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14. ABSTRACT
Epidemiology studies have linked estrogen to breast carcinogenesis. However, the question of how estrogen acts as a carcinogen is basically unanswered. We hypothesized that estrogen signaling through the estrogen receptor alpha (ERa) may induce a mutator phenotype by suppressing DNA repair activity in ERa positive mammary epithelial cells. To determine the effect of estrogen and/or ERa on DNA repair activity in the ERa positive human breast cancer cells, we have developed a method to measure DNA repair activity and DNA mutation rate in live cells. Measurements of DNA repair efficiency showed that ERa positive cells had significantly lower DNA repair activity than ERa negative cells. Ectopic expression of ERa in ERa negative breast cancer cells reduced DNA repair activity. Treatment of ERa positive breast cancer cells with estrogen inhibited DNA repair and increased mutation rate. Our results suggest that estrogen/ER may induce genetic instability by suppressing DNA repair activity resulting in an increased DNA mutation rate.

15. SUBJECT TERMS
Estrogen, mutagenesis, mutation rate, mammary epithelial, cells

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Introduction

Breast cancer is the most frequently diagnosed neoplasm in American women. It is also the leading cause of cancer death among non-smoking females (1). While many risk factors have been indicated or implicated, cumulative lifetime exposure to estrogen links most known risk factors (except for radiation) for breast cancer (2). Approximately, two-thirds of breast cancer express estrogen receptor (ER) (3, 4). However, the question of how estrogen causes breast cancer is basically unanswered although different hypotheses have been proposed (2, 5, 6). While epidemiology studies linking estrogen to breast cancer risk factors suggest that estrogen may be a tumor initiator, laboratory studies thus far have not elucidated how estrogen can act as a carcinogen (5). Since estrogen is known to stimulate ER-mediated cell proliferation in mammary epithelial cells, its carcinogenicity is attributed to an accumulation of genetic mutations resulting from stimulated cell proliferation. However, many studies have shown that the accumulation of mutations in a cancer cell has to come from either genotoxic carcinogens or mutator phenotypes due to the low spontaneous mutation rates in mammalian cells (7). Mutator phenotypes are normally acquired early during neoplastic transformation and are believed to be responsible for genetic instabilities observed in vast majority of cancer (8). There are two major types of genetic instabilities. One is subtle sequence changes that are due to defects of either nucleotide excision repair or DNA mismatch repair (MMR). The other involves gross changes of chromosome sequence or number, which we can collectively call chromosome instability. While the molecular basis of chromosome instability remains undefined in most human cancers, it appears to arise from subtle, intragenic mutations of the genes, whose products play a key role in maintaining chromosome stability (8). Thus, it is possible that a transient, conditional suppression of DNA MMR activity may contribute to the initiation of chromosome instability, since subtle DNA mutations due to defects of nucleotide excision repair are produced by extrinsic factors such as u.v. irradiation. Therefore, we hypothesized that estrogen might induce a transient mutator phenotype by repressing MMR activity in ER positive (ER+) cells. To determine the effect of estrogen and/or ER on DNA MMR activity in the ER positive human breast cancer cells, we have developed a method to measure DNA MMR activity in live cells. We have also developed a method to measure mutation rate as a function of estrogen/ER signaling. Our goals were to compare the DNA MMR efficiency between ER+ and ER- cells, to determine the effect of estrogen on MMR efficiency in ER+ human breast cancer cells and ER- breast cancer cells that express an ectopic ER, and to determine the effect of estrogen on DNA mutation rate.

Body

Our research accomplishments for the entire award period are described below.

1. Development of a method to measure DNA MMR activity in live cells.

Loss of DNA mismatch repair (MMR) function leads to the development and progression of certain cancers. Currently, the assays for detection of DNA MMR activity involve the use of extracted cell content and are often tedious and costly. In order to determine whether 17β-estradiol signaling through ER inhibits DNA MMR activity, we developed a method that can quantitatively assess MMR efficiency in live cells and is less labor-intensive. A G-G or T-G mismatch was introduced into ATG start codon of the enhanced green fluorescent protein (EGFP) gene. Repair of the G-G or T-G mismatch to G-C or T-A, respectively in the heteroduplex plasmid generates a functional EGFP gene expression. The heteroduplex plasmid and a similarly constructed homoduplex plasmid were transfected in parallel into the same cell line and the number of green cells counted by flowcytometry. Relative EGFP expression was calculated as the total fluorescent intensity of cells transfected with the heteroduplex construct divided by that of cells transfected with the homoduplex construct. We have tested several cell lines from both MMR-deficient and MMR-proficient groups using this method including a colon carcinoma cell line HCT116 with defective hMLH1 gene and a derivative complemented by transient transfection with hMLH1 cDNA. Results show that MMR-proficient cells have significantly higher EGFP expression than MMR-deficient cells, and that transient expression of hMLH1 alone can elevate MMR activity in HCT116 cells. This method can be potentially useful in comparing and monitoring MMR activity in live cells under various growth conditions. The details of this work are described in the appended manuscript, which is published in *Nucleic Acid Research*. This method was used to measure DNA MMR activity in ER+ and ER- human breast cancer cells as described below.

2. ER+ human breast cancer cells have a significantly lower DNA MMR activity than ER- human breast cancer cells.

Three ER+ human breast cancer cell lines, MCF-7, T47D and ZR75-1, and one ER- human breast cancer cell line MDA-MB-231 were used in our study. In addition, human cervical cancer cell line Hela was used as a MMR proficient positive control whereas human colon cancer cell line HCT116 was used as a MMR deficient negative control. HCT116 cells were cultured in a serum-free McCoy's 5A medium whereas the other cell lines were cultured in a McCoy's 5A medium containing 10% fetal bovine serum as described previously (9). To confirm that the ER+ cell lines are sensitive to estrogen, we tested whether 17β-estradiol can stimulate transcriptional activation of an estrogen-responsive promoter-luciferase reporter construct. The construct was transiently co-transfected with a beta-galactosidase expression plasmid into the three ER+ cell lines and the cells were treated with or without 10^{-7} M 17β-estradiol for 48 hours. The cell lysate was analyzed for the activity of luciferase and beta-galactosidase. Luciferase activity normalized by beta-galactosidase activity was plotted in Fig. 1 as relative luciferase unit (RLU), which was significantly ($P<0.05$ with Student *t* tests) increased by the treatment of 17β-estradiol. Thus, these three cell lines express ER. We next measured DNA MMR activity in the ER+ and ER- cell lines. Fig. 2 shows that the MMR efficiency of the ER+ breast cancer cell lines is statistically the same as that of the MMR deficient HCT116 cells and significantly lower than that of the ER- MDA-MB-231 cells. In contrast, the MMR efficiency of the ER- MDA-MB-231 cells was statistically the same as that of the MMR proficient Hela cells. We have since used Western blot to confirm that indeed the ER+ breast cancer cells have a lower MMR activity than Hela cell as reflected by the level of EGFP protein expressed from repaired heteroduplex plasmid relative

to that from homoduplex plasmid (Fig. 3). These results suggest that ER appears to inhibit DNA MMR activity.

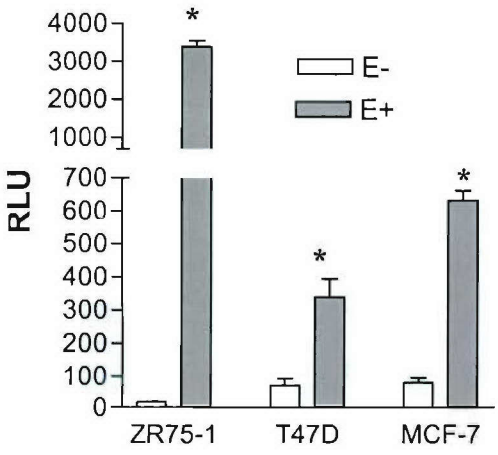


Fig. 1. Estrogen responsiveness of the three human breast cancer cell lines. Exponentially growing cells were plated in 12-well plates. After 24 hr, a estrogen-responsive promoter-luciferase reporter construct was transiently co-transfected with a beta-galactosidase expression plasmid into the cells, which were then treated without (E-) or with (E+) 10^{-7} M 17b-estradiol for 48 hr. The activities of luciferase and beta-galactosidase were assayed in the cell lysate. Luciferase activity normalized with beta-galactosidase activity is plotted as relative luciferase unit (RLU). Each data point is the mean±SE from three wells. The asterisk "*" denotes significant difference from the corresponding control at $P<0.05$ with Student *t* test.

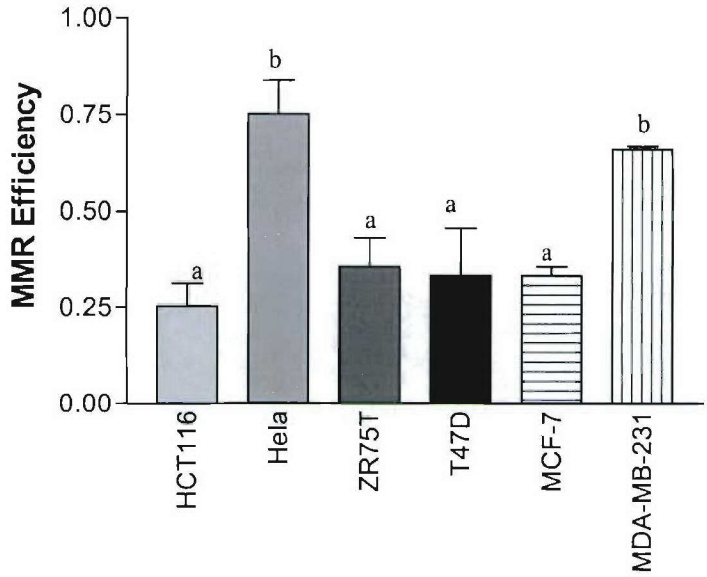


Fig. 2. MMR efficiency in breast cancer cell lines and in MMR deficient HCT116 and MMR proficient Hela cell lines. Exponential growing cells were plated in 60-mm dish. After 24 hr, they were transfected with a nicked heteroduplex or homoduplex EGFP expression plasmid. A red fluorescent protein expression plasmid was co-transfected for the normalization of transfection efficiency. Twenty-four hour after transfection, the cells were harvested for flowcytometry analysis. Each data point represents the mean±SE of 3 or 4 independent experiments. The columns bearing a different letter are significantly different at $P<0.05$ by one-way analysis of variance and Newman-Keuls tests.

3. Ectopic expression of ER significantly inhibits MMR activity in MDA-MB-231 cells.

The ER negative MDA-MB-231 cell line was shown to become responsive to estrogen when transfected with an ectopic ER (10). We have obtained both the control and ER-transfected (named s-30) MDA-MB-231 cell lines from Dr. V.C. Jordan. To test their estrogen sensitivity, we transfected the estrogen responsive promoter-luciferase reporter construct into the two-matched cell lines. Fig. 4 shows that while the control cell line

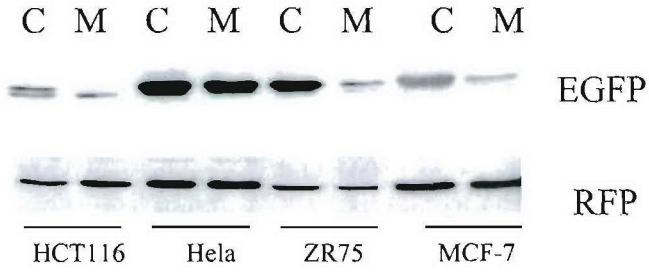


Fig. 3. Comparison of MMR activity in ER+ breast cancer cells with that in Hela and HCT116 Cells. The cells were plated in 60mm dish and transfected with homoduplex (C) or heteroduplex (M) EGFP plasmid together with RFP plasmid. The cells were harvested 24 hr after transfection and cell extracts were used for Western blot using anti-EGFP or anti-RFP antibodies. As in the figure 2, the upper band in the EGFP blot represents EGFP protein level. The lower one is non-specific.

is not responsive to estrogen, the s-30 cell line is highly responsive. Interestingly, re-expression of ER significantly ($P<0.05$) reduced the MMR efficiency in the c-30 cell line (Fig. 5). Thus, ectopic ER appears to act similarly to the endogenous ER in regulating MMR activity.

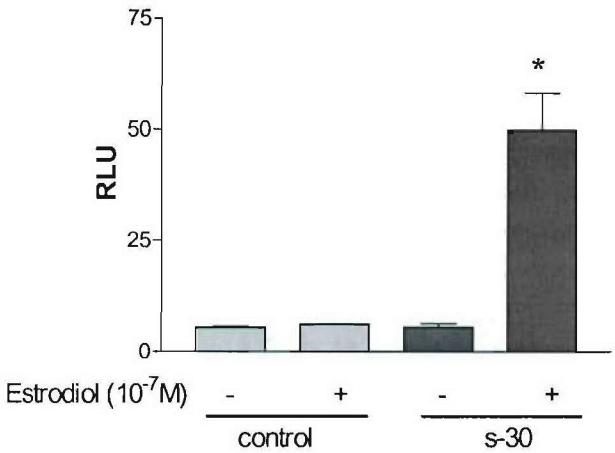


Fig. 4. Comparison of estrogen responsiveness between MDA-MB-231 wild type control cells and ectopic ER-transfected s-30 cells. Cells were plated in triplicates in 12-well plates and transfected with an estrogen-responsive luciferase reporter construct and beta-galactosidase expression plasmid 24 hr later. The transfected cells were treated with or without 10^{-7} M 17β-estradiol four hours after transfection. The activities of luciferase and beta-galactosidase were assayed in the cell lysate after 24 hr of the treatment. Luciferase activity normalized with beta-galactosidase activity is plotted as relative luciferase unit (RLU). Each data point is the mean±SE from three wells. The asterisk "*" denotes significant difference from the control with Student *t* test at $P<0.05$.

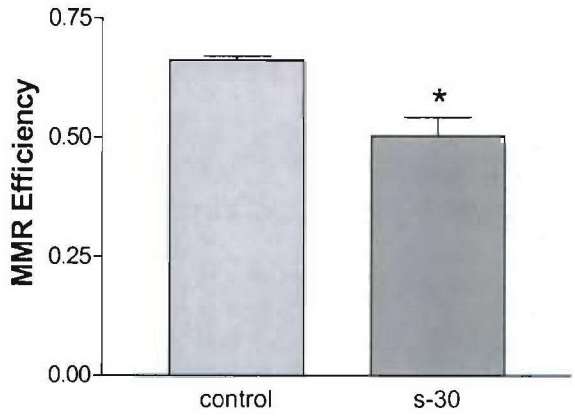


Fig. 5. Inhibition of MMR efficiency by ectopic expression of the alpha type ER in MDA-MB-231 cell line. MDA-MB-231 control and ER-transfected s-30 cells were plated in 60-mm dishes. On next day, they were transfected with a nicked heteroduplex or homoduplex EGFP expression plasmid. A red fluorescent protein expression plasmid was co-transfected for the normalization of transfection efficiency. Twenty-four hour after transfection, the cells were harvested for flowcytometry analysis. Each data point represents the mean±SE of 3 independent experiments. The asterisk "*" denotes significant difference from the control with Student *t* test at $P<0.05$.

4. Effect of 17β-estradiol on MMR activity.

ER is known to function in an estrogen-dependent manner. Therefore, we cultured MCF-7 and ZR75 cell lines in an estrogen-free medium and tested whether treatment of the cells with 17β-estradiol can reduce MMR efficiency. Fig. 6 shows that 17β-estradiol appears to have a biphasic effect on the MMR activity in MCF-7 cells. The concentrations at 10^{-9} and 10^{-11} M appears to be more effective in inhibiting MMR activity than either 10^{-7} or 10^{-13} M. Similar results were obtained from another ER+ breast cancer ZR75 cells as shown in Fig. 7. We also found that the treatment of the MDA-MB-231 s-30 cell line with 17β-estradiol at 10^{-7}M also reduced MMR efficiency as shown in Fig. 8.

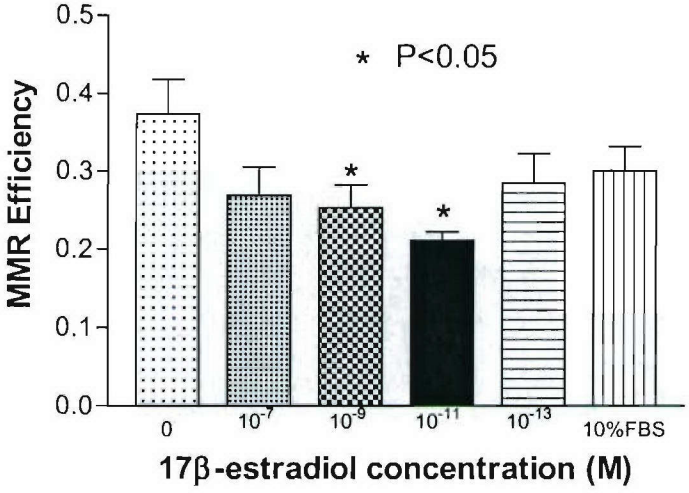


Fig. 6. Effect of 17β-estradiol on MMR efficiency in MCF-7 cells. MCF-7 cells were cultured in a McCoy's 5A medium containing 10% fetal bovine serum (10% FBS) and an estrogen-free medium (McCoy's 5A without phenol red and with 10% charcoal stripped FBS) containing different concentrations of 17β-estrodial for 4 days. Then the cells were plated in 60mm dish at 400,000cell/dish. After 24 hr, the homoduplex or heteroduplex EGFP plasmid was co-transfected with RFP plasmid with FuGene6. The cells were harvested for flow cytometry analysis after an additional 24 hr. MMR efficiency are plotted as mean±SE from three independent experiments. Asterisk "*" indicates statistical difference from control, without estrogen treatment, with Student *t* tests.

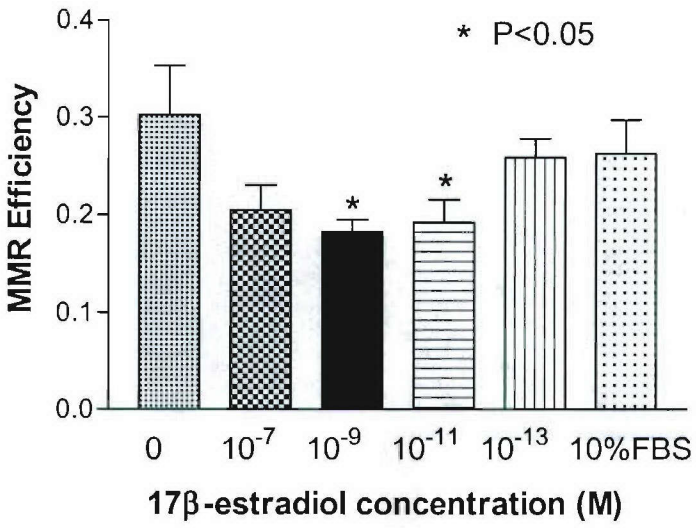


Fig. 7. Effect of 17β-estradiol on MMR efficiency in ZR75 cells. The experiments were performed following the same protocol as described in Fig. 4 legend. The data are presented as as mean±SE from three independent experiments. Asterisk "*" indicates statistical difference from control, without estrogen treatment, with Student *t* tests.

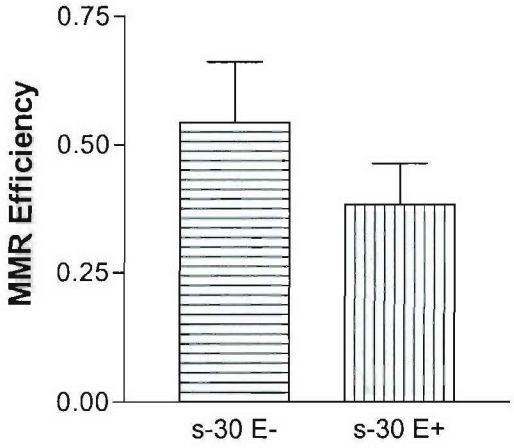


Fig. 8. MMR efficiency of MDA-MB-231 cells cultured with or without estrogen. The cells were cultured in a phenol red free medium without (E-) or with (E+) 10-7 M 17β-estradiol for 1 week. They were than plated in 60-mm dishes in the same medium for the measurement of MMR efficiency as described in the legend of Fig. 2. Each data point is the mean±SE from 3 independent experiments.

5. Effect of 17β-estradiol on the expression of MMR enzymes.

In order to determine how estrogen inhibits MMR, we have determined the effect of 17β-estradiol on the expression of a number of enzymes involved in MMR. As shown in Fig. 9, among MLH1, PMS2, and MSH2, only MLH1 is inhibited by estrogen. It is interesting to note that the most effective

concentration in inhibiting MLH1 expression is 10^{-11} M 17β-estradiol. We have also found that the expression of another MMR enzyme, MSH6, is not affected by 17β-estradiol (data not shown).

6. Effect of 17β-estradiol on the expression of hMSH3 and PCNA.

Since overexpression of hMSH3 was shown to reduce single-base MMR activity (11, 12), we originally hypothesized that estrogen may stimulate hMSH3 expression, thus suppressing single-base MMR activity in ER+ cells. Therefore, we measured the effect of 17β-estradiol treatment on MSH3 protein level with Western blot. As shown in Fig. 10, 17β-estradiol does not appear to stimulate MSH3 expression.

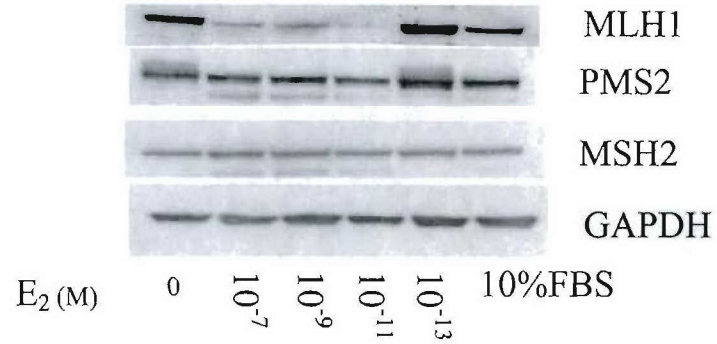


Fig. 9. Inhibition of hMLH1 expression by 17β-estradiol (E_2) in MCF-7 cell. MCF-7 cells were cultured in the regular 10%FBS medium or the estrogen-free medium with different concentration of 17β-estrodial for 6 days. The cells were harvested and extracted for Western blotting. Anti-MLH1, PMS2 and MSH2 antibodies were purchased from Oncogene. Anti-GAPDH antibody was from Ambion.

Another hypothesis was that estrogen might inhibit the expression of proliferating cell nuclear antigen (PCNA) since PCNA is required for DNA MMR (13). Therefore, we have also measured the effect of 17β-estradiol treatment on the expression of PCNA. Similar to MSH3, PCNA protein level was not altered after 17β-estradiol treatment (Fig. 10).

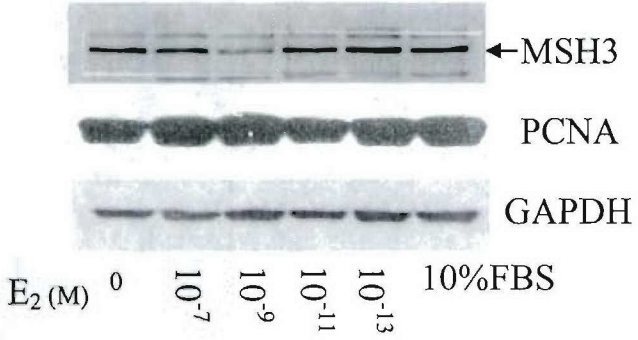


Fig. 10. Effect of 17β-estradiol (E_2) treatment on the expression of MSH3 and PCNA in MCF-7 cell. MCF-7 cells were cultured in the regular 10%FBS medium or the estrogen-free medium with different concentrations of 17β-estrodial for 6 days. The cells were harvested and extracted for Western Blot. Anti-MLH3 and anti-PCNA antibodies were purchased from Oncogene. Anti-GAPDH antibody was from Ambion.

7. Effect of tamoxifen and ectopic expression of MLH1 on estrogen-induced MMR inhibition.

To determine whether the inhibitory activity of 17β-estradiol can be reversed by the anti-estrogen, tamoxifen, we treated 17β-estradiol-treated MCF-7 cells with tamoxifen and then measured MMR efficiency. As shown in Fig. 11, while 17β-estradiol reduced MMR efficiency, tamoxifen appears to reverse the inhibition. This experiment was repeated once and tamoxifen again increased MMR efficiency in the presence of 17β-estradiol (Fig. 12).

Since the treatment with 17β-estradiol was shown to inhibit MMR activity and the expression of human MLH1 (hMLH1) (Fig. 9), we have determined whether ectopic expression of hMLH1 can reverse the inhibition by 17β-estradiol. As shown in Fig. 11, ectopic expression of hMLH1 did reverse 17β-estradiol-induced inhibition of MMR suggesting that the inhibition of MMR activity by estrogen is apparently due to the inhibition of hMLH1 expression.

Fig. 11. Tamoxifen and ectopic expression of hMLH1 elevate MMR activity inhibited by 17b-estradiol (E2). MCF-7 cells were cultured with or without 17b-estrodial for 4 days. They were then plated at 400,000 cells/60mm dish for MMR assay. One dish was treated with tamoxifen (TAM) plus E2. The homo- or hetero-duplex EGFP plasmid was co-transfected with RFP plasmid 24 hr later. For another two dishes, either control or hMLH1 expression plasmid was also transfected. The flowcytometry analysis was performed after additional 24 hr. MMR efficiency is presented as mean±SE from three separate experiments. Asterisk "*" denotes significantly different at $P<0.1$ from E2 treatment alone.

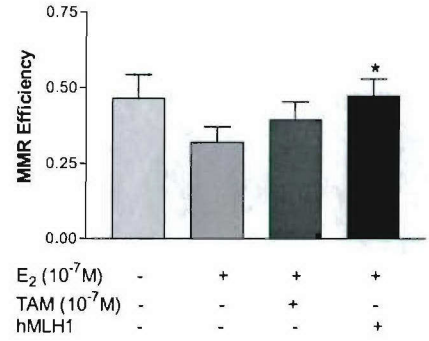
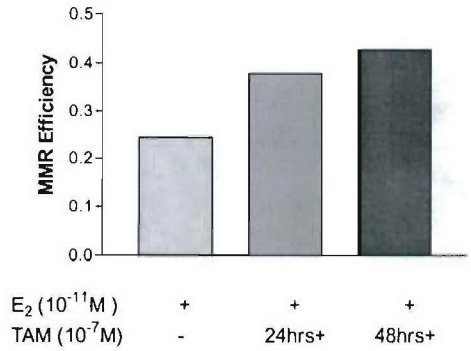


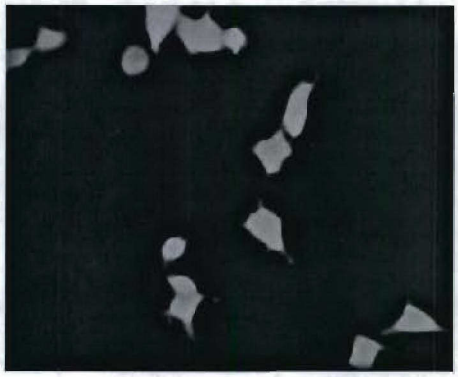
Fig. 12. Increase MMR efficiency in MCF-7 cells with the treatment of tamoxifen. MCF-7 cells were cultured in the estrogen-free media plus E2 (10^{-11} M) for 4 days. They were then plated at 400,000 cells/60mm dish and treated with or without tamoxifen (10^{-7} M) for 24 or 48 hr. The cells were then used for MMR assay. Each value is from one dish.



8. Determine the effect of 17β-estradiol on microsatellite mutation in MCF-7 cells

To examine whether the reduced MMR efficiency is associated with increased microsatellite instability (MSI) in MCF-7 cells, we engineered a microsatellite sequence consisting of an 11-adenine track downstream from the start codon of the enhanced green fluorescence protein gene to generate a frame-shift mutation. Stable transfection of this faulty 11-A GFP does not yield green cells, whereas stable transfection of an EGFP gene with a 12-adenine track downstream from the start codon expressed EGFP (Fig. 13). As such, mutation of the poly 11-A microsatellite through insertion of one nucleotide or deletion of two should restore coding sequence for EGFP expression. The 11-A EGFP expression plasmid was stably transfected into the MCF-7 cells as well as human colon carcinoma Lovo (an MMR deficient cell) and SW480 (an MMR proficient cell) cells. The stable clones were ring-cloned and expanded in the regular 10F medium. We first measured the emergence of percent green cells as a function of cell proliferation with flowcytometry, and calculated the mutation rate, which is the percent green cells divided by population doubling. As shown in Fig. 14, the MMR deficient Lovo clone (cl. 5) had a much higher mutation rate than the MMR proficient SW480 clone (cl. 9), consistent with the notion that MMR deficiency increases the mutation rate. An MCF-7 11-A clone (cl. 4) showed much higher mutation rate than both Lovo 11-A clone and SW480 11-A clone. Further studies revealed that the MCF-7 clone had 4 copies of the A-11 EGFP gene where as the Lovo and SW480 clones had 2 copies. This explains why the MCF-7 clone showed much a higher mutation rate than Lovo and SW480. To determine the effect of 17β-estradiol (10^{-7} M) on the mutation rate of the 11-A EGFP gene in the MCF-7 clone, the cells were equally divided and cultured in estrogen-free medium with or without 10^{-7} M 17β-estradiol or in the regular 10F medium for one week before cells were scored for EGFP expression with a flowcytometer using un-transfected cells as negative controls. Fig. 15 shows that the mutation rate was almost doubled in 10F medium and almost tripled in 17β-estradiol medium when compared with the cells in the estrogen-free medium from three separate experiments. Thus, 17β-estradiol appears to induce MSI. This is in agreement with a report showing that estrogen can generate MSI during estrogen-induced kidney tumorigenesis in hamster (14).

Fig. 13. Expression of A-12 EGFP in Hela cells. An EGFP expression plasmid was mutated to contain a 12-adenine sequence down stream from the start codon of EGFP. The modified plasmid was transfected into Hela cells. The expression of EGFP in the transfected cells was visualized under a fluorescence inverted microscope and the image was taken with a digital camera.



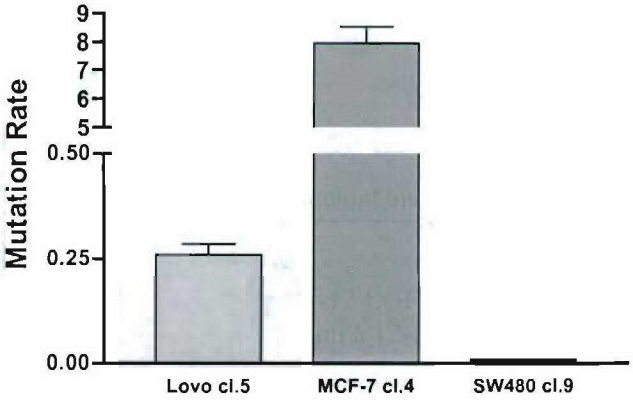


Fig. 14. Pre-sorted non-green cells from A11-EGFP stably transfected cell clones were cultured for 2 weeks. Cells were harvested and analyzed by flowcytometry. Mutation rate was calculated according to green cell percentage and cell population doubling time. The values are presented as mean±SEM from three determinations.

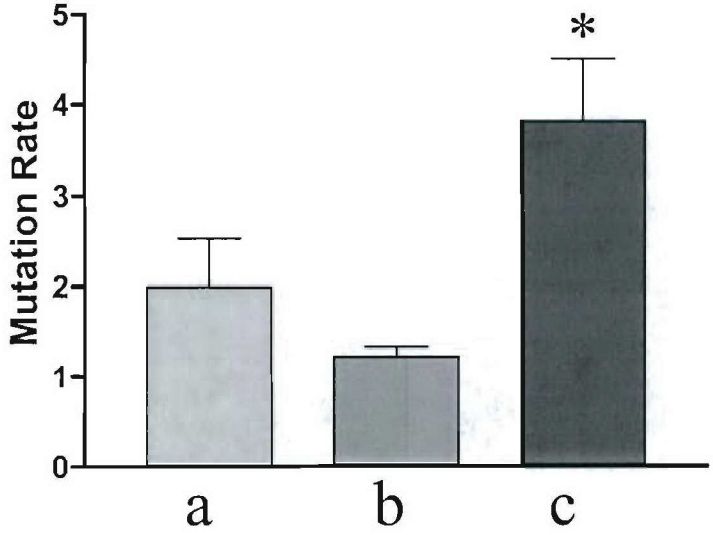


Fig. 15. Presorted non-green cells of A11-EGFP stable transfected MCF-7 clone were cultured in regular 10% FBS medium (a), phenol red free medium containing 10% charcoal/dextra-treated FBS without 17β-estradiol (b), or with 10^{-7} M 17β-estradiol (c) for 1 week. The percentage of green cells was analyzed by flowcytometry. The values are presented as means±SEM from triplicate measurements. The asterisk "*" denotes significant difference ($P<0.05$) between with or without 17β-estradiol treatment with one-way ANOVA.

KEY RESEARCH ACCOMPLISHMENTS

1. Developed a method that can effectively and conveniently measure DNA MMR activity in live cells.
2. Determined that ER positive human breast cancer cell lines have a significantly lower DNA MMR activity than ER negative breast cancer cells.
3. Determined that ectopic expression of ER in ER negative breast cancer cells inhibits MMR activity.
4. Determined that treatment with estrogen can further decrease MMR activity in ER positive cells.
5. Determined that the anti-estrogen, tamoxifen, can elevate MMR activity inhibited by estrogen.
6. Determined that the inhibition of DNA MMR by estrogen appears due to estrogen-induced repression of hMLH1 expression.
7. Developed a method that can conveniently measure DNA mutation rate in live cells.
8. Demonstrated that the 11-polyA-containing EGFP can be used to differentiate MMR proficient cell from MMR deficient cell.
9. Demonstrated that the treatment with estrogen can increase mutation rate in MCF-7 cells as reported by the reversion of mutated EGFP gene.

REPORTABLE OUTCOMES

1. Manuscript

   Lei, X., Y. Zhu, A. Tomkinson, and L-Z. Sun. Measurement of DNA mismatch repair activity in live cells. *Nucleic Acids Research* 32 (12): e100, 2004.

2. Abstracts

   Sun, L-Z. and X. Lei. High mutation rate in human estrogen receptor positive breast cancer cells. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Orlando, FL. Sept. 25-28, 2002. Abstract #P7-35.

   Lei, X. and L-Z. Sun. Estrogen Suppress the DNA Mismatch Repair Activity in Human Estrogen Receptor Positive Breast Canccer Cells. The 13th Annual Symposium on Cancer Research in San Antonio and South Texas, TX. Nov. 11, 2004. Abstract #27.

   Lei, X. and LuZhe Sun. Estrogen Suppresses the DNA Mismatch Repair Activity in Human Estrogen Receptor Positive Breast Canccer Cells. Proceedings of 96th Annual Meeting of American Association for Cancer Research, Abstract #1785. April 2005. Anaheim, CA.

3. Methods developed

   a) Measurement of DNA mismatch repair activity in live cells. This method has received a great deal of attention by the scientific community. The plasmids constructed for the method has been requested by fifteen laboratories inside and outside of the U.S.

   b) Measurement of DNA mutation rate in live cells. An expression plasmid of the enhanced green fluorescent protein was modified to contain a poly A tract mimicking the microsatellite DNA in mammalian cells.

4. Cell lines

   Stable cell lines transfected with the 11-A EGFP expression plasmid have been developed that can be used to measured DNA mutation rate with a convenient method. The cell lines include MCF-7, Lovo, and SW480.

5. Promotions

   During the award period, the PI, LuZhe Sun, was promoted to full professor with tenure and the postdoctoral fellow, Xiufen Lei, was promoted to Research Instructor.

PERSONNEL RECEIVING PAY FROM THE AWARD

LuZhe Sun, Ph.D. – Principle Investigator
Xiufen Lei, M.D., Ph.D. – Postdoctoral Fellow/Instructor (Research)
Tracy Gilmore, B.S. – Research Assistant
Jennifer Thy Nguyen, B.S. – Research Assistant
Thy Le, B.S. – Research Assistant

CONCLUSIONS

Defective DNA MMR system has been shown to cause microsatellite instability and gastrointestinal carcinogenesis (15). Since the mechanism(s) by which estrogen and its receptor cause breast carcinogenesis is not clear and microsatellite instability has been observed in breast cancer cells (16-18), we hypothesized that estrogen and ER may generate transient MMR deficient phenotype. To test our hypothesis, we needed to screen a number of ER+ and ER- cell lines. Since the current in vitro methods to measure MMR activity are tedious and often costly, we needed a more robust assay to accomplish our goal. Therefore, we developed a method to measure DNA MMR activity in live cells with the support from the current award. Since this method can quantitatively measure MMR activity in a live, single cell, it offers two potential utilities. First, it can be used to monitor changes of MMR activity in live cells as they are subjected to varying growth conditions. Hence, it should aid the study of whether and how MMR activity is regulated under different physiological and/or pathological conditions. Second, it may be used clinically for the diagnosis of MMR status in tumor cells. Using this method, we found that the ER+ breast cancer cell lines have significantly lower MMR efficiency than ER- cells. Treatment of ER+ cells with estrogen appeared to further reduce MMR efficiency, this can be reversed by the treatment with anti-estrogens such as tamoxifen. We also found that estrogen appears to inhibit the expression of hMLH1 and that ectopic expression of hMLH1 can reverse the inhibition of MMR activity by estrogen. Our results suggest that estrogen inhibits DNA MMR by suppressing hMLH1 expressing. To determine the consequence of the inhibition of MMR activity by estrogen on genetic stability, we investigated the effect of estrogen on microsatellite stability. We found that the mutation rate of an engineered microsatellite sequence was significantly increased by estrogen treatment. Our results thus far implies that estrogen and ER may inhibit DNA MMR activity in mammary epithelial cells to generate a MMR deficient phenotype, which in turn causes mutation of key genes involved in breast carcinogenesis.

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APPENDIX

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Measurement of DNA mismatch repair activity in live cells

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ABSTRACT

Loss of DNA mismatch repair (MMR) function leads to the development and progression of certain cancers. Currently, assays for DNA MMR activity involve the use of cell extracts and are technically challenging and costly. Here, we report a rapid, less labor-intensive method that can quantitatively measure MMR activity in live cells. A G–G or T–G mismatch was introduced into the ATG start codon of the enhanced green fluorescent protein (EGFP) gene. Repair of the G–G or T–G mismatch to G–C or T–A, respectively, in the heteroduplex plasmid generates a functional EGFP gene expression. The heteroduplex plasmid and a similarly constructed homoduplex plasmid were transfected in parallel into the same cell line and the number of green cells counted by flow cytometry. Relative EGFP expression was calculated as the total fluorescence intensity of cells transfected with the heteroduplex construct divided by that of cells transfected with the homoduplex construct. We have tested several cell lines from both MMR-deficient and MMR-proficient groups using this method, including a colon carcinoma cell line HCT116 with defective hMLH1 gene and a derivative complemented by transient transfection with hMLH1 cDNA. Results show that MMR-proficient cells have significantly higher EGFP expression than MMR-deficient cells, and that transient expression of hMLH1 alone can elevate MMR activity in HCT116 cells. This method is potentially useful in comparing and monitoring MMR activity in live cells under various growth conditions.

INTRODUCTION

The mismatch repair (MMR) system has been well studied in prokaryotes, especially in *Escherichia coli* (1–3). Nucleotides misincorporated during DNA replication that escape the proof-reading activity of DNA polymerase can be recognized by the MutS protein. Binding of MutS to the mismatch site leads to recruitment of other proteins, including MutL and MutH, and triggers a series of enzymatic reactions resulting in the removal of the misincorporated nucleotides. The MMR system appears more complicated in humans than in *E.coli* (3–5). Multiple homologs of MutS and MutL have been identified and cloned in human. Three important MutS homologs are hMSH2, hMSH6 and hMSH3. hMSH2 can heterodimerize with either hMSH6 or hMSH3, forming two different protein complexes, designated hMutSα or hMutSβ, respectively (6–8). Several MutL homologs including hMLH1, hPMS1, hPMS2 and hMLH3 have been shown to form heterodimers, such as hMLH1-hPMS2 (hMutLα), hMLH1-hPMS1 (hMutLβ) and hMLH1-hMLH3 (9–12).

MMR defects have been strongly associated with certain types of cancer, especially hereditary non-polyposis colorectal cancer (HNPCC) and sporadic colorectal cancer (13,14). More than 70% of HNPCC patients have been found to have germline mutations in either the *hMSH2* or the *hMLH1* gene, and a small percentage have defective *hPMS2* or *hPMS1* gene (15). More recently, germline mutations of *hMSH6* were identified in patients with hereditary colorectal cancer (16,17). Cancer cells from most HNPCC patients show a phenotype of replication error (RER^+) or microsatellite instability (MSI^+) with a high frequency of mutations in microsatellite sequences. While it is widely believed that MSI is a marker for defective MMR, a significant portion of patients with MSI^+ cancers do not have mutations of known MMR genes (13). In fact, several studies have shown that the most common mechanism causing MSI in sporadic colon cancer is transcriptional silencing of the *hMLH1* gene by methylation of the *hMLH1* gene promoter (18–21). A limited number of studies have also reported changes of expression of MMR genes or MMR activity during cell cycle progression, by growth factor stimulation, or under different growth conditions (22–24). While these studies suggest that MMR activity may be regulated, it is not clear what physiological or pathological conditions might result in reduced MMR activity in various types of cells. Furthermore, few studies have addressed the significance of altered MMR activity in carcinogenesis. One roadblock in addressing these issues appears to be the lack of a simple and effective assay to compare and monitor MMR activity in various types of live cells under various physiological and pathological conditions.

In this study, we have devised an assay utilizing the reversion of a modified start codon of the enhanced green fluorescent protein (EGFP) gene to quantitatively measure

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MMR activity in live cells. This approach has previously been used for measuring other types of DNA repair (25,26). Our results indicate that this assay can effectively distinguish MMR^- cells from MMR^+ cells. Also, using this method, we show that ectopic expression of hMHL1 in HCT116 cells significantly elevates MMR activity.

MATERIALS AND METHODS

Modification of an EGFP expression plasmid

Two modifications were introduced to plasmid pEGFP-N1 (Clontech). First, a DNA fragment (230 bp) encompassing the SV40 replication origin was deleted from pEGFP-N1 by digesting the plasmid with StuI and SexAI, purifying the large fragment, filling in the ends with Klenow (New England Biolabs) and circularizing the large fragment with ligase. Second, two restriction enzyme sites, EcoRV and NruI, were engineered around the start codon ATG of EGFP gene using a PCR-based site-directed mutagenesis method (27). Briefly, two primers, 5′-ggtggcgaccgggatatcccgggccc-3′ and 5′-atggtgagcaatcgcgaggagctgttc-3′, were designed in such a way that they can anneal to the adjacent positions on opposite strands of pEGFP-N1. The underlined sequences are the introduced restriction sites, EcoRV and NruI, respectively. The PCR reaction contained 20 pmol of both primers, 59 ng pEGFP-N1, 50 nmol dNTPs and 2.5 U cloned *Pfu* polymerase (Strategene) in 1× cloned pfu buffer. After 20 cycles (94°C for 30 s, 72°C for 13 min), the PCR product was purified by phenol/chloroform extraction and ethanol precipitation, and then dissolved in 20 µl T4 ligase buffer with 400 U of T4 ligase (New England Biolabs). The ligation reaction was incubated at 16°C for 2 h and then heated at 65°C for 20 min to inactivate T4 ligase. DNA was precipitated by ethanol, re-dissolved in 20 µl DpnI buffer and digested with 20 U DpnI (New England Biolabs) for 2 h to destroy the methylated, wild-type DNA template. One microliter of the digestion mixture was used to transform an *E.coli* strain, JM110, and several transformed clones were expanded. The DNA prepared from these clones was first checked with restriction analyses and then sequenced. We selected one clone with the desired mutations and named it p95-1.

Construction of heteroduplex and homoduplex plasmids

The p95-1 (3 µg) was digested with ApaI (Life Technologies), whose site is within the multiple cloning site of pEGFP-N1, and NruI (New England Biolabs) (Figure 1). The larger fragment was purified from an agarose gel with NA45 membrane (Schleicher & Schuell Inc). Purified DNA was equally divided into three aliquots and subjected to three parallel ligations to generate a T–G or G–G mismatched heteroduplex plasmid, a homoduplex plasmid and a negative control. To construct the T–G heteroduplex plasmid, two oligonucleotides, EGFP3-11 (5′-cgggatccaccggtcgccaccatggtgagcaatcg-3′) and EGFP3-12 (5′-cgattgctcaccgtggtggcgaccggtggatcccgggcc-3′) were phosphorylated by T4 polynucleotide kinase. The phosphorylated oligonucleotides (20 pmol each) were then mixed together in 20 µl T4 ligase buffer, heated at 92°C for 3 min, and allowed to slowly cool down to room temperature, forming a double-stranded oligonucleotide with a T–G mismatch. Of the oligonucleotide mixture, 2 µl was mixed with 1 µg of p95-1 larger fragment, 5 µl ligase buffer, 42 µl H_2O and 400 U T4 ligase, and incubated at 16°C for 10 min. The reaction was then further diluted by 4-fold with T4 ligase buffer and was

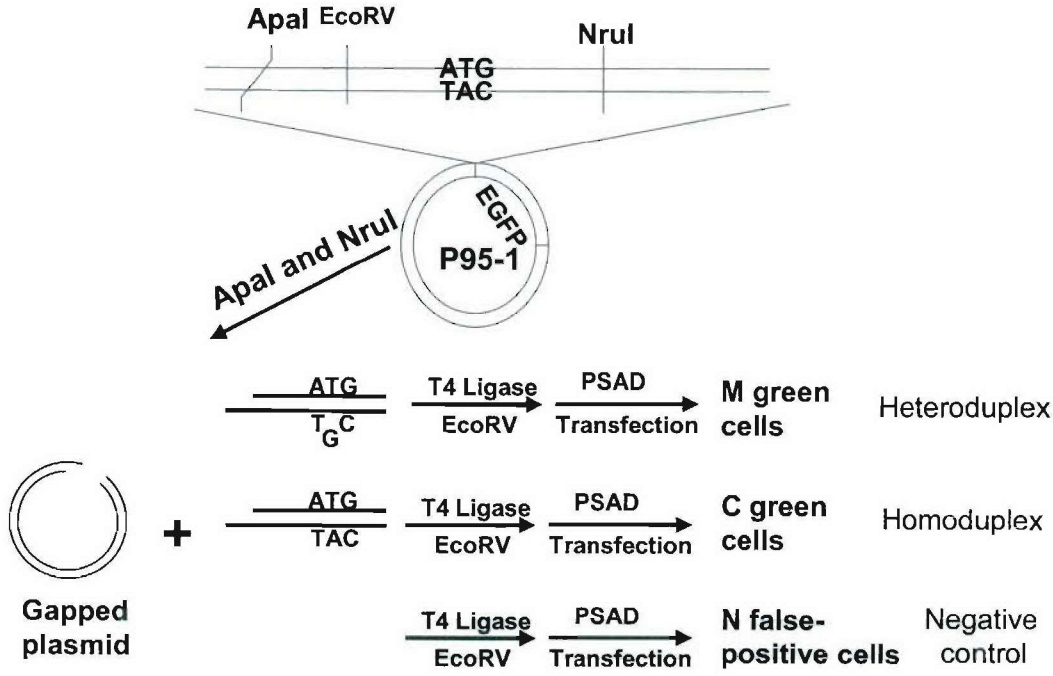

Figure 1. Flow chart of protocol for the quantitation of DNA MMR *in vivo*. MMR efficiency as measured with relative EGFP expression was calculated using the equation of $(M \cdot I_M - N \cdot I_N)/(C \cdot I_C - N \cdot I_N)$, where M, N or C is the percentage of green cells for heteroduplex, negative control or homoduplex transfection, respectively, and I_M, I_N or I_C is the mean fluorescence intensity of green cells for M, N or C, respectively. PSAD stands for plasmid-safe ATP-dependent DNase.

kept at 16°C overnight. A G–G heteroduplex plasmid was constructed by replacing the oligonucleotide EGFP3-12 in the T–G mismatch construction with oligonucleotide EGFP3-15 (5′-cgattgctcacgatggtggcgaccggtggatcccgggcc-3′). The homoduplex plasmid was constructed in the same way as the heteroduplex plasmid except that the oligonucleotide EGFP3-12 was replaced with oligonucleotide EGFP3-13 (5′-cgattgctcaccatggtggcgaccggtggatcccgggcc-3′). For the negative control, the ligation reaction was carried out in the absence of any primer.

After ligation, DNA was precipitated with ethanol. To destroy the residual parental p95-1 plasmid that might not be separated from the p95-1 larger fragment, we subjected the precipitated DNA to EcoRV digestion. Since the linear p95-1 large fragment and the circularized heteroduplex plasmid in the ligation mixture may undergo homologous recombination after being transfected into cells, we also digested the precipitated DNA with the plasmid-safe ATP-dependent DNase (PSAD) from Epicenter. Precipitated DNA from all three ligation reactions was resuspended in 50 µl PSAD buffer containing 20 U PSAD and 5 U EcoRV. After 30 min of digestion at 37°C, the reactions were heated at 80°C for another 30 min to inactivate the enzymes. Before transfection, a small aliquot of the reaction was subjected to electrophoresis in agrose gel to estimate DNA concentration.

Construction of nicked heteroduplex and homoduplex plasmids

The CMV promoter- EGFP gene cassette in pEGFP-N1 was subcloned into pGEM5Z(+) (Promega). This plasmid, pGEM5Z(+)-EGFP, was used to generate single-stranded circular DNA (ssDNA) containing the coding strand of EGFP with a helper phage (New England Biolab) according to the manufacturer's instruction. The ssDNA was annealed to BstXI-linearized pGEM5Z(+)-EGFP in 1.5-fold excess molar ratio to generate a homoduplex plasmid with a nick in the EGFP template strand located 692 bp 3′ upstream from the start codon. To generate a nicked heteroduplex plasmid with a G–G mismatch, we mutated the start codon in the EGFP template strand from 3′ TAC to 3′ TAG. Annealing of the ssDNA containing the coding strand of the wild-type EGFP with BstXI-linearized, mutated pGEM5Z(+)-EGFP produced a G–G mismatch at the start codon of EGFP with a nick located 694 bp 3′ upstream from the mismatch. After annealing, the mixture was digested with PSAD to degrade linearized DNA and ssDNA.

Cell lines and culture

The HCT116 human colon carcinoma cell line was kindly provided by Dr Michael Brattain and cultured in McCoy's 5A medium supplemented with bovine pancreas insulin, human transferrin, epidermal growth factor, pyruvate, vitamins, amino acids and antibiotics (28). LoVo, SW480 and HeLa were originally obtained from American Type Culture Collection. These four cell lines were adapted to McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), pyruvate, vitamins, amino acids and antibiotics (29). AA8, CHO EM9 and 46BR.1G1 cell lines were also cultured in this medium. HCT116+ch3 and HCT116+ch5 cell lines were kindly provided by Dr Minoru Koi. HCT116+ch3 was cultured in DMEM medium supplemented with 10% FBS and 325 µg/ml active G418 (Life Technologies). HCT116+ch5 was cultured in DMEM supplemented with 10% fetal calf serum and 6 µg/ml Blasticidin S (Calbiochem). Working cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and routinely checked for mycoplasma contamination.

Transfection

Lipofectamine and lipofectamine PLUS reagents (Life Technologies) were used to transfect DNA into cultured cells. Cells were plated on 60 mm dishes at densities ranging from 0.4×10^6 to 1×10^6 cells/dish depending on the cell type. Transfections were performed the following day according to the protocols from the manufacturer. In some experiments, a red fluorescent protein (RFP) expression plasmid (1 µg), pDsRed1-N1 (Clontech), was co-transfected with the homoduplex or heteroduplex EGFP plasmid (0.75 µg) to ascertain that the transfection efficiency of the two kinds of EGFP plasmid was similar. The cells were trypsinized 24 h after transfection, and resuspended in PBS at a concentration of 0.5×10^6 cells/ml. In our preliminary experiments, 24 h incubation yielded higher relative EGFP expression in HeLa cells than a shorter incubation period such as 8, 12 or 16 h. The number of green cells and the intensity of the fluorescence were then determined by flow cytometry.

Flow cytometry

From each transfection, 30 000 cells were counted and their green fluorescence intensity at 530 ± 30 nm wavelength was measured using FACSCalibur (Becton Dickinson). The laser is a 15 mW, 488 nm, air-cooled argon-ion laser. The marker to identify positive cells was set in such a way that 0.2% cells transfected with the negative control DNA were considered as positive.

Calculation of relative EGFP expression and statistical analyses

MMR efficiency in each cell line was measured with relative EGFP expression. It was calculated according to the formula, relative EGFP expression = $(M \cdot I_M - N \cdot I_N)/(C \cdot I_C - N \cdot I_N)$, where M, N and C are the percentages of green cells for heteroduplex, negative control and homoduplex transfection, respectively. I_M, I_N and I_C are the mean fluorescence intensities of positive cells for M, N and C, respectively. $N \cdot I_N$ was omitted in the calculation when it is negligible. When the red fluorescent protein expression plasmid was co-transfected with the heteroduplex or homoduplex plasmid, the percentage of red cells was used in the calculation to normalize transfection efficiency. Since the same amount of heteroduplex or homoduplex plasmid is transfected into each cell line, the maximal value of relative EGFP expression should be 100%. When comparing relative EGFP expression between two groups, we used one-tailed student t-test. When comparing multiple groups, we used ANOVA and Newman–Keuls test (30).

Western blot

Cells recovered from the flow cytometer were rinsed twice with ice-cold PBS and lysed in 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 µg/ml

phenylmethylsulfonyl fluoride and 1% Nonidet P-40. Equal amounts of proteins (50 μg of each extract) were separated by SDS–PAGE and transferred to a nitrocellulose membrane (Amersham Corp.). Membranes were blocked in TBST [100 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.05% Tween-20] containing 5% nonfat powder milk, and then incubated with mouse monoclonal anti-hMLH1 antibody (Oncogene Science) at a final concentration of 1.0 μg/ml. After three washes with TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at 1:1000 dilution (New England Biolabs) and washed again. Antigen–antibody complexes were detected by chemiluminescence according to the manufacturer's instructions (New England Biolabs). Following the same procedure, living colors DsRed monoclonal antibody (Clontech) was used to detect the red fluorescent protein, whereas living colors A.v. peptide antibody–HRP conjugate (Clontech) was used to detect green fluorescent protein.

RESULTS

Description of the method

The method used to measure DNA mismatch repair activity *in vivo* is shown schematically in Figure 1. In comparison with the parental plasmid pEGFP-N1, p95-1 does not have an SV40 replication origin, preventing its replication in cells expressing SV40 large T antigen. Moreover, it has two more restriction enzyme sites, EcoRV and NruI, flanking the start codon of EGFP. After digestion with ApaI and NruI, the larger fragment (gapped plasmid) was isolated by gel purification. The gapped plasmid was then incubated in three parallel ligation reactions. In the first reaction, the gapped plasmid was ligated to a heteroduplex oligonucleotide that is identical to the small ApaI/NruI DNA fragment except that it has a T–G mismatch and no EcoRV site. In the second reaction, the gapped plasmid was ligated to a homoduplex oligonucleotide that has no EcoRV site. No oligonucleotide was added to the third reaction. The ligation mixtures were then subjected to digestion by EcoRV and PSAD DNase. EcoRV linearized any residual parental plasmid p95-1. PSAD DNase was used to digest linear DNA molecules so that the possibility of homologous recombination after transfection between the gapped plasmid and the ligated, heteroduplex circular plasmid to generate wild-type EGFP sequence was eliminated.

To confirm the formation of circular homoduplex and heteroduplex plasmids, the ligation products were digested with NcoI restriction endonuclease. There are three NcoI restriction sites throughout p95-1, one of which encompasses the start codon of EGFP. After NcoI digestion and agarose gel electrophoresis, the homoduplex plasmid should yield 3 bands of 2285 bp, 1904 and 317 bp, while the heteroduplex plasmid should only yield 2 bands of 2602 bp and 1904 bp because NcoI cannot recognize and cut the mismatched site at the start codon. As shown in Figure 2A, NcoI digested the homoduplex plasmid into two larger fragments (2.3 and 1.9 kb), which were visible, and one smaller fragment (317 bp), which was not visible after photography. On the other hand, NcoI digestion of the ligation product of the gapped plasmid and the mismatched oligonucleotide only generated two fragments of 2.6 and 1.9 kb confirming the formation of the heteroduplex plasmid.

To compare DNA MMR activity measured with a circular heteroduplex plasmid versus a nicked, circular heteroduplex plasmid, we also constructed nicked, circular homoduplex and heteroduplex plasmids as described in the Materials and Methods. Similar to the intact, circular plasmids, digestion of the nicked, circular homoduplex plasmid with NcoI generated three expected fragments, 3.3, 1.0 and 317 bp, whereas digestion of the nicked, circular heteroduplex plasmid only generated two fragments, 3.3 and 1.3 kb (Figure 2B).

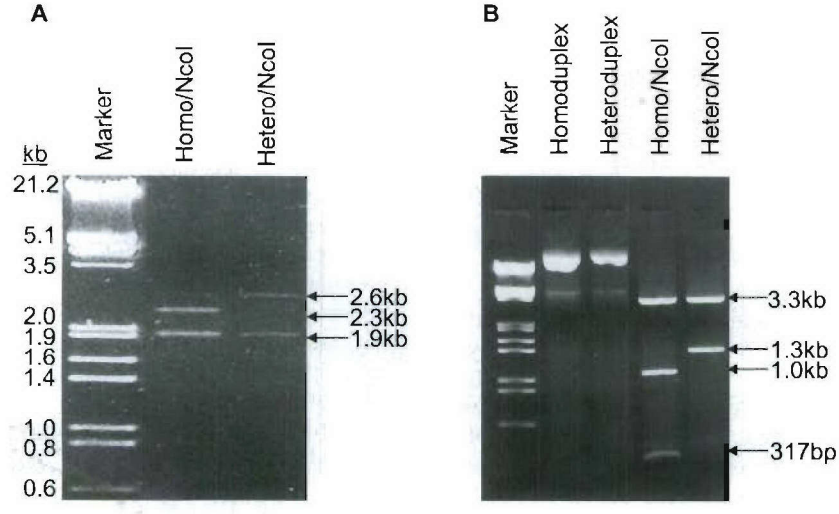

Figure 2. Restriction analysis of ligation products. (**A**) Digestion of un-nicked homoduplex and heteroduplex plasmids with NcoI. The ligation products was digested with NcoI endonuclease and then electrophoresed in 1% agarose gel. The homoduplex plasmid was digested into three fragments of 2.3, 1.9 and 317 bp. The ethidium bromide staining of the 317 bp fragment was too weak to be photographed. A faint band at 2.6 kb is likely the incompletely digested product that generates 2.3 and 317 bp fragments. The heteroduplex plasmid was digested into two fragments of 2.6 and 1.9 kb. (**B**) Digestion of nicked homoduplex and heteroduplex plasmids with NcoI. Homoduplex was digested into three fragments of 3.3, 1.0 and 317 bp, whereas the heteroduplex was digested into two fragments of 3.3 and 1.3 kb.

MMR$^+$ cell lines showed significantly higher relative EGFP expression than MMR$^-$ cell lines

To determine whether the heteroduplex plasmid can be used to measure DNA MMR efficiency, we compared relative EGFP expression in two MMR deficient cell lines HCT116 and LoVo, and two MMR proficient cell lines HeLa and SW480. HCT116 cells harbor a nonsense mutation in exon 9 in hMLH1 gene (31–33). LoVo does not express hMSH2 protein (7,34). Nicked or un-nicked homoduplex or heteroduplex EGFP plasmid was co-transfected with RFP plasmid into the cells. Green and red fluorescent images of the transfected cells were taken before the cells were lifted and analyzed with flow cytometry. As shown in Figure 3A, EGFP was largely co-expressed with RFP in HeLa cells transfected with either homo- or heteroduplex EGFP plasmid. In contrast, many of RFP-expressing HCT116 cells did not show EGFP expression when transfected with the heteroduplex EGFP plasmid while the cells co-transfected with the homoduplex EGFP plasmid and RFP plasmid co-expressed the two fluorescent proteins. Similarly, flow cytometry analysis of HeLa and HCT116 cells after homoduplex or heteroduplex plasmid transfection showed that while the EGFP positive populations of HeLa cells transfected with either homo- or heteroduplex were very similar, the EGFP positive population of HCT116 cells transfected with the heteroduplex was considerably lower than that of HCT116 cells transfected with the homoduplex indicating a deficiency in MMR (Figure 3B). The percentage of gated EGFP- and RFP-positive cells and mean fluorescence intensity of EGFP-positive cells from the flow cytometry analysis in Figure 3B are presented in Table 1 for the calculation of relative EGFP expression. Repeated and independent analyses with flow cytometry showed that the relative EGFP expression of the two MMR$^+$ cell lines, HeLa and SW480, was significantly ($P < 0.001$) higher than that of the two MMR$^-$ cell lines, HCT116 and LoVo (Figure 4A). To confirm that the lower number of green cells and reduced intensity of green fluorescence in MMR- cells was due to lower expression levels of EGFP, we quantified EGFP and RFP protein levels in HCT116 and HeLa cells that were transfected with nicked homoduplex or heteroduplex EGFP plasmid by western blotting. The EGFP level in the heteroduplex plasmid-transfected HCT116 cells was noticeably lower than that in the homoduplex plasmid-transfected HCT116 cells (Figure 4B). In contrast, the EGFP expression levels in the homoduplex and heteroduplex plasmid-transfected HeLa cells were very similar. Density analysis of the EGFP bands indicated that the EGFP expression from the heteroduplex plasmid was about a quarter of that from the homoduplex plasmid in HCT116 cells (Figure 4C).

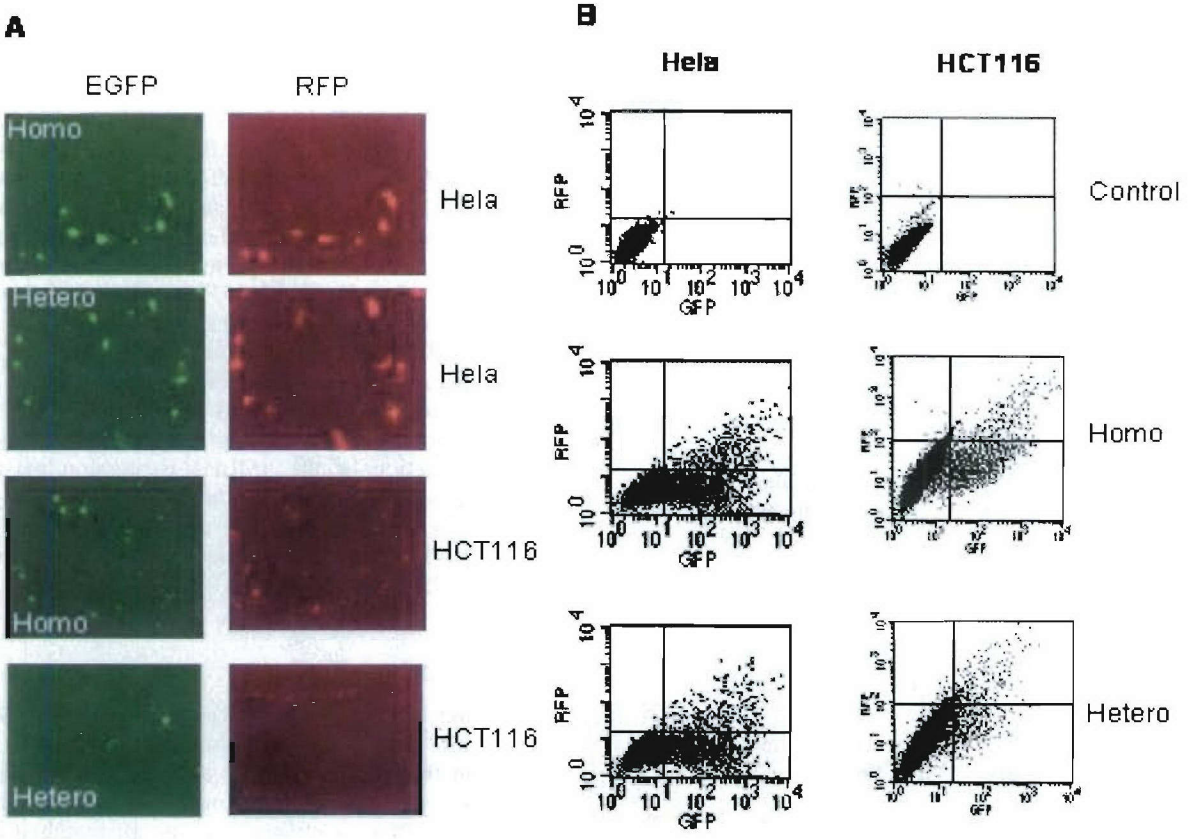

Figure 3. Heteroduplex EGFP plasmid is effectively repaired in HeLa cell, but not in HCT116 cell. (**A**) Fluorescent images of HeLa and HCT116 cells after co-transfection of nicked homo- or heteroduplex EGFP plasmid and RFP plasmid (pDsRed1-N1). (**B**). A typical flow cytometry data set of HeLa and HCT116 cells after co-transfection with nicked homo- or heteroduplex EGFP plasmid and RFP plasmid.

Table 1. Calculation of relative EGFP expression using flow cytometry data corresponding to Figure 3B

	Quadrant	Control % Gated	Mean	Homoduplex % Gated	Mean	Heteroduplex % Gated	Mean	Relative EGFP expression[a]
HeLa	UL	0.03	9.37	0.13	9.36	0.14	9.44	1829.99/1918.87 = 0.954
	UR	0.02	32.19	4.46	751.36	3.73	672.30	
	LL	99.95	3.43	72.44	5.37	77.44	5.31	
	LR	0.00	***	22.97	237.55	18.70	244.62	
HCT116	UL	0.03	6.35	0.19	10.81	0.36	11.77	234.69/1353.42 = 0.173
	UR	0.01	793.45	1.64	766.25	1.59	155.75	
	LL	99.95	3.20	89.62	4.90	94.55	4.70	
	LR	0.01	20.82	8.54	142.87	3.50	60.00	

[a]EGFP expression from homoduplex or heteroduplex plasmid transfection was calculated using the following equation.

$$\frac{(\text{LR \% gated} \times \text{LR mean}) + (\text{UR \% gated} \times \text{UR mean})}{\text{UL \% gated} + \text{UR \% gated}}$$

Relative EGFP expression = EGFP expression from heteroduplex/EGFP expression from homoduplex.

Relative EGFP expression from a nicked heteroduplex was similar to that from an un-nicked heteroduplex in live cells

Since a single-strand nick was shown to be necessary for strand-specific repair of a mismatched base pair in nuclear extracts of human cells *in vitro* (35), we tested whether this is also the case in live cells. A homoduplex or heteroduplex EGFP plasmid with a single-strand nick was constructed such that the nick was placed in the template strand of EGFP. Preferential repair of the G–G mismatch in the template strand will result in the correct expression of EGFP and should consequently increase the relative EGFP expression. However, the presence of the nick had no significant effect on relative EGFP expression in MMR-proficient HeLa and SW480 cells, and MMR-deficient HCT116 cells (Figure 4A). It is possible that the nicked plasmid is efficiently ligated prior to the action of the MMR enzymes. To test this hypothesis, we measured relative EGFP expression in cell lines with reduced levels of either DNA ligase I (46BR.1G1 cell) or DNA ligase IIIα (CHO EM9 cells) activity. As shown in Figure 5, the relative EGFP expression was significantly higher in the CHO EM9 cells that have reduced levels of DNA ligase IIIα protein and activity (36). This suggests that the transfected nicked plasmid DNA is efficiently ligated by DNA ligase IIIα.

The advantage of using the un-nicked circular heteroduplex plasmid DNA is that various kinds of mismatched heteroduplex oligonucleotides can be ligated into the gapped plasmid (see Figure 1) for MMR studies. To extend the results obtained with the G–G mismatched heteroduplex, we also measured relative EGFP expression from a T–G mismatch in an un-nicked circular plasmid in the four cell lines. The overall MMR efficiency of the T–G mismatch was lower than that of the G–G mismatch for all of the four cell lines (compare Figure 6A with Figure 4A). However, the ratios of relative EGFP expression on either G–G or T–G mismatch between MMR^- cells and MMR^+ cells remained basically the same. The mean ratios of HCT116's MMR efficiency to HeLa cells' MMR efficiency on G–G and T–G are 34.7 and 39.7%, respectively. Therefore, T–G and G–G mismatches can both be used to differentiate MMR^+ and MMR^- cells.

Complementation of MMR genes significantly elevated MMR activity in MMR^- cells

HCT116 does not express functional hMLH1 protein and therefore has an impaired MMR system. Previous studies have shown that transfer of chromosome 3, which carries a copy of the normal *hMLH1* gene, into HCT116 cells restores MMR activity to cell extracts (37). This effect is chromosome specific, since transfer of chromosome 2 or 5 did not elevate MMR activity in cell extracts. To confirm that MMR complementation can also be observed *in vivo*, we tested our method using HCT116+ch3 and HCT116+ch5 cells. As expected, the relative EGFP expression of HCT116+ch5 cells was similar to that of HCT116 cells (Figure 6B). In contrast, HCT116+ch3 had a significantly higher ($P < 0.01$) relative EGFP expression, which was similar to those of HeLa and SW480 cells as shown in Figure 6A. Thus, the presence of chromosome 3, but not chromosome 5, significantly elevates *in vivo* MMR activity in HCT116 cells.

Complementation of the MMR defect in HCT116 cells by the *hMLH1* gene or cDNA alone has not been demonstrated because healthy HCT116 clones were not produced (38). To examine whether the expression of hMLH1 alone is sufficient to increase MMR activity in HCT116 cells, we transiently co-transfected HCT116 cells with an *hMLH1* cDNA expression plasmid and either the homoduplex or G–G heteroduplex plasmid. Expression of hMLH1 protein was detected by western blotting 48 h after transfection (Figure 7A). Even though the transfection efficiency was <10%, we were able to detect a significant ($P < 0.02$) increase of MMR activity as reflected with an increase of relative EGFP expression in the hMLH1-transfected cells when compared with the MMR efficiency of the cells transfected with an empty vector, pRC/CMV (Figure 7B). Transfection of the hMLH1 expression plasmid into SW480 cells had no effect on the relative EGFP expression. These results indicate that our method can detect restored MMR activity by the expression of hMLH1 protein in HCT116 cells.

DISCUSSION

The methods that are currently used to measure MMR activity can basically be grouped into two categories. The first one is an *in vitro* method using cell-free extracts (35,39). Nuclear or cytoplasmic extracts from various cells are incubated with mismatched DNA substrates, such as mismatched M13mp2 phage DNA, and MMR activity is measured either directly by endonuclease restriction analyses and/or DNA sequencing or indirectly by further transforming MMR^- bacteria with the

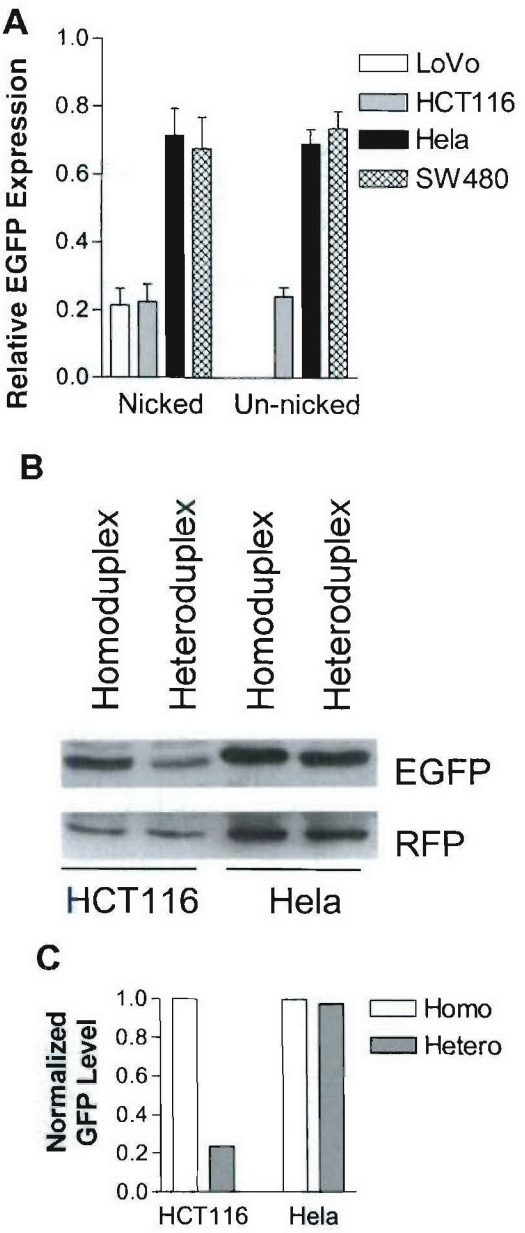

Figure 4. MMR$^+$ cell lines showed significantly higher relative EGFP expression than MMR$^-$ cell lines. (**A**) Nicked or un-nicked homo- or heteroduplex (with a G–G mismatch) plasmids were transfected into the cell lines depicted. Percentage of green cells and their mean intensity from each transfection were obtained from flow cytometry, and relative EGFP expression was calculated as described in Materials and Methods. Each column represents mean ± SEM from five independent measurements for HCT116 and HeLa, and from three independent measurements for LoVo and SW480. (**B**) HCT116 and HeLa cells were co-transfected with nicked homoduplex or heteroduplex EGFP plasmid and RFP plasmid. Twenty-four hours later, the cells were harvested and western blot was done as described in Materials and Methods. (**C**) The density of EGFP bands was measured with Image-Pro Plus software (Media Cybernetics), normalized with that of the corresponding RFP bands, and plotted in an arbitrary unit.

repaired phage DNA mixture. The need for a relatively large number of cells for the preparation of the cell-free extract makes this type of assay expensive. Furthermore, it cannot be utilized to measure the dynamics of MMR activity in live cells in response to the changes in the intracellular and/or

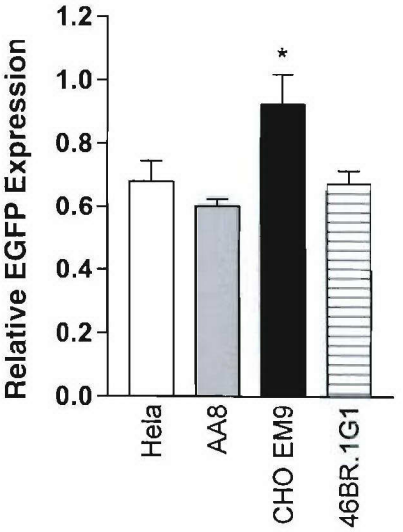

Figure 5. DNA ligase IIIα deficient cell line, CHO EM9, showed higher relative EGFP expression. Nicked homo- and heteroduplex plasmids were transfected into the cell lines depicted in the figure. CHO EM 9 was derived from the parental cell AA8, which is ligase IIIα-proficient. Relative EGFP expression was presented as mean ± SEM from three independent experiments. The asterisk indicates significant difference ($P \leq 0.05$) from other mean values with student t tests.

extracellular environment. For the second category, mismatched or heteroduplex DNA, including both plasmid and viral DNA, is introduced into cells and then later retrieved for analysis (40–43). In this case, the retrieval process is usually laborious and tedious. The method we describe here can effectively differentiate MMR$^+$ cells from MMR$^-$ cells and is relatively fast and inexpensive. More importantly, it can be used to monitor the regulation of MMR activity in live cells.

In our equation for the calculation of the relative EGFP expression, we incorporated the mean intensity of green fluorescence. This is because multiple copies of the heteroduplex plasmid might be transfected into a single cell and because the MMR$^-$ cells transfected with the heteroduplex plasmid consistently displayed a small number of green cells with low fluorescence intensity (Table 1). We assumed that an MMR$^+$ cell should correct more mismatched copies of the heteroduplex plasmid than an MMR$^-$ cell if they are transfected with multiple copies of the heteroduplex plasmid. We also assumed that fluorescence intensity is proportional to the copy number of the corrected EGFP gene. Therefore, by using an intensity-weighted formula, the calculated MMR efficiency reflects the percentage of correctly repaired heteroduplex DNA copies instead of the percentage of cells that correctly repaired the heteroduplex DNA.

Theoretically, the MMR efficiency of MMR$^-$ cells should be around zero. However, our study as well as previous studies using *in vitro* assays for MMR$^-$ cells (34,44) or embryo fibroblasts from MMR gene knockout mouse (45) have detected a significant residual MMR activity. With our method, one contributing factor was that some EGFP expression was independent of the start codon. When a modified EGFP expression plasmid with a fixed mutation at its start codon was transfected into HeLa and HCT116 cells, a small number of green cells with low intensity were observed. After flow cytometry

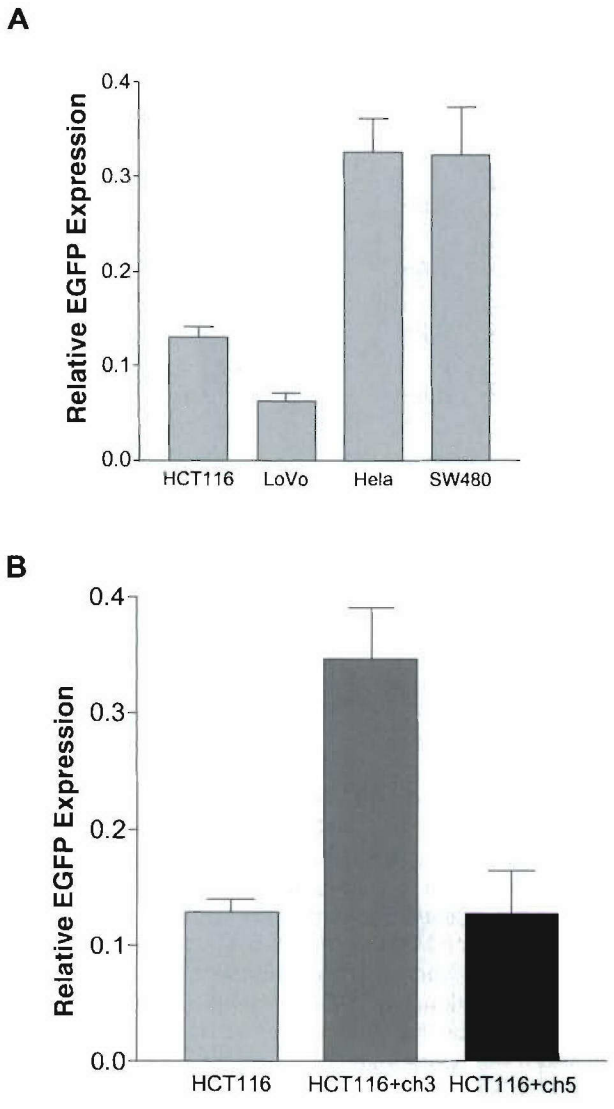

Figure 6. Relative EGFP expression from a T–G mismatch and the effect of chromosome 3 complementation on MMR in HCT116 cell. (**A**) The same amount of un-nicked heteroduplex (with a T–G mismatch), homoduplex, and negative control DNA were separately transfected into the depicted cell lines. Percentage of green cells and their mean intensity from each transfection were obtained from flow cytometry, and relative EGFP expression was calculated as described in Materials and Methods. Each column represents mean ± SEM from seven independent measurements for HCT116, HeLa and SW480, and six independent measurements for LoVo. According to Newman–Keuls multiple comparison test, the MMR efficiencies of HCT116 and LoVo are significantly different from those of HeLa or SW480 ($P < 0.001$), whereas the MMR efficiency between the two MMR- cell lines or between the two MMR$^+$ cell lines is not significantly different from each other ($P > 0.05$). (**B**) Comparison of T–G MMR in HCT116, HCT116+ch3 and HCT116+ch5 cells. The relative EGFP expression in HCT116 is 0.13 ± 0.01 from 7 independent measurements. The relative EGFP expression in HCT116+ch3 and HCT116+ch5 is respectively 0.35 ± 0.05 and 0.13 ± 0.04 from 5 independent measurements. The data are presented as mean ± SEM. According to Newman–Keuls multiple comparison test, the MMR efficiency of HCT116+ch3 is significantly higher from those of HCT116 and HCT116+ch5 ($P < 0.01$).

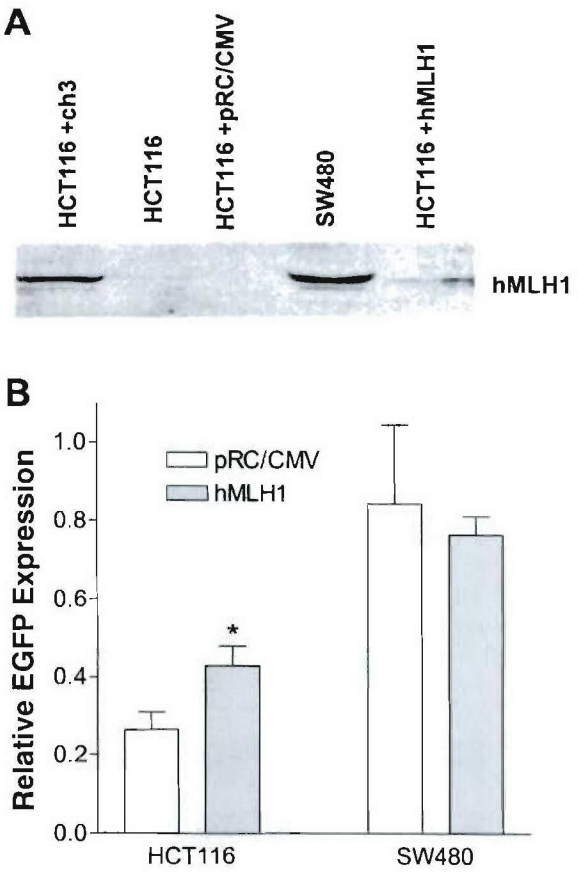

Figure 7. Ectopic expression of hMLH1 increased MMR efficiency in HCT116 cells. (**A**) Detection of hMLH1 protein with western blotting. HCT116 and SW480 were co-transfected with 1 μg pCMV-MLH1 or 0.6 μg pRC/CMV (control) and the homo- or heteroduplex (with a G–G mismatch) plasmid. Forty-eight hours later, the transfected cells were lifted and analyzed with flow cytometry. Cells recovered from the flow cytometer, along with HCT116+ch3 and SW480 cells, were lysed. Proteins were extracted, separated on 8% SDS-PAGE, and immunoblotted by a hMLH1 antibody. (**B**) Effect of hMLH1 transfection on G–G MMR in HCT116 and SW480 cells. Relative EGFP expression was calculated after flow cytometry analysis as described in the Materials and Methods. According to student *t*-test, HCT116 transfected with hMLH1 expression plasmid has a significant higher ($P < 0.02$) MMR efficiency (0.43 ± 0.05) than HCT116 transfected with pRC/CMV (0.26 ± 0.05). Each column represents mean ± SEM of three independent measurements.

analysis, we found that the AUG-independent expression accounted for 5% of the calculated relative EGFP expression. As such, ~25–50% of MMR activity measured with our method in HCT116 cells was apparently due to AUG-independent expression of the EGFP because the relative EGFP expression in HCT116 cell is ~10–20% as shown in the figures. Another possibility for the residual MMR activity may be due to other cellular DNA repair mechanisms such as base excision repair, nucleotide excision repair or even an unknown repair system (46,47). However, it is likely that these other repair mechanisms are less effective than the MMR system. In support of this idea, when a heteroduplex DNA was incubated with cell extract for a short duration of 15 min, the repair efficiency of cell extract from MMR-proficient cells was found to be 10-fold or greater than that of cell extract from MMR-deficient cells (34,48). In the current study, the transfected heteroduplex was inside the cell for ~24 h to allow for the repair of the mismatch and subsequent expression of EGFP. While this relatively long period of time was optimal for maximal repair and expression of EGFP in the MMR-proficient cells, an

appreciable amount of the heteroduplex was apparently also being repaired by systems other than MMR during this time period in the MMR-deficient cells. Altogether, both AUG-independent GFP expression and alternative repair systems could account for the limited difference of MMR efficiency (3-fold) between MMR-proficient and MMR-deficient cells.

A key feature of the MMR system is that repair is directed to the newly synthesized strand (1). In the repair assay with cell extracts, a nick can act as the strand-discrimination signal (35). Unexpectedly, nicked and intact plasmid DNAs were repaired with equal efficiency in our *in vivo* assay. One possible explanation for this observation is that the transfected nicked plasmid is rapidly ligated and the resultant intact circular plasmid subsequently acted upon by the MMR pathway. In support of this model, *in vivo* MMR was more efficient in the DNA ligase III-deficient Chinese hamster ovary cell line, EM9. The observation that DNA ligase III—but not DNA ligase I—deficiency increases *in vivo* MMR efficiency is consistent with a study showing that DNA ligase III is the predominant DNA nick-joining ligase in proliferating mammalian cells (49).

For the G–G mismatch, we obtained relative EGFP expression of 65 and 70% for HeLa and SW480 cells, respectively. However, it decreased to 48 and 47% for HeLa and SW480, respectively, when they were transfected with the T–G heteroduplex. Since previous studies have shown that both G–G and T–G mismatches are optimal substrates for the MMR system (23,35,50), it is not likely that the repair of T–G mismatch was less efficient than the repair of G–G mismatch in these two cell lines. The difference may be explained by the fact that the T–G mismatch can be repaired by both MMR and a DNA glycosylase-initiated repair system (50). When we constructed the T–G mismatch, we deliberately engineered the G in the antisense (template) strand. Since the T–G DNA glycosylase always converts T–G to C–G, its repair of the T–G mismatch would create a fixed mutation in the EGFP gene that prevents expression. We suspect that the T–G glycosylase competes with the MMR system for the T–G substrates, leaving the MMR system with fewer substrates to act upon than in the case of G–G mismatch. Thus, while both the T–G and G–G mismatched substrates can be used to distinguish MMR^+ from MMR^- cells, the calculated relative EGFP expression from G–G mismatch may be a more reliable indicator of the MMR activity in live cells.

The low MMR activity in the hMLH1-deficient cell line HCT116 was complemented after introduction of a normal human chromosome 3, which carries the *hMLH1* gene (37). Our *in vivo* MMR assay reiterated this result. However, it has not been unequivocally demonstrated that the correction of the MMR defect in HCT116 cells is due to the expression of *hMLH1* alone or *hMLH1* plus additional genes on the chromosome 3. Investigators have attempted to transfect and re-express *hMLH1* cDNA to complement the MMR deficiency. In one study, stable expression of hMLH1 protein in Mlh1-deficient mouse embryonic fibroblasts was shown to complement DNA mismatch repair defects (51). However, when transfecting *hMLH1* or *hMSH2* into MMR^- human cell lines, investigators have difficulties in obtaining stably transfected clones. This is apparently due to the fact that overexpression of *hMSH2* or *hMLH1* can induce apoptosis in both MMR^+ and MMR^- cells (52), and overexpression of *hMLH1* can also suppress cell growth (38). Since MMR activity is scored in individual, live cells within a relatively short period of time using our method, we reasoned that we should be able to detect the effect of transiently expressed *hMLH1* on MMR efficiency. Indeed, the MMR efficiency was significantly elevated 48 h after transient co-transfection of *hMLH1* expression plasmid with the heteroduplex plasmid into HCT116 cells. Thus, our method can detect the restoration of MMR activity in the MMR-deficient cell.

Since the method we describe here can quantitatively measure MMR activity in a live single cell, it offers two potential utilities. First, it can be used to monitor changes of MMR activity in live cells as they are subjected to varying growth conditions. Hence, it should aid the study of whether and how MMR activity is regulated under different physiological and/or pathological conditions. Second, it may be used clinically for the diagnosis of MMR status in tumor cells. It appears highly feasible that a small number of tumor cells from a primary culture can be microinjected or transfected with the homoduplex or heteroduplex plasmid and the MMR activity in each injected/transfected cell be scored by quantitative fluorescence microscopy. We are currently exploring these possibilities.

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