western Blot analysis were carried out as described (24). Mouse anti-DNMT-1 antibody (Cell Signaling Technology, Danvers, Ma.) and anti-actin antibodies (Santa Cruz, Santa Cruz, Ca.) were used for the western blot analysis.

Analysis of Globin Chain Synthesis

Biosynthetic radiolabelling of baboon erythroid progenitor cell cultures was performed 72 hours following nucleofection to determine levels of synthesis of individual globin chain. Cells ($2-5 \times 10^5$) were incubated in leucine-free alpha-MEM (Invitrogen) containing 50µCi/ml L-[4, 5- 3 H] leucine (GE; TRK510, 163 Ci/mmol, Amersham, GE Healthcare, Piscataway, N.J.) for 24 hours (25). Following radiolabelling, cells were suspended in H₂O containing nonradioactive carrier hemoglobin and lysed by multiple freeze-thaw cycles in dry ice-methanol baths. Separation of globin chains was performed by HPLC with a LiChroCART 250-4 column (VWR) using a Spectra System HPLC (Thermo Finnegan, West Pa Beach, Fl.) with acetonitrile-methanol gradients as described (26). Quantitation of radioactivity in collected fractions was determined by liquid scintillation counting using a Packard Tricarb 1600TR liquid scintillation analyzer.

RESULTS

Effect of siDNMT1 in CID-Dependent Mouse β YAC BM cells

Nucleofection conditions were established that reproducibly transfected 80-90% of the CID-dependent mouse β YAC BM cells (Figure 1A). Cells were transfected with 3 different siRNA molecules targeting mouse DNMT1 (siDNMT1), Ambion s65071 (siDNMT#1), s65072 (siDNMT#2), and s65073 (siDNMT#3). Real time PCR analysis of DNMT1 mRNA levels 48 hours post transfection (Figure 1B) showed that all three siRNAs targeting DNMT1 decreased DNMT1 mRNA levels to 70-80% of the level of untreated controls. No effect on DNMT3A was observed. Increased DNMT3B was observed in cells treated with

siDNMT1. Western blot analysis showed marked loss of DNMT1 protein in cells treated with siRNAs targeting DNMT1 72 hours post-transfection (Figure 1C).

To test the effect of siDNMT1 on globin gene expression, four independent experiments were performed using siDNMT1 s65071 that showed the greatest effect on DNMT1 expression. Cells were transfected with siDNMT1 or nonsilencing siRNA as a negative control, or treated with decitabine (5×10^{-7} M) as a positive control, and the effect on globin gene expression determined. Transfection efficiency was monitored in all experiments by introduction of pMaxGFP and showed that $79.9 \pm 4.5\%$ of cells were GFP positive 24 hours post-transfection. RNA samples analyzed 48 hours post-transfection showed that DNMT1 expression was decreased $80.5 \pm 4.7\%$ in cells treated with siDNMT1 compared to cells treated with nonsilencing siRNA.

Expression of total human β -like globin RNA was increased 10.09 ± 5.88 fold and 10.46 ± 7.35 fold (normalized to mouse α -globin RNA) in cells treated with siDNMT1 and decitabine, respectively, compared to untreated controls, while the minor change in cells treated with nonsilencing siRNA (1.52 ± 0.36 fold) was not significant. The ratio of human β -globin RNA/total ($\epsilon + \gamma + \beta$) human β -like globin RNA was high in untreated control cells ($0.95 \pm .04$) and cells treated with nonsilencing siRNA ($0.97 \pm .01$), but was decreased in cells treated with siDNMT1 (0.48 ± 0.10 , $p < .0001$, $n = 4$), and decitabine (0.51 ± 0.08 , $p < .0001$, $n = 4$, Figure 2). Decreased β -globin expression was associated with an increase in the γ -globin/total human β -like globin mRNA ratio in cells treated with siDNMT1 (0.29 ± 0.04 , $p < .0001$, $n = 4$) and in cells treated with decitabine (0.23 ± 0.11 , $p < .01$, $n = 4$) compared to cells treated with either with nonsilencing siRNA ($0.01 \pm .01$, Figure 2) or untreated control cells ($0.01 \pm .01$), respectively. The ϵ -globin RNA/total β -like globin mRNA ratio was also increased in cells treated with siDNMT1 (0.24 ± 0.10 , $p < .01$, $n = 4$) and decitabine (0.26 ± 0.14 , $p < .02$, $n = 4$) compared to cells treated with nonsilencing siRNA ($0.03 \pm .01$) or untreated controls ($.04 \pm .03$), respectively. In cells treated with siDNMT1 the mean change in both ϵ -globin (84 fold; range = 27 to 170) and γ -globin (117 fold; range = 44 to 260 fold; normalized to mouse α -globin) was far greater than the effect on β -globin (5 fold; range 1.16 to 10). In decitabine-treated cells the mean increase in both ϵ -globin (115 fold; range = 29 to 145) and γ -globin expression (62 fold; range 33-590) were similar to those observed in siDNMT1-treated cells. Smaller changes in human β -globin expression (5.4 fold; range 2.8 to 10.8) were observed. These results show that both siDNMT1 and decitabine preferentially increase ϵ - and γ -globin gene expression in CID-dependent mouse β YAC BM cells.

Bisulfite sequence analysis showed that 5 CpG residues in the 5' γ -globin promoter region were highly methylated (98.0% dmC) in untreated control cells. Decreased DNA methylation of the γ -globin gene promoter region was observed in cells treated with either siDNMT1 (53.3% dmC) or decitabine (68.0%; Figure 3A). Similar effects on ϵ -globin promoter DNA methylation were observed. In untreated control cells the ϵ -globin promoter was highly methylated (78.7% dmC). Methylation was reduced following treatment with either siDNMT1 (33.1% dmC) or decitabine (42.4% dmC, Figure 3B).

Effect of siDNMT1 in Primary Baboon Erythroid Progenitor Cell Cultures

Experiments were then performed to determine whether siDNMT1 had similar effects on globin gene expression in primary erythroid progenitor cell cultures. CD34+ cells were purified from baboon BM aspirates and grown in Iscove's media containing 30% FBS, dexamethasone, epo and SCF. Baboon cells were used because the effect of decitabine has been well characterized in the baboon in vivo model, the growth and analysis of globin gene expression in cultured baboon erythroid progenitors has been well characterized in our laboratory, and the availability of baboon BM aspirates provided a reliable source of cells for these experiments. Initial experiments established nucleofection conditions that reproducibly achieved 45-55% transfection efficiency in d7-d8 erythroid progenitor cultures (Figure 4A). Four siRNAs targeting human DNMT1 were tested for effect on DNMT1 expression. Two siRNAs, s4216 and s4217, decreased DNMT1 mRNA levels >80%. Western blot analysis of DNMT1 protein levels in cells treated with s4216 and s4217 compared to nonsilencing siRNA and normalized to actin showed a greater decrease in DNMT1 using s4217 siDNMT1 (64%) compared to s4216 (41%; Figure 4B). Therefore s4217 was chosen for further studies.

Cells were transfected with s4217 siDNMT1 or control nonsilencing siRNA by nucleofection on two successive days of culture (d7 and 8), preceding the time of peak hemoglobin synthesis (d11-12). RNA was purified 24 hours and 72 hours following the last nucleofection to determine the effect on DNMT1 expression and globin gene expression. Expression of DNMT1 was decreased $80.1 \pm 3.47\%$ in cells treated with siDNMT1 compared to those treated with nonsilencing siRNA. No significant difference in the ratio of α -globin/total β -like globin mRNA was observed between untreated control cells and cells treated with either siDNMT1 or decitabine (0.5×10^{-7} M). Treatment with siDNMT1 increased the γ -globin /total β -like globin RNA ratio to 0.41 ± 0.08 compared to 0.28 ± 0.08 in cells treated with nonsilencing siRNA ($p < .01$, $n=4$, Table 1; Figure 4A). The γ -globin / total β -like globin RNA ratio was also increased in cultures treated with decitabine ($0.48 \pm .09$; Table 1, Figure 4A) compared to untreated control cells ($.31 \pm .04$, $p < .001$, $n=6$). In contrast, siDNMT1 decreased the β -globin / total β -like globin RNA ratio ($.47 \pm .10$) compared to cells treated with nonsilencing siRNA ($.67 \pm .09$, $p < .01$, $n=4$). The β -globin / total β -like globin RNA ratio was also decreased in decitabine-treated cultures ($0.48 \pm .09$) compared to untreated controls ($0.67 \pm .04$, $p < .001$, $n=6$). The ϵ -globin/ total β -like globin mRNA ratio was increased in cells treated with siDNMT1 ($0.10 \pm .06$) compared to cells treated with nonsilencing siRNA ($.04 \pm .04$, $p < .02$, $n=4$) and was also increased in cultures treated with decitabine ($.04 \pm .03$, $p < .01$, $n=6$) compared to untreated controls ($.002 \pm .001$)

HPLC analysis of biosynthetically radiolabelled globin chains confirmed the effect of siDNMT1 on γ -globin expression (Table 2; Figure 4B, Figure 5). In cells transfected with siDNMT1, the $\gamma/\gamma + \beta$ synthetic ratio was 0.51 ± 0.12 and was significantly increased ($p < .01$) compared to cells treated with nonsilencing siRNA (0.32 ± 0.10 , Table 2, Figure 4B). Notably, the $\gamma/\gamma + \beta$ chain ratio was not significantly different between cells treated with siDNMT1 (0.51 ± 0.12) and decitabine (0.62 ± 0.07) while the difference between cells treated with decitabine and untreated cells (0.32 ± 0.06) was significant ($p < .0001$).

Bisulfite sequence analysis showed that the level of DNA methylation of CpG residues within the 5' γ -globin region in cells treated with siRNA targeting DNMT1 (45.4% dmC) was decreased compared to cells treated with nonsilencing siRNA (70.1 % dmC) and untreated control cells (77.8% dmC). A similar decrease in DNA methylation of the γ -globin promoter was observed in decitabine-treated cultures (46.8%). The level of ϵ -globin gene promoter DNA methylation was also decreased in cells treated with siDNMT1 (52.8% dmC) and decitabine (75.0% dmC) compared to untreated control cells (100% dmC) and cells treated with nonsilencing siRNA (100% dmC). Therefore increased expression of ϵ -globin and γ -globin mRNA and increased $\gamma/\gamma+\beta$ globin chain ratios in cells treated with siDNMT1 and decitabine were associated with loss of DNA methylation of the ϵ - and γ -globin gene promoters.

DISCUSSION

The ability of siRNA-mediated downregulation of DNMT1 to increase expression of the ϵ - and γ -globin genes and decrease DNA methylation of the ϵ - and γ -globin promoter regions in CID-dependent mouse β YAC BM cells and in primary cultures of erythroid progenitor cells derived from CD34+ baboon BM strongly suggests DNMT1 is directly involved in both the maintenance of high levels of DNA methylation of these promoter regions and the repression of these genes in adult-stage erythroid cells. Recent results have shown that BCL11A and SOX6 mediate γ -globin repression. Interactions of BCL11A and SOX6 with the Mi2/NURD repressive complex (27, 28), suggests that Mi2/NURD is involved in γ -globin repression. Other reports in β YAC transgenic mice have also suggested the involvement of the NURD complex in γ -globin repression (29). The methyl domain binding proteins MBD2 and MBD3 are components of Mi2/NURD complexes and β YAC MBD2-/- transgenic mice reactivate human γ -globin (7). Therefore it is possible that DNA methylation is involved in the recruitment or stable binding of this complex to the β -globin gene locus to mediate γ -globin repression.

Our results are in agreement with a previous report (19) that demonstrated that DNMT1 was involved in maintenance of DNA methylation of the γ -globin gene promoter in human erythroid cells. In that report, however, increased γ -globin expression was not observed when DNMT1 levels were decreased using RNAi. Although the reasons for this are not clear, differences in the culture system and/or delivery system for siRNA could be involved. Although human BFUE can express moderate to high levels of γ -globin, the human culture system used in those experiments expressed low, physiological levels of HbF. The increased expression of HbF in cultured baboon erythroid progenitors in our experiments is not dissimilar, however, from other human BFUE culture systems where factors present in fetal bovine serum (30-32), and growth factors such as SCF have been shown to increase HbF (33-36). Because expression of γ -globin may require not only loss of repressive factors but also the presence of appropriate levels of positively-acting factors, the timing of treatments that reduce levels of DNA methylation must coincide with the presence of appropriate levels of positively-acting factors in order to achieve increased γ -globin expression. In our experiments delivery of siDNMT1 by nucleofection resulted in a greater reduction in DNA methylation at critical times coinciding with peak γ -globin gene expression than the lipid delivery systems or viral vector employed in the human culture experiments. In addition,

polymorphisms within the baboon γ -globin promoter that eliminate two CpG residues at -51 and +16 and thus reduce the overall methyl CpG density of the baboon gene with respect to the human gene could render the baboon gene more sensitive to changes in DNA methylation (1). Interestingly, both polymorphic sites of the baboon are within functional stage-selector elements shown to favor γ -globin over β -globin gene transcription in artificial constructs introduced into K562 cells (37).

These results show that DNMT1 is required to maintain DNA methylation of the γ -globin gene promoter and repress γ -globin gene expression in the CID-dependent β YAC mouse BM cell model and in adult-stage baboon erythroid cells. Decitabine and siDNMT1 induced similar levels of γ -globin expression. Therefore DNMT1 is likely the primary cellular target affected by decitabine to achieve increased γ -globin expression. Another report (20), based on experiments conducted in the K562 leukemic cell line, has suggested that recruitment of DNMT3A, in response to PRMT5-mediated methylation of histone H4 arg 3, induces DNA methylation and repression of the γ -globin gene. The level of DNA methylation of the γ -globin gene, as determined by quantitative pyrosequencing is quite low in K562 cells (3.0±0.7%) compared to erythroid cells derived from human BFUe cultures (89±6.0%) (38). Also, an aberrant pattern of DNA methylation in K562 cells (39) suggests the presence of abnormalities in the DNA methylation machinery. The specific role of DNMT3A remains to be confirmed in primary cells. Because DNMT1 acts as a maintenance methylase while DNMT3A acts as a de novo methylase, it is possible that each enzyme acts independently at different stages of differentiation to establish and maintain DNA methylation of the γ -globin promoter and repression of γ -globin gene expression, or alternatively, the enzymes act in a cooperative manner, as has been observed in other systems (40, 41). In this respect, cooperative effects of DNMT3B and DNMT1 in the regulation of ribosomal RNA, another PRMT5 target gene, have been reported (42, 43). Further studies should be conducted to delineate the functional roles of each specific DNMT in primary erythroid cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIGURE 1.
A. Flow cytometric analysis of GFP content of CID-dependent mouse β YAC BMCs following nucleofection with pMAXGFP. **B.** Effect of 3 siRNAs targeting DNMT1 on expression of DNMT1. Cells were transfected with s65071, s65072, or s65073 siRNAs targeting DNMT1 or nonsilencing siRNA. Levels of DNMT1 (filled bars), DNMT3A (checked bars), and DNMT3B (unfilled bars) 48 hours post-transfection were analyzed by real time PCR with mouse GAPDH as the standard. **C.** Western blot analysis showing reduction in DNMT1 protein in CID-dependent mouse β YAC BMCs 72 hours post-transfection with siRNAs targeting DNMT1 compared to untreated control cells, cells subjected to nucleofection procedure in the absence of siRNA, and cells transfected with siRNA targeting G9A and nonsilencing siRNA as negative controls. As positive control for loss of DNMT1, cells were treated with decitabine (0.5×10^{-7} M) for 72 hours.

FIGURE 2.

A. siRNAs targeting DNMT1 increase ϵ - and γ -globin RNA in CID-dependent mouse β YAC BMCs. Real time PCR analysis of ϵ -, γ -, and human β -globin expression in untreated parental CID-dependent β YAC BM cells, following transfection with nonsilencing siRNA, and siDNMT1, and following treatment with decitabine (0.5×10^{-7} M) cells. Results are shown as RNA/total ($\epsilon + \gamma + \beta$) human β -like globin RNA (mean $\pm$ SD).

FIGURE 3.

Levels of DNA methylation of CpG residues within the 5' γ -globin promoter region (**A**) and ϵ -globin promoter region (**B**), are decreased following transfection of siDNMT1 and decitabine treatment in CID-dependent β YAC BM cells. Each row represents the sequence analysis of a single pCR4 clone containing an amplicon produced from bisulfite-treated DNA. Methylated sites (red); unmethylated sites (green).

FIGURE 4.

A. Flow cytometric analysis of GFP content of a baboon erythroid progenitor cell culture transfected with the pMAXGFP vector by nucleofection on d8. Cells were analyzed 24 hours post-transfection. Flow cytometric analysis showed that 54.6% of the cells were GFP +. **B.** Western blot analysis of DNMT1 72 hours following transfection of baboon erythroid progenitor cells with either nonsilencing siRNA (NS), or the s4216 and s4217 siRNAs targeting DNMT1. The blot was reprobbed with anti-actin antibody as a loading control. **C.** siDNMT1 and decitabine increase ϵ - and γ -globin RNA expression in erythroid progenitor cultures derived from CD34+ baboon BM cells. RNA was analyzed by real time PCR and presented as RNA/total ($\epsilon + \gamma + \beta$) β -globin like RNA (mean $\pm$ SD). **D.** siDNMT1 and decitabine increase the $\gamma/\gamma + \beta$ globin chain synthetic ratio in erythroid progenitor cell cultures derived from CD34+ baboon BM. Cells were incubated with [3 H] leucine in leucine-free media 72 hours following transfection with nonsilencing siRNA, siDNMT1, or decitabine treatment. Lysates were separated by HPLC and fractions collected. Radioactivity in fractions was determined by scintillation counting. Results are shown as $\gamma/\gamma + \beta$ ratios (mean $\pm$ SD).

PA 7472

$$\gamma/\gamma+\beta=0.37$$

$$\gamma/\gamma+\beta=0.63$$

PA 7484

$$\gamma/\gamma+\beta=0.21$$

$$\gamma/\gamma+\beta=0.39$$

FIGURE 5.

Representative HPLC chromatograms from two biosynthetic radiolabelling experiments showing the pattern of globin chain synthesis in two baboon erythroid progenitor cell cultures treated with either nonsilencing siRNA or siDNMT1.

A. γ -globin

B. ϵ -globin

FIGURE 6.

Levels of DNA methylation of 5 CpG residues within the 5' γ -globin promoter region in erythroid progenitor cultures following treatment with nonsilencing siRNA, siDNMT1, and decitabine compared to untreated control cultures. Each row represents the sequence analysis of a single pCR4 clone containing an amplicon produced from bisulfite-treated DNA. Both the γ -globin (A) and ϵ -globin promoter (B) were analyzed. Methylated sites (red); unmethylated sites (green); polymorphic sites where the methylatable CpG residues is not present (yellow).

Table 1

Effect of siDNMT1 and decitabine on γ -globin gene expression in erythroid progenitor cell cultures.

Exp	Animal	γ / total β -like ($\epsilon + \gamma + \beta$) globin RNA			
		Untreated Control	Nonsilencing siRNA	siDNMT1	Decitabine
1	7482	0.221	0.235	0.319	ND
2	7470	0.349	0.398	0.520	ND
3	7472	0.297	0.250	0.389	ND
4	7472	ND	0.253	0.398	ND
5	7254	0.361	ND	ND	0.441
6	7254	0.290	ND	ND	0.401
7	7484	0.291	ND	ND	0.461
8	7254	0.280	ND	ND	0.422
9	7254	0.360	ND	ND	0.537
10	7256	0.32	ND	ND	0.570
Mean $\pm$ SD		0.31 $\pm$.04	0.28 $\pm$.08	0.41 $\pm$.08	0.48 $\pm$.09

Table 2

Effect of siDNMT1 and decitabine on globin chain synthesis.

Exp	Animal	γ -globin chain synthesis ($\gamma/\gamma+\beta$)			
		Untreated Control	Nonsilencing siRNA	siDNMT1	Decitabine
1	7482	0.23	0.32	0.43	ND
2	7484	0.23	0.21	0.39	ND
3	7472	0.38	0.37	0.63	ND
4	7472	0.39	0.41	0.59	ND
5	7254	0.40	ND	ND	0.55
6	7254	0.29	ND	ND	0.59
7	7482	0.32	ND	ND	0.70
8	7484	0.34	ND	ND	0.65
Mean $\pm$ SD		0.32 $\pm$ 0.06	0.32 $\pm$ 0.10	0.51 $\pm$ 0.12	0.62 $\pm$ 0.07