CHAPTER IV
RESULTS

Endogenous DNA double-strand breaks (EDSBs) are generally hypermethylated. Notably, hypomethylated EDSBs may arise from methylated DNA carries a higher rate of EDSB production. However, in a screen for EDSB hot spots experiment, we found that the mechanism inducing EDSBs hot spots were DNA methylation independent (11). Therefore, we hypothesized that hypermethylated EDSBs may arise from a lower rate of repair in methylated EDSB. Thus, the aim of this thesis was to study DNA methylation is associated with EDSB repair pathway in G0 and S phase.

In order to determine quantity of EDSB, we performed a technique for the detection of EDSBs, called L1-EDSB-LMPCR. Locus-specific EDSBs can be detected using ligation-mediated polymerase chain reaction (LMPCR) (Figure 4). EDSBs are believed to occur rarely and arbitrary throughout the genome. Using repetitive sequences that randomly scatter throughout the human genome, genome-wide EDSBs are detected in close to LINE1-repetitive sequences. Then, detected the methylation status by PCR combined with bisulfite restriction analysis (COBRA) of L1s (COBRA-L1) (Figure 5). COBRA-L1-EDSB assay can measure the methylation level of L1s near EDSBs, which reflects the methylation level of EDSBs in a genome-wide fashion. The degree of methylation between genomic L1 and L1-EDSB sequences was examined by COBRA-L1 and COBRA-L1-EDSB, respectively (11).

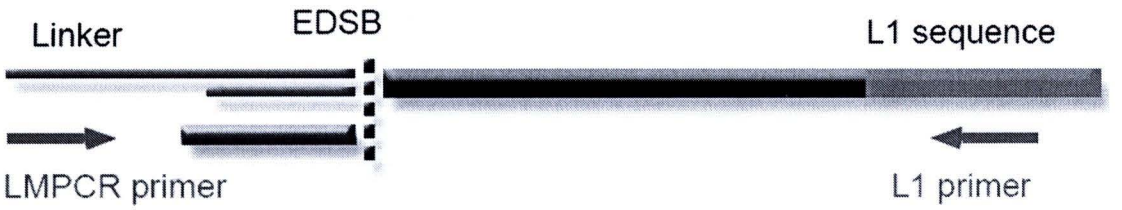

Figure 4. Schematic Illustration of L1-EDSB-LMLPCR. L1 sequence ligated by linker at EDSB. The white rectangle is Taqman probe complementary to LMPCR linker. Arrows are PCR primers.

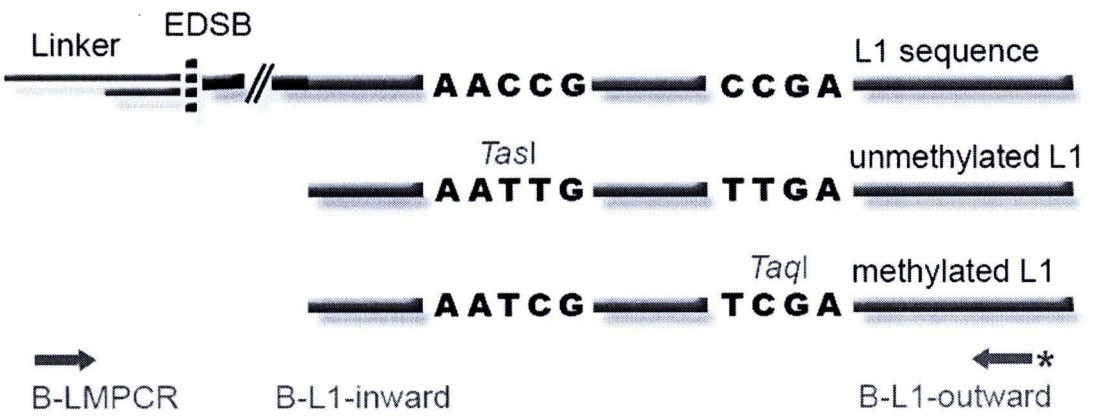

Figure 5. Schematic Illustration of COBRA-L1 and COBRA-L1-EDSB. L1 sequence ligated by linker at EDSB. Arrows are PCR primers, with star indicating 5' labeled primer with ^{32}P for COBRA. AACCG and CCGA are L1 sequences; when treated with bisulfite and PCR, unmethylated AACCG will be converted to AATTG (*Tas*I site) and methylated CCGA to TCGA (*Taq*I site).

Ku86 in NHEJ did not prefer to repair methylated EDSBs

From the previous study, we observed that HeLa cells in G0 phase process the least EDSBs and EDSBs were hypermethylated in most examined cell phases. Especially, G0 phase showed the most statistical significance. Therefore, we interested in methylated EDSB repair depend on NHEJ pathway in G0 phase since this pathway is the main DSB repair pathway in mammalian cells and major repair in this phase. NHEJ pathway depends on DNA-PK complex, which is compose of DNA-PKcs, Ku70 and Ku86. Ku86 is DNA binding protein that specifically binds to broken end of DSBs, which is a key protein in this pathway (20, 83). We hypothesized that if hypermethylated EDSBs involve with NHEJ repair pathway in G0, methylation level of EDSBs will increase in defected NHEJ repair pathway. To prove the hypothesis, we compared the methylation level of EDSBs between normal and defected NHEJ cell. NHEJ was inhibited by depleted Ku86 using siRNA technique. SiRNA system is composing of hygromycin resistant gene, which is selectable marker to select the stable Ku86 siRNA cell line. Control siRNA cells were used to avoid the false positive results and composed of scramble sequence in plasmid that is no match to any sequence in human genome. In order to confirm the method, we used the western blot technique to detect decreased protein level of Ku86. As show in Figure 6, Ku86 protein level of Ku86 siRNA cell was evidently lower than control siRNA cell.

Then, we could perform L1-EDSB-LMLPCR and COBRA-L1-EDSB to detect the level and percent methylation of EDSBs. The result showed that down-regulation of Ku86 protein did not affected both level and percent methylation of EDSBs (Figure 7, 8). However, not only Ku86 dependent NHEJ but also ATM dependent NHEJ (7, 8) and back up NHEJ (B-NHEJ) (84) can function in G0 phase.

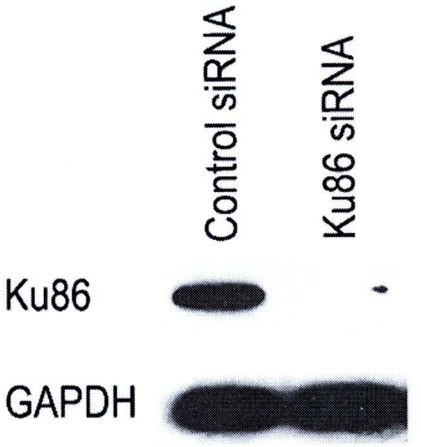

Figure 6. Western Blotting of Ku86 in HeLa cells transfected with siRNA. The analysis of Ku86 expression in HeLa cells transfected with Ku86 specific siRNAs constructs and G3PDH is used as control for protein loading.

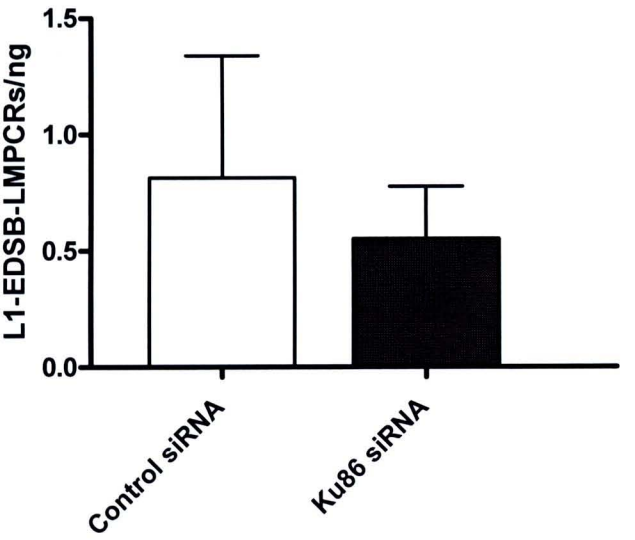

Figure 7. L1-EDSB-LMPCR Quantities in Ku86 defected cells. L1-EDSB-LMPCR quantities from control and Ku86 siRNA cells at G0 phase.

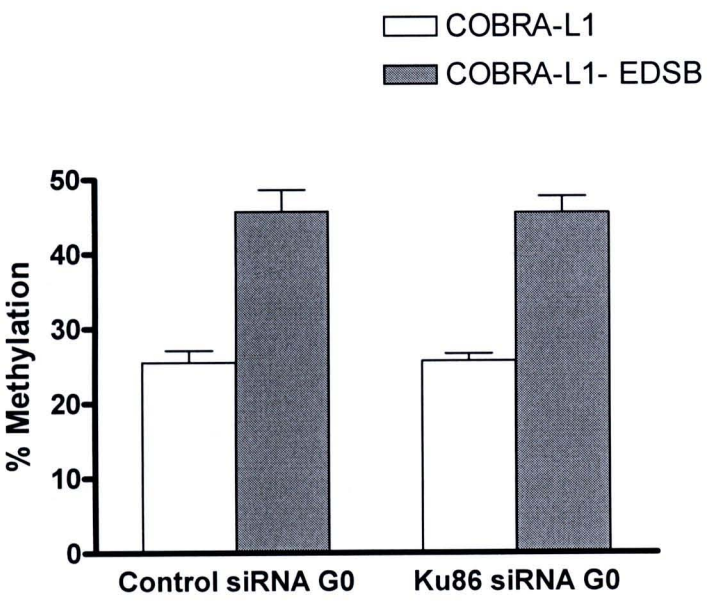

Figure 8. Inhibition of Ku86 did not affect the EDSBs methylation status. COBRA-L1 and COBRA-L1-EDSB showed methylation level of genomic DNA and EDSBS in both control and stable Ku86 siRNA cells. Control siRNA is nonspecific siRNA sequence. Data represent non statistic significant between two cells in G0 phase.

ATM-dependent NHEJ preferentially repair methylated EDSBs

From the result, it indicated that Ku86 down-regulation cannot be explained whether DNA-PK-dependent pathway effect to repair the methylated EDSBs because back-up pathway can repair EDSBs while Ku86 is depleted. However, mammalian cells do not use this pathway for DNA repair since Ku86 usually express in normal cells. Therefore we interested in other candidate proteins in NHEJ pathway including ATM and DNA-PKcs. DNA-PK-dependent NHEJ is thought to repair the majority of DSBs and involves error-prone religation of the two broken DNA ends. Recently, a sub-pathway of precise NHEJ that can repair DSBs with high fidelity has been proposed (85). Besides, ATM and DNA-PKcs are phosphatidylinositol-3-kinase and both of them can activate downstream repair proteins in DSB repair pathway (15, 16). While DNA-PKcs, a subunit of DNA-PK complex as same as Ku86, is required for general NHEJ, ATM acts jointly with checkpoint kinase 2 (CDK2) and BRCA1 in controlling the fidelity of DNA end-joining by precise NHEJ (7). We found that the level of EDSBs in control cells higher than NHEJ-defected cells in G0 phase (Figure 10). Hence, we compared EDSB methylation level between deprived DNA-PKcs and ATM cells. The result demonstrated that EDSB methylation levels remarkably increased when ATM was genetically deprived by stably transfecting siRNA (Figure 11). The mechanism of increasing EDSB levels in control siRNA is not known. Nevertheless, this interference is not lesser our conclusion that there is the significant influence of decreased ATM in increasing methylation level of EDSBs. Stably transfection of DNA-PKcs siRNA in HeLa cells causes down-regulation not only DNA-PKcs but also ATM (Figure 8) as has previous been observed (86). EDSB methylation levels of DNA-PKcs siRNA cells were significantly lower than that of ATM siRNA cells, especially in G0 phase, as if the loss of DNA-PKcs compensated the influence of ATM deficiency on the methylation level of accumulated EDSBs (Figure 9). These results suggest that DNA-PKcs is more important in the repair of unmethylated EDSBs whereas ATM is more important for methylated DNA.

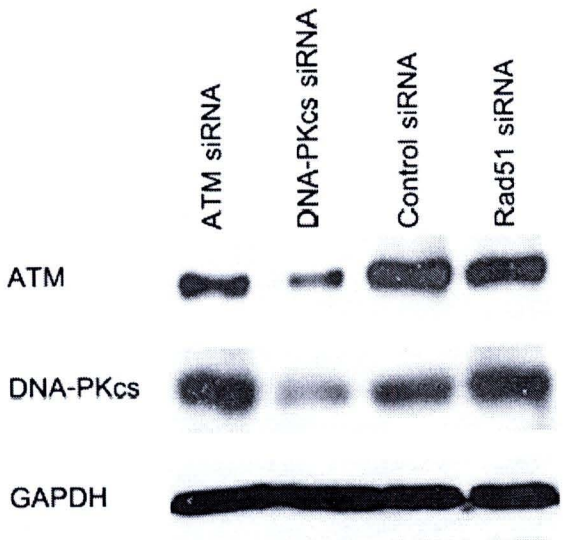

Figure 9. Western Blotting of DNA-PKcs and ATM in HeLa Cells transfected with siRNA. The analysis of DNA-PKcs and ATM expression in HeLa cells transfected with DNA-PKcs-, ATM-specific siRNAs constructs or nonspecific siRNA (control and Rad51) vector.

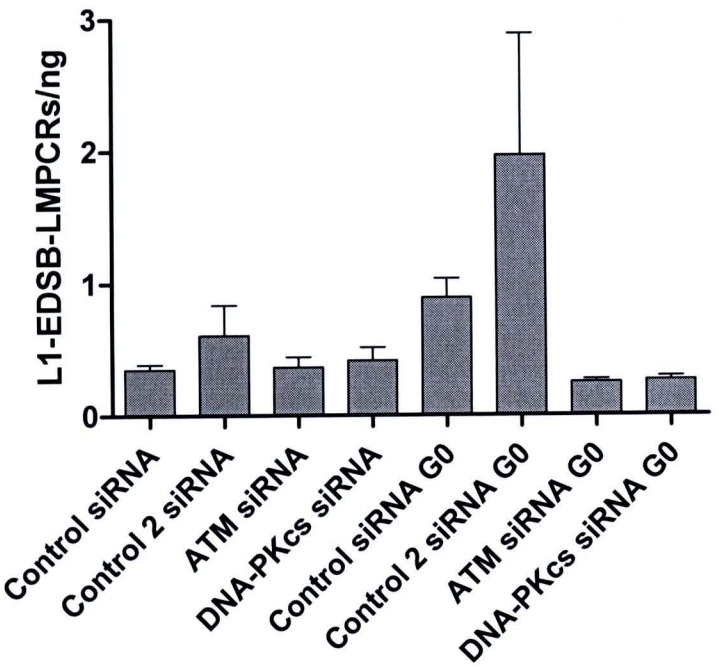

Figure 10. L1-EDSB-LMPCR Quantities in NHEJ defected cells. L1-EDSB-LMPCR quantities from control DNA-PKcs and ATM siRNA cells at G0 phase.

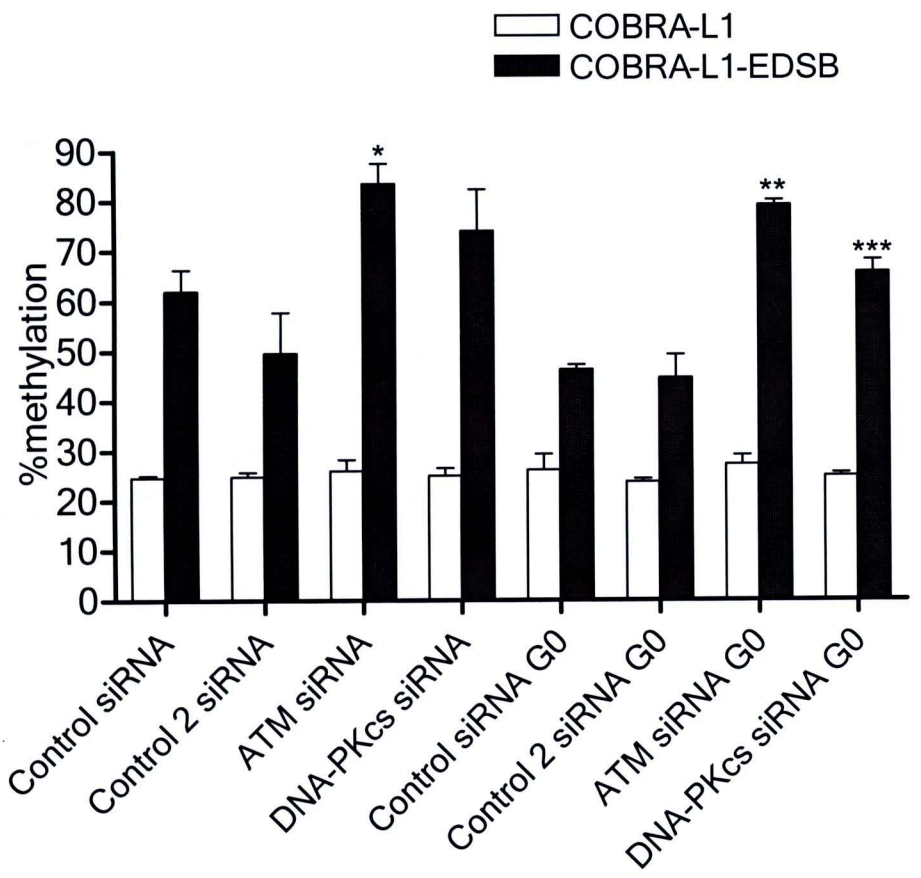

Figure 11. Inhibition of DNA-PKcs Leads to Accumulation of Unmethylated EDSBs while Downregulation of ATM Increases Methylation Level of EDSBs. COBRA-L1 and COBRA-L1-EDSB of stable DNA-PKcs and ATM siRNA transfected HeLa cells. Control siRNA and control2 siRNA are nonspecific siRNA transfected in 2 different experiments. Data represent means ±SEM, with statistical significance determined by paired 2-tailed t-test, *$P<0.05$, **$P<0.001$ when compared with control siRNAs, and ***$P<0.01$ with ATM siRNA G0.

Hypermethylated EDSBs and HR repair

From the previous study, we found that EDSBs are generally hypermethylated in most cell cycles (11). Besides G0 phase, we want to know whether HR pathway in S phase prefer to repair hypermethylated EDSBs. Rad51 is the candidate protein in this pathway because it catalyses the invasion of the broken ends of the DSB into the intact sister chromatid. In this experiment, we inhibited Rad51 expression by siRNA method after that measured EDSB and methylation level in both normal and defected HR cell. As show in Figure 2, protein level of RAD51 in Rad51 siRNA cell was evidently lower than control siRNA (Figure 10).

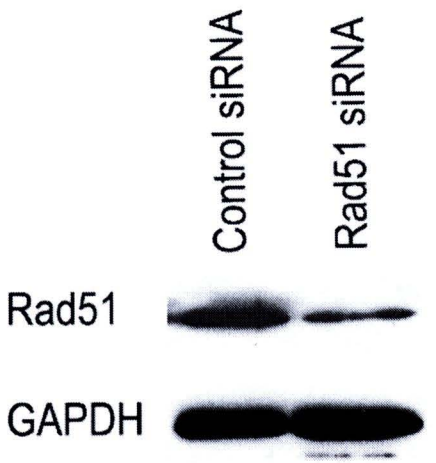

Figure 12. Western Blotting of RAD51 in HeLa Cells Transfected with siRNA. The analysis of RAD51 expression in HeLa cells transfected with RAD51 specific siRNAs constructs and G3PDH is used as control for protein loading.

We assessed the amount of EDSBs and their methylation status in several cell cycle phases, G0, G1/S and S, in HeLa cells. HeLa cells were synchronized in G1/S and S phase by thymidine block and in G0 phase by serum deprivation. Then, we measured EDSB level and mehtylation status by L1-EDSB-LMPCR and COBRA-L1 EDSB, respectively. For S phase, we observed EDSB quantity and methylation status in S phase. The results demonstrated that the level of EDSBs was not significantly different between control and Rad51-defected cells. Notably, we found that G0 phase possesses the least EDSBs when compared with other phase of cell cycles (Figure 11).

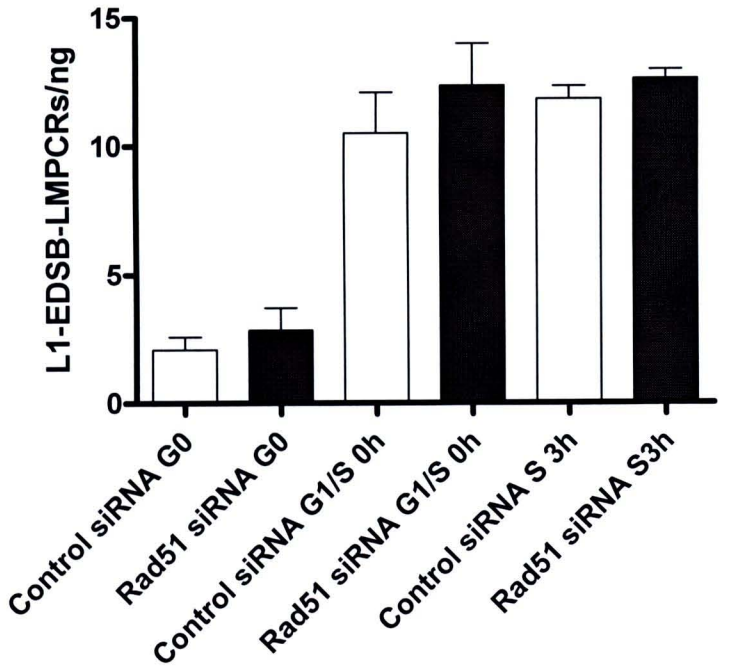

Figure 13. L1-EDSB-LMPCR Quantities in HR defected cells with cell Synchronization. L1-EDSB-LMPCR quantities from control and Rad51siRNA cells at G0, G1/S at 0h, and S phases at 3 h after the release into S phase from thymidine block.

We compared the methylation levels between Rad51 and control siRNA and found that the methylation level was not significantly different between two cells in each phase of cell cycle (Figure12). This result suggested that methylated EDSB repair did not depend on HR pathway.

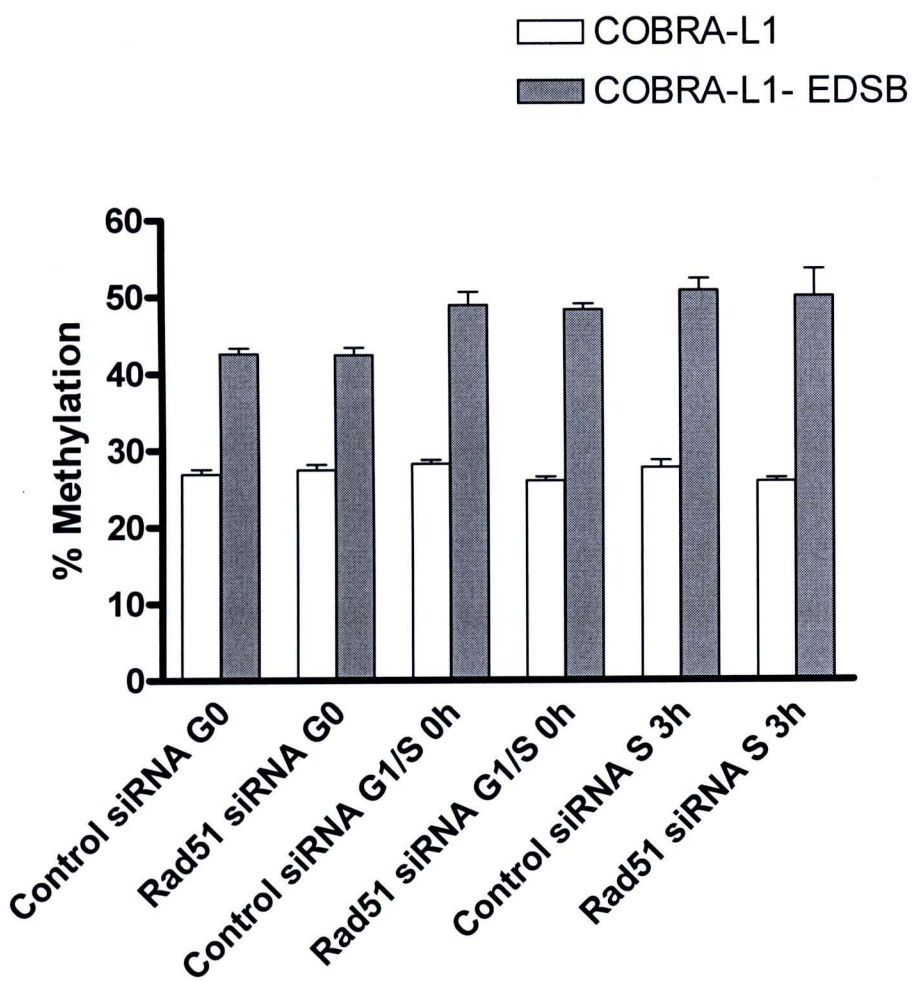

Figure 14. Inhibition of Rad51 did not affect the EDSBs methylation status. COBRA-L1 and COBRA-L1-EDSB of stable Rad51 siRNA transfected HeLa cells. Control siRNA is nonspecific siRNA transfected. Data represent. Non statistic significant between two cells in any phase of cell cycle.