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GADD45α modulates DNA methylation induced by DNA damage during homologous recombination

GADD45a modula la metilación del ADN inducido por el daño del ADN durante la recombinación homóloga

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Abstract— Homologous recombination is one of the major pathways for repairing DNA double strand breaks, the most deleterious of DNA lesions. Recent studies suggest that DNA methylation events target homologous recombination segments; however, the underlying mechanism of DNA methylation during homologous recombination is not understood. In this work, we show that GADD45α, a protein involved in cell cycle control, growth arrest, and apoptosis, plays some role in the epigenetic of homologous recombination. Specifically, it is suggested that dimerization of GADD45α monomers is required. Several point mutants of GADD45α were constructed and analyzed to show defects in self-association. Among them, the GADD45α mutant, CE83AA, lacked the ability to dimerize or oligomerize, which suppressed DNA methylation at homologous recombination sites in vivo. Based on this, we propose a model in which the dimerization (or oligomerization) of GADD45α is involved in strand specific DNA methylation that attends homologous recombination.

Keywords— DNA damage, DNA methylation, GADD45α, Homologous recombination.

Resumen— La recombinación homóloga es una de las principales vías para la reparación de la ruptura de doble cadena del ADN, la más grave de las lesiones del ADN. Estudios recientes sugieren que la metilación del ADN apunta hacia segmentos de recombinación homóloga; sin embargo, el mecanismo de metilación del ADN durante la recombinación homóloga no es claro. En este estudio, mostramos que GADD45α, una proteína que se encuentra relacionada con el control del ciclo celular, el ceso del crecimiento y la apoptosis, juega un papel en la epigenética de la recombinación homóloga. Específicamente, se ha sugerido que es requerido un dímero de monómeros de GADD45α. Varios puntos mutantes de GADD45α fueron construidos y analizados para mostrar defectos en la libre asociación. Entre ellos, el mutante GADD45α, CE83AA, carecía de la habilidad de dimerización u oligomerización, lo cual suprimió in vivo la metilación del ADN en los sitios de recombinación homóloga. Con base en esto, proponemos un modelo en el cual la dimerización (u oligomerización) de GADD45α está involucrada en la cadena específica de metilación del ADN que lleva a la recombinación homóloga.

Palabras clave— Daño del ADN, Metilación del ADN, GADD45α, Recombinación homóloga.

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I. Introduction

DNA double-strand breaks (DSBs) are regarded as perhaps the most dangerous form of DNA damage, as chromosomal breakage may result in an extreme loss of genetic integrity [1-2]. DSBs are more common than was once expected and are estimated to occur at levels of 10 per cell per day in mammals. This is because they are caused not only by environmental sources, such as ionizing radiation and exposure to genotoxic compounds, but also and more importantly, from endogenous sources, such as replication fork collapse during DNA replication and repair events [3]. DSBs are generated when the two complementary strands of the DNA double helix are broken simultaneously at nearby sites. Physically dissociated ends can recombine inappropriately with other genomic sites, leading to chromosomal translocations. Of the various forms of DNA damage, this one is the most hazardous to the cell [1]. Inefficient resection and persistence of DSBs can result in cell cycle arrest and apoptosis or attempts at DNA repair. Incorrect repair of DSBs may cause genomic instability in the form of gross chromosomal loss, amplification or rearrangements that can lead to cancer [2-3].

Because of threats posed by DSBs, eukaryotic cells have evolved complex, highly conserved systems to rapidly and efficiently detect these lesions, signal their presence, and bring about their repair [1,4-5]. Two major forms of DSBs repair are non-homologous end-joining (NHEJ) and homologous recombination (HR) [3,6]. These pathways are largely distinct from one another and function in complementary ways [4-5,7].

NHEJ includes the ligation of two DNA and it is error prone because no undamaged DNA is involved. In contrast, HR involves an intact DNA as a template and is error free [7]. HR can lead to gene conversion, a conservative process in which a donor DNA sequence with homology to both sides of the DSB supplies genetic information to repair the DSB. In this process, often called gene conversion, a donor DNA sequence with homology to both sides (e.g. a homologous chromosome or a sister chromatid) of the DSB supplies genetic information to repair the DSB. The homologous sequence is copied into the broken locus, making the repaired locus an exact copy of donor sequence, without altering the donor sequence [1,4,7].

DNA methylation is a covalent modification of genomic DNA [7]. It has been implicated in development and differentiation, X chromosome inactivation, imprinting and cancer [1]; and is an essential epigenetic mark that controls gene expression [8]. The DNA of mammals can be methylated on cytosines within the CpG dinucleotides. The added methyl groups protrude in the major groove of DNA. When the DNA is symmetrically methylated, both methyls face the same direction and are close to one another. The addition of methyl groups changes the biophysical characteristics of the DNA and has two effects: it inhibits the recognition of DNA by some proteins and permits the binding of others. The modification is brought about by enzymes called DNA methyltransferases (DNMTs) [8-9]. There are three such enzymes in mammals: DNMT1, DNMT3a, and DNMT3b [8, 9]. Biochemical and genetic data support a model in which all three enzymes have de novo and maintenance activities and cooperate to establish and maintain genomic methylation patterns. Of the methyltransferases, DNMT1 has the highest expression level in vivo and the highest specific activity in vitro, and mutations in DNMT1 have the most severe phenotypes [9].

DNA methylation often induces a transcriptionally silent state of methylated regions and interferes the transcription by preventing the binding of basal transcription machinery or other transcription factors that require contact with cytosine residues [7]. A total of two types of DNA methylation can be distinguished. Stable methylation is inherited through generations in a male or female-specific fashion and is responsible for both mono- and bi-allelic imprinting. Metastable methylation is variable and generates different methylation patterns among individual cells and cell types. It is modified by environment and changes during the lifetime of individual cells [1].

DNA methylation can be erased in passive manner by inhibiting the methylation of newly synthesized daughter strand during DNA replication, but in plants, the genomic methylation patterns can be changed in part by active demethylation. This active demethylation in mammals is controversial due to the lack of a confirmed mechanism and proteins. Recently, it was proposed that GADD45α (growth arrest and DNA-damage inducible gene 45 alpha) promotes active demethylation of CpG-methylated DNA by a DNA repair based mechanism [7]. The GADD45 proteins are small and acidic proteins (165 amino acids residues) located primarily in the nucleus [10-11] that were originally isolated from Chinese Hamster Ovarian (CHO) cells treated with UV irradiation [7] and are recognized as important tumor and autoimmune suppressors [12]. Three isoforms α (GADD45), β (MyD118) and γ (CR6) have been identified, and amino acid sequence identities among the three isoforms range from 50% to 60% [10]. Expression of these proteins is induced in response to a number of DNA-damaging agents and genotoxic stresses including hyperosmotic stress and UV irradiation [13]. All three genes are inducible by cellular stress, but their expression profile is distinct in various tissues. GADD45α is involved in maintenance of genomic stability, DNA repair, cell cycle check point and suppression of cell growth [7].

In previous studies, it was proposed that methylation followed by HR marks the repaired region [1]. The induction of DNA methylation during HR was strand-specific, that is, only the coding strand of the repaired region was methylated. However, the mechanism for strand-specific methylation remained to be elucidated. In this study, a GADD45α mutant showing defects in self-association failed to inhibit the DNA methylation suggesting that the dimerization or oligomerization of GADD45α is required for its role in strand-specific methylation during HR. Here, we propose that GADD45α coordinates the DNA methylation at the repaired DNA segments during HR and plays a role in passive demethylation events.

II. Materials and methods

2.1 Cell culture

HeLa-DR-GFP (Green Fluorescent Protein) stable lines were cultured in RPMI medium supplemented with 10% fetal bovine serum (Invitrogen). The cell line was grown at 37°C in 5% CO_2. HEK293FT cells (Invitrogen) were grown in Dulbecco's modified Eagle's medium (Gibco) with 10% fetal bovine serum. The cell lines were grown at 37ºC in 5% CO_2.

2.2 Plasmids

To clone the V5 tagged GADD45α and its point mutants, first, a pCMV-nV5 plasmid was constructed by inserting a V5 linker containing one copy of epitope into pcDNA3.1(+) (Invitrogen) using the HindIII and BamHI sites (HindIII site was dead by inserting V5 linker). Each point mutants of Myc-GADD45α was generated by two consecutive amino acids residues with alanine residues using site-directed mutagenesis kit (Stratagene) [7]. GADD45α and its point mutants PCR products were digested with EcoRV and XbaI and cloned into pCMV-nV5 plasmid.

2.3 Transient transfection

HeLa-DR-GFP cells were transfected with lipofectamine2000 as recommended by the manufacturer (Invitrogen) with 2 μg of pSceI plasmid along with Myc-GADD45α or Myc-GADD45α CE83AA plasmid. Cells were harvested 2 or 4 days after transfection.

2.4 Flow cytometry analysis for GFP positive cells

DR-GFP stable cells were transfected with pSceI and Myc-GADD45α (or Myc-GADD45α CE83AA). After 2 or 4 days, cells were treated with trypsin and collected. After two washes with PBS, cells were resuspended in 500 μl of 1X PBS. Fluorescence-activated cell sorter (FACSCalibur, BD Biosciences) analysis was used to reveal the percentage of cells expressing GFP, using $6x10^4$ cells per analysis. This FACSCalibur uses the CellQuest alias program and performs *n* to each sample analysis for the final draw an average.

2.5 GADD45α self-association, co-immunoprecipitation and western blotting

HEK293FT cells were transiently transfected with Myc-tagged GADD45α mutants and corresponding V5-tagged each GADD45α mutant and harvested after 24 h. Whole cell extracts were prepared by lysing cells in RIPA buffer (1X PBS pH 7.5, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 10% glycerol) containing 1X protease inhibitor cocktail (Roche), 1 mM DTT, and 1 mM PMSF. The whole cell extracts was diluted 5 times with 0.5X PBS containing 1X protease inhibitor cocktail, 1 mM DTT, 1 mM EDTA, and 1 mM PMSF. 0.5 μg of Myc-antibody (Upstate) was added to samples for 3 h, followed by 20 μl of protein A Sepharose 4B beads (Zymed) overnight. Beads were washed four times with 1X RIPA buffer. Bound proteins were eluted with sample buffer and analyzed by western blotting. Co-immunoprecipitated V5 tagged GADD45α and point mutants were detected using anti-V5 antibody (Invitrogen). The immune complex was detected with Amersham's enhanced chemiluminescence kit. Signals were quantified using a GeneTools (Syngene) program.

III. Results and discussions

3.1 I-SceI induces a homologous recombination in HeLa-DR-GFP cells

This recombination assay uses the DRGFP plasmid developed by M. Jasin [7,14] which contains two mutated GFP genes separated by a drug selection marker, the puromycin N-acetyltransferase gene (Fig. 1). The 5' GFP gene (cassette I) has a recognition site for I-SceI, a rare-cutting endonuclease that barely cut several eukaryotic genomes tested [15]. The I-SceI site is introduced at BcgI site by substituting 11bp by site-directed mutagenesis. This substitution also includes two in-frame stop codons inactivating GFP gene in cassette I. The downstream (3') GFP gene contains truncation at its 5' and 3' position having only about 800bp of GFP gene. This part of GFP could be used as a template for HR. HeLa cells containing DRGFP in the genome was generated and selected by puromycin resistance. Puromycin resistant pools of HeLa cells were then transiently transfected with a plasmid expressing I-SceI. The expression of I-SceI induced DSBs in the genome of HeLa-DRGFP cells.

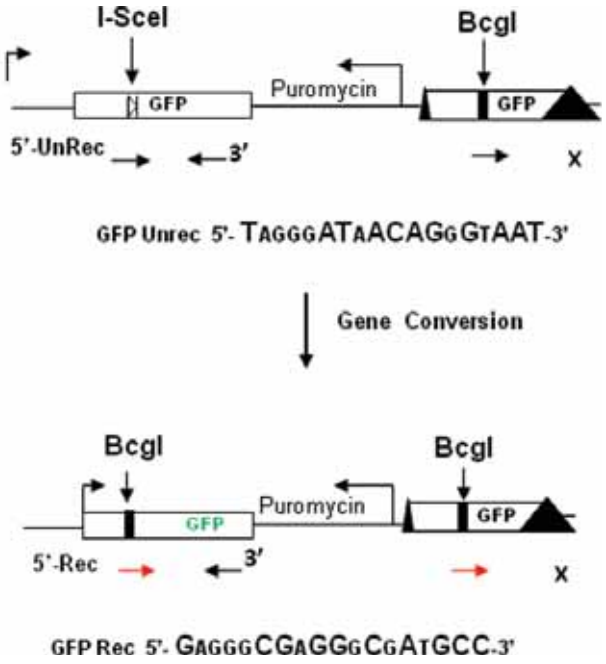

Fig. 1. Formation of a functional GFP gene by recombination initiated at a specific DSB [7].

The resultant DSBs triggered HR and recombinants products were analyzed (Fig. 2). After 3 days, FACS analysis was used to detect cells expressing GFP, which is a resultant product of gene conversion in this system. The percentage of GFP positive cells (GFP+) was counted.

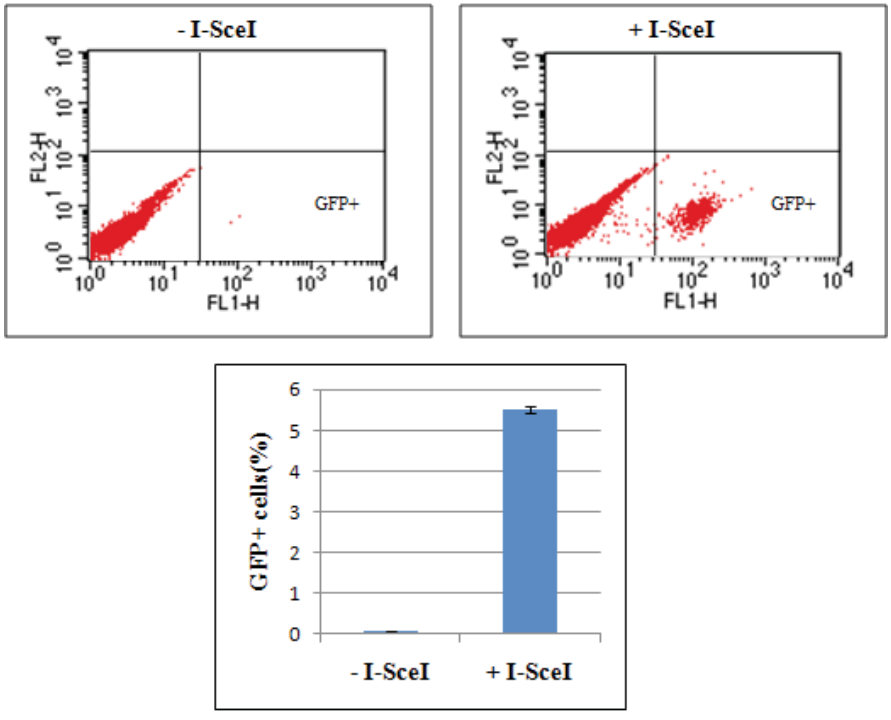

Fig. 2. Recombination assay. About 5.5% of cells expressed GFP and the GFP expression was observed in cells transfected with I-SceI plasmid. Control cells, which were no transfected with I-SceI plasmid showed background level fluorescence revealing that HR was induced by DSB.

3.2 GADD45α RT34AD or CE83AA mutation reduces its self-association

In previous report, we reported that DNMT1 is involved in the methylation of repaired region during HR [1]. Interestingly, the initial DNA methylation seems to be introduced on only one strand and subsequent cell division generates two populations of GFP positive cells, higher expression and lower expression. In higher expression cells, the recombined region was hypomethylated and in lower expression cells, the region was hypermethylated. This raised the question of how only one strand is methylated during HR. One intriguing idea is that DNMT1 might methylate both strands, then one strand is demethylated or one strand is protected from the methylation by other factors. As a putative factor for coordinating DNA methylation, we chose a GADD45α, which is induced upon DNA damage and interacts with DNMT1 [7]. Since GADD45α has been known that it forms dimerization or oligomerization, which is required for its biological function [16], we hypothesized that the dimer or oligomerization of GADD45α might be required for its role in HR. To investigate the role of GADD45α dimerization (or oligomerization), we constructed four V5-epitope tagged GADD45α point mutants, RT34AD (R34A, T35D), LC56AA (L56A, C57A), ED63AA (E63A, D64A), and CE83AA (C83A, E84A) (Fig. 3) using Myc-epitope tagged point mutants as a template. We performed co-immunoprecipitation experiments. HEK293FT cells were transfected with Myc tagged GADD45α mutants along with V5 tagged corresponding each point mutants. After 24h, cells were harvested and lysed. Whole cells extracts were subjected to immunoprecipitation using anti-Myc antibody to bring down Myc tagged GADD45α mutants. The immune complexes were eluted and separated on SDS-PAGE followed by western blot using anti-V5 antibody to check co-immunoprecipitated V5- GADD45α. As clearly shown in Fig. 4, wild type Myc- GADD45α and V5- GADD45α interact with each other (Fig. 4A, lane 1). However, all point mutants showed reduced self-association compared to that of wild type (Fig. 4A). The level of dimerization or oligomerization was reduced up to 30% (Fig. 4B). Especially, the self-association of RT34AD and CE83AA mutations severely decreased. This implicates that those amino acid residues are important for GADD45α dimerization of oligomerization.

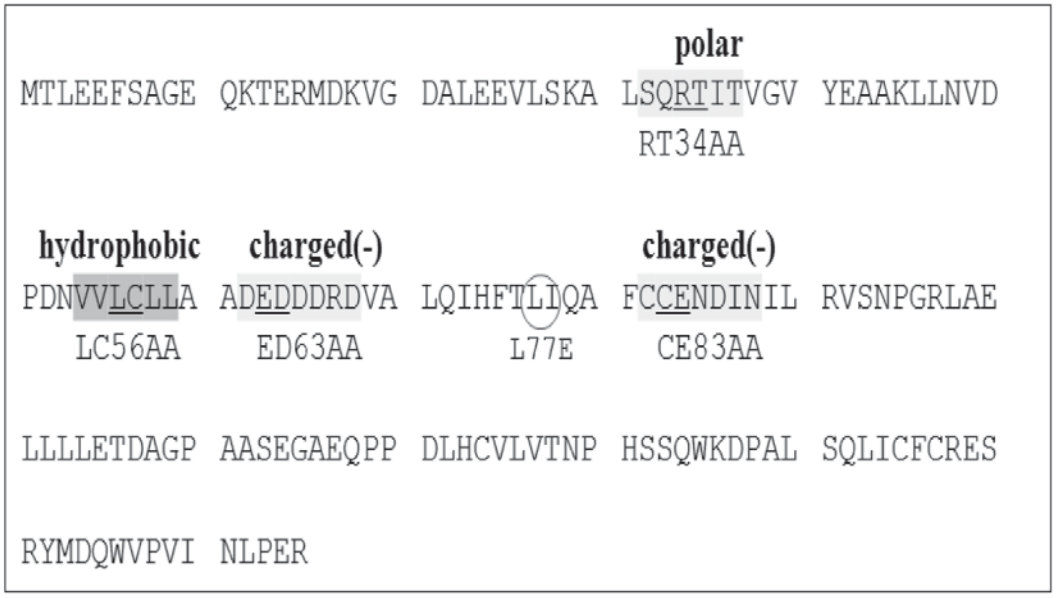

Fig. 3. The position of point mutation in GADD45α point mutants. The GADD45α protein is a small, acidic protein of 165 amino acid residues. It does not have any distinct motif or domain. To determine the region of GADD45α involved in interaction with DNMT1, we systematically changed two consecutive amino acids, which reside in clustered charged, polar, or hydrophobic regions. We generate four point mutants, RT34AD (R34A, T35D), LC56AA (L56A, C57A), ED63AA (E63A, D64A) and CE83AA (C83A, E84A). Two consecutive amino acid residues were replaced with two alanine residues.

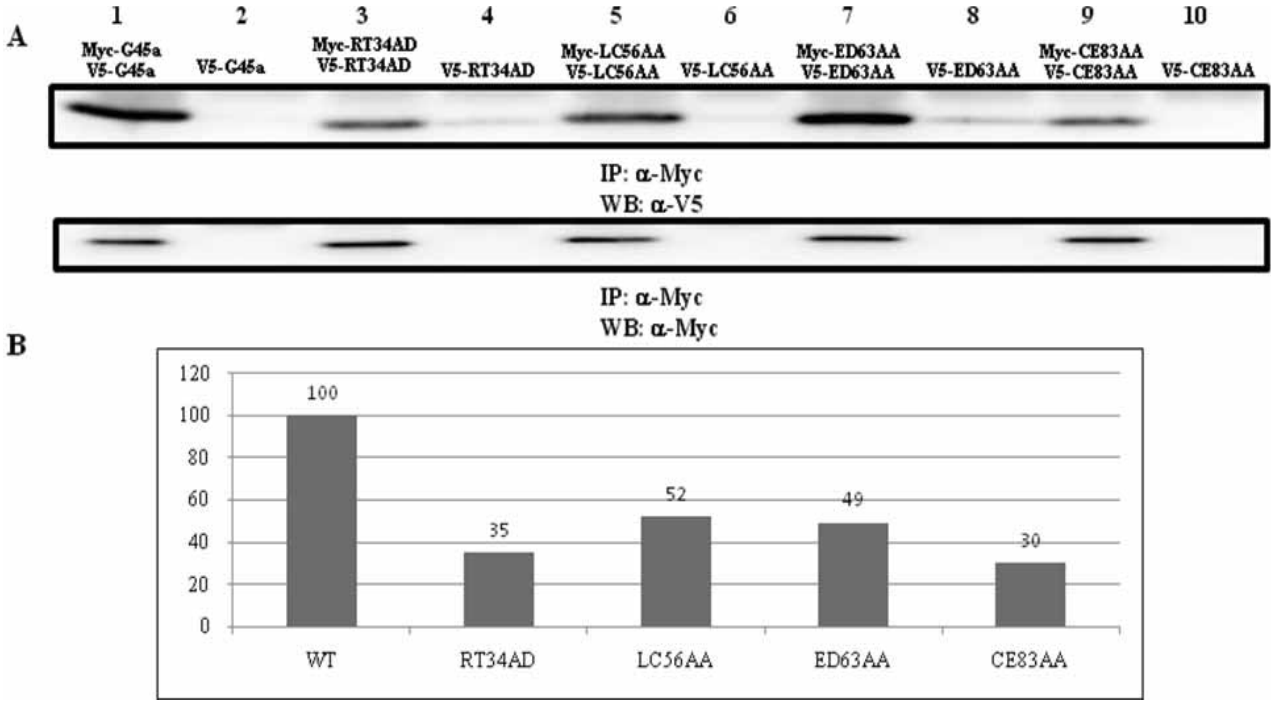

Fig. 4. The self-association of each GADD45α point mutants. A) Co-immunoprecipitation of each GADD45α point mutants. B) Quantification of band intensity. The amount of co-immunoprecipitated each GADD45α point mutants was normalized by the amount of immunoprecipitated each GADD45α point mutants and the relative level of each band against wild type was plotted.

3.3 The dimerization or oligomerization of GADD45α is required for regulating DNA methylation during HR

To further investigate the role of GADD45α self-association, we analyzed the GFP expression of HeLa-DRGFP cells in the presence or absence of GADD45α or GADD45α CE83AA mutant, which has shown least dimer or oligomerization ability (Fig. 5). HeLa-DRGFP cells were transfected with pSceI plasmid along with Myc-GADD45α or Myc- GADD45α CE83AA. Cells were further incubated for 7 days and GFP+ cells were analyzed by FACS. Co-expression of Myc- GADD45α significantly increased the number of GFP+ cells (Fig. 5). The increase of GFP+ cells was proportional to the amount of Myc- GADD45α plasmid up to 0.5 μg. Above 0.5 μg, the rate of increase was not proportional (see Fig. 5 for SceI + Myc- GADD45α 1 μg sample). However, Myc-GADD45α CE83AA mutant did not increase the number of GFP+ cells. Interestingly, CE83AA mutant decreased the GFP+ cells at high dosage. That is, the cells transfected with 0.25 μg DNA showed the increase in GFP+ cells and comparable to that of wild type. However, cells transfected with more than 0.5 μg DNA showed the decrease in GFP+ cells. Currently, it is not clear how CE83AA decrease the GFP+ cells. Probably, the high dosage of mutant completely removes the action of endogenous GADD45α from HR events. Taken together, the stimulation of recombinant GFP gene expression by GADD45α suggests that the methylation of recombinant gene was inhibited by GADD45α resulting in more expression of recombinant GFP genes and this inhibition requires GADD45α dimerization or oligomerization.

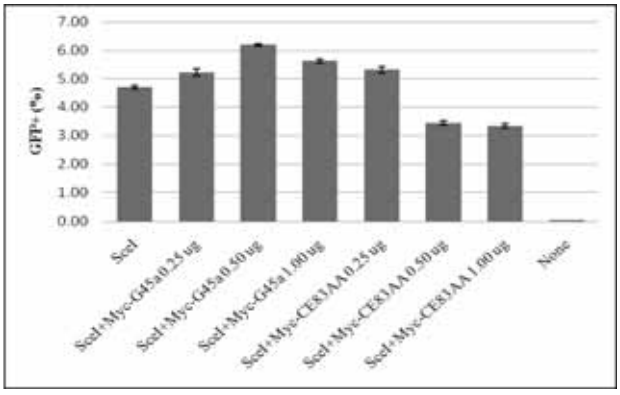

Fig. 5. The stimulation of recombinant GFP gene expression by GADD45α. HeLa-DRGFP cells were transfected with 2 mg of SceI plasmid and different amount of Myc-GADD45α or Myc-GADD45α CE83AA. After 7 days, GFP+ cells were analyzed by FACS. Co-expression of Myc-GADD45α significantly increased the number of GFP+ cells, while CE83AA was ineffective.

IV. Conclusion

In previous work, we demonstrated that HR was accompanied by DNA methylation and the induction of methylation was strand-specific. However, the mechanism of strand-specific methylation during HR is currently unknown. Here, we are proposing that DNMT1 and GADD45α coordinate the methylation of repaired genes during homologous recombination.

DNMT1 mediates the hypermethylation of one strand near to the HR region, leaving the opposing strand hypomethylated [1]. How this strand-specific methylation could be achieved? As a putative regulator of this process, we tested GADD45α. GADD45α is a small acidic protein and is well known as a growth suppressor upon genotoxic stress. Interestingly, it has been implicated in methylation/ demethylation events [17-18]. A role for GADD45α in the strand-specific methylation during HR can be summarized as follows. First, GADD45α physically interacts with the catalytic domain of DNMT1 [7]. Second, the silencing of recombinant DNA following HR was partially reversed by high level expression of GADD45α GFP expression was reproducibly increased when GADD45α was overexpressed. Third, GADD45α CE83AA mutant, showing reduced self-association, did not increase the GFP expression. In summary, we provide new data implicating that GADD45α might regulate the epigenetic silencing following homologous recombination. Based on these data we are proposing a model depicting DNMT1/ GADD45α interplay during HR (Fig. 6). The HR repair involves strand assimilation, excision of ½ of I-SceI site and recruitment of DNMT1 to upper strand as a complex with GADD45α (dimer or oligomer) [3]. At HR sites, GADD45α might modulate DNMT1 activity by inhibiting its enzymatic activity or preventing the access of DNMT1 to the bottom strand. As a result, the methylation will be introduced on only upper strand leading two populations of GFP expression cells (Fig. 6). Hypermethylation will silence the HR site and unmethylation will produce high GFP expression. In this process, the dimer or oligomerization of GADD45α and stoichiometric ratio between GADD45α dimer or oligomer and DNMT1 seem to be important. CE83AA mutant showed weak self-association. When this mutant was expressed at low gene dosage, it behaved like wild type and increased GFP expression (Fig. 5). However, at high gene dosage, CE83AA mutant decreased GFP expression rather than unaffecting GFP expression. This implicates that C83AA might bind to endogenous GADD45α and inhibit its binding to DNMT1, when it is expressed above certain level, resulting in the decrease of GFP expression (Fig. 5). Although the detail mechanism of DNMT1/ GADD45α interplay is not clear currently, our data suggest, that GADD45α plays a role in modulating epigenetic silencing at HR site.

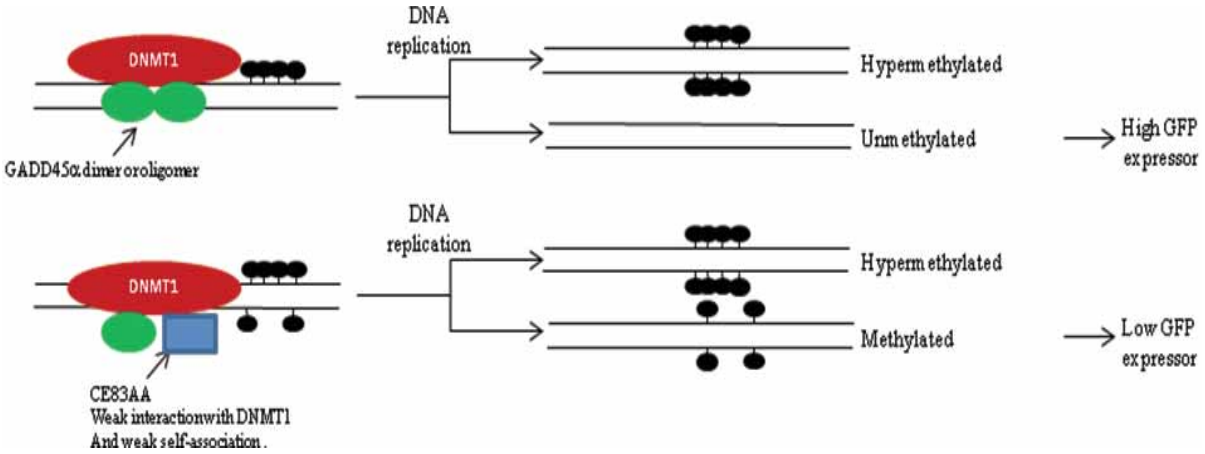

Fig. 6. A model for DNMT1/GADD45α interplay during HR repair. The figure illustrates the possible interplay between DNMT1 and GADD45α during HR leading to silencing, low expression, or high expression of HR DNA segments. Filled rollipop represent methylated CpGs induced by HR. In this model, GADD45α modulates DNMT1 activity at HR site. Depends on availability of GADD45α, HR DNA segments could be hyper- or hypomethylated.

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