CHAPTER I

INTRODUCTION

Background and Rationale

Global hypomethylation is one of the most common molecular events in the multistep carcinogenesis (1-3). This process leads to chromosome instability, characterized by a higher rate of chromosomal mutations and DNA deletion. Interestingly, DNA double-strand breaks (DSBs) are intermediate products of the spontaneous global hypomethylation-related mutations (4-6). In 2003, Vilenchik and Knudson estimated the existence of endogenous DSBs (EDSBs) and concluded that EDSBs could account for a substantial fraction of oncogenic events in human carcinomas (7). If EDSBs do not arise uniformly or are not processed at equal rates across the genome, mutation hot spots should be present (7). Therefore, genome wide methylation depletion may cause genomic instability if DNA methylation influences EDSB processes.

I. Endogenous DNA Double-strand Breaks and Chromosomal Instability

DNA damage can occur by intrinsic insults including base pair mismatches during DNA replication, collapse of replication forks, and attack by reactive oxygen species produced during normal cellular metabolism. Moreover, DNA damage can also be induced by extrinsic insults such as exposure to ultraviolet light, ionizing radiation, or environmental mutagens. One particularly harmful form of DNA damage is DSBs. In normal cells, DSBs occur spontaneously at background levels, which are termed EDSBs. These breaks are induced by a number of mechanisms including intermediates of different DNA recombination processes such as decatenation of intertwined DNA molecules by topoisomerase II and LINE-1 retrotransposition, programmed cleavage by specific endonuclease during immunoglobulin gene rearrangement, and products converted from single-strand lesions (SSLs) (7-10). Previously, Knudonson *et al.* estimated that in normal human cells about 1% SSLs were converted to about 50 EDSBs per cell in each cell cycle. This number is calculated as if EDSBs are produced by 1.5-2.0 Gy of sparsely ionizing radiation, which is a physiologic dose of normal cellular environment (7). The production of such breaks is a dynamic process that occurs in two

steps. The first step is the abundant production of SSLs of different types during normal cell cycle. These lesions cause collapse of replication forks from single-strand breaks (SSBs). The second step is the conversion of some SSLs into EDSBs durings the S phase of cell cycle (7).

EDSBs are particularly dangerous lesions if they occur during the replication of the genome and during the segregation of duplicated chromosomes into daughter cells. Proper genome duplication is hampered by EDSBs. If broken chromosomes are carried through mitosis, the acentric chromosome fragments will not partition evenly between daughter cells. Therefore, eukaryotes have developed several checkpoints to prevent cells from starting DNA replication (the G1/S checkpoint), from progressing with replication (the intra S checkpoint), or from going into mitosis (the G2/M checkpoint), if they contain damaged DNA (11, 12). Furthermore, all eukaryotes have evolved several mechanisms to deal with DSBs, which indicates the importance and difficulty of repairing this type of DNA injury. The two main pathways are homologous recombination (HR) and non-homologous end-joining (NHEJ). These two repair modes differ in their requirement for a homologous template DNA and in the fidelity of DSB repair. Whereas, HR ensures accurate DSB repair, NHEJ does not. Although HR is important to the maintenance of genomic stability in DNA containing DSBs, almost all chromosomal DSBs in human cells are repaired by NHEJ (13).

The fidelity of EDSB repair is importance to the fate of the cell. The failure to repair EDSBs or their inaccurate repair can lead to chromosomal instability (CIN) that contributes to carcinogenesis. CIN phenotype is characterized by the gross rearrangement of chromosomes. Common chromosomal aberrations include the loss or gain of whole chromosomes or chromosomal fragments, and the amplification of chromosomal segments (14). Numerous studies employing mouse models and cellular models have demonstrated the correlation between the formation of DSBs and the generation of chromosomal aberrations. The chicken B-cell line DT40 cells without functional Ku70 and/or Rad54 were generated as a cellular model for NHEJ and HR, respectively (13). Disruption of the *RAD54* gene, which encoded a component of the HR pathway, caused radiosensitivity, whereas inactivation of *Ku70*, which encoded a

component of the NHEJ pathway, was not detectable effect on survival after γ-irradiation. Disruption of *RAD54* also increased the rates of chromosomal aberration, mainly in the form of chromatid type breaks. A low dose of γ-irradiation markedly increased the number of breaks in this mutant. The *Ku70* mutation did not significantly affect chromosomal instability in DT40 cells, which suggested that HR is the main pathway to repair DSBs in these cells. Other evidence for the involvement of DSBs in chromosomal aberrations came from studies in mouse model. The first know DSB-repair-defective mouse mutant was the SCID (severe combined immunodeficiency) mouse, which carried a spontaneous mutation that prevents the production of mature B and T cells, owing to a defect in joining the DSB intermediate in V(D)J recombination. These mice not only had a defect in the development of their immune system, but also were hypersensitive to ionizing radiation (15).

II. Global Hypomethylation and Chromosomal Instability

In mammals, DNA methylation is essential for normal embryonic development, as it plays an important role in the regulation of gene expression, X chromosome inactivation, genomic imprinting, chromatin modification, and silencing of endogenous retroviruses. DNA methylation, the addition of a methyl group to the carbon-5 position of cytosine residues, is the only common covalent modification of human DNA and occurs almost exclusively at cytosines that are immediately followed by a guanine (so-called CpG dinucleotides) (16). Alterations in DNA methylation are regarded as epigenetic changes, although they affect the structure of DNA, but not materially affect the genetic code (16). DNA methylation patterns are established and maintained by a complex interplay among three DNA methyltransferases (DNMTs), DNMT1, DNMT3A, and DNMT3B. DNMT1 is the main enzyme in mammals. It is responsible for the post-replicative restoration of hemi-methylated sites to full methylation, referred to as maintenance methylation. Whereas DNMT3A and DNMT3B are thought to be involved primarily in methylating new sites, a process called de novo methylation (17).

Human cancer cells often display abnormal pattern of DNA methylation. Two distinct changes in DNA methylation status have been observed, termed

"hypermethylation" and "hypomethylation". Hyper- and Hypomethylation are most often used to describe relative status, best understood in relation to an expected, "normal" setting or degree of methylation (i.e., the level of methylation that is seen in nonmalignant cells). Therefore, Hyper- and Hypomethylation imply aberration level of methylation, which are associated with functional changes in the genome that have the potential to oncogenic mechanisms (18). The hypermethylation usually occurs in the CpG-rich promoter regions of tumor suppressor genes, causing their transcriptional inactivation. In contrast, the hypomethylation reactivates protooncogenes and retrotransposon elements (16, 18, 19). Additionally, genomic hypomethylaion has a causal role in tumor formation by promoting CIN. Evidence for this notion came from the frequently observed global hypomethylation in tumor cells, and from a previous study suggesting that defects in DNA methylation might contribute to the CIN of some colorectal tumor cell lines. Several studies from mutation experiments also support the evidence that DNA methylation maintains the stability of the genome. The first studied showed that Murine embryonic stem cells carrying defect of *DNMT1* gene exhibited significantly elevated mutation rates (6). Similarly, the second one studied in mice carrying mutant *DNMT1* allele developed aggressive T cell lymphomas and cancer cells displayed chromosomal aberrations (5). These studies imply an important role of mammalian DNA methylation in maintaining genomic stability. However, the mechanism of how global hypomethylation causes CIN is remained unknown.

III. Conclude Hypotheses

Both EDSBs and global hypomethylation have been proposed to cause CIN, which is crucial for cancer development process. This study was to explore the association between EDSBs and global hypomethylation. This thesis aimed to establish techniques for detecting the quantity, methylation level, and location of EDSBs, and to study the association between EDSBs and DNA methylation.

In order to determine how DNA methylation affects EDSBs, we first developed a set of novel techniques to analyze the extent and methylation level of genomic EDSBs. Locus-specific EDSBs can be detected using ligation-mediated polymerase chain

reaction (LMPCR), a commonly used PCR technique designed for the analysis of EDSBs during lymphoid development, such as V(D)J recombination (20) and somatic hypermutation (21). Since general EDSBs are believed to occur rarely and randomly throughout the genome (7), repetitive sequences that widely intersperse in the human genome can be applied in a similar assay for the detection of EDSBs in their proximity, which would represent genome wide EDSBs. Therefore, we combined LMPCR with interspersed repetitive sequence PCR (IRSPCR) (22) using LINE-1 (L1) human retrotransposons (L1-EDSB-LMPCR). Linker oligonucleotides are ligated to EDSBs in high molecular weight DNA preparation and quantitatively analyzed by realtime PCR using an L1 primer and a Taqman probe complementary to the linker. Additionally, methylation status of L1s have been extensively studied in several cancers and normal tissues by PCR combined with bisulfite restriction analysis (COBRA) of L1s (COBRA-L1) (3). Treatment with bisulfite converts unmethylated cytosines, but not methylated cytosines, to uracils and then thymines after PCR, thus generates detectable methylation-dependent changes in the restriction pattern of PCR-amplified L1 sequences. Methylation level is then calculated and presented as a percentage of total DNA. Therefore, by combining L1-EDSB-LMPCR with COBRA-L1 through the treatment of linker-ligated DNA with bisulfite prior to PCR with L1 and linker primers and restriction analysis (COBRA-L1-EDSB), we can measure the methylation level of L1s adjacent to EDSBs, which reflects the methylation level of EDSBs in a genome wide fashion.

Additionally, we evaluated if EDSBs occur at methylated and unmethylated DNA differently. We screened for EDSB hot spots and identified if they possessed methylation bias by L1-EDSB-LM methylation specific PCR (L1-EDSB-LM-MSP). When amplified toward L1 sequence, L1-EDSB-MSP was interpreted by analyzing the band patterns of methylated and unmethylated EDSBs as DNA fingerprinting for mapping the locations of EDSBs.

According to our study by COBRA-L1-EDSB, we found that the majority of EDSBs were methylated. Hypermethylated EDSBs may arise from one of two causes: either methylated DNA carries a higher rate of EDSB production or a lower rate of EDSB repair. To distinguish between these two hypotheses, we first examined EDSB production. We

hypothesized that EDSBs were preferentially produced in S phase from the conversion of single strand lesions. For DNA repair, we hypothesized that there is preferential cellular response to unmethylated EDSBs. One of the earliest DSB repair responses is the phosphorylation of histone, H2AX, at serine 139, yielding a focal product, γ-H2AX (23). Therefore, we compared the methylation status of γ-H2AX-bound L1s DNA, COBRA-L1-H2AX, with COBRA-L1-EDSB. γ-H2AX-bound DNA was obtained by chromatin immunoprecipitation (ChIP) (24). DNA methylation is usually associated with heterochromatin and histone deacetylation (25, 26). Furthermore, DSB repair can be regulated by histone acetylation, which can enhance phosphorylation of H2AX in the context of nucleosomes. We therefore hypothesized that methylated EDSBs that escape the γ-H2AX response are retained within heterochromatin. To test this hypothesis, we converted heterochromatin of HeLa cells into euchromatin with a histone deacetylase inhibitor, trichostatin A (TSA).

Research Questions

1. Are there significant amount of EDSBs exist and associated with DNA methylation?
2. Are EDSBs associated with DNA methylation in cis?
3. Why are EDSBs preferentially methylated?
4. Are there γ-H2AX free methylated EDSBs retained within heterochromatin?

Objectives

1. To establish techniques for detecting the quantity, methylation level and location of EDSBs.
2. To describe the general characteristics of EDSBs including quantity, methylation level and location among cell types.
3. To compare percentage of methylation level between EDSBs and genomic DNA.
4. To detect the amount and methylation status of EDSBs during cell cycles.

5. To explore the physiologic mechanism by which methylated EDSBs are retained.
6. To compare between the amount and methylation status of EDSBs and DNA binding to γ-H2AX.
7. To prove if EDSBs are retained within heterochromatin by evaluate alteration of the amount and methylation status DNA binding to γ-H2AX after TSA treatment.

Hypotheses

1. EDSBs are randomly arisen throughout the whole genome.
2. EDSBs are associated with their methylation statuses.
3. EDSBs are preferentially produced in S phase from the conversion of single strand lesions.
4. There is preferential cellular response to euchromatin, nonmethylated EDSBs.
5. Methylated EDSBs that escape the γ-H2AX response are retained within heterochromatin.

Key Words

Endogenous DNA double-stand breaks, chromosomal instability, global hypomethylation

Expected Benefit

The results of this study will help us to understand the mechanism of tumorigenesis.

Conceptual framework

1. Are there significant amount of EDSBs exist and associated with DNA methylation?

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I. To establish techniques for detecting the quantity, methylation level and location of EDSBs.

II. To describe the general characteristics of EDSBs including quantity, methylation level and location among cell types.

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Established new methods

- **L1-EDSB-LMPCR**

To measure the actual quantity of EDSBs.

- **COBRA-L1**

To detect the level of DNA methylation in whole genome.

- **COBRA-L1-EDSB**

To determine the amount of methylated and unmethylated EDSBs.

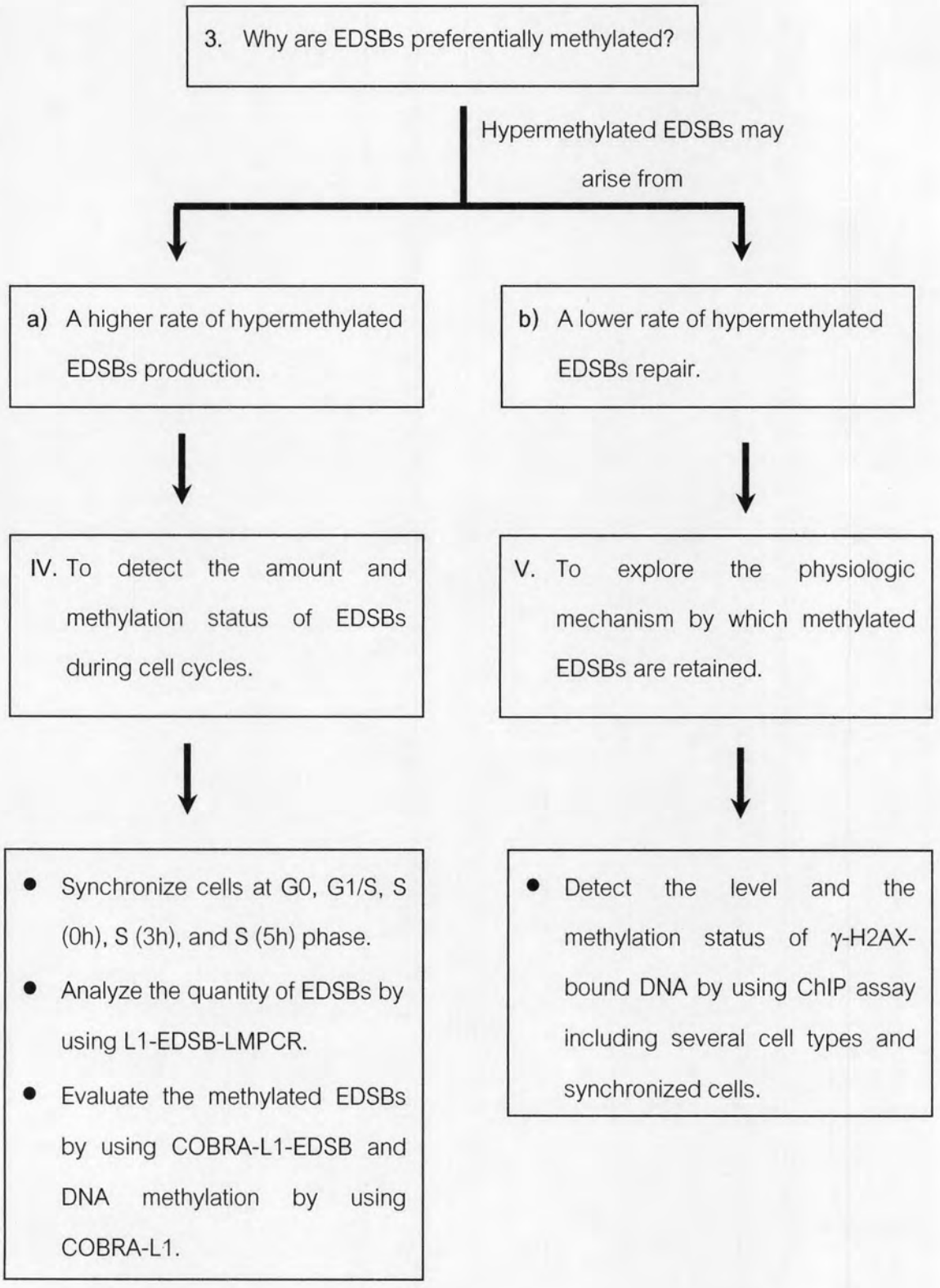
- **L1-EDSB-LM-MSP**

To analyze the band patterns of methylated and unmethylated EDSBs for mapping the locations of EDSBs.

2. Are EDSBs associated with DNA methylation in cis?

III. To compare percentage of methylation level between EDSBs and genomic DNA.

- Detect the methylated EDSBs by using COBRA-L1-EDSB and DNA methylation by using COBRA-L1 in several cancer cell lines and normal cells.


3. Why are EDSBs preferentially methylated?
Hypermethylated EDSBs may arise from
a) A higher rate of hypermethylated EDSBs production.
b) A lower rate of hypermethylated EDSBs repair.
IV. To detect the amount and methylation status of EDSBs during cell cycles.
V. To explore the physiologic mechanism by which methylated EDSBs are retained.
• Synchronize cells at G0, G1/S, S (0h), S (3h), and S (5h) phase.
• Analyze the quantity of EDSBs by using L1-EDSB-LMPCR.
• Evaluate the methylated EDSBs by using COBRA-L1-EDSB and DNA methylation by using COBRA-L1.
• Detect the level and the methylation status of γ-H2AX-bound DNA by using ChIP assay including several cell types and synchronized cells.

4. Are there γ-H2AX free methylated EDSBs retained within heterochromatin?

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VI. To evaluate the amount of DNA binding to γ-H2AX and methylation status in TSA treatment.

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- Convert heterochromatin of Hela cells into euchromatin with TSA.
- Analyze the methylated EDSBs by using COBRA-L1-EDSB and DNA methylation by using COBRA-L1.
- Measure the level and the methylation status of γ-H2AX-bound DNA by using ChIP assay.