

Assessment of Global DNA Methylation in Stem Cells with Reverse-Phase High Performance Liquid Chromatography

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ABSTRACT

Objective: We tested the possibility of the use of reverse-phase high performance liquid chromatography (RP-HPLC), a sensitive, simple and cost effective technique, for evaluation of global DNA methylation alteration in stem cells.

Methods: We detected genomic methylation in four cell lines of mouse embryonic stem cells and in the cell at various developmental stages, including mouse embryonic fibroblast (n=5) and mouse tissues (n=9), using RP-HPLC. The samples were extracted for single nucleosides and investigated for the concentrations of deoxy-cytidine (dC), deoxy-guanosine (dG) and deoxy-methyl-cytidine (5-dmC) using RP-HPLC. The methylation levels were obtained by a ratio of the entire genomic 5m-C to dC.

Results: Our results demonstrated that the RP-HPLC technique can be used for analysis of global methylation on the stem cell genome. It can display the discrepancy of methylation levels among the cells at different degrees of cell differentiation.

Conclusion: We present the evidence that the RP-HPLC technique can be used for the entire-genomic methylation detection in stem cells and should be further developed as a strategy for monitoring the global DNA methylation of stem cells in advanced research.

Keywords: DNA methylation, epigenetics, methyl cytidine, reverses phase-HPLC, stem cells

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INTRODUCTION

Epigenetics, the controlling system of gene activity without change of DNA sequence, conducts gene expression and repression in cells.¹ Epigenetics play important roles in determination of cell activities, including cell growth and development.¹ The system of epigenetics consists of several mechanisms such as DNA methylation, modifications of histones and remodeling of chromatin. All mechanisms operate together to regulate gene activity in cells. A major mechanism of the epigenetic system is DNA methylation, which generally refers to adding a methyl group at cytosine on CpG dinucleotides and changes cytosine (C) to 5-methyl cytosine (5-dmC). The 5-methyl group on cytosine base results in preventing DNA from binding with transcription factors leading to gene silencing.¹ DNA methylation is always designed to

investigate for epigenomics studies owing to its co-action with other epigenetics mechanisms and also being a key mechanism to regulate the activity of some epigenetic modification, such as gene imprinting.² For example, reports by Reik et al. in 2003³ and Suteevun et al. in 2006⁴ demonstrated the working together between methylation on DNA and deacetylation of histones, which leads to the condensation of the DNA into a chromatin conformation and also modulate gene repression and heterochromatic regions.

Epigenetic modifications act to maintain the characteristics and quality of stem cells. At a pluripotent state, an epigenetic system has critical influence in controlling stem cells to sustain an undifferentiated status by the up-regulating of the genes involving maintenance of cell pluripotency and the silencing of the genes motivating cell differentiation.⁵ The study of epigenetics variation via DNA methylation can provide more informative understanding of stem cell development.

The investigation of DNA methylation in cells was reported by various methods.⁶⁻⁹ The use of each analytical technique depends upon the objectives of each study. However, most of those techniques needed the complicated

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instruments and high-cost chemical reagents. In 2003, Cezar *et al.*,¹⁰ reported the use of reverse phase high performance liquid chromatography (RP-HPLC) for DNA methylation level investigation in animal tissues. The entire-genomic detection of monomeric nucleosides using RP-HPLC technique was first developed by Gehrke *et al.*¹¹ This technique uses a common equipment which is available in the chemistry laboratory facilitating a wide set up of DNA methylation studies. Owing to its uncomplicated approach and affording a highly sensitive result with cost effectiveness, the RP-HPLC is an interesting DNA methylation detection technique, which may be able to be used for DNA methylation observation in stem cells.

In this study, we aimed to examine the possibility for using RP-HPLC to investigate genome-wide DNA methylation in stem cells and apply it to observe the altering of genomic methylation of *in vitro* extended stem cells. The RP-HPLC was designed to measure global DNA methylation in mouse embryonic stem cell (mESC) lines and to be comparative to cells at different developmental stages, including fully differentiated cells (heart, liver and skin) and differentiating cells (mouse embryonic fibroblasts).

MATERIALS AND METHODS

Samples collection

For genomic methylation investigation in cells at different developmental stages, three different samples types were provided, including (1) mouse embryonic stem cells (mESCs) lines (n=4), which are *in vitro* cultured on mouse feeder cells using mESC culture medium (α -MEM supplemented with 20% ES-FBS, 2mM L-glutamine, 1,000 unit/ml leukemia inhibitory factor (LIF), 0.1mM non-essential amino acids, 0.1mM β -mercaptoethanol, 100 unit/ml penicillin /100 μ g/ml streptomycin). The method for providing mouse feeder cells has been described elsewhere in Bryja *et al.*¹², (2) fresh tissues from heart, skin and liver of three mice at 8-week-old, (3) mouse embryonic fibroblasts (mEFs) were obtained from mouse embryos at 13.5 day post coitum of a pregnant mouse (n=5).

For analysis of genomic methylation patterns in mESCs during *in vitro* culture, each mESC line was *in vitro* extended for ten subculture passages and the cells were subsequently collected. After that, the mESCs were spontaneously differentiated via embryoid body formation and re-cultured under mESC culture medium without LIF¹³. Each experiment was contributed using four mESC lines. The experiments were done in triplicate.

All procedures using animals were approved by the Animal Care and Use Committee Guidelines at Mahidol University's equivalent to the Animal Use and Care Committee in Thailand.

DNA extraction

The fresh tissues were cut into small pieces and then pestle crushed under cell lysis buffer (5M NaCl, 1M Trizma base and 0.5M EDTA, pH 8). The cells and tissue samples were extracted for DNA. The DNA extraction was built up using a twice phenol-chloroform technique. Following this technique, samples were incubated in cell lysis buffer, 10% SDS and Proteinase K and then precipitated with 8M potassium acetate before mixing with Phenol-Chloroform.

Single nucleoside preparation

Twenty-five micrograms of DNA in fifty microliters

of distilled water was denatured at 95°C for 5 minutes and immediately placed on ice. Ten units of ribonuclease H and ribonuclease A (Ambion, St. Austin, TX) were added to each sample to degrade RNA from RNA:DNA hybrids. To obtain single nucleoside suspension, DNA was digested by 10 units of Nuclease P1 (US Biology, MA) and followed by 150 units of calf intestinal phosphatase (New England Biolab, MA). Single nucleoside suspensions were kept at -20°C until HPLC analysis.

Reverse phase HPLC analysis

For chromatographic analysis, an Alliance model of Waters HPLC system (Waters Corporation, Milford, MA) was used along with a Water Alliance-2695 Autosampler and a Water Alliance-2996 Detector. The detector was set to monitor the absorption at 280 nm. Peak areas and retention times were detected and the concentrations were calculated based on the standard curve of 2-deoxy Guanosine, 2-deoxy Cytidine and 5-methyl Cytidine by Empower software system. The method for this analysis was modified from Gehrke *et al.*¹¹ A two buffer gradient was used on a Atlantis dC18, 5 μ m, 4.6250 mm column (part no.186001346) with Atlantis dC18 pre-column (part no.186001323). Buffer A (0.05M KH_2PO_4 , 2.5% (v/v) methanol, pH 4.0) was pumped for the first 21 minutes followed by 9 minutes of buffer B (0.05M KH_2PO_4 , 8.0% (v/v) methanol, pH 4.0). The 70% methanol was run for eight minutes to flush the column. This was followed by an immediate switch back to buffer A which re-equilibrated the column for 20 minutes. The total run time was 58 minutes. A flow rate of 0.75 was maintained throughout the method.

Standard curve analysis

The standard curve of 2-deoxy Guanosine and 2-deoxy Cytidine were contributed at five concentrations of 100, 10, 1, 0.1 and 0.01 μ M using 2-Deoxyguanosine hydrate (Sigma-Aldrich, MO) and 2-Deoxycytidine hydrate (Sigma-Aldrich, MO), respectively. The standard curve of 5-methyl Cytidine (5-dmC) was made at the concentrations of 10, 1, 0.1, 0.01 and 0.001 μ M using 5-methyl-2-Deoxycytidine (MP Biomedical, Illkirch, France).

Calculation and statistics

Genomic dC, dG and 5-dmC concentrations in each sample were based on integrating the peak areas of each genomic nucleoside to the peak area of a known concentration of a standard nucleoside on a standard curve. Each of the nucleoside peaks from every sample was confirmed by analysis of its specific absorbent pattern generated by a Photodiode Array (PDA) detector as compared with a standard nucleoside. The global 5-dmC nucleoside concentration was obtained from the peak areas of 5-dmC and dC by this formula : $5\text{-dmC} \times 100 / (5\text{-dmC} + \text{dC})$. The peak area of (5-dmC+dC) and dG by this formula: $(5\text{-dmC} + \text{dC}) / \text{dG} = 1$, was used as the cut off for indicating acceptable data. The difference of global DNA methylation levels among sample types was statistically indicated by student T-test and one-way ANOVA at p-value < 0.05.

RESULTS

With using RP-HPLC detection, genomic DNA of mouse ESC line (n=4), mouse embryonic fibroblast (n=5) and three types of tissues derived from three mice (n=9) respectively demonstrated the peak areas of dC, 5-dmC

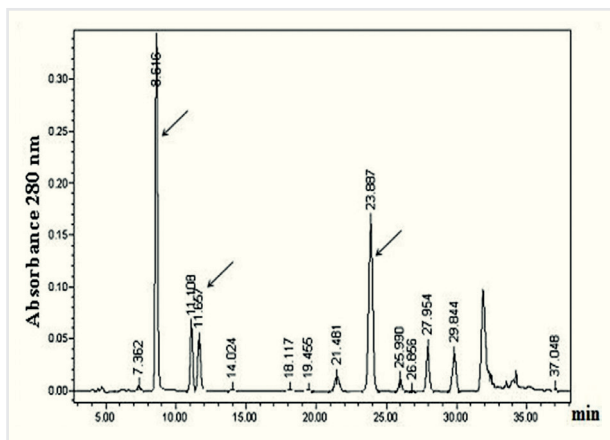


Fig 1. dC, dG and 5-dmC peaks by RP-HPLC.

Each peak presents an amount of genomics components, which are separate by RP-HPLC technique. The arrows show the peak of cytosine (C), methylated cytosine (5-dmC) and guanosine (G) at the specific retention time around 8.6, 11.6 and 23.9 min. Each of these three peaks was used for areas under peak investigation, which peak areas will be calculated for the nucleoside concentrations for dC, dG and 5-dmC in each sample. The percentages of global 5-dmC nucleoside derived from peak area of 5-dmC and dC by formula: $5\text{-dmC} \times 100 / (5\text{-dmC} + \text{dC})$.

and dG at 8.6, 11.6 and 23.9 minutes as shown in Fig 1. Most of the DNA samples (94%) showed the ratio of $(5\text{-dmC} + \text{dC}) / \text{dG}$ at close to 1. For the result of technique sensitivity, we found that the use of RP-HPLC in methylation study can display the dissimilarity of genomic methylation levels among the cells at undifferentiated-, differentiating- and fully differentiated stages. The undifferentiated mESCs showed hypomethylation as compared to the samples at higher degrees of cell differentiation. The percentages of genomic 5-dmC contents in mESC lines showed an average at 3.9% with a range of 2.1-4.2%. For genomic methylation detection in cells at a differentiating stage, the mEFs showed a broad range of detected genomic 5-dmC contents with 3.44% to 6.67%. Their average of 5-dmC contents was 4.8%. For the genomic 5-dmC investigation in the fully differentiated cells, the cells derived from mouse fibroblast, liver and heart tissues, the average 5-dmC content of each differentiated cell was 6.28%. The average percentage of 5-dmC content of mouse liver cells was 6.4%, with a range of 4.24% to 7.6%. The cells from mouse hearts had an average genomic 5-dmC content of 6.0%, with a range of 5.92% to 6.28%. The mouse skin fibroblasts had an average of 6.9%, ranging from 6.76% to 8.21%. The genomic 5-dmC content of all samples from three different adult organs were subjected to statistical analysis and showed no significant difference ($P=0.84$).

The genomic 5-dmC contents obtained within each group of undifferentiated mESCs, differentiating mEFs and fully differentiated cells were compared. Although, the results showed no statistically significant difference of genomic 5-dmC contents among these three groups, we observed the discrepancy of genomic 5-dmC levels in undifferentiated mESCs as compared with the cells in the fully differentiated group (Fig 2).

With the above results of monitoring global DNA methylation using RP-HPLC, we tested the stability of the methylation detection in *in vitro* prolong stem cells. Four mESC lines were expanded and methylation

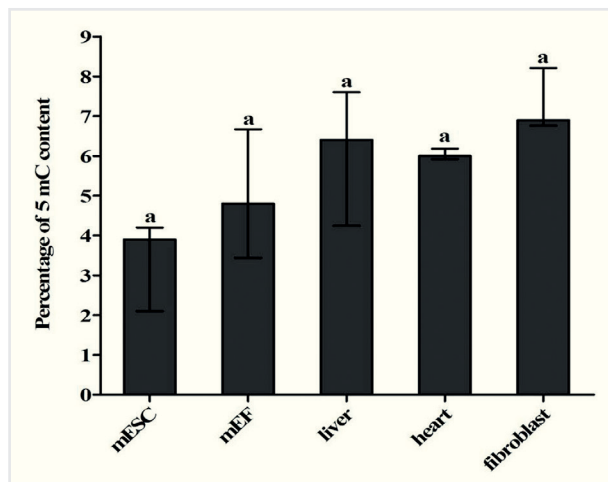


Fig 2. Comparison of global DNA methylation level from each sample type.

Discrepancy of global DNA methylation level has been observed in three sample types, including mESCs, mEFs and adult tissues, with using HPLC technique. The increasing of methylation level relates with the degree of cell differentiation in sample studied. Bars with the same alphabet (a) indicates no statistical difference in each other ($P>0.05$).

analyzed throughout ten subculture passages. The spontaneous differentiated cells of those mESC lines, which showed morphologic change with no oct-4 expression by RT-PCR, were applied as differentiated cell controls for this analysis. The result presented the steady patterns of global DNA methylation throughout multiple subculture passages studied. The 5-dmC percentages derived from ten studied passages of four mESC lines in three experiments were found within a range of 2.1-4.1 and at an average of 3.29 ± 0.85 . The 5-dmC percentages obtained from the spontaneous differentiated cells showed a wide range of 3.8-7.1, and at the average of 5.4 ± 1.14 . The hypermethylation was found in the spontaneous differentiated cells. All experiments exhibited the similar methylation patterns.

DISCUSSION

The epigenetic system has been thought to play a crucial role in the maintenance of the stem cell characteristics.⁵ Analysis of epigenetics leads understanding of stem cell development and the progression of stem cell therapy. Our study revealed for the possibility of using a RP-HPLC technique to investigate the epigenetics change in stem cell genomes via the mechanism of DNA methylation. The RP-HPLC was used to evaluate the level of DNA methylation by measurement of 5-methyl-cytidine and cytidine contents in an overall genome. Discrepancy of 5-methyl-cytidine and cytidine amount ratios observed among undifferentiated stem cells and the cells at various degrees of differentiation indicates the ability of using the RP-HPLC technique in identification of the cell developmental stage along with the altering of DNA methylation levels.

The concentration of global methylation is derived by the formula of $(5\text{-dmC} \times 100) / (5\text{-dmC} + \text{dC})$. The increasing of monomeric Cytidine (C) content derived from RNA contamination in DNA suspension samples can lead to false hypomethylation. Hence, the DNA extraction from

samples is a critical process for the methylation investigation using the RP-HPLC technique. We found that the RP-HPLC results obtained from the triplicate analysis of a sample with RNA contamination showed hypomethylation and low precision (data not shown). This appearance is consistent with the previous evidences explained in reports by Brena et al in 2006.¹⁴ To avoid the RNA contamination, the samples used in our study were limited to only the OD260/280 at 1.80-1.85. Using this selection, we found that our triplicate studies show higher precise results. However, the approach to avoid RNA contamination in RP-HPLC samples needs to be further provided. An idea for prevention of the contamination of monomeric ribonucleic acid molecules in RP-HPLC samples may be modifying RNA in the samples from single strand to double strands. The Nuclease P1 enzyme, which was used in preparation of single nucleoside suspension, hydrolyses phosphodiester bonds only on single stranded RNA or denatured DNA, but not double stranded RNA or DNA. This definition will make double strand RNA free from hydrolysis of this enzyme leading to no contamination of monomeric molecules of RNA in RP-HPLC samples.

Besides, DNA methylation plays an important role in the maintenance of stem cell characteristics, this methylation, which occurs on CpG dinucleotides of the mammalian genome, is believed to sustain the normality of genome activities.¹⁵ A huge number of CpG dinucleotides are located in interspersed repetitive sequences (IRS). The methylation at cytosine of the CpG dinucleotides in the IRS inhibits the activity of the IRS by controlling the gene mechanisms and RNA production. The loss of genome-wide methylation is commonly found in cancer cells. Consistently, Lister et al.,¹⁶ found that controlling of gene activity via the DNA methylation mechanism in pluripotent embryonic stem cells produces a unique pattern. A vast number of DNA methylations in embryonic stem cells can be found at the non-CpG sites, whereas, it usually occurs at the CpG sites in differentiated cells and other cell types. From this information, the observation of DNA methylation in stem cells should be a genome-wide detection, not a site-specific methylation detection. We, thus, provide a technique to investigate DNA methylation in term of entire-genomic methylation. Although, a number of techniques for genome-wide DNA methylation study have been made available, i.e. confocal microscopic detection, pyrosequencing and microarray,¹⁷⁻¹⁹ those techniques were limited with complicated and unavailable equipment. The genomic methylation detection using RP-HPLC technique uses a common equipment, which enables the possibility to apply in global DNA methylation study in stem cells. Our work aimed to develop a strategy for using this RP-HPLC technique for global methylation detection in stem cells. Four cell lines of mES and five individual mEF samples and nine adult tissues were provided for this investigation. Although, a small sample size was used in this analysis, the obtained results within each type of sample studied show a consistent outcome indicating the possibility to use this analysis in further advanced work. This number of mES cell lines has also been used in several mES cell studies for many aspects.²⁰⁻²³

In conclusion, we report the first evidence that the RP-HPLC technique can be used for global DNA methylation assessment in stem cells. This methylation detection technique should be further developed by the uses to estimate the genomic methylation and the developmental degree in stem cell research.

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