Suppression of DNMTs Accelerates the *In Vitro* Erythropoietic Differentiation of Human CD34$^+$ Progenitor Cells

Seok-Ho Kim[1], Hee-Young Yang[1], Dong Kee Jeong[2], Sang Ryeul Lee[1], Zae Young Ryoo[3] and Tae-Hoon Lee[1,†]

[1]*Department of Oral Biochemistry, The 2nd Stage of BK21 Program for Dental School, Dental Science Research Institute, Chonnam National University, Gwangju 500-757, Korea*

[2]*Department of Animal Science & Biotechnology, College of Applied Life Science, Cheju National University, Jeju 690-756, Korea*

[3]*School of Life Science and Biotechnology, Kyungpook National University, Daegu 702-701, Korea.*

ABSTRACT

Epigenetic modification dependent DNA methyltransferases (DNMTs) play an important role in tissue- and stage-specific gene regulation and normal mammalian development. In this study, we show that DNMTs are expressed at different levels during hematopoietic stem cell (HSC) differentiation to proerythrocytes. DNMT1, DNMT3A, and DNMT3B were highly expressed at day 7 after differentiation. We used specific siRNA as a tool to probe the relationship between the expression of DNMTs and erythropoietic differentiation. When introduced siRNA of DMNT1 and DMNT3b in human CD34$^+$ cells, these more differentiated into erythrocytes. This was confirmed by glycophorin A (GPA) positive cell analysis and globin gene expression. GPA$^+$ cells increased up to 20～30%, and ɣ- and ε-globin genes increased in siRNA transfected cells. Therefore, our data suggest that suppression of DNA methylation can affect positively differentiation of HSC and may contribute to expression of erythrocyte lineage genes including GPA and globins.

(Key words : Erythropoiesis, DNA methyltransferase, Hematopoietic stem cell, *In vitro* differentiation, siRNA)

INTRODUCTION

DNA methylation serves as an epigenetic marker and also regulates tissue specific gene expression, cell differentiation, genomic imprinting, X-chromosome inactivation, chromatin structure, carcinogenesis, and aging. DNA methylation is an epigenetic event in which the addition of a methyl group to the fifth carbon position of a cytosine residue occurs frequently in CpG dinucleotide (Bird, 2002). This event is closely associated with the modification of chromatin structure directly linked DNA such as gene promoter regions and also plays an important role in regulating gene expression in normal cells (Bird, 2002). It was known that methylation of CpG dinucleotides was mediated by at three DNA methyltransferase (DNMT) families, including DNMT1, DNMT3a, and DNMT3b (Bestor, 2000). D-NMT1 is required to maintain DNA methylation and ubiquitously expressed in proliferating cells. It localizes to DNA replication foci (Leonhardt *et al.*, 1992). However, *de novo* methylases, DNMT3a and DNMT3b might contribute to an initial reprogramming of DNA methylation patterns for developing genomes (Okano *et al.*, 1999; Leu *et al.*, 2003). These enzymes are highly expressed in embryonic cells but are lowly expressed in adult cells (Bestor, 2000).

In the previous study, it was reported that DNMTs are highly expressed in human CD34$^+$ cells and are detected in the hematopoietic lineage cells such as peripheral neutrophils, T lymphocytes, total bone marrow cells (BMCs), and various colonies derived from the hematopoietic stem cell (HSC) (Mizuno *et al.*, 2001). It is estimated that DNMTs might be required to maintain HSC self-renewal capacity and multipotentiality into their multiple hematopoietic lineages. For example, DNMTs in hematopoietic differentiation played a role in human disease such as immunodeficiency, centromere instability, and facial anomalies (ICF) syndrome and acute and chronic myelogenous leukemia (Maraschio *et al.*, 1988; Mizuno *et al.*, 2001).

In human, developmental switch occurs in β -globin gene locus. In fetal stage, ε -globin gene is switched to ɣ-globin, whereas the ɣ-globin is switched to β -globin in adult stage (Cao *et al.*, 2004; Stamatoyannopoulos, 2005). After switching, activated globin promoter

* This work was supported by Korea Research Foundation Grant (KRF-2004-015-E00238).

† Corresponding author : Phone: +82-62-530-4842, E-mail: thlee83@chonnam.ac.kr

gene is un-methylated, whereas the inactivated globins are highly methylated in cells. This phenomenon explains that the human globin gene is controlled through DNA methylation. The γ-globin gene of fetal hemoglobin can be reactivated in red blood cells at adult stage by chemical reagents, such as 5-aza-2′-deoxycytidine and 5-azacytidine (Cao *et al.*, 2004; Saunthararajah *et al.*, 2004). Also, inhibitors of DNA methylation could modify HSC fate and reactivate the erythroid differentiation potential of the myeloid-restricted cell line (Baiocchi *et al.*, 2003; Milhem *et al.*, 2004). A number of pharmacological agents are currently available for clinical trial to treat the patient with sickle cell disease (SSD) and β -thalassimia. However, the question of controversial carcinogenic effect of those reagents remains unsettled (Saunthararajah *et al.*, 2004). Therefore, to ensure a safer system to patients, the need to develop therapeutic approach is increasing.

In this study, we studied the differential expression of DNMTs during human $CD34^+$ cells differentiation into proerythrocytes. Furthermore, we investigated whether the expression levels of DNMTs connected with erythropoietic gene expression in human $CD34^+$ cells, differentiated progenitor cells, and K562 cell lines. Finally, we identified the relationship between DNMTs and erythropoiesis.

MATERIALS AND METHODS

Isolation and Culture Conditions of Human $CD34^+$ Cells

Umbilical cord blood (UCB) samples were donated from mothers of newborns with the written consents. The consents were approved by the Institutional Review Board at Chonnam National University Hospital. Isolation of human hematopoietic progenitor cells was achieved by magnetic cell sorting (MACS; Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's protocol. Mononuclear cells contained in the blood were isolated using Lymphoprep gradient (AXIS-SHIELD, Oslo, Norway) and washed twice with phosphate-buffered saline (PBS) supplemented with 2 mM EDTA (Sigma-Aldrich, St. Louis, MO, USA) and 0.5% bovine serum albumin (BSA; Sigma-Aldrich). Human $CD34^+$ cells were isolated by positive selection using magnetic beads tagged with anti-CD34 antibodies and a VarioMacs magnet (Miltenyi Biotec). The purity of the recovered cells was typically more than 85%. Human $CD34^+$ cells were cultured in serum-free liquid medium using QBSF-60 media (Quality Biological, Gaithersburg, MD, USA) supplemented with 10 U/ml penicillin/streptomycin (Sigma-Aldrich) and a cocktail of cytokine (R&D Systems, Minneapolis, MN, USA) as follows: days 0 to 3, 10 ng/ml interleukin-3 (IL-3; Sigma-Aldrich) and 10 ng/ml stem cell factor (SCF; Sigma-Aldrich): days 3 to 7, 10 ng/ml IL-3, 10 ng/ml SCF and 2 U/ml erythropoietin (EPO; Sigma-Aldrich): and after day 9, 2 U/ml EPO. After day 9, media were refreshed every 3 to 4 days and cell density was maintained below 1×10^6/ml.

Cell line and Erythropoiesis Induction

The K562 cell line was maintained in RPMI1640 medium (JBI, Wel-gene, Korea) supplemented with 10% fetal bovine serum (FBS; Life Technologies Laboratories, Grand Island, NY, USA) plus 10 U/ml penicillin, and 10 μ g/ml streptomycin. Erythropoietic differentiation was induced in K562 cells by treatment with 40 μ M hemin (Sigma-Aldrich) for 4 days.

Construction of siRNA Vectors for DNMT1, DNMT3b and Transfection in K562 and Human $CD34^+$ Cells

pSilence 2.1-U6 hygro vector (Ambion, Austin, TX, USA) was used for DNA vector based siRNA synthesis. Target sequence of *DNMT1* and DNMT3b were as follows (Leu *et al.*, 2003): DNMT1: 5′-CCGGATCCTCTGTCCGTTCACATGTGTTTCAAGAGAACACATGTGAACGGACAGATTTTTTAAGCTTCCG-3′, DNMT3b: 5′-GCCGGATCCAGATGACGGATGCCTAGAGTTCAAGAGACTCTAGGCATCCGTCATCTTTTTTAAGCTTCCG-3′. These oligonucleotides contain a sense strand of 19 nucleotides followed by a loop sequence (TTCAAGAGA), the antisense strand, and six timidines that act as a RNA polymerase III transcriptional stop signal. Also, the oligonucleotide pairs were designed to contain *BamH I* and *Hind III*. The paired oligonucleotides were annealed. Each mixture was first incubated at 94℃ for 5 min, then at 25℃ for 3 min and at 72℃ for 3 min at three cycles. It was then ligated to pSilence 2.1-U6 hygro vector. The vector was transfected into K562 cells by electroporation apparatus (BTX, San Diego, CA, USA) according to the manufacturer's protocol. Stable siRNA expression clones were selected by culturing the cells in 500 μ g/ml geneticin for 2 weeks. Human $CD34^+$ cells were electroporated using a Human CD34 cell Nucleofector Kit (Amaxa Biosystems, Gaithersburg, MD, USA) according to the manufacturer's protocol.

Fluorescence Activated Cell Sorter (FACS) Analysis of Antigen Expression

Phenotypic differentiation was monitored using the following antibodies: fluorescein isothiocyanate (FITC) or phycoetythrin (PE) conjugated CD34, FITC conjugated CD36, and PE conjugated GPA. Cells were analyzed using FACS (Beckman Coulter, Fullerton, CA, USA). All analyses were paired with the corresponding matched isotype control of the specific mAb used. All staining and washes were performed in PBS,

0.1% BSA, and 0.1% sodium azide (Sigma-Aldrich). Approximately 1 × 10^5 cells were collected, washed with FACS buffer and incubated with antibody at 4℃.

Reverse-Transcription (RT) PCR and Quantitative Real-Time PCR

RNA isolation was performed using Trizol (MRC, Cincinnati, OH, USA) and Oligo dTs were used to prime cDNA synthesis. Total RNA of cells was reverse-transcribed into cDNA by AMV reverse transcriptase (Roche Applied Science, Indianapolis, ID, USA). Specific oligonucleotide primers were designed using Primer Express Software (Applied Biosystems, Warrington Cheshire, UK). RT-PCR reaction was carried out in a 40 μ l reaction using TaqMan Reverse Transcription Reagent (Applied Biosystems) and real-time reaction was performed with the SYBR Green PCR master mix (Applied Biosystems). Quantitative real-time PCR was performed with ABI Prism 7300 Real Time PCR System (Applied Biosystems). The primer used for the real-time PCR is as follows: DNMT1: sense strand, 5′-TGTGAGGTTCGCTTATATCAAC-3′; antisense strand, 5′-CTCATACAGTGGTAGATTTG-3′, DNMT3b: sense strand, 5′-CCAGGGCAAGC AGAAGAGAA-3′, antisense strand, 5′-TACTCTAATAGGTCCCGTGC-3′.

Western Blotting

Cells were obtained as pellet after centrifugation at 800 × g for 5 min. The pellets were resuspended in lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM $Na_4O_2P_7$, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μ g/ml leupeptin, 1 mM PMSF] at 4℃ for 1 hr. Protein concentrations in resuspended pellets were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Proteins in resuspended lysates were fractionated in 10% SDS-polyacrylamide gels and transferred onto PVDF membranes (Bio-rad, Hercules, CA, USA). The membranes were blocked by incubation in 5% dried skim milk in TBST (0.05% Tween-20 in TBS) and probed with DNMT1, DNMT3b, and β -actin antibody, respectively. Antibody binding was detected by using an enhanced chemiluminescence kit for western blotting detection with hyper-enhanced chemiluminescence film (Amersham, Piscataway, NJ, USA).

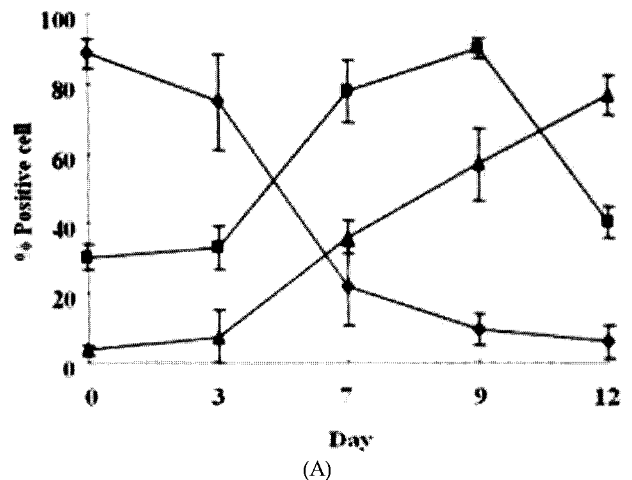
Statistical Analysis

Data are shown as averages and standard deviations. P values were calculated by unpaired Student's *t*-test. All statistical analyses were analyzed with SigmaPlot 9.0.

RESULTS

Differentiation of Human CD34$^+$ Cells Originating from Human UCB into Proerythrocytes

To identify influence of DNMTs during erythropoiesis, we established *in vitro* erythropoietic differentiation system of human CD34$^+$ cells with UCB. This system was identified in previous research (Yang *et al.*, 2007). Namely, human CD34$^+$ cells were isolated from UCB and stimulated in serum-free liquid medium supplemented with SCF, IL-3, and EPO for 12 days. Human CD34$^+$ cells, when cultured with medium supplemented with IL-3 and SCF for 3 days, lead to the full expansion and erythropoietic differentiation at early stage. Increasing the proportion of human CD36$^+$ cells, committed erythroid precursor having cell surface marker, GPA, was observed by FACS analysis (Fig. 1A). Cells were cultured with additional EPO and cytokine on the same media. The proportion of human CD34$^+$ cells was less than human CD36$^+$ cells at day 7, and GPA$^+$ cells were increased after day 3. It means that such cell population is primarily pronormoblast, which is an intermediate stage in erythropoietic differentiation. After that stage, the population of GPA$^+$ cells was constantly increased up to about 90% at day 12. GPA is a marker of more ma-



(A)

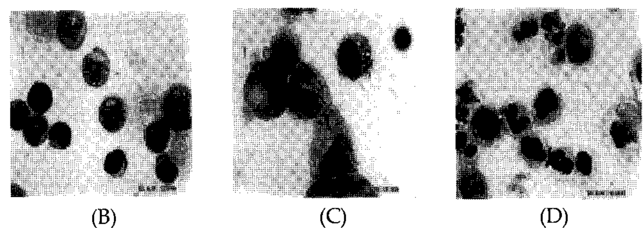

(B) (C) (D)

Fig. 1. Stem cell differentiation and morphological changes. (A) *In vitro* erythropoiesis of human CD34$^+$ cells originated from UCB. FACS analysis shows the percentage by using cell surface marker, CD34-FITC (◆), CD36-PE (■), and GPA-FITC (▲). (B), (C), and (D) Cell morphology during erythropoietic differentiation by Wright giemsa stain. The mean change (±S.D.) of three independent experiments.

ture erythroid cells. This molecule appears at the basophilic normoblast stage at which the population of CD36$^+$ cells decreased during erythropoiesis. Also, it was performed that a number of basophilic normoblasts matured into polychromatic normoblasts. Erythrocyte morphology change during *in vitro* erythropoietic differentiation was identified by microscopy (Fig. 1B, C, and D). During 12 days culture of human CD-34$^+$ cells in cytokine regimen, the expansion level of these increased up to ~60 fold. Therefore, this result demonstrates that our culture system provides a useful model of *in vitro* erythropoiesis.

Expression of DNMTs during HSC Differentiation

Our data showed that the level of DNA methylation of Line-1 tended to increase in the whole genome during erythrocyte differentiation (Fig. 2). It is explained by the fact that DNA methylation during erythropoiesis is influenced by DNMTs, including DNMT1, DNMT3a, and DNMT3b. Therefore, we examined the gene expression patterns of DNMT1, DNMT3a, and DNMT3b, during erythropoiesis by real-time PCR and western blotting with DNMT1, DNMT3a, and DNMT-3b antibody (Fig. 3). The expression level of DNMT1, DNMT3a, and DNMT3b mRNA was detected during erythropoiesis. Interestingly, DNMT3b was expressed to higher levels than DNMT1 at day 7 (Fig. 3A). However, after determination of erythroid lineage, we observed that mRNA level of DNMT1 was increased compared to DNMT3b in erythroid cells (Fig. 3A). DNMTs were detected during *in vitro* CD34$^+$ cells differentiation (Fig. 3B). We confirmed that DNMTs were highly expressed at day 7 (Fig. 3B). These indicate that DNMTs are important to lineage commitment during erythropoiesis and may regulate several genes. In differentiation of K562 cells, these are representative of terminal differentiation of erythropoiesis. DNMT1 was spontaneously expressed during erythropoiesis (Fig. 2C), whereas DNMT3a and DNMT3b were expressed at day 3. These results also indicate that epigenetic modification throughout DNMTs might play a role in erythropoiesis.

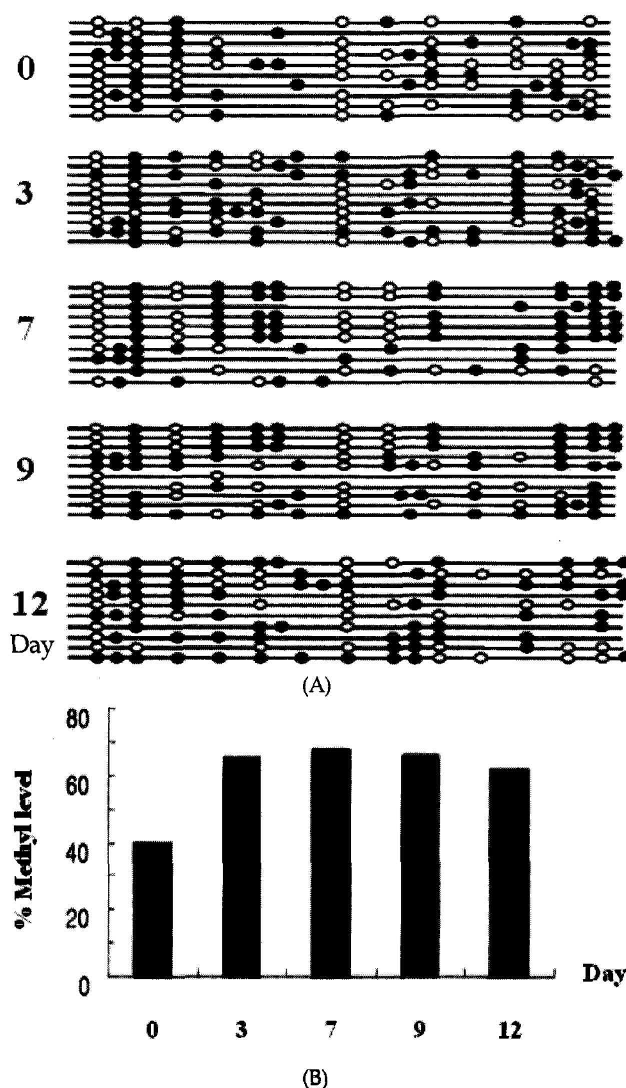


Fig. 2. DNA methylation level during erythropoiesis. (A) Methylation patterns are profiled depending on the LINE repetitive sequence during erythropoiesis. (B) After bisulfite treatment, individually sequenced PCR products were obtained from each stage during erythropoietic differentiation. Blank and filled rectangles indicate unmethylated and methylated CpGs, respectively. Percent methylation is the proportion of methylated CpG sites.

Increment of Erythropoiesis in K562 and Human CD34$^+$ Cells that Suppressed DNMTs

We suppressed the expression of DNMTs using siRNA targeted to respective mRNA in K562 cells. We constructed two separate siRNA vectors that targeted to DNMT1 and DNMT3b whose sequences were homologous to the respective 3′-end untranslated region of the genes, as described, in "Materials and Methods." We introduced DNMT1 siRNA, DNMT3b siRNA, and mock vector at 10 μ M into K562 cells using electroporation, and established stable cell lines by geneticin for 2 weeks. We identified protein level of DNMTs in transfected cells by western blotting. Amounts of DNMT1 and DNMT3b in K562 cells transfected with DNMT1 siRNA and DNMT3b siRNA, and both of them, respectively, were significantly lower than those in wild type K562 cells (Fig. 4A). In order to induce expression of globin genes on the transfected cells, we differentiated the cells using hemin. As shown in Fig. 3B and C, mRNA expression of γ- and ε -globin in K562 cells co-transfected with two types of siRNA was significantly increased compared to in K562 cells transfected with mock vector or single siRNA. Moreover, γ-globin in DMNT3b siRNA transfected cells was 2-fold decreased than mock and

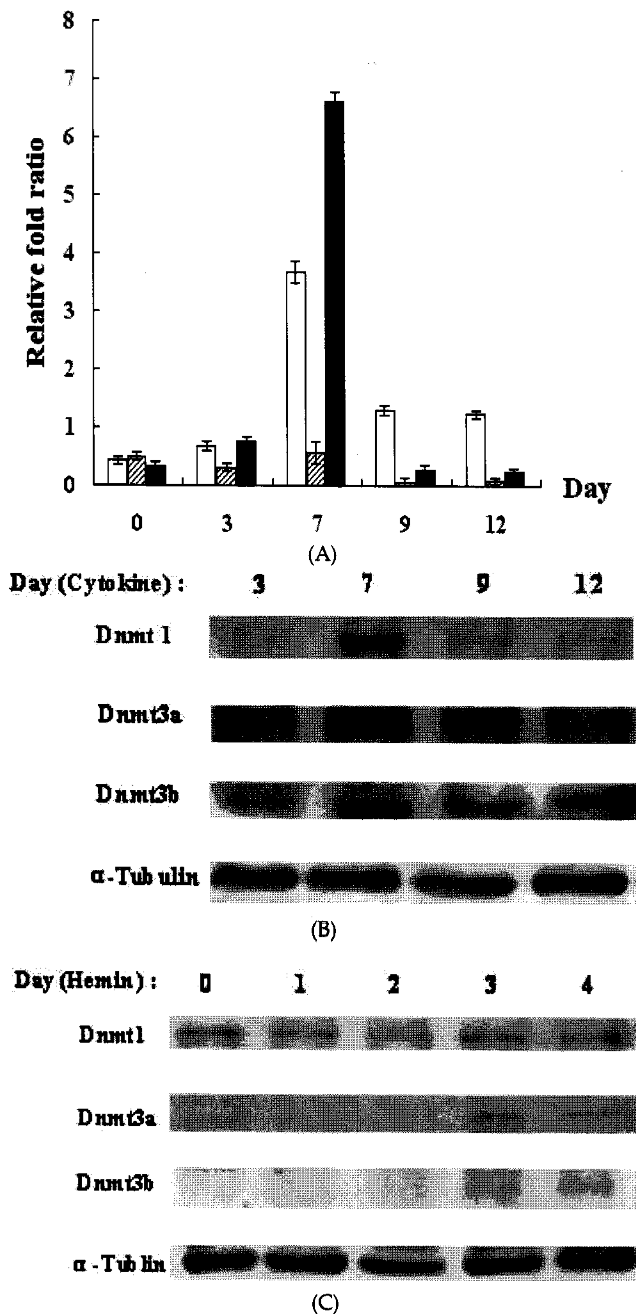


Fig. 3. DNMTs expression during *in vitro* erythropoiesis. (A) Real-time PCR analysis to DNMT1 (□), DNMT3a (▨), and DNMT3b (■) expression at days 0, 3, 7, 9, and 12. The mean change (±S.D) of 3 dependent experiments. (B) DNMTs expression during *in vitro* erythropoiesis of human CD34+ cells induced by cytokines. (C) DNMTs expression during erythropoiesis of K562 cells induced by hemin. Both (B) and (C) were analyzed by western blotting analysis using DNMT1, DNMT3b and α-tubulin antibody.

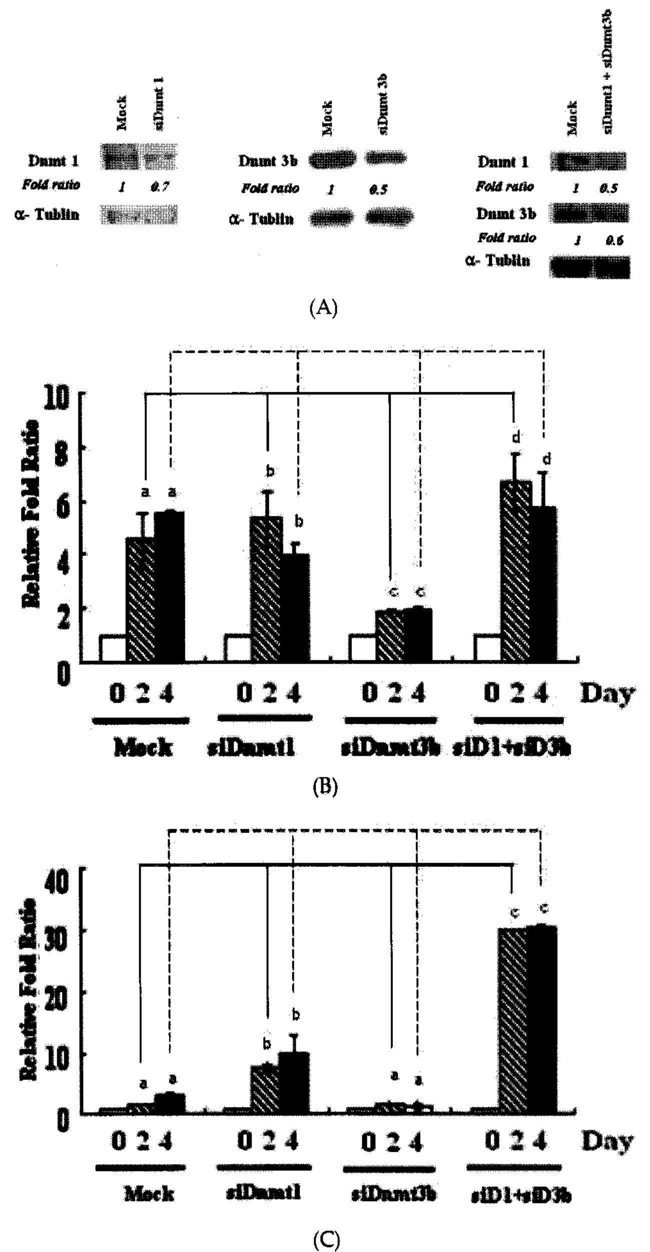


Fig. 4. Expression of globins by siRNA of DNMT genes in K562 cells. (A) Western blot analysis by antibody for either DNMT1 or DNMT3b and α- tubulin on stable siRNA transfected K562 cells. Fold ratio means the expression ratio of each gene to tubulin. (B) γ-globin and (C) ε-globin expression pattern by real-time PCR using primer on stable transfectants. The mean change (±S.D) of three independent experiments. The values are significantly different on the same day ($p < 0.05$).

DNMT1 siRNA transfectant, whereas ε -globin in DNMT3b siRNA transfected cells was 3-fold decreased than mock and DNMT1 siRNA transfectants.

In order to test whether down-regulation of DNMTs has the same effect on the maturation of primary cells, we examined inhibition of DNMT1 and DNMT3b transcription using the same siRNA on primary human cells. DNMT1 siRNA and DNMT3b siRNA were transfected into human CD34+ cells using Nucleo-

fector (Amaxa biosystems). A mock vector was used as a negative control. Evaluation of transduction efficiency with green fluorescent protein (GFP) as a control revealed that about 60% of the cells were transfected. This was confirmed by FACS analysis (Fig. 1A). At day 7, erythroid differentiation of transfected cells was evaluated according to GPA expression. The rate of GPA expression in the cells transfected with mock vector was 25.5±11%, whereas co-transfectants with DNMT1 and DNMT3b siRNA were 51±9.7% (Fig. 5A). As the rate of GPA expression in siRNA co-transfected cells was increased, human CD34$^+$ cells population was also decreased. DNMT1 siRNA and DNMT3b siRNA co-transfected cells showed not only an enhanced erythroid differentiation but also an increased expression of globins, especially ε -globin (Fig. 5B). However, DNMT1 or DNMT3b siRNA transfection to CD34$^+$ cells was not effective to erythropoietic differentiation (data not shown). Also, these were not effective to the γ- and ε -globin expression (data not shown).

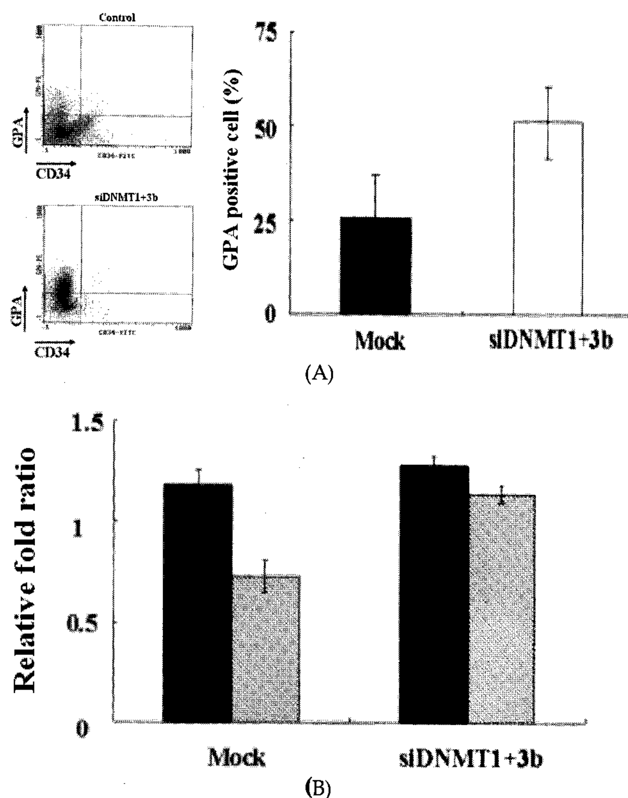


Fig. 5. Effect of DNMT1 and DNMT3b siRNA for human CD34$^+$ HSCs differentiations. (A) Erythropoietic differentiation by FACS analysis using anti-GPA (PE) and anti-CD34 (FITC) on mock, or DNMT1 and DNMT3b siRNA co-transfection at day 7. The mean change (±S.D) of 3 dependent experiments. (B) The quantification of the γ-(■) and ε-globin(▧) mRNAs from DNMT1 and DNMT3b siRNA co-transfected cell at day 7 by real-time PCR. Results were normalized to the level of β-actin mRNA. The mean change (±S.D) of three independent experiments.

These results suggest that co-transfection of DNMT1 siRNA and DNMT3b siRNA might regulate the globin expression during erythropoiesis.

DISCUSSION

Epigenetic events of DNA and histone markers, DNA methylation, in totipotent early embryos and pluripotent germ cells play important roles in many biological functions. Adult stem cell assumes an aspect about epigenetic reprogrammings. In previous study, it was reported that DNMT1, DNMT3a, and DNMT3b were expressed at detectable levels in several hematopoietic lineage cells (Mizuno *et al.*, 2001). We profiled expression level of DNMTs at each differentiation stage during *in vitro* erythropoiesis, and identified the relationship of DNMTs and the differentiation of human CD34$^+$ cells into proerythrocytes. Human CD34$^+$ cells possess that the biological properties are easily influenced by regulatory factor as epigenetic modification. For instance, myeloperoxidase, which is essential for differentiation to myeloid (Lubbert *et al.*, 1991), γ-globin which is activated in the fetal stage of development (Cao *et al.*, 2004), EPO (Yin *et al.*, 2000), c-fms which is methylated in macrophage, and G-CSF receptors (Felgner *et al.*, 1999) are controlled by lineage or site specific methylation. Although epigenetic events in the adult stem cell has been recently described throughout methylation status of specific molecules, the pattern of global DNA methylation during differentiation has not been yet studied. In this study, isolated human CD34$^+$ cells underwent a stepwise progression of epigenetic events during erythropoiesis that HSC lead to methylation according to differentiation into erythroid lineage (see Fig. 3). This data suggested epigenetic machinery such as *de novo* methylases, including DNMT3a and DNMT3b that are thought to process a part in the biological incident of human CD34$^+$ cells and may regulate gene expression, which is essential for erythropoietic differentiation. Especially, expression of DNMT3b was significantly higher than it of DNMT1 at day 7. There is the same result as higher expression of DNMT3b in human CD34$^+$ cells transcriptosome database (Gomes *et al.*, 2002). However, DNMT3a is lowly expressed compared to DNMT1 or DNMT3b at day 7. Also, protein level of DNMT3a is uncertainly expressed during erythropoiesis. Therefore, it is estimated that DNMT3b, *de novo* methylase, contribute to erythropoiesis by inducing methylation. Also, we are convinced that HSC may possess the ability for epigenetic reprogramming.

In this study, we provided an evidence for the in-

volvement of DNMTs in the regulation of erythropoietic differentiation using siRNA. siRNA, known as silencing of gene expression by RNA interference, has become a tool to identify functionally relevant molecules. Several reports show that siRNA of several DNMTs, especially DNMT1 and DNMT3b leads to reactivation of silenced gene and differentiation (Suzuki *et al.*, 2004; Oridate *et al.*, 2005). We found that although the interference of DNMT1 mRNA had some effects on DNA demethylation of globin genes, here, the combined interference of DNMT3b and DNMT1 mRNAs further enhanced the γ and ε -globin gene expression (see Fig. 4). The interference of DNMT3b mRNA alone showed only a minimal effect on DNA demethylation and gene reactivation. Taken together, these results suggest that whereas DNMT3b acts an accessory to support DNMT1 function, DNMT1 is the key maintenance methyltransferase in this cell line. In the previous study, physical evidence to support this idea comes from an immunocytochemical and pull-down experiment using antibodies specific for DNMT1 showed that both DNMT1 and the de novo methyltransferases were included in the immunoprecipitated complex (Leu *et al.*, 2003). These phenomena were identified in our results. When DNMTs siRNAs are introduced into human $CD34^+$ cells, expression level of GPA and globins was increased in normal erythroid cells during erythropoiesis (see Fig. 5). Especially, ε -globin was more increased in both human $CD34^+$ cells and K562 cells than γ-globin. It is inferred that DNA methylation on ε -globin promoter region may be more easily demethylated through suppression of DNMTs than the other globin promoter regions. It indicated that DNMTs suppression by siRNA could enhance erythropoietic differentiation as revealed by expression of GPA and globins (see Fig. 5). In our data, the reversibility of epigenetic state offers exciting opportunities for adjustment of cell fate. To date, it was reported that epigenetic inhibitors could also modulate the commitment of multiple lineages throughout regulation of gene expression (Laird, 2005). There is an important marker in gene modulation based on results of therapy for human disease. 5-azacytidine and 5-aza-z'-deoxy-cytidine were widespreadly used as demethylating reagent (Li *et al.*, 1970). These can effectively activate silenced genes throughout inhibition of DNMTs. These are used for selective chemotherapy agents against cancer, including SSD. SSD is an inherited disease of red blood cells and is caused by abnormal hemoglobin. A number of pharmacological agents are currently available for clinical trial to treat the patient with SSD (Saunthararajah *et al.*, 2003; Saunthararajah *et al.*, 2004). Because γ-globin gene of fetal hemoglobin can be reactivated by 5-aza-2`-deoxycytidine and 5-azacytidine in adult red blood cells, these are used for therapy for SSD and β -thalassemia (Cao *et al.*, 2004; Saunthararajah *et al.*, 2004). However, these are still in controversy regarding carcinogenic potential (Saunthararajah *et al.*, 2004).

In conclusion, the DNMTs were generally expressed in differentiation of HSC into proerythrocytes. Suppression of DNMTs by siRNA stimulates erythropoietic differentiation by reactivating silenced genes, including globin and GPA. Therefore, erythropoietic differentiation by suppression of DNMTs using siRNA can be used as basic data for the possibility to explore new therapy to treat the patient with SSD and β -thalassemia.

ACKNOWLEDGMENT

We thank Dr. J. Hur at Eden Hospital in Gwangju, Korea for meaningful discussions.

REFERENCES

1. Baiocchi M, Di Rico C, Di Pietro R, Di Baldassarre A, Migliaccio AR (2003): 5-azacytidine reactivates the erythroid differentiation potential of the myeloid-restricted murine cell line 32D Ro. Exp Cell Res 285:258-267.
2. Bestor TH (2000): The DNA methyltransferases of mammals. Hum Mol Genet 9:2395-2402.
3. Bird A (2002): DNA methylation patterns and epigenetic memory. Gene Dev 16:6-21.
4. Cao H, Stamatoyannopoulos G, Jung M (2004): Induction of human gamma globin gene expression by histone deacetylase inhibitors. Blood 103:701-709.
5. Felgner J, Heidorn K, Korbacher D, Frahm SO, Parwaresch R (1999): Cell lineage specificity in G-CSF receptor gene methylation. Leukemia 13:530-534.
6. Gomes I, Sharma TT, Edassery S, Fulton N, Mar BG, Westbrook CA (2002): Novel transcription factors in human CD34 antigen-positive hematopoietic cells. Blood 100:107-119.
7. Laird PW (2005): Cancer epigenetics. Hum Mol Genet 14(S1):R65-76.
8. Leonhardt H, Page AW, Weier HU, Bestor TH (1992): A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. Cell 71: 865-873.
9. Leu YW, Rahmatpanah F, Shi H, Wei SH, Liu JC, Yan PS, Huang TH (2003): Double RNA interference of DNMT3b and DNMT1 enhances DNA demethylation and gene reactivation. Cancer Res 63: 6110-6115.
10. Li LH, Olin EJ, Buskirk HH, Reineke LM (1970): Cytotoxicity and mode of action of 5-azacytidine

on L1210 leukemia. Cancer Res 30:2760-2769.

11. Lubbert M, Miller CW, Koeffler HP (1991): Changes of DNA methylation and chromatin structure in the human myeloperoxidase gene during myeloid differentiation. Blood 78:345-356.
12. Maraschio P, Zuffardi O, Dalla Fior T, Tiepolo L (1988): Immunodeficiency, centromeric heterochromatin instability of chromosomes 1, 9, and 16, and facial anomalies: the ICF syndrome. J Med Genet 25:173-180.
13. Milhem M, Mahmud N, Lavelle D, Araki H, DeSimone J, Saunthararajah Y, Hoffman R (2004): Modification of hematopoietic stem cell fate by 5-aza 2'deoxycytidine and trichostatin A. Blood 103:4102-4110.
14. Mizuno S, Chijiwa T, Okamura T, Akashi K, Fukumaki Y, Niho Y, Sasaki H (2001): Expression of DNA methyltransferases DNMT1, 3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia. Blood 97(5):1172-1179.
15. Okano M, Bell DW, Haber DA, Li DA (1999): DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation and mammalian development. Cell 99:247-257.
16. Oridate N, Lotan R (2005): Suppression of DNA methyltransferase 1 levels in head and neck squamous carcinoma cells using small interfering RNA results in growth inhibition and increase in Cdk inhibitor p21. Int J Onco 26:757-761.
17. Saunthararajah Y, Hillery CA, Lavelle D, Molokie R, Dorn L (2003): Effects of 5-aza-2'-deoxycytidine on fetal hemoglobin levels, red cell adhesion, and hematopoietic differentiation in patients with sickle cell disease. Blood 102:3865-3870.
18. Saunthararajah Y, Lavelle D, DeSimone J (2004): DNA hypo-methylating agents and sickle cell disease. Brit J Haematol 126:629-636.
19. Stamatoyannopoulos G (2005): Control of globin gene expression during development and erythroid differentiation. Exp Hematol 33:259-271.
20. Suzuki M, Sunaga N, Shames DS, Toyooka S, Gazdar AF, Minna JD (2004): RNA interference-mediated knockdown of DNA methyltransferase 1 leads to promoter demethylation and gene re-expression in human lung and breast cancer cells. Cancer Res 64:3137-3143.
21. Yang HY, Kim SH, Kim SH, Kim DJ, Kim SU, Yu DY, Yeom YI, Lee DS, Kim YJ, Park BJ, Lee TH (2007): The suppression of zfpm-1 accelerates the erythropoietic differentiation of human $CD34^+$ cells. Biochem Biophys Res Commun 353(4):978-984.
22. Yin H, Blanchard KL (2000): DNA methylation represses the expression of the human erythropoietin gene by two different mechanisms. Blood 95:111-119.

(Received: 4 October 2007/ Accepted: 15 November 2007)