Cell Death Caused by Single-Stranded Oligodeoxynucleotide-Mediated Targeted Genomic Sequence Modification

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Targeted gene repair directed by single-stranded oligodeoxynucleotides (ssODNs) offers a promising tool for biotechnology and gene therapy. However, the methodology is currently limited by its low frequency of repair events, variability, and low viability of "corrected" cells. In this study, we showed that during ssODN-mediated gene repair reaction, a significant population of corrected cells failed to divide, and were much more prone to undergo apoptosis, as marked by processing of caspases and PARP-1. In addition, we found that apoptotic cell death triggered by ssODN-mediated gene repair was largely independent of the ATM/ATR kinase. Furthermore, we examined the potential involvement of the mismatch repair (MMR) proteins in this "correction reaction-induced" cell death. Result showed that while defective MMR greatly enhanced the efficiency of gene correction, compromising the MMR system did not yield any viable corrected clone, indicating that the MMR machinery, although plays a critical role in determining ssODN-directed repair, was not involved in the observed cellular genotoxic responses.

Introduction

Targeted gene repair mediated by single-stranded oligodeoxynucleotides (ssODNs) offers a promising tool for biotechnology and gene therapy (Alexeev et al., 2002; Agarwal et al., 2003; Liu et al., 2003). This approach has vast potentials, and in particular, to correct or introduce subtle mutations in desirable genomic DNA loci without introducing unwanted exogenous sequences. Despite its numerous advantages, its utility as a universal methodology for clinical therapeutic and research purposes is currently limited by its low frequency of repair events, variability amongst different experimental settings, and most importantly, low viability of "corrected" cells.

While much effort has been devoted to increasing the rates of ssODN-based sequence correction and the understanding of its mechanisms, little is known with regard to the fate of the corrected cells. Previously, Olsen et al. reported that the majority of corrected CHO-mEGFP cells were arrested in G2/M phase, and that only 1%–2% cells were capable of forming viable colonies 24 hours after ssODN transfection. Although treatment of cells with caffeine, an ATM/ATR

inhibitor, attenuated the checkpoint arrest and a population of cells entered mitosis, the number of viable colonies did not increase (Olsen et al., 2005a). Using HeLa-F5 and HEK-mEGFP cells as model systems, we previously reported a similar G2/M arrest phenomenon in corrected cells, and this inhibition of cell cycle progression was not noticeably affected by pretreatment of ATM/ATR inhibitors caffeine or pentoxifylline (Wang et al., 2006). More recently, the presence of phosphorylated H2AX was shown to be associated with this cell cycle arrest (Olsen et al., 2009).

The mechanism involved in ssODN-based gene repair has been postulated to occur as a 2-step process, namely DNA strand pairing and DNA repair. During the strand pairing event, ssODN are introduced into cells and aligned with its complementary DNA sequence at the target locus. It is now clear that this step is catalyzed by enzymes involved in homologous recombination (HR), such as RAD51 (Yanez and Porter, 1999; Thorpe et al., 2002) and XRCC (Olsen et al., 2003). This step creates a 3-stranded intermediate, D-loop (Igoucheva et al., 2003). The event subsequent to the DNA strand pairing is the processing of these recombination intermediates,

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leading to modifications of the target sequence. There is an increasing body of evidence suggesting that during DNA replication, D-loops can be enveloped or bypassed by the replication fork, generating a strand with a mismatched base pair complex and an uncorrected copy of the gene (Olsen et al., 2005b; Parekh-Olmedo et al., 2005; Radecke et al., 2006). The mismatched base pair complex might remain and even be passed onto daughter cells. Notably, it has been demonstrated that the aberrant processing of mismatches can lead to mismatch repair (MMR)-dependent G2/M checkpoint activation and apoptosis (Kaina et al., 1997; Yan et al., 2003; Stojic et al., 2004).

The eukaryotic MMR system comprises five major subunits, including hMSH2, hMSH3, hMSH6, hMLH1, and hPMS2. hMutS-α (heterodimer of hMSH2 and hMSH6) binds to mismatches and small insertion/deletion loops, whereas hMutS-β (heterodimer of hMSH2 and hMSH3) recognizes larger insertion/deletion loops (Genschel et al., 1998; Guerrette et al., 1998). Following the recognition step, both hMutS dimers recruit another heterodimeric complex, MutLα, which is composed of hMLH1 and hPMS2. The formation of hMutS–hMutL complex activates the subsequent repair process. In addition to their functions in repair of mismatches, MMR proteins also play an important role in recognizing and signaling DNA damage responses (Palombo et al., 1995; Lin et al., 2004). However, the specific mechanisms by which MMR proteins activate cytotoxic responses remain elusive.

In this study, we characterized the cell death that occurred during gene correction reaction mediated by ssODN. We also examined the possibility that the MMR pathway might function as activators of DNA damage responses associated with gene correction events. We found that the low viability of corrected cells caused by ssODN-mediated gene correction reaction correlated with increased apoptosis, which occurred via a caspase-dependent but an ATM/ATR-independent pathway. Finally, we found that the MMR proteins limited the efficiency of targeted gene correction, but did not trigger the repair-associated genotoxic response.

Materials and Methods

Cell lines

To detect ssODN-mediated gene repair, pmEGFP (Wu et al., 2005) with a mutant EGFP expression cassette was linearized by *Apa*LI and transfected into HeLa, HEK293T-Lα (kindly offered by Prof. Josef Jiricny) (Cejka et al., 2003), or LoVo cells (kindly offered by Prof. Stefan Zeuzem) (Brieger et al., 2002). The cells were cultured for 24 hours before being seeded with the density of 100 cells/well in a 96-well plate and cultured in selective medium (supplemented with 600 μg/mL G418) for 2 weeks. At least 10 resistant clones were picked and examined for the ability of gene repair. Cell clones HeLa-F5 (Wu et al., 2005), HEK293T-Lα-mEGFP, and LoVo-13-mEGFP cells were selected for further experiments. In the case of HEK293T-Lα-mEGFP, before transfection with the ssODN, cells were grown for at least 8 days in medium containing Tet Systems Approved FBS (Clontech, Palo Alto, CA) either in the absence or presence of 50 ng/mL doxycycline (Sigma, St. Louis, MO) to induce or repress expression of the central MMR protein, hMLH1.

Target gene correction assay

Cells were seeded at 2×10^5 in 12-well plates and grown for 24 hours before transfection. For ssODN-mediated gene repair assay, a 25-mer phosphothioate-modified (underlined) antisense ssODN (E6: CCTTGCTCACCATGGTGGCGGAATT) was transfected into HeLa-F5, HEK293T-Lα-mEGFP, or LoVo-13-mEGFP cells. For each well, 0.8 μg DNA was transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). zVAD-fmk (50 μM), caffeine (3 mM), or pentoxifylline (3 mM) (Sigma, St. Louis, MO) was added to culture medium before transfection, where indicated. To measure gene repair efficiency, cells were trypsinized 36 hours post-transfection and directly subjected to flow cytometry (Beckman Coulter, Fullerton, CA) using a 488-nm argon-ion laser. Single cell population was ensured by FS/SS gating during analysis.

Analysis of apoptosis

Apoptosis was detected by performing the 4′6-diamidino-2-phenylindole (DAPI) staining, the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay, and the poly (ADP-ribose) polymerase (PARP) cleavage assay.

For DAPI staining, harvested cells were washed with PBS, incubated in PBS containing 0.1% Triton X-100 for 10 minutes. And then cells were spinned down and resuspended in 4% paraformaldehyde solution containing 10 μg/mL DAPI (Sigma). The morphology of the cells' nuclei was observed using a fluorescence microscope (Olympus, Center Valley, PA). Apoptotic nuclei were identified by the fragmented morphology of nuclear bodies. More than 200 green cells and 500 normal cells were counted.

TUNEL was performed as described (Li and Darzynkiewicz, 1995). In brief, after transfection, cells were harvested at the indicated time points, washed, fixed, permeabilized, and then resuspended in TdT reaction solution containing 80 μM BrdUTP (Sigma) and 12.5 U TdT (Roche, Basel, Switzerland). To distinguish corrected cells (GFP fluorescence) from apoptotic cells, cells were labeled with PE-conjugated anti-BrdU monoclonal antibody (BD, Franklin Lakes, NJ). Similarly, PARP cleavage assay was carried out with anti-PARP p85 fragment antibody (Promega, Madison, WI) (Li and Darzynkiewicz, 2000) and Alexa Fluor 633-labeled secondary antibody (Invitrogen, Carlsbad, CA). For both TUNEL and PARP cleavage assays, about 300,000 cells were analyzed on flow cytometer (Beckman Coulter, Fullerton, CA). All assays were carried out in triplicate.

Western blot

Protein extracts were prepared and subjected to western blot analysis as described previously (Liu et al., 2007).

Clonogenic survival assay

Cells were seeded at 8×10^5 per 60-mm dishes and grown for 24 hours to reach 60%–80% confluence. Thirty-six hours after transfection with ssODN, corrected cells were sorted by FACS, a total of 500 corrected (green) cells were seeded onto 10-cm dishes and allowed to attach overnight. Colonies were observed and counted after 7 to 14 days under the confocal microscope (Olympus).

Sequencing of genomic DNA

Genomic DNA was extracted from survival fluorescent colonies. A nested polymerase chain reaction (PCR) was used to amplify specific regions containing the target locus, followed by subcloning, and sequencing as described previously (Wu et al., 2005).

Statistical analysis

Data was analyzed with unpaired 2-tailed Student's *t*-test or 1-way ANOVA followed by Tukey's multiple comparison test with GraphPad Prism software (San Diego, CA). Data were expressed as mean $\pm$ SD derived from at least 3 independent experiments. Differences were considered significant at $P < 0.05$.

Results and Discussion

Corrected cells were more prone to undergo apoptosis than uncorrected cells

An increasing number of studies have shown that the majority of corrected cells does not divide, and these cells died eventually (Olsen et al., 2005a, 2009; Ferrara and Kmiec, 2006). To corroborate these findings, we first measured the time course of the corrected cells. As shown in Figure 1A, the percentage of corrected/green HeLa-F5 cells was maximal at 36 hours (0.13%) after transfection with ssODN,

and decreased to 0.027% by 96 hours post-transfection. Similarly, using HeLa-F5 or HEK293T-Lα-mGFP as model systems, microscopic analyses revealed a substantial number of corrected green cells began to round up at 72 hours and eventually detached from the plate. This indicated that ssODN-mediated correction reaction was associated with cell death.

Our previous cell cycle analysis of corrected/green cells showed that the vast majority of the green (89.34%) cells were arrested at G2/M phase and failed to recover normal proliferation 24 hours after transfection (Wang et al., 2006). Transfection with either a control ssODN or a wild-type egfp plasmid did not affect cell cycle progression (data not shown), indicating that the G2/M arrest was not a result of the introduction of ssODN or the expression of the functional egfp gene, but was specifically happened in the corrected cells. So the cause of the cell cycle arrest herein might be the activation of DNA damage response pathways induced by the ssODN-mediated correction reaction.

Recently, DNA damages, particularly DNA double-strand breaks (DSBs), have been directly detected in corrected cells (Olsen et al., 2009). The permanent existence of DNA damages led to cell death including apoptosis (Plesca et al., 2008). Several attempts have been made to detect apoptosis in various cell types (Olsen et al., 2003, 2009; Ferrara et al., 2007). However, apoptosis was only observed in CHO-mGFP cells when the cells were transfected with branched ssODN (Olsen et al., 2003). To determine whether the low
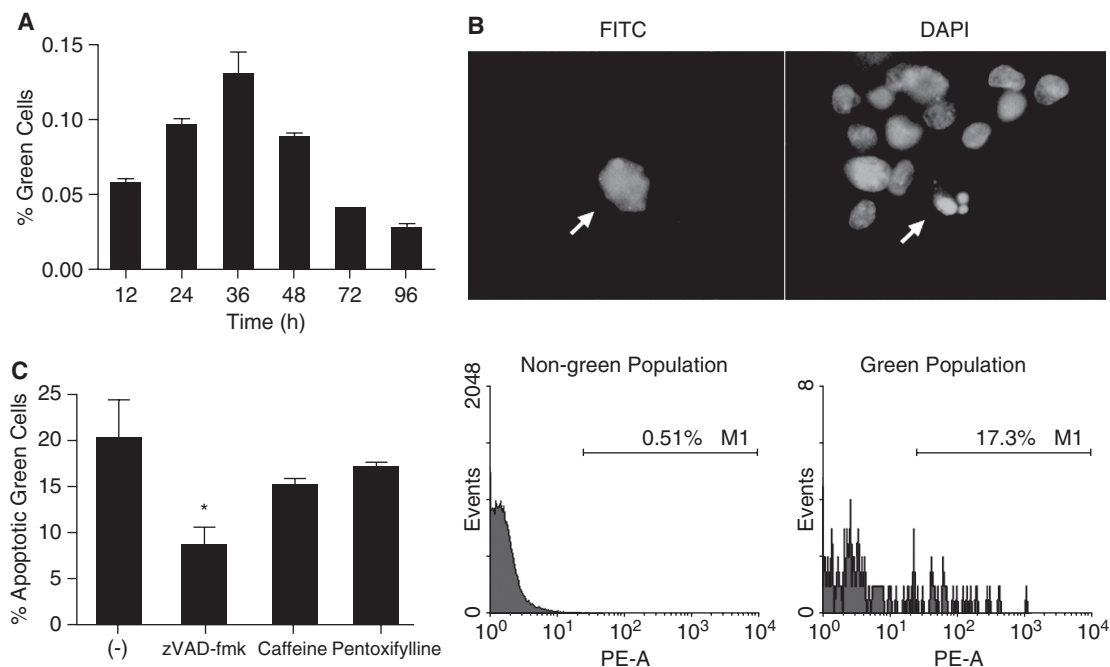

FIG. 1. (**A**) Time course of green/corrected cells produced in single-stranded oligodeoxynucleotides (ssODN)-mediated target gene repair. HeLa-F5 cells were transfected with the ssODN-E6 and the number of green/corrected cells was quantified by flow cytometry at the indicated time points after transfection. (**B**) Measurements of apoptosis in green cells. HeLa-F5 cells were analyzed by 4′6-diamidino-2-phenylindole (DAPI) staining (*upper panel*) and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay (*lower panel*) 12 hours and 48 hours, respectively, after transfection with the ssODN-E6. (**C**) Effects of caspases or ATM/ATR inhibitions on apoptosis caused by ssODN-mediated targeted gene repair. HeLa-F5 cells were pretreated with zVAD-fmk (50 μM), caffeine (3 mM), or pentoxifylline (3 mM) for 1 hour and then transfected with ssODN-E6. Apoptosis was measured 48 hours after transfection using TUNEL assay. *$P < 0.05$ vs. control. Data were representative of 3 independent experiments.

viability of corrected cells was related to apoptosis, we first evaluated the formation of apoptotic bodies using DAPI staining, which is characteristic for apoptotic cell death (Fig. 1B). By direct counting, 17.23% green cells showed signs of morphological nuclear damage and formation of apoptotic body as early as 12 hours after introduction of corrective oligonucleotides, which was significantly higher than that of non-green cells (1.34%). To further verify apoptosis induced by ssODN-mediated correction reaction, TUNEL assays were performed. As shown in Figure 1C, only 0.5% total cells underwent apoptosis, whereas >20% green cells were TUNEL-positive (Fig. 1B) 48 hours after transfection. These results indicated that ssODN-corrected cells were much more prone to undergo apoptosis than uncorrected cells. Our data also suggested that activation of apoptosis might be a very early event.

The apoptosis of corrected cells was caspase-dependent and ATM/ATR-independent

To determine the involvement of caspase in the observed apoptosis, zVAD-fmk, a pan-caspase inhibitor (Slee et al., 1996), was employed to examine its ability to prevent apoptosis of green cells. zVAD-fmk treatment significantly increased the number of green cells by around 1.5 times (data not shown). When green population was selected for analysis, untreated and zVAD-fmk-pretreated cells showed about 20.1% and 8.41% apoptosis index, respectively (Fig. 1C). These data demonstrated that zVAD-fmk significantly reduced the apoptotic percentage of corrected cells by 58%, indicating that the majority of apoptotic green cells may undergo caspase-dependent apoptosis. The finding was verified by PARP cleavage assay. Early in apoptosis, PARP is cleaved by caspases, primarily by caspase-3. Hence this assay can be used to determine the activation of caspase-3 (Li and Darzynkiewicz, 2000). Results showed that the respective values for cleaved PARP were 8.97% for green cells and 0.92% for total cells.

ATM/ATR-dependent G2/M arrest has been suggested, at least in part, to be responsible for the abnormal cell cycle profile of green CHO-mGFP cells (Olsen et al., 2005a). Likewise, Ferrara et al. demonstrated that ATM/ATR downstream mediators Chk1 and/or Chk2 were specifically activated in corrected DLD-1 cells (Ferrara and Kmiec, 2006). However, in HeLa-F5 cells, we did not observe obvious release of arrested green cells by caffeine and pentoxifylline treatments (Wang et al., 2006). Consistently, exposure of HeLa-F5 cells to these 2 ATM/ATR inhibitors slightly but not significantly protected green cells from apoptosis (Fig. 1C), implying ATM/ATR-mediated pathways does not play an important role in this apoptotic cell death.

hMLH1 deficiency increased gene correction efficiency, but not affected the viability of corrected cells

Our interest in exploring a role of MMR was based on the notion that the tolerance to MMR of a base mismatch is the prerequisite of the targeted sequence alteration. And the aberrant processing of mismatches can lead to MMR-dependent G2/M checkpoint activation and apoptosis (Kaina et al., 1997; Yan et al., 2003; Stojic et al., 2004).

It was reported that hMLH1-deficient cells were less susceptible to apoptosis induced by lipoplatin than hMLH1-proficient cells (Fedier et al., 2006). To examine the possibility that MMR proteins function as activators of genotoxic responses to cell death induced by ssODN-mediated gene repair, we first isolated a clone of HEK293T-Lα-mEGFP cells with the megfp gene stably integrated into the chromosome of HEK293T-Lα cells. The expression of hMLH1 in this cell line can be controlled by doxycycline (Cejka et al., 2003). When HEK293T-Lα-mEGFP cells were treated with doxycycline for 8 days, the expression of hMLH1 was significantly reduced (Fig. 2A). To measure ssODN-mediated gene repair efficiency, hMLH1-deficient or -proficient cells were transfected with ssODN-E6 and were subjected to FACS analysis. A relatively low but reproducible repair frequency (0.02%) was observed in hMLH1-proficient cells. It has been shown that a defective MMR system would increase the repair efficiency (Dekker et al., 2003; Olsen et al., 2005b, 2009; Parekh-Olmedo
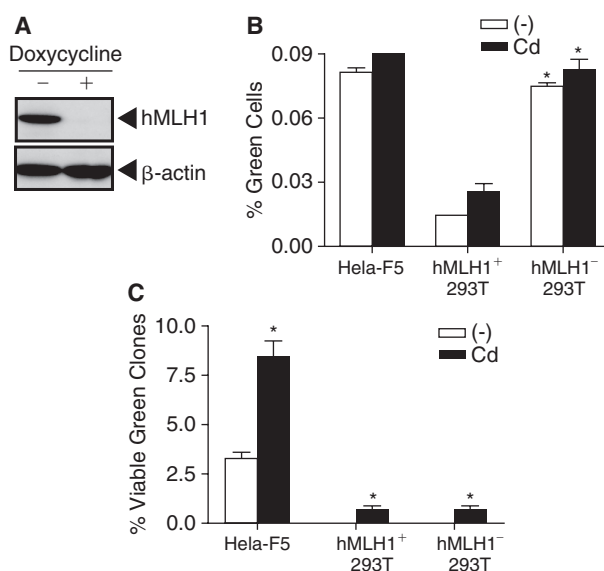

FIG. 2. (A) hMLH1 expression was silenced by doxycycline. HEK293T-Lα-mGFP cells were cultured for 8 days in the absence or presence of 50 ng/mL doxycycline, and hMLH1 expressions were measured with MLH1 polyclonal antibody. Blot shown is representative of at least 3 independent experiments. (B) Effects of zcadmium treatment or hMLH1 silencing on targeted gene repair efficiency. HeLa-F5, hMLH1-proficient, or hMLH1-deficient cells were incubated with or without cadmium (0.5 μM) for 1 hour and then transfected with ssODN-E6. The numbers of green cells were quantified by flow cytometry 36 hours after transfection. HEK293T-Lα-mGFP cells were labeled as 293T for short. *P < 0.05 vs. hMLH1-proficient HEK293T-Lα-mGFP cells. Data were representative of 3 independent experiments. (C) Effects of cadmium treatment or hMLH1 silencing on viability of green/corrected cells. HeLa-F5, hMLH1-proficient, or hMLH1-deficient HEK293T-Lα-mGFP cells were incubated with or without cadmium (0.5 μM) for 1 hour, followed by transfection with the ssODN-E6. Green colonies were counted by clonogenic survival assay after 7 days of continuous culture. *P < 0.05 vs. control. Data are representative of 3 independent experiments.

et al., 2005; Radecke et al., 2006). As expected, comparing with hMLH1-proficient cells, a 5.4 times higher repair frequency was obtained in hMLH1-deficient cells. However, in clonogenic survival assay, neither hMLH1-deficient nor -proficient cells could proliferate into visible colonies from at least five independent attempts (Fig. 2C). Results indicated that hMLH1 is not responsible for the observed cell death induced by ssODN-mediated gene repair.

Cadmium treatment did not affect the viability of corrected cells

It is known that the heavy metal cadmium can inhibit MMR by interfering with the binding of MSH2 and MSH6 complexes to mismatched DNA, and by inhibiting ATP hydrolysis of MSH6 (Clark and Kunkel, 2004). To assess if cadmium might prevent the cell death as described earlier, we next treated HeLa-F5 and HEK293T-Lα-mEGFP cells with 0.5 μM cadmium 1 hour prior to transfection. After 36 hours co-incubation with cadmium, green cells were sorted by FACS

FIG. 3. (**A**) Colony formation of viable green cells. With cadmium treatment, green HeLa-F5 cells (*upper panel*) or hMLH1-deficient HEK293T-Lα-mGFP cells (*lower panel*) were imaged at ×20 original magnification after 7 days of continuous culture. Colonies shown are representatives of at least 3 independent experiments. (**B**) Sequencing of mgfp gene fragments from viable green clones of HeLa-F5 or hMLH1-deficient HEK293T-Lα-mGFP cells. Single-stranded oligodeoxynucleotide-E6 was designed to be complementary to the untranscribed strand except for a "T–T" mismatch at the target site. The position of the spontaneous mutation that results in a fake start codon is underlined.

and seeded in 10-cm dishes with fresh medium containing the same concentration of cadmium. Unlike the effects of MLH1 deficiency, cadmium treatment did not significantly increase the repair efficiency (Fig. 2B), but enhanced the viability of green cells. In HeLa-F5 cells, there was a significant increase in the number of viable green clones in cadmium-treated cells compared with control cells ($P < 0.05$) (Figs. 2C, 3A). In the case of HEK293T-Lα-mEGFP cells, after 7 days of continuous cultures, 2–3 out of 500 green cells formed viable colonies in cadmium-treated cells (Figs. 2C, 3A). As described previously (Wu et al., 2005), the target sequence was indeed repaired when the green cells were directly subjected to sequence analysis after sorting. Nevertheless, sequence analysis revealed that the megfp genes of all viable green clones, including both HeLa-F5 and HEK293T-Lα-mEGFP cells, were not corrected at the desired site (Fig. 3B). An "A-T" point mutation was found at 10-bp downstream of the target site. This single point mutation resulted in start codon for a significant part of the megfp gene, causing it to be transcribed and translated into a functional GFP polypeptide missing 3 amino acids (Met-Val-Ser) at its N-terminus. The defective repair system induced by cadmium treatment likely increased the spontaneous mutation rate in these cells. As a result, the "A-T" single point mutation was induced and caused the cells to turn green. Furthermore, these spontaneous mutants likely survived because the mutation might have bypassed or did not trigger the DNA damage responses. In addition, in Fig. 3B, a normal "A" signal was found beneath the mutated "T" one. Considering 2 copies of megfp genes were stably integrated in the chromosome of HeLa-F5 cells (Yin et al., 2005), the suggested spontaneous mutation might only occur in 1 copy of them, leaving the other copy unchanged.

Accordingly, MMR proteins might not be required for the herein cellular genotoxic responses. This result was reminiscent to those using LoVo-mEGFP cells, which were generated by stably integrating the megfp gene into the genome of MSH2-deficient LoVo cells (Brieger et al., 2002). Although a routine repair frequency of 0.05% was obtained 36 hours after transfected with ssODN-E6, no green colony was observed in clonogenic survival assay (data not shown).

Taken together, in this ssODN-mediated gene repair event, activation of toxicity in corrected cells proceeded at least through the rapid activation of apoptosis and the cell cycle arrest. Neither ATM/ATR kinases nor MMR proteins play an important role. The mechanism underlying this cell death phenomenon is under investigation.
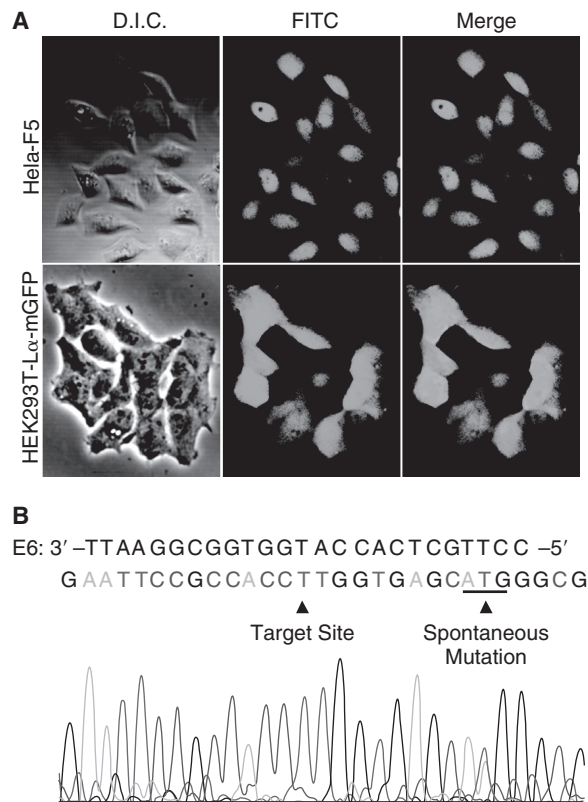
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