Published in final edited form as:
Science. 2011 November 11; 334(6057): 799–802. doi:10.1126/science.1207306.

Global DNA Demethylation During Erythropoiesis *in vivo*[*]

Jeffrey R. Shearstone[1], **Ramona Pop**[1], **Christoph Bock**[2,3,4], **Patrick Boyle**[2], **Alexander Meissner**[2,3,4], and **Merav Socolovsky**[1,#]

[1]Department of Pediatrics and Department of Cancer Biology, University of Massachusetts Medical School, Worcester Massachusetts, USA

[2]Broad Institute, Cambridge, Massachusetts, USA

[3]Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, Massachusetts, USA

[4]Harvard Stem Cell Institute, Cambridge, Massachusetts, USA

Abstract

In the mammalian genome, 5'-CpG-3' dinucleotides are frequently methylated, correlating with transcriptional silencing. Genome-wide demethylation is thought to occur only twice during development, in primordial germ cells and in the pre-implantation embryo. These demethylation events are followed by *de novo* methylation, setting up a pattern inherited throughout development and modified only at tissue-specific loci. Here we studied DNA methylation in differentiating mouse erythroblasts *in vivo* using genomic-scale reduced representation bisulfite sequencing (RRBS). Demethylation at the erythroid-specific β-globin locus was coincident with global DNA demethylation at most genomic elements. Global demethylation was continuous throughout differentiation and required rapid DNA replication. Hence, DNA demethylation can occur globally during somatic cell differentiation, providing an experimental model for its study in development and disease.

The formation of enucleated red cells, or erythropoiesis, first occurs in the murine fetal liver between embryonic day 11 (E11) and E15 and is dependent on the hormone erythropoietin (Epo). We labeled mouse fetal liver with the cell surface markers CD71 and Ter119 and identified six subsets of cells, S0 to S5, which form a sequence of increasingly mature erythroid cells (Fig. 1A) (1). Subsets S1 to S5 contain only erythroid cells. S0 contains erythroid progenitors (70%), which we further enrich (>95%) by negative selection for cells expressing the cell surface markers CD41, Mac-1 and Gr-1 (1). We recently found that transition of erythroid progenitors from S0 to S1 marks a key commitment step that takes place during S phase and requires S phase progression (1). It comprises the synchronous onset of Epo dependence, activation of the erythroid master transcriptional regulator GATA-1, and a conformational switch in chromatin at the β-globin locus control region (LCR) (1). Using freshly-sorted cells from subsets S0 to S4/5, we found rapid loss of methylation at 6 CpGs in Hypersensitive Sites 1 and 2 (HS1, HS2) of this locus (1), beginning with the transition from S0 to S1 (Fig. S1A) (1). We also found significant and

---

[*]This manuscript has been accepted for publication in *Science*. This version has not undergone final editing. Please refer to the complete version of record at http://www.sciencemag.org/. The manuscript may not be reproduced or used in any manner that does not fall within the fair use provisions of the Copyright Act without the prior, written permission of AAAS.

[#]Corresponding author: Merav Socolovsky, University of Massachusetts Medical School, Department of Pediatrics, Hematology/Oncology Division, and Department of Cancer Biology, 364 Plantation Street, LRB Room 403, Worcester MA 01605, office (508) 856 3743; lab (508) 856 3704; cell (617) 797 1633; fax (508) 856 1310, merav.socolovsky@umassmed.edu.

progressive loss of methylation at the promoter regions of the PU.1 and Fas genes, whose expression declines with erythroid differentiation (1, 2) (Fig. S1B, C).

We therefore examined the possibility that DNA methylation may be lost globally during erythropoiesis. We used a 5-methylcytosine-specific antibody in an Enzyme-Linked Immunosorbent Assay (Fig. S1D), and the Luminometric Methylation Assay (3) (Fig. S1E), which compares cleavage at CCGG sites by the isoschizomers *HpaII* and *MspI*, enzymes that are methylation sensitive and insensitive, respectively (4). Both approaches showed significant global demethylation with differentiation.

We next examined loci that are usually stably methylated in somatic cells. The imprinted H19 Differentially Methylated Region (DMR) aids the paternal-specific expression of the Igf2 gene (5). We amplified this region from bisulfite-converted genomic DNA of S0 or S4/5 cells and sequenced individual clones (Fig. 1B). S0 clones formed a bimodal distribution consistent with imprinting. This distribution was partly lost in S4/5, with methylation falling from 61% in S0 to 42% in S4/5 (Fig. 1B). Pyrosequencing of the bulk PCR product at the H19 DMR and at two additional imprinted loci showed a similar, significant demethylation accompanying differentiation (Fig. S2A).

LINE-1 retrotransposons are present at ~100,000 chromosomal sites in the mouse genome and are usually highly methylated (6). Methylation of a 5' LINE-1 tandem repeat region (7) decreased from 90% in S0 to 70% in S4/5 ($p<0.001$) (Figures 1C, S2B), with methylation remaining lower in pyrenocytes (nuclei) extruded from mature erythroblasts (Fig. S2C). LINE-1 methylation levels were also low in yolk-sac erythrocytes (Fig. S2C) and in adult bone-marrow erythroblasts (Fig. S2D), suggesting that erythroid demethylation is not limited to the fetal liver.

To characterize DNA methylation on a genomic scale, we used reduced representation bisulfite sequencing (RRBS) (8) in freshly sorted fetal liver subsets. We examined 5kb non-overlapping tiles with RRBS coverage of ~10% of the genome (Figures 2A, S3A). A plot of the mean methylation level of tiles in subsets S1 to S4/5, against their methylation level in S0, shows that nearly all tiles fall increasingly below the middle diagonal (Fig. 2A). Overall, there is progressive DNA demethylation across a broad range of genomic elements, with median levels falling from 79% in S0 to 55% in S4/5 (Fig. S3B, C). The largest losses are in regions whose S0 methylation level is high, such as promoters with low CpG frequency (Fig. S3B, C; see supplementary website, http://erythrocyte-demethylation.computational-epigenetics.org/). The fractional loss across most genomic elements is 25%-30% of their initial methylation in S0, though imprinted regions are relatively protected, losing only 13% of initial methylation (Fig. S3D). Although the rate of demethylation at the β-globin LCR is faster than the rate of global demethylation, the two processes are tightly correlated throughout differentiation (Fig. 2B). Therefore, a shared mechanism is likely to be responsible for demethylation at both erythroid-specific and genome-wide loci.

We asked whether global demethylation results in a global increase in transcription. Quantitative RT-PCR showed no significant increase in the LINE-1 transcript (Fig. S4A). Further, expression microarrays from S1 and S3 suggest that more genes are downregulated than upregulated during differentiation (Fig. S4B). RRBS analysis showed demethylation associated with both upregulated and downregulated genes (Fig. 2C). The β-globin and Fas gene loci provide representative examples of both gene categories (Fig. S4C). Nevertheless, upregulated genes had a lower initial methylation level in S0 (Fig. 2C), consistent with the documented link between gene-specific DNA demethylation and expression in erythropoiesis (1, 9–11). The absence of a global increase in transcription is in agreement

with previous studies and consistent with demethylation being only one of several concerted functions in chromatin required for gene activation (12, 13).

We examined the expression of DNA methylation regulators (Figures 3, S5). The maintenance methylase DNA methyltransferase 1 (Dnmt1) mRNA and protein did not alter with differentiation. By contrast, both *de novo* methylases Dnmt3a and Dnmt3b, which may also contribute to maintenance methylation (14–16), were markedly downregulated (Figures 3, S5). Of the regulators implicated in active demethylation (17–20), Gadd45a was significantly upregulated (Fig. S5). To examine the significance of these observations, we retrovirally-transduced sorted S0 cells with Dnmt3a or Dnmt3b (15) (Fig. S6). Re-expression of the Dnmt3 enzymes did not, however, prevent demethylation (Fig. S6C), nor did shRNA-mediated 'knockdown' of either Gadd45a or Mbd4 (Fig. S7). Therefore, expression changes in Dnmt3 or Gadd45a, by themselves, are not sufficient to account for the observed loss in methylation. We also attempted to retrovirally over-express Dnmt1 in differentiating erythroblasts, with little consequent effect on demethylation (Fig. S8).

We recently found that the transition from S0 to S1 is S phase-dependent and that S1 cells are in S phase of the cycle (Fig. 4A, (1)). Further, a brief pulse of the nucleotide analogue bromodeoxyuridine (BrdU) *in vivo* showed a 50% higher rate of intra-S phase DNA synthesis in S1 cells than in S-phase cells in previous cycles in S0 (Fig 4A, (1)) (21). To investigate the potential role of DNA replication in demethylation, we arrested the cycle in late G1 or in S phase, respectively with mimosine (22) or with aphidicolin, an inhibitor of DNA polymerases (23). Either drug reversibly prevented demethylation at both the β-globin LCR and at LINE-1 elements (Fig. S9). Global demethylation therefore requires DNA replication, and likely results from inadequate methylation of nascent DNA. We also specifically decelerated intra-S phase DNA synthesis in S1 cells, without affecting the number of S phase cells, by using a low aphidicolin concentration (0.1μM, Fig 4B, S9C). The resulting slower DNA synthesis rate prevented demethylation at LINE-1 elements and inhibited global demethylation as judged by genomic-scale RRBS. It only partially reduced demethylation at the β-globin LCR (Figures 4B, S9C, D, S10).

S1 cells are susceptible to leukemic transformation (24, 25), making it unlikely that global demethylation is the result of lax genomic integrity maintenance. To examine the potential function of global demethylation, we examined whether it affects expression of a subset of erythroid genes that, like β–globin, are massively induced with differentiation (Fig. S1A). The rapid induction of these genes was indeed compromised when we inhibited global demethylation using 0.1μM aphidicolin (Fig. S11). Addition of 5-aza 2'-deoxycytidine (5-aza), an inhibitor of maintenance methylation (26), rescued the rapid induction of most of these genes (Fig. S11C). As an alternative approach, we 'knocked-down' Dnmt1 in fetal liver cells, finding accelerated erythroid gene induction with differentiation (Fig. S12). Global cellular mechanisms that lead to global demethylation may therefore function to accelerate removal of methylation marks at sites of massively induced erythroid genes.

Taken together, erythropoiesis is associated with genome-wide loss of one third of all DNA methylation at nearly all genomic loci, within a space of ~3 cell divisions. Quantitatively, this degree of demethylation is less severe than that measured recently in primordial germ cells (18). Mechanistically, erythroid global demethylation is dependent on DNA replication, resembling global demethylation of the maternal genome in the early zygote. In addition, it requires a rapid rate of DNA synthesis, a finding of potential relevance to other instances of global demethylation including cancer. We conclude that DNA demethylation can occur globally during somatic cell differentiation, providing an experimental model for its study in development and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Ralph Scully, Peter A. Jones, Rudolf Jaenisch, Job Dekker, Craig Peterson and Schahram Akbarian for helpful discussions, Daniel Hidalgo for technical assistance, Dr. E. Li for Dnmt cDNAs, Richard Konz, Ted Giehl and the UMass FACS core, and Stephen Baker for statistical analysis. This work was funded by NIH/NHLBI RO1 HL084168, ACS grant RSG06-051-01, NIH CA T32-130807 and Diabetes Endocrinology Research Center grant DK32520. The RRBS analysis may be accessed at http://erythrocyte-demethylation.computational-epigenetics.org/. Affymetrix microarray expression data for the S1 and S3 subsets are deposited in the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/), GEO accession number GSE32214.

References and notes

1. Pop R, et al. PLoS Biol. 2010; 8
2. Socolovsky M, et al. PLoS Biol. Sep 25.2007 5:e252. [PubMed: 17896863]
3. Karimi M, et al. Exp Cell Res. Jul 1.2006 312:1989. [PubMed: 16624287]
4. Cedar H, Solage A, Glaser G, Razin A. Nucleic Acids Res. 1979; 6:2125. [PubMed: 223125]
5. Lopes S, et al. Hum Mol Genet. Feb 1.2003 12:295. [PubMed: 12554683]
6. Silver, LM. Mouse Genetics. Oxford University Press; 1995. p. 5.4
7. Loeb DD, et al. Mol Cell Biol. Jan.1986 6:168. [PubMed: 3023821]
8. Meissner A, et al. Nature. Aug 7.2008 454:766. [PubMed: 18600261]
9. Broske AM, et al. Nat Genet. Nov.2009 41:1207. [PubMed: 19801979]
10. Singh M, et al. Exp Hematol. Jan.2007 35:48. [PubMed: 17198873]
11. Dover GJ, Charache SH, Boyer SH, Talbot CC Jr, Smith KD. Prog Clin Biol Res. 1983; 134:475. [PubMed: 6198661]
12. Michalowsky LA, Jones PA. Mol Cell Biol. Mar.1989 9:885. [PubMed: 2471061]
13. Jackson-Grusby L, et al. Nat Genet. Jan.2001 27:31. [PubMed: 11137995]
14. Liang G, et al. Mol Cell Biol. Jan.2002 22:480. [PubMed: 11756544]
15. Chen T, Ueda Y, Dodge JE, Wang Z, Li E. Mol Cell Biol. Aug.2003 23:5594. [PubMed: 12897133]
16. Jones PA, Liang G. Nat Rev Genet. Nov.2009 10:805. [PubMed: 19789556]
17. Barreto G, et al. Nature. Feb 8.2007 445:671. [PubMed: 17268471]
18. Popp C, et al. Nature. Feb 25.2010 463:1101. [PubMed: 20098412]
19. Rai K, et al. Cell. Dec 26.2008 135:1201. [PubMed: 19109892]
20. Bhutani N, et al. Nature. Feb 25.2010 463:1042. [PubMed: 20027182]
21. Gratzner HG. Science. Oct 29.1982 218:474. [PubMed: 7123245]
22. Lalande M. Exp Cell Res. Feb.1990 186:332. [PubMed: 2153561]
23. Ikegami S, et al. Nature. Oct 5.1978 275:458. [PubMed: 692726]
24. Kowal-Vern A, et al. Am J Hematol. Sep.2000 65:5. [PubMed: 10936857]
25. Pinkus GS, Said JW. Am J Pathol. Mar.1981 102:308. [PubMed: 7011040]
26. Christman JK. Oncogene. Aug 12.2002 21:5483. [PubMed: 12154409]
27. McGrath KE, et al. Blood. Feb 15.2008 111:2409. [PubMed: 18032705]
28. Heintzman ND, et al. Nature. May 7.2009 459:108. [PubMed: 19295514]
29. von Lindern M, et al. Blood. Jul 15.1999 94:550. [PubMed: 10397722]
30. Rohde C, Zhang Y, Reinhardt R, Jeltsch A. BMC Bioinformatics. 11:230. [PubMed: 20459626]
31. Simon R, et al. Cancer Inform. 2007; 3:11. [PubMed: 19455231]
32. Socolovsky M, Fallon AEJ, Wang S, Brugnara C, Lodish HF. Cell. 1999; 98:181. [PubMed: 10428030]

33. Naviaux RK, Costanzi E, Haas M, Verma IM. J Virol. Aug.1996 70:5701. [PubMed: 8764092]

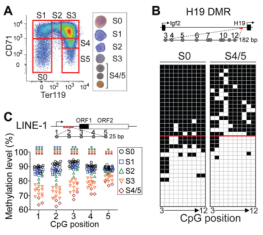

Figure 1. Global DNA demethylation in erythropoiesis

A Erythoid differentiation subsets in freshly isolated fetal liver, defined by the CD71/ Ter119 flow cytometric profile. Box on right shows examples of cytospin cells from each subset, stained with Giemsa (blue) and diaminobenzidine (for hemoglobin, brown).

B DNA methylation of individual CpGs (columns) in individual clones (rows) at the H19 DMR, in genomic DNA from sorted S0 and S4/5 subsets. The median clone is indicated (red line). Methylation fell from 61% (S0) to 42% (S4/5, p=0.007, two-tailed Mann-Whitney test).

C Methylation at the 5' tandem repeat of LINE-1 retrotransposons. A genomic map (not to scale) shows tandem repeats in red, and numbered CpGs within one expanded repeat sequence. Data points are methylation level of individual CpGs in specific differentiation subsets within multiple replicate experiments. ***p<0.001, **p<0.01, *p<0.05 (linear mixed model). See also Fig. S2B.

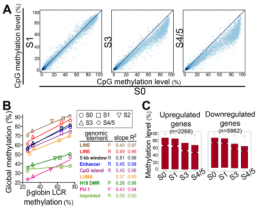

Figure 2. RRBS analysis shows global demethylation

A DNA methylation of 5kb non-overlapping tiles across the genome in increasingly mature S1, S3 and S4/5 erythroblasts plotted against S0 progenitors. Data are mean of 2 experiments, shown for tiles with sufficient RRBS coverage (≥5 CpGs, with ≥5 valid sequencing reads per CpG, Fig. S3A).

B Linear correlation across S0 to S4/5 between global methylation at various genomic elements, and methylation at the β-globin LCR. 'P', pyrosequencing; 'R', RRBS. Data pooled from Figures 1C, 2A, S1C, S1E, S2A, S2B, S3B, S3C.

C DNA methylation at 5kb tiles associated with genes that are either upregulated or downregulated with the transition from S1 to S3, by gene expression microarrays. Boxplots correspond to center quartiles, with indicated median (black bar). Whiskers extend to the most extreme data point, which is no more than 1.5 times the interquartile range from the box.

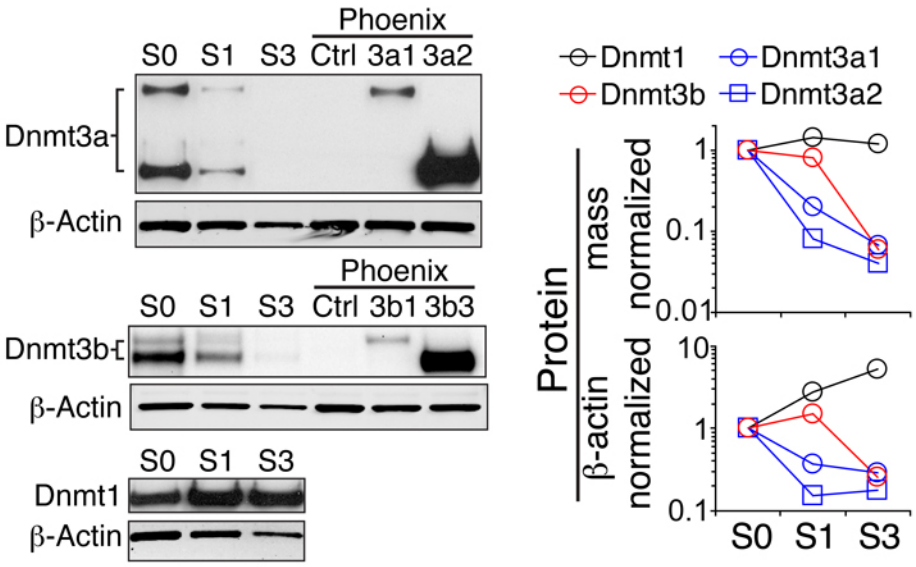

Figure 3. Decreased Dnmt3 expression with differentiation

Western blotting for Dnmts in freshly sorted erythroid subsets (left panels), with corresponding quantitation (right panels). An equal mass of cell lysate was loaded in each lane. Phoenix cells (derived from Large T antigen-transformed human embryonic kidney '293T' cells), were either untransfected (Ctrl) or transduced with vectors encoding Dnmt3a1, Dnmt3a2, Dnmt3b1, or Dnmt3b3. Representative of two independent experiments.

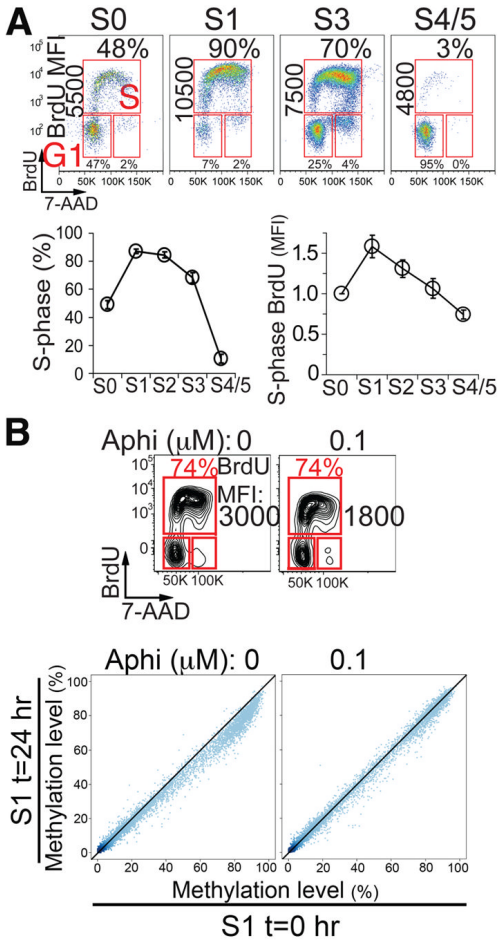

Figure 4. Global demethylation is dependent on rapid DNA replication

A Cell number in S phase (%) in subsets S0 to S4/5, and corresponding intra-S phase DNA synthesis rate as measured by BrdU Median Fluorscence Intensity (MFI) in the S phase gate. Pregnant mice were pulsed with BrdU 30' prior to harvest of fetal livers. Data in lower panels are mean ± s.e.m of 8 -15 replicates.

B Global methylation (RRBS, 5kb tiles, lower panels) of S1 cells following differentiation in Epo ± Aphi (0.1 μM) for t=24 hr, compared with their methylation levels at the time of sorting (t=0). Data are mean of duplicate experiments with sufficient RRBS coverage. Upper panel: a representative cell cycle analysis of S1 cells cultured in Epo ± Aphi (0.1 μM, 16hr) showing the fraction of cells in S phase (%) and intra-S phase DNA synthesis (BrdU MFI). See also Fig. S9C, D.