Gene Ther Mol Biol Vol 9, 445-456, 2005

Caffeine affects the level of gene repair in mammalian cells; implications for a role of DNA replication in the correction of single base mutations

Research Article

Julia U. Engstrom and Eric B. Kmiec*

Department of Biological Sciences, Delaware Biotechnology Institute, University of Delaware, Newark, DE 19716

***Correspondence**: Eric B. Kmiec, Department of Biological Sciences, Delaware Biotechnology Institute, University of Delaware, Newark, DE 19716; Tel: 302-831-3420; Fax: 302-831-3427; e-mail: ekmiec@udel.edu

Key words: repair, caffeine, oligonucleotides, cell cycle, replication, DLD-1

Abbreviations: Fluorescent, (FAM); homologous recombination, (HR); single-stranded DNA, (ssDNA)

Received: 31 August 2005; Revised: 15 November 2005;
Accepted: 22 November 2005; electronically published: December 2005

Summary

Single stranded DNA oligonucleotides have been used to direct the correction of point mutations in mammalian cells. The introduction of a single-stranded oligonucleotide sets in motion a cascade of events that eventually leads to a stalling of DNA replication forks, which enables the oligo-mediated correction event to occur more readily. This is due to the fact that cells in S phase are more amenable to gene correction and a role for DNA replication in the gene repair reaction has been established. Here, we develop a mechanistic view of how cell cycle arrest might influence the gene repair reaction using the radiosensitizing agent, caffeine, as a tool. First, we demonstrate that ATM activation is enhanced and sustained by caffeine then we identify multiple roles for caffeine in the modulation of the gene repair reaction. Finally, we show that caffeine-treated cells retain the oligonucleotides for longer periods of time, perhaps enabling the oligo to assimilate into the target site more readily. Our results may define ATM activation upon the entry of the oligonucleotide as a likely block to the proliferation of corrected cells.

I. Introduction

Targeted gene repair is a process that leads to the genetic alteration of a mammalian genome. While the pathway is not fully elucidated, the first phase involves the homologous alignment and assimilation of a short single-stranded oligonucleotide at a specific DNA sequence (Andersen et al, 2002). This DNA pairing event results in the formation of a transient three stranded structure containing a single mismatched base pair (Drury and Kmiec, 2003). The mismatch is resolved using DNA repair and/or DNA replication pathways, leading to the conversion of a mutant base to wild type in at least one allele (Brachman and Kmiec, 2005) (Parekh-Olmedo et al, 2005). A number of factors have been shown to influence the frequency and accuracy of the nucleotide exchange event including the transcriptional activity of the gene (Liu et al, 2002; Igoucheva et al, 2003), the polarity of the target strand (Hu et al, 2005) and the *presence or absence* of proteins involved in mismatch repair (Gamper et al, 2000; Rice et al, 2001; Dekker et al, 2003; Kren et al, 2003; Pierce et al, 2003). The frequency of gene repair has also been found to increase when the level of homologous recombination activity is elevated, either through exogenous over-expression of RAD51 (Thorpe et al, 2002) or by the introduction of DNA damage (Ferrara et al, 2004; Ferrara and Kmiec, 2004; Wu et al, 2005). In the latter case, agents such as etoposide (VP16) or camptothecin that induce double-strand breaks seem to stimulate the frequency several fold. Recently, we showed that the mechanism of stimulation relies more on the presence of stalled or slowed DNA replication forks in response to the breakage than to the breakage event itself (Brachman and Kmiec, 2005).

Homologous recombination, in response to DNA damage, is initially regulated through the activation of the ATM protein, an event that is known to be blocked by the radio-sensitizing agent, caffeine (Ferrara et al, 2004; Ferrara and Kmiec, 2004; Sarkaria et al, 1999). The activation of ATM is also seen when single-stranded DNA is transformed into mammalian cells, as free ends induce the DNA damage response (Nur-E-Kamal et al, 2003; Olsen et al, 2003, 2005; Parekh-Olmedo et al, 2005).

Thus, it is not surprising that as part of the gene repair reaction, the oligonucleotide activates ATM in the absence of chromosomal damage. This response is blocked quite effectively by caffeine when present prior to the introduction of the oligonucleotide, resulting in reduced levels of gene correction (Ferrara and Kmiec, 2004). Paradoxically, when caffeine is added *after* the oligonucleotide enters the cell, the level of gene repair remains constant or is elevated.

Caffeine has a plethora of effects on cells and cellular metabolism. It increases the influx of external Ca^{+2} by binding to L-type channels on plasma membranes and it also causes an emptying of internal Ca^{+2} stores by interacting with Ryanodine Receptors on the ER (see Zucchi and Ronca-Testoni, 1997 for review). The endogenous levels of Ca^{+2} ions are known to regulate cell cycle progression, G2/M transition and exit from mitosis (Kahl and Means, 2003; Munaron et al, 2004). In addition, caffeine inhibits the *in vitro* protein kinase activity of ATM and ATR, with an IC_{50} of 0.2 and 1.1mM, respectively (Sarkaria et al, 1999), and concentrations greater than 3mM are known to inhibit DNA synthesis directly (Murnane et al, 1981).

We have been studying the regulation of gene repair by the activation or suppression of the DNA damage response pathway and showed recently that ATM is activated by the intracellular accumulation of the oligonucleotide, extending the work of Nur-E-Kamal et al, 2003). In the present paper, we expand our studies on caffeine in the hope of gaining more insight into how the correction process itself is regulated. We uncover a number of reaction parameters affecting cell cycle arrest and progression and define several roles for caffeine in sustaining the levels of gene repair.

II. Materials and methods

A. Cell line and culture conditions

DLD-1 cells were purchased from American Type Cell Culture (ATCC, Manassas, VA). The integrated DLD-1 clone one (DLD-1-1) was obtained by integrating the pEGFP-N3 vector containing a single point mutation (TAG) in the chromophore region of the eGFP gene, and "clone one" was isolated through serial dilution and by Southern blot analysis, was shown to contain 2-4 copies of the eGFP gene (Hu et al, 2005). For these experiments, cells were grown in RPMI 1640 medium supplemented with 2 mM glutamine, 4.5 g/l glucose, 10 mM HEPES, 1 mM sodium pyruvate, and supplemented with 10% FBS. Cells were maintained at 37 °C and 5% CO_2 and kept under selection by the addition of 200 μg/ml G418 (Gibco, Invitrogen Co., Carlsband, CA) to the media.

B. Oligonucleotides designs

EGFP3S/47NT and EGFP3S/47T-FAM (IDT, Coralville, IA) are single stranded DNA oligonucleotides 47 nucleotides long containing three phosphorothioate linkages at both the 5' and 3' ends (**Figure 1**). The 47NT was designed to be complimentary to the non-transcribed strand and contains a mismatch at the site of the desired base change; the 47T oligo is complimentary to the transcribed strand and has been previously shown to promote minimal levels of gene correction (Hu et al, 2005). The 6-FAM™ labeled oligonucleotide has a 6-carboxylfluorescein (single isomer derivative of fluorescein) dye attached to the 5' end of the EGFP3S/47T oligonucleotide.

Figure 1. *Sequence of wild type and mutant eGFP gene and targeting oligonucleotides.* The sequence of the wild-type and mutant eGFP genes are presented with the targeted codon in bold and the position of the mutation in red. Also shown are the DNA sequences of the correcting 47-mer oligonucleotide (EGFP3S/47NT), which is directed to the non-transcribed strand, and the 47-mer oligonucleotide (EGFP3S/47T), which is directed to the transcribed strand; the 47T has a conjugated 6-FAM™ to the 5' end. The location of the mismatch to the mutant eGFP gene is in red and asterisks indicate the position of the phosphorothioate linkages; all the oligos are depicted in a 5' to 3' orientation. The correcting oligonucleotide converts the mutant stop codon to a tyrosine, which restores the expression of eGFP in corrected cells.

C. eGFP gene targeting and oligonucleotide retention

For the eGFP targeting experiments, DLD-1 cells were washed with PBS, trypsinized, harvested by centrifugation and re-plated at a density of $1.5x10^6$ cells per 100 mm dish, 24 h prior to targeting. Following this incubation, cells were washed with PBS, trypsinized, centrifuged, and re-suspended in serum-free medium at a concentration of $2x10^6$ cells/100 µl; 100 µl of the cell suspension was transferred to a 4 mm gap cuvette (Fisher Scientific, Pittsburgh, PA). The EGFP3S/47NT oligonucleotide was added at a concentration of 5 µM and the cells were electroporated (LV, 250 V, 13 ms, 2 pulses, 1 s interval) using a BTX Electro Square Porator™ ECM830 apparatus (BTX, Holliston, MA). The cells were then transferred to a 60 mm dish containing fresh medium supplemented with 10% FBS and incubated for 24 h at 37 °C before harvesting for FACS analysis. For oligonucleotide retention, cells were prepared as described above except 5 µM of the EGFP3S/47T-FAM oligo was added in place of EGFP3S/47NT, to ensure that any fluorescence was derived from the fluorescein and not due to correction events, and cells were electroporated as described. After electroporation, cells were plated in a 100 mm dish containing fresh medium supplemented with 10% FBS and incubated for 16-72 h at 37 °C before harvesting for FACS analysis

D. Treatment of DLD-1 cell cultures with caffeine

Caffeine was obtained from Sigma-Aldrich (St. Louis, MO.) with a stock solution prepared in distilled water to a final concentration of 200 mM and added to incubating cells at a final concentration of 4 mM. The term "pre-treatment" indicates addition of caffeine at the start of the 24 h incubation prior to electroporation; "post-treatment" refers to the addition of caffeine to the 60 mm or 100 mm dishes following electroporation.

E. Flow cytometry analysis

eGFP fluorescence of corrected cells and fluorescence of FAM-labeled oligonucleotides were measured by a Becton Dickinson FACS calibur flow cytometer (Becton Dickinson, Rutherford, NJ.). For eGFP fluorescence, cells were harvested 24 h after electroporation (unless otherwise indicated) and resuspended in FACS buffer (5% BSA, 2 mM EDTA, 2 µg/ml propidium iodide in PBS). 50,000 cells were analyzed for each and the cells that were GFP positive and PI negative were scored as corrected cells. For FAM-labeled oligonucleotides, cells were harvested at the indicated time periods and were prepared for FACS analysis like the eGFP samples. Settings for the FACS calibur were determined as previously described (Ferrara et al, 2004).

F. Confocal microscopy

Presence of FAM-labeled oligonucleotide was confirmed by confocal analysis. Following electroporation of the EGFP3S/47T-FAM oligonucleotide, cells were seeded onto microscope slides and treated with 4 mM caffeine for 24 and 40 h in a 37 °C incubator. The cells were washed once with PBS and fresh medium was added as well as 5 µg/ml Hoechst 33342 nuclear dye (Molecular Probes, Eugene, OR).

For detection of activated ATM, cells were plated on microscope slides and treated with 4 mM caffeine for 18-48 h, as indicated. Following this treatment, cells were washed in PBS for 5 minutes at 37 °C and fixed in 3.7% (w/v) paraformaldehyde for 20 min at room temperature. Cells were washed twice in PBS and permeabilized in PBS-T (PBS containing 0.5% (v/v) Triton X-100) for 10 min at room temperature and blocked by 10% normal goat serum (Zymed Laboratories, San Francisco, CA.) for 10 min and by PBS-10% milk solution for 30 min at room temperature. The rabbit anti-ATM pS1981 antibody (Rockland, Gildertsville, PA) was added at a 1:400 dilution in PBS-10% milk for 16 h at 4 °C. Cells were washed four times with PBS-T (15 minutes each, room temperature) and incubated for 1 h at room temperature with a Cy-3-conjugated goat anti-rabbit antibody (Zymed Laboratories, San Francisco, CA) at a dilution of 1:500 in PBS plus 10% milk. The slides were washed four times in PBS-T (15 min each) and mounted using a SlowFade Antifade Kit containing DAPI (Molecular Probes, Eugene, OR).

All images were obtained with a Zeiss LSM 510 NLO inverted confocal microscope using a 10x Plan-Apochromat (NA 0.45) or a 40X Plan-Neofluar (NA 1.3) oil immersion objective, as noted. FAM oligonucleotide was detected using 488nm excitation from a 25 mW Argon laser and a 505 long pass emission filter (Omnichrome, Chino, CA); A 543nm excitation from a 1 mW helium-neon laser with a 560 long pass emission filter for the Cy-3 conjugated secondary antibody; and the for the DAPI and Hoescht 33342 nuclear dyes, 737 nm excitation using a 5 watt pumped Mira 900F femtosecond mode-locked titanium:sapphire laser with a with a 390-465 IR band pass emission filter (Coherent, Inc., Santa Clara, CA) was used.

G. BrdU cell replication assay, MTT cell viability/proliferation assay, and cell cycle analysis

BrdU incorporation was analyzed using a Roche in situ cell proliferation kit, FLUOS (Indianapolis, IN). Cells were grown in 60 mm dishes following electroporation of the oligonucleotide and at 24 and 48 h, 10 µM BrdU was added to the growth medium. Cells were incubated for 30 min at 37 °C and then washed three times with PBS. They were then trypsinized, washed with PBS, resuspended in 0.5 ml PBS and fixed by the addition of 5 ml cold 70% ethanol while vortexing. The cell suspension was incubated at 4 °C for one hour, centrifuged at 2000 rpm for 5 min, washed in PBS, and re-centrifuged. The pellet was then resuspended in 500 µl of 4 M HCl and incubated for 15 minutes at room temperature. 2 ml of PBS was added and cells were centrifuged for 10 minutes at 2000 rpm, followed by a second PBS wash. 500 µl of incubation buffer (0.5% BSA – 0.1% Tween 20 in PBS) was added and cells were incubated for 20 min at room temperature. Cells were spun down and pellet was resuspended in 100 µl of antibody solution [Anti-bromodeoxyuridine fluorescein (Roche: 1 202 693) 3µl/sample in PBS-0.1% BSA] and incubated for 20 min at room temperature, protected from light. Cells were washed twice with PBS and resuspended in 500 µl PBS and prepared for FACS analysis.

To measure cell proliferation, three separate conditions were examined: cells not electroporated, cells electroporated without oligonucleotides, and cells electroporated with 5 µM oligonucleotides. Under these conditions, cells were plated at a density of $0.5x10^5$ cells per well in a 6-well plate, with or without 4 mM caffeine, in triplicate. At the indicated times, cells were washed once with PBS and 500 µl MTT [Methylthiazolyldiphenyl-tetrazolium bromide (Sigma: M-2128) in PBS at 5 mg/ml (50X stock)], diluted to 0.1 mg/ml in serum-free medium, was added to each sample. Following a two hour incubation in a CO_2 incubator, the MTT solution was removed and 1 ml DMSO was added and plates were placed on a rotator for 1 h, protected from light. 200 µl of the DMSO was removed, in triplicate, and transferred to a 96 well plate and read on a Victor^3 V Plate reader (PerkinElmer, Wellesly, MA) with a 570 nm test filter.

For cell cycle analysis, cells were split and grown as for targeting experiments and were electroporated as described previously, with 5 μM oligo. After a 24 h incubation either with or without 4 mM caffeine, cells were washed once with PBS, trypsinized and washed a second time with PBS. Cells were spun down at 1200rpm for 5 min and re-suspended in 500 μl cold PBS per $1x10^6$ cells. 5 ml (per 500μl of PBS) of 70% cold ethanol was added drop wise while vortexing. Cells were then incubated at 4 °C overnight followed by a 4 °C centrifugation at 2000rpm for 5 min. They were washed with cold PBS, re-centrifuged and cell pellet was re-suspended in 300 μl of FACS buffer (50 μg/ml RNase A, 2.5 μg/ml propidium iodine, 1% FBS in PBS). Samples were first incubated at 37 °C for 1 h and then returned to 4 °C overnight, prior to FACS analysis. Cell cycle distribution was determined by ModFit LT software.

III. Results

A. Functional assay for gene repair activity

Gene repair was monitored by the correction of a single point mutation in an integrated eGFP gene in DLD-1 cells. The mutation, which is located in the chromophore region of the encoded protein, creates a stop codon (TAG); correction of this mutation (to TAC) restores functionality to eGFP and the resulting green fluorescence is measured by FACS (Drury and Kmiec, 2003; Brachman and Kmiec, 2004, 2005; Drury and Kmiec, 2004; Ferrara and Kmiec, 2004; Ferrara et al, 2004; Hu et al, 2005). The oligonucleotide (EGFP3S/47NT) designed to direct the repair of the mutation is 47 bases in length, contains three phosphorothioate linkages at each terminus and hybridizes to the non-coding strand of the mutant gene. The DNA sequence of the wild type gene, the mutant gene and the oligonucleotide are presented in **Figure 1**. Previous data have shown that neither a perfectly matched oligonucleotide nor an oligonucleotide bearing no sequence complementarity to the target is able to direct detectable levels of repair of the mutant eGFP gene (Hu et al, 2005; Parekh-Olmedo et al, 2003).

The gene repair reaction is initiated by electroporating EGFP3S/47NT into a clonally isolated DLD-1 cell line (D-1) containing several copies of the mutant eGFP gene (Hu et al, 2005). Cells are plated 24 h prior to electroporation and correction of the eGFP gene is measured 24 to 72 h later; the percentage of cells expressing functional eGFP is calculated by a Cell Quest and GFP/PI analysis. Routinely, fifty thousand cells are gated and analyzed for green fluorescence and cell viability (by PI staining). This protocol has been used to assess gene repair activity at the phenotypic level in several cell lines (see Brachman and Kmiec, 2005 and references therein).

In a previous study, caffeine was shown to *inhibit* gene repair activity if added to the cells 24 hours before electroporation of the oligonucleotide (Ferrara and Kmiec, 2004). Caffeine, a xanthine derivative, has a number of activities acting as a radiosensitizer by inhibiting the phosphorylation of p53 (ser-15) by ATM. As a result, the homologous recombination (HR) pathway remains largely dormant and the levels of gene repair activity are reduced (Ferrara and Kmiec, 2004). The ATM protein is one of the central regulators of the cells' response to DNA damage (Sancar et al, 2004), which often manifests itself in the form of free DNA ends. These ends can be generated either by resection of double-strand breaks or by the exogenous addition of single-stranded DNA (ssDNA). As described above, Nur-E-Kamal et al, 2003 demonstrated that ssDNA alone induces an increase in ATM expression and functional activation. Since gene repair is initiated by the introduction of ssDNA, we wondered if the addition of caffeine at the time of ssDNA entry into the cell would affect ATM activation. We further explored how caffeine might influence the cell cycle profile and the frequency of gene repair.

B. Caffeine's dual role in the gene correction reaction

We designed a series of experiments in which 4 mM caffeine was added 24 h prior to electroporation and then washed out, added at the time of electroporation, or both. Correction in the DLD-1 cell line was measured by FACS 24 h after the introduction of the oligonucleotide and **Figure 2** illustrates the results. Pre-incubation with caffeine partially blocks gene repair whether it is washed out or not, but, addition of caffeine after electroporation does not block gene repair at all; in fact it stimulates correction efficiency significantly ($p<0.001$). Thus, the inhibitory effect of caffeine is observed only when added prior to oligonucleotide transfer; it is not seen when the cells are exposed to caffeine after electroporation.

Since the induction of ATM is associated with an up-regulation of HR, and appears to be a keystone feature of the cell's response to DNA damage, we next measured the cellular levels of ATM induced by ssDNA in the presence and absence of caffeine. DLD-1 cells were electroporated with or without 5 μM EGFP3S/47NT and in the presence or absence of 4 mM caffeine. At 18 and 24 h post electroporation, the cells were stained with an antibody directed against the phosphorylated (activated) form of the ATM protein. As shown in **Figure 3A**, electroporation of an oligonucleotide leads to ATM protein activation (panels i and iii), consistent with the data of Nur-E-Kamal et al, 2003. Under reaction conditions in which electroporated cells are placed in 4 mM caffeine after electroporation, ATM protein levels are observed to be elevated even further at both the 18 (ii) and 24 h (iv) time points. These results lead us to conclude that when caffeine is added after the introduction of the oligonucleotide, ATM is either hyperactivated or it's activation state is maintained. These data are consistent with observations of (Cortez, 2003), who showed that caffeine can inhibit checkpoint responses and retard cell proliferation *without* inhibiting ATM in the presence of DNA damage. Caffeine also seems to sustain the protein's activation, as high levels of ATM can be observed only in cells exposed to the agent, as examined by confocal microscopy 48 h after electroporation of the ssDNA (**Figure 3B**). Panel (i) reveals a modest level of ATM while panel (ii), which represents cells that were incubated with 4 mM caffeine immediately following oligonucleotide addition, presents higher levels of nuclear staining ATM. **Figure 3C** represents several control experiments in which we observed no activation of ATM by electroporation alone in the absence or presence of 4

mM caffeine. Taken together, we believe that ATM activation is dependent on the introduction of the oligonucleotide, an action that is enhanced by caffeine.

C. Oligonucleotide retention is enhanced in the presence of caffeine

Based on results described above, one might predict that the sustained presence of oligonucleotide could also serve as a source for constant reactivation of ATM. We therefore wondered if the enhanced ATM activation results from an elongated half-life of the oligonucleotide in the cells treated with caffeine. In other words, does caffeine increase or prolong ATM activation by causing the cell to retain the oligonucleotide for a longer period of time? To address this question, we synthesized an oligonucleotide containing a fluorescent (FAM) dye conjugated on the 5' end of the molecule to enable us to monitor the presence of the oligonucleotide within the population of cells after electroporation. The DLD-1 cells were thus electroporated in the presence or absence of 4 mM caffeine and the number of cells containing the FAM-labeled oligonucleotide was assessed by FACs at various time-points. **Figure 4A** displays the percentage of cells containing the fluorescent oligonucleotide in each sample. The data reveal that a significantly higher level of oligonucleotide is present in cells that have been incubated with caffeine after electroporation. Confocal microscope images (**Figure 4B**) taken at the 24 (i and ii) and 40 (iii and iv) hour time points confirm that the oligonucleotide is not only retained longer in cultures containing caffeine (ii and iv) but is present at much higher levels, as determined by fluorescence intensity. In this figure, the FAM oligo is green and the DAPI nuclear stain is red, so that the co-localization of the oligo within the nuclei of the cells can be easily identified by the yellow fluorescence. Taken together, these data suggest that treatment with caffeine leads to an increase in the percentage of cells with oligonucleotides or perhaps to allow cells to posses an increased capacity to retain the oligonucleotides. Thus, enhanced ATM activation may be accentuated by the longer half-life of the oligonucleotide in cells incubated with caffeine.

D. Treatment with caffeine decreases DNA replication activity, leading to stable levels of gene correction

The data presented to this point indicate that the addition of caffeine to the gene repair reaction immediately *after* oligonucleotide electroporation elevates correction efficiency (**Figure 2**). This result is perplexing considering the fact that pre-incubation with caffeine reduces the correction efficiency. One explanation may be that a 24 h pre-incubation would be long enough to slow the rate of replication of cells to levels that are not conducive or optimal for the gene repair reaction to take place. In contrast, when caffeine is added with the oligo, there is a slowing of replication, which takes place gradually. We have shown previously that a *gradual* reduction in fork movement results in high levels of gene repair (Brachman and Kmiec, 2005).

We decided to test this prediction using several types of assays that measure cell proliferation and/or DNA replication activity. First, an MTT assay system (Ochs and Kaina, 2000) was used to examine the overall rate of cell proliferation is reduced in the presence of caffeine..

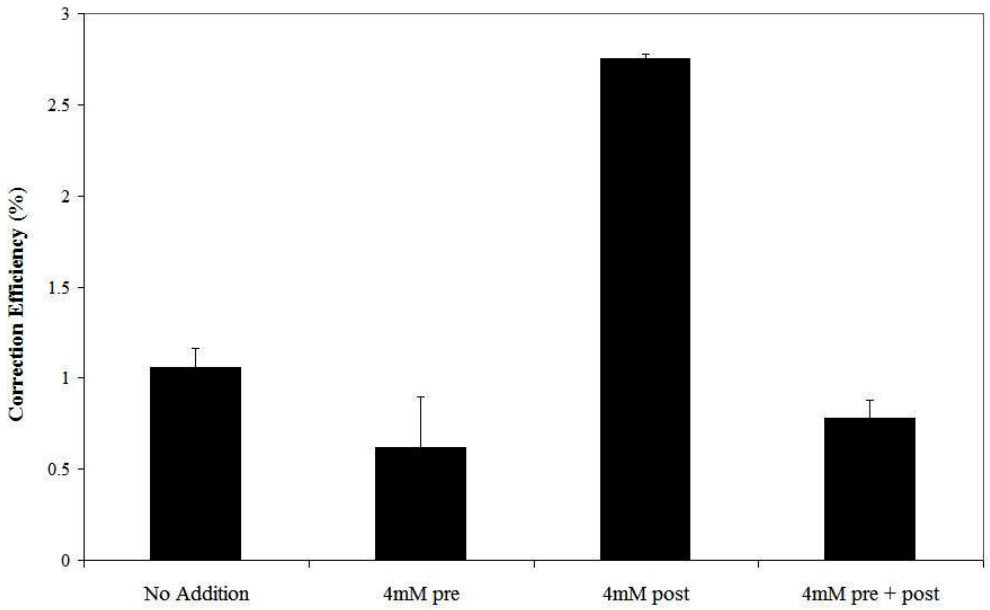


Figure 2. *Caffeine has two distinct effects when added prior to or after electroporation of the oligonucleotide.* 4 mM caffeine was added to the growth medium for a period of 24 hours either before cells were electroporated with 5 μM EGFP3S/47NT (4 mM pre), after the electroporation (4 mM post), or a combination of the two treatments (4 mM pre + post). Correction efficiency: the percentage of green fluorescent cells is presented as a function of the total number of cells counted (50,000 for each sample); means and standard deviations were calculated based on the results of three independent experiments and are depicted on the graph. T-test was performed between no treatment and 4 mM post treatment (**p<.001).

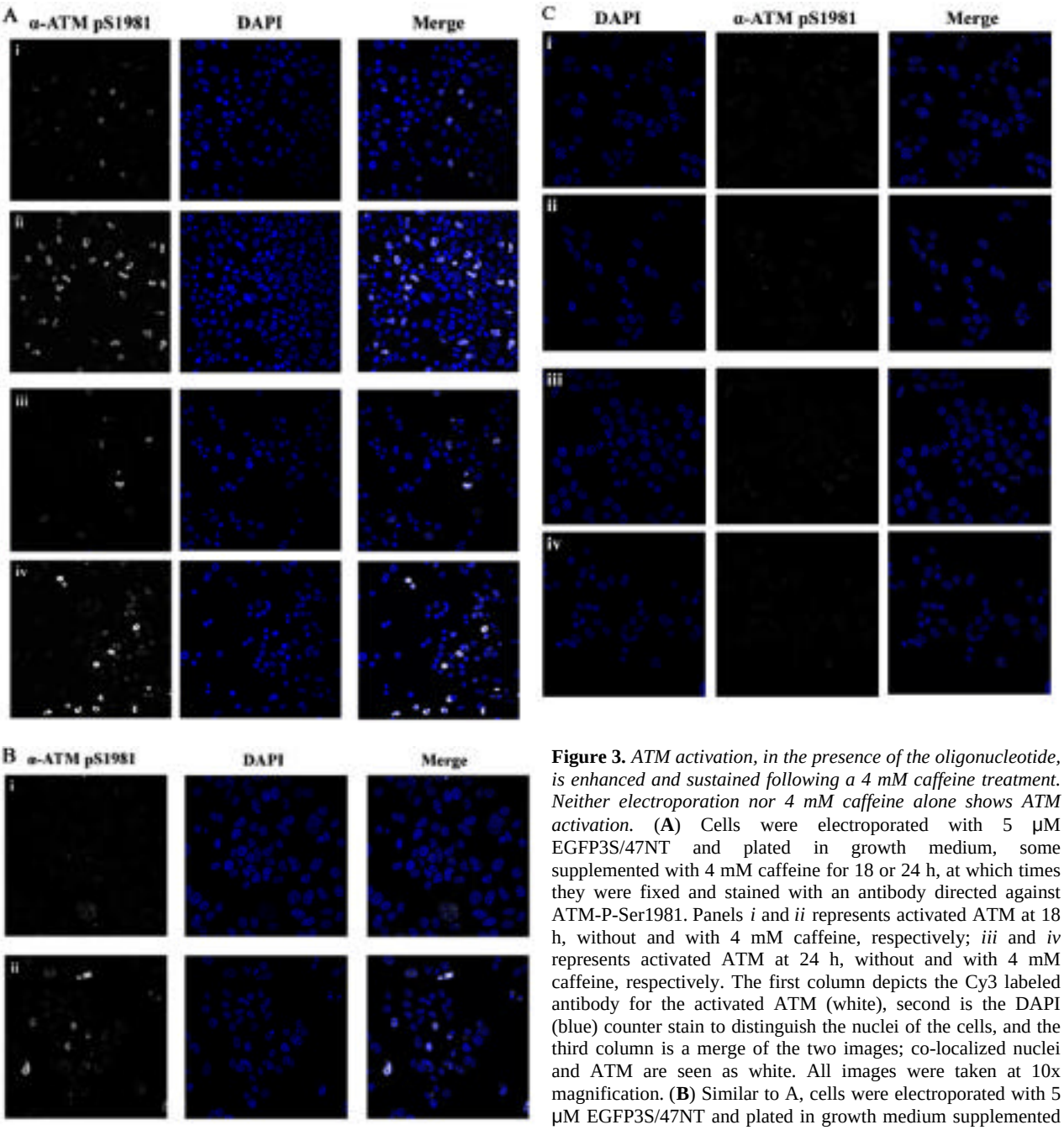
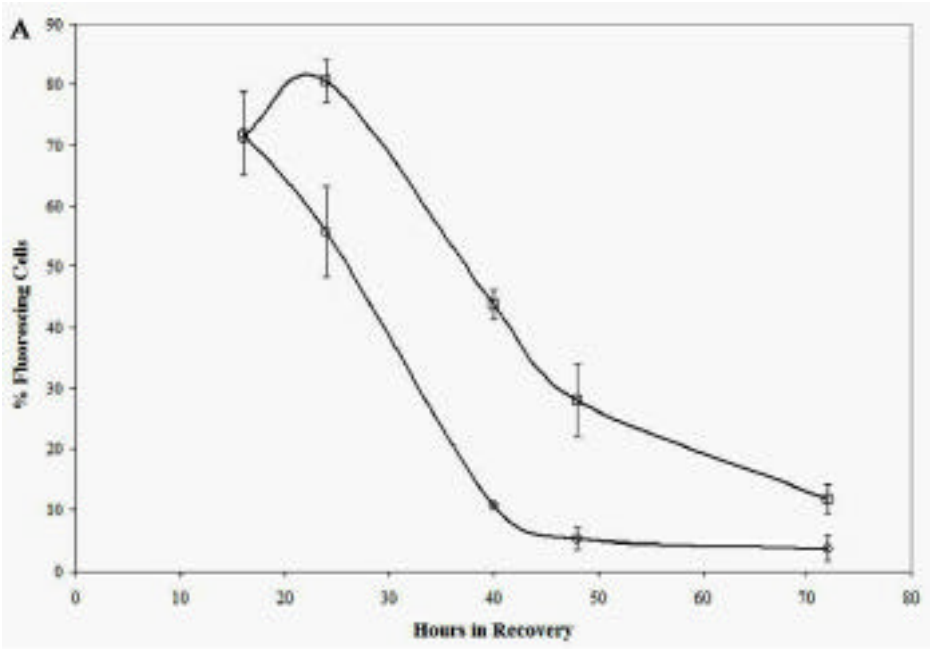


Figure 3. *ATM activation, in the presence of the oligonucleotide, is enhanced and sustained following a 4 mM caffeine treatment. Neither electroporation nor 4 mM caffeine alone shows ATM activation.* (**A**) Cells were electroporated with 5 μM EGFP3S/47NT and plated in growth medium, some supplemented with 4 mM caffeine for 18 or 24 h, at which times they were fixed and stained with an antibody directed against ATM-P-Ser1981. Panels *i* and *ii* represents activated ATM at 18 h, without and with 4 mM caffeine, respectively; *iii* and *iv* represents activated ATM at 24 h, without and with 4 mM caffeine, respectively. The first column depicts the Cy3 labeled antibody for the activated ATM (white), second is the DAPI (blue) counter stain to distinguish the nuclei of the cells, and the third column is a merge of the two images; co-localized nuclei and ATM are seen as white. All images were taken at 10x magnification. (**B**) Similar to A, cells were electroporated with 5 μM EGFP3S/47NT and plated in growth medium supplemented with or without 4 mM caffeine for 48 h, at which point they were fixed and stained with an antibody against ATM-P-Ser1981, and counter-stained with DAPI. Panel *i* shows cell magnified at 40x that did not receive 4 mM caffeine for the duration of the 48 h, panel *ii* shows cells at the same magnification following the caffeine incubation. As in A, activated ATM is depicted in white and DAPI in blue. (**C**) Cells were electroporated without any oligonucleotide and plated with or without 4 mM caffeine for 18 or 24 h, at which times they were fixed and stained with an antibody directed against ATM-P-Ser1981. Panels i and ii represents activated ATM at 18 h without and with 4 mM caffeine, respectively; iii and iv shows activated ATM at 24 h without and with 4 mM caffeine, respectively. The first column depicts the nuclear stain, DAPI (blue), while the second column shows the ATM antibody (shown in white); column three is the merge of the two images. Images were taken at 10X magnification..



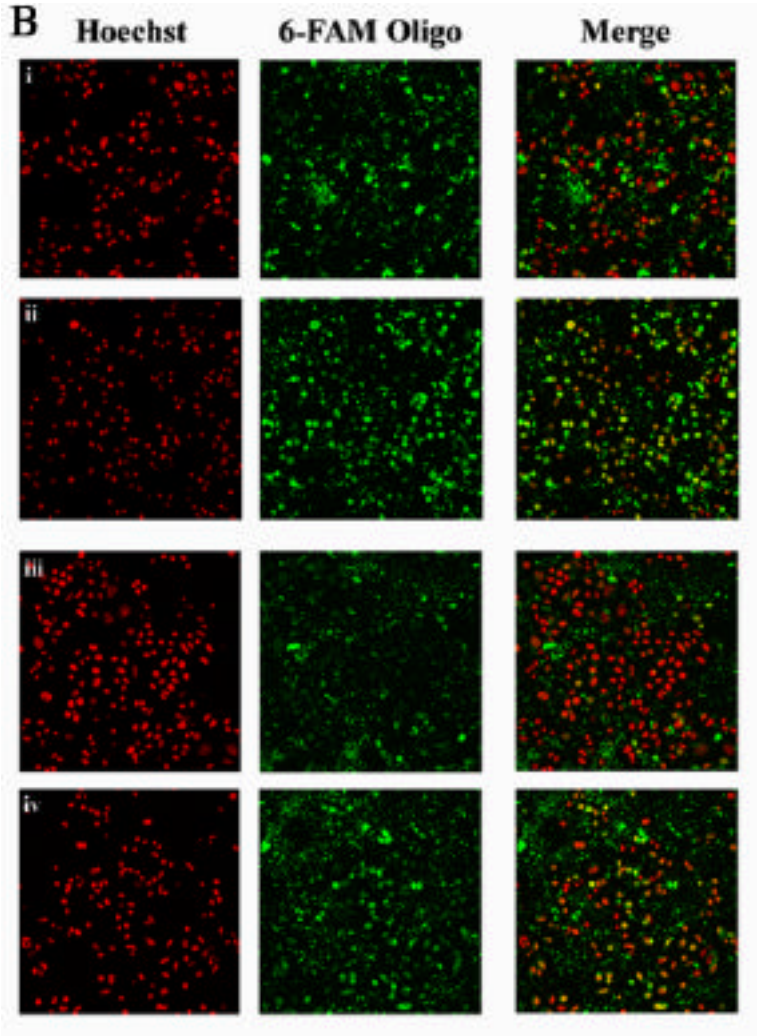


Figure 4. *The presence of 4 mM caffeine allows for the retention of the oligo for a longer duration and at a greater quantity.* (**A**) DLD-1 cells were electroporated with 5 μM EGFP3S/47T 6-FAM labeled oligo and were incubated with (□) or without (○) 4 mM caffeine for varying periods of time, at which point they were collected and prepared for FACS. The percentage of cells containing the FAM oligo is calculated as the number of cells out of the total 50,000 cells analyzed. Mean and standard deviations were calculated based on the results of 4-5 independent experiments, and are depicted on the graph. (**B**) To corroborate the FACS data, cells were plated in chambers following electroporation of the tagged oligo with or without 4 mM caffeine supplemented medium for either 24 (panels i and ii) or 40 h (panels iii and iv); samples not treated with 4 mM caffeine in the recovery are depicted in panels i and iii, those with the 4 mM treatment in ii and iv. After these incubations, the cell nuclei were stained with Hoechst 33342 dye and examined for fluorescence under a confocal microscope at 10X magnification. Images depict Hoechst nuclei stain visualized in red, the 6-FAM labeled oligo as green, and a co-localization of the two is seen as yellow in the merged column.

Table 1.

		Hours in culture		
		24	**48**	**72**
No Electroporation	0 mM	1.45 ± .05	2.13 ± .10	5.08 ± .10
	4 mM	1.00 ± .04	1.24 ± .04	1.30 ± .03
Electroporation No Oligo	0 mM	1.12 ± .03	2.08 ± .07	4.26 ± .07
	4 mM	0.76 ± .04	0.91 ± .08	1.11 ± .05
Electroporation Oligo	0 mM	1.02 ± .08	1.41 ± .04	2.66 ± .06
	4 mM	0.77 ± .06	0.68 ± .01	0.76 ± .13

Cells were treated in three different conditions: non-electroporated, electroporated, or electroporated with 5 μM EGFP3S/47NT oligo. Cells were then plated in separate 6-well plates for each time point and the caffeine treatments, either 0 or 4 mM, were added in triplicate for the duration of the respective incubations. At these time points, plates were removed and analyzed for cell proliferation using the MTT assay, as described in the methods section. Values represent the average OD at 570 nm for three individual samples for each treatment, standard deviation is also noted.

We tested this by electroporating cells and treating them under a variety of conditions as outlined in **Table 1**, with cell proliferation measured at 24 h, 48 h and 72 h, respectively. The data suggests that the presence of caffeine reduces cell proliferation under all reaction conditions, particularly under those that promote high levels of gene repair (+ oligo, + caffeine).

Next, we examined DNA replication activity directly in electroporated cells that had been incubated for 24 or 48 h with caffeine. To do so, a BrdU uptake and staining method was utilized to explore correlations between DNA replication activity and gene repair (Brachman and Kmiec, 2005). BrdU staining provides a measure of the level of cells with actively replicating template. The protocol was carried out 24 or 48 h after cells had been electroporated with 5 μM of EGF3S/47NT, with or without 4 mM caffeine in the recovery. At both time points, the number of actively replicating DNA templates was reduced in the reactions that contained caffeine (**Figure 5A**), with a decrease of approximately 50%. This observation is consistent with data presented above showing that cellular proliferation is retarded in the presence of 4 mM caffeine. Our observations also support previous studies wherein caffeine was found to have a significant impact on cell proliferation (Kaufmann et al, 2003).

Finally, we profiled cell cycle in the presence of 4 mM caffeine and the results are presented in **Figure 5B**. In untreated, but electroporated (+ oligo) cells, approximately 50% of the DLD-1 cells are in S phase, consistent with previous observations (Brachman and Kmiec, 2005). But after a 24 h treatment of 4 mM caffeine, the percentage cells in S phase and G2 is reduced significantly with a build up in G1. Thus, caffeine effectively removes a large percentage of cells that are amenable to gene repair, which are cells containing active DNA replication templates.

Olsen et al, 2003, 2005 recently reported that, the percentage of cells containing corrected genes gradually decreases with time, a phenomenon due perhaps, to the fact that *corrected* cells may specifically divide more slowly than their non-corrected counterparts. Thus, if our hypothesis about the inhibition of cell proliferation by caffeine via a reduction in DNA replication is valid, then the correction efficiency should be maintained for an extended period of time. In this case, the overall population of cells treated with caffeine would neither be actively replicating nor dividing. We tested this idea by measuring correction efficiency at longer time points after the introduction of the oligonucleotide, by allowing the cells to react for 24, 48, or 72 h following the electroporation. During these time periods, some of the samples were incubated in 4 mM caffeine, and as shown in **Table 2**, the level of gene repair remains fairly constant throughout the entire period of the reaction. Our data suggest that the percentage of corrected cells in a treated population, electroporated with ssDNA and then recovered in 4 mM caffeine, remains constant because caffeine exerts its influence by slowing the replication and division of *all* cells in the population preventing a continual dilution of cells containing a repaired eGFP gene.

IV. Discussion

Among its many cellular effects, caffeine is known to inhibit the phosphorylation of p53 by the ATM protein and as a result, inhibit the activation of homologous recombination (see Lundin et al, 2003 and references therein). Caffeine has been used routinely as a tool to uncover possible roles for homologous recombination in a variety of cell processes including the response to DNA damage. Previously, we showed that caffeine can inhibit gene repair activity if added prior to the transfer of the single-stranded DNA vector into target cells (Ferrara and Kmiec, 2004; Ferrara et al, 2004). But, if added after the introduction of the vector, the suppressive effects on gene repair were minimized and a reproducible enhancement of repair was observed. In this paper, we expand on the observation and explore the regulatory role of DNA replication and cell proliferation in gene repair activity.

The entry of the single-stranded vector into mammalian cells induces ATM activation, a process that can be visualized by cellular staining using an antibody specific for the activated form of the protein (Bakkenist and Kastan, 2003; Nur-E-Kamal et al, 2003) (**Figure 3**). When the cells are exposed to caffeine *after* ssDNA electroporation, we observe that the level of activated

ATM in the nuclei rises. Similar observations have been made in studies where the effect of caffeine on cells exposed to radiation was evaluated (Blasina et al, 1999; Sarkaria et al, 1999). These workers observed that caffeine added to cells in culture prior to radiation prevented the induction of Chk2 and p53, proteins that are normally activated through the kinase property of ATM. But when caffeine was added after radiation, almost no suppression was seen (Blasina et al, 1999). This suggests that inhibitory effects of caffeine on ATM are minimized when

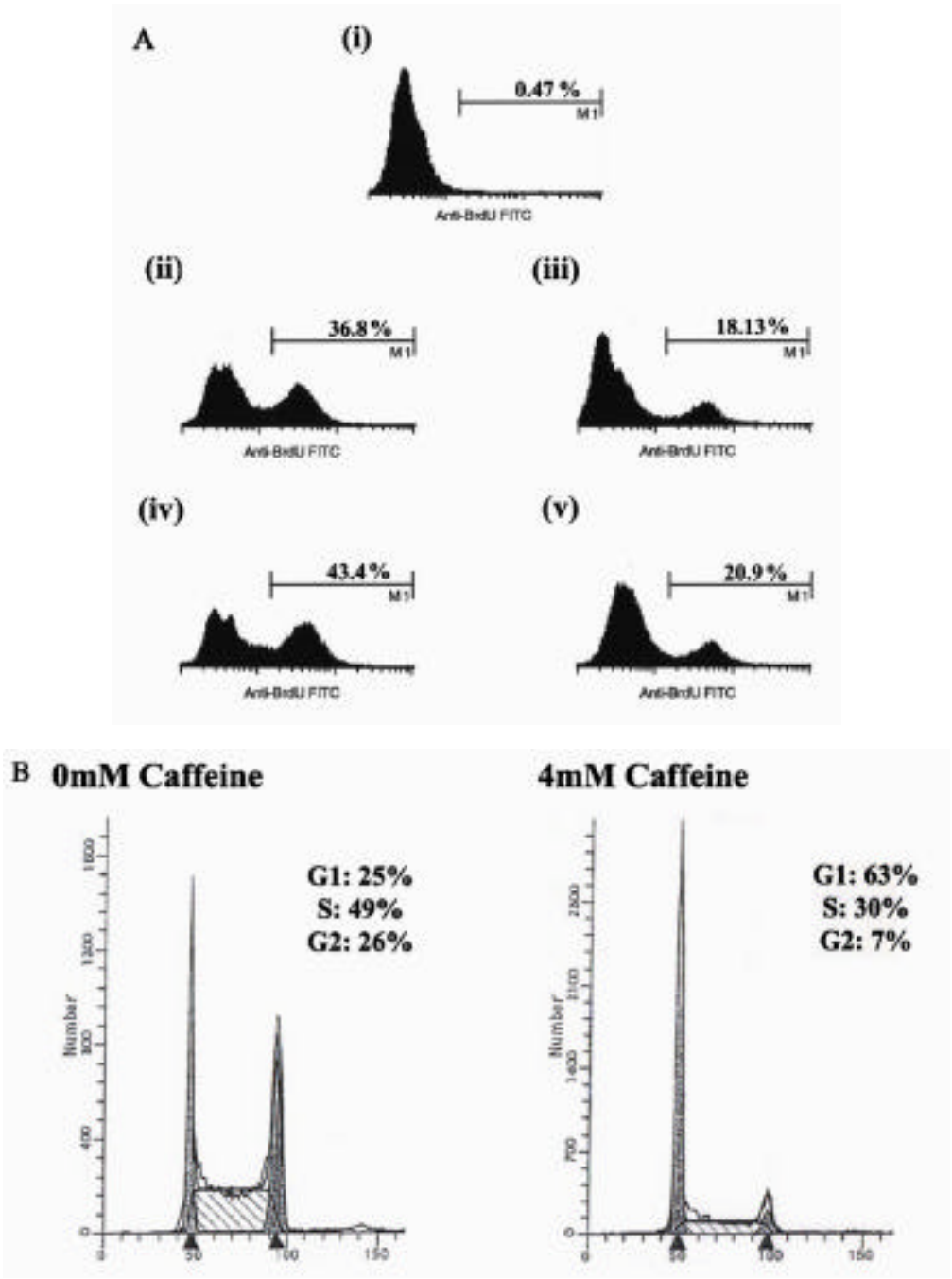


Figure 5. *Cell replication is retarded by the addition of 4 mM caffeine.* (**A**) Replication of caffeine treated cells. Cells were collected and electroporated with 5 μM EGFP3S/47NT oligo and recovered in medium either supplemented with (iii and v) or without (ii and iv) 4 mM caffeine for a duration of 24h (ii and iii) and 48h (iv and v). At these time points, the BrdU incorporation protocol was followed (see methods and materials) and cells were later analyzed by FACS for the presence of the modified nucleotide. Panel (i) shows background fluorescence, as these were not treated with BrdU. Values indicate the percent of the 20,000 cells analyzed that scored positive for BrdU incorporation. (**B**) Cell cycle analysis of caffeine treated cells. Cells were electroporated with 5 μM EGFP3S/47NT oligo and incubated in the presence or absence of 4 mM caffeine for 24 h, at which point they were processed for cell cycle analysis. The profiles were generated using the ModFit LT software.

Table 2.

	Hours in culture		
	24	48	72
No treatment	1.12 ± .06	0.91 ± .11	0.059 ± .04
4 mM Caffeine	2.76 ±.01	2.63 ± .07	2.23 ± .03

DLD-1 cells were electroporated with 5 μM EGFP3S/47NT and plated into medium supplemented with or without 4 mM caffeine for a period of 24 to 72 h. At the termination of these recovery incubations, the cells were collected and analyzed by FACS, as described in the methods section. Numbers relate to the average percent of GFP positive cells in a total of 50,000 cells analyzed of two experiments; standard deviations are also included.

ATM is in its activated form; in that case by radiation, in our case, by the entry of single-stranded DNA into the mammalian cells. Regardless of how it becomes activated, ATM phosphorylates a large array of proteins with a plethora of functions. Among the most important downstream events, however, is the arrest of the cell cycle, which provides a window a time for DNA damage repair to take place.

Caffeine is also known to partially inhibit DNA synthesis (Deplanque et al, 2000) with effects seen in some systems within 30 minutes (Kaufmann et al, 2003). These observations are likely to offer an explanation for the increase in correction efficiency seen when caffeine is added *after* the electroporation of the oligonucleotide. Recently, Majumdar et al, 2003, Hu et al, 2005, and Brachman and Kmiec, 2005 showed that cells traversing S-phase are most amenable to the gene repair reaction. Furthermore, other groups including our own lab (Brachman and Kmiec, 2005; Wu et al, 2005), have demonstrated that a gradual reduction in the rate of DNA synthesis leads to an increase in gene correction efficiency, presumably by expanding the window of time in which the oligonucleotide can gain access to the daughter strand of a replication fork (see Parekh-Olmedo et al, 2005 for model). The gradual reduction in the rate of DNA synthesis caused by caffeine could be acting in a similar fashion and within the same window of time; the slowdown of replication allowing for the assimilation of the oligonucleotide into the target site more readily. In addition, caffeine is also known to regulate the firing of late origins of replication (Shechter et al, 2004), which would enable the oligonucleotide to find the target site more easily.

The sustained presence of the oligonucleotide in cells incubated with caffeine and the effect of caffeine on DNA replication certainly offers an explanation for the suppression of cell proliferation. With the oligo present in the cell for longer times, a constant activation or reactivation of ATM would be occurring, leading to a more pronounced arrest of DNA replication. In addition, as shown in Table II, the inhibition of cell division by caffeine maintains the correction efficiency at a high level throughout the time course of the experiments. We observed that an untreated population of cells progressively reduces its inherited correction efficiency, presumably by a dilution effect of the corrected cells, observations consistent with Olsen et al, 2003, 2005. By our experiments, caffeine acts to block *all* cells in the population from proliferating robustly, thus maintaining the corrected and uncorrected cell ratio; the result of this reaction is the apparent maintenance of correction efficiency. Monitoring the level of ATM activated by the oligonucleotide may provide a reasonable way to predict how probable it is that the corrected cells will proliferate.

Our primary goal in this work was to examine the observation that caffeine did not inhibit the gene repair reaction when added to cells after the introduction of the single-stranded oligonucleotide. These results now demonstrate that the initial stimulation of gene repair frequency by caffeine is due, in all likelihood, to the gradual slowing of DNA replication. Our work with caffeine also provides an explanation for the decrease in correction efficiency appearing over time (Olsen et al, 2005). We find that ATM activation may be the keystone event that confers this phenotype onto corrected cells. Furthermore, the presence of caffeine in targeted cultures maintains high levels of correction by blocking DNA replication and cell proliferation. Thus, it is now imperative to focus on protocols that avoid the activation of high levels of ATM when considering gene repair as a therapeutic approach to inherited disorders.

References

Andersen MS, Sorensen CB, Bolund L and Jensen TG **(2002)** Mechanisms underlying targeted gene correction using chimeric RNA/DNA and single-stranded DNA oligonucleotides. **J Mol Med** 80, 770-781.

Bakkenist CJ and Kastan MB **(2003)** DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. **Nature** 421, 499-506.

Blasina A, Price BD, Turenne GA and McGowan CH **(1999)** Caffeine inhibits the checkpoint kinase ATM. **Curr Biol** 9, 1135-1138.

Brachman EE and Kmiec EB **(2004)** DNA replication and transcription direct a DNA strand bias in the process of targeted gene repair in mammalian cells. **J Cell Sci** 117, 3867-3874.

Brachman EE and Kmiec EB **(2005)** Gene repair in mammalian cells is stimulated by the elongation of S phase and transient stalling of replication forks. **DNA Repair (Amst)** 4, 445-457.

Cortez D **(2003)** Caffeine inhibits checkpoint responses without inhibiting the ataxia-telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR) protein kinases. **J Biol Chem** 278, 37139-37145.

Dekker M, Brouwers C and Te RH **(2003)** Targeted gene modification in mismatch-repair-deficient embryonic stem

cells by single-stranded DNA oligonucleotides. **Nucleic Acids Res** 31, E27.

Deplanque G, Vincent F, Mah-Becherel MC, Cazenave JP, Bergerat JP and Klein-Soyer C **(2000)** Caffeine does not cause override of the G2/M block induced by UVc or gamma radiation in normal human skin fibroblasts. **Br J Cancer** 83, 346-353.

Drury MD and Kmiec EB **(2003)** DNA pairing is an important step in the process of targeted nucleotide exchange. **Nucleic Acids Res** 31, 899-910.

Drury MD and Kmiec EB **(2004)** Double displacement loops (double d-loops) are templates for oligonucleotide-directed mutagenesis and gene repair. **Oligonucleotides** 14, 274-286.

Ferrara L and Kmiec EB **(2004)** Camptothecin enhances the frequency of oligonucleotide-directed gene repair in mammalian cells by inducing DNA damage and activating homologous recombination. **Nucleic Acids Res** 32, 5239-5248.

Ferrara L, Parekh-Olmedo H and Kmiec E **(2004)** Enhanced oligonucleotide-directed gene targeting in mammalian cells follwoing treatment with DNA damaging agents. **Exp Cell Res** 300, 170-179.

Gamper HB, Parekh H, Rice MC, Bruner M, Youkey H and Kmiec EB **(2000)** The DNA strand of chimeric RNA/DNA oligonucleotides can direct gene repair/conversion activity in mammalian and plant cell-free extracts. **Nucleic Acids Res** 28, 4332-4339.

Hu Y, Parekh-Olmedo H, Drury M, Skogen M and Kmiec EB **(2005)** Reaction parameters of targeted gene repair in Mammalian cells. **Mol Biotechnol** 29, 197-210.

Igoucheva O, Alexeev V, Pryce M and Yoon K **(2003)** Transcription affects formation and processing of intermediates in oligonucleotide-mediated gene alteration. **Nucleic Acids Res** 31, 2659-2670.

Kahl CR and Means AR **(2003)** Regulation of cell cycle progression by calcium/calmodulin-dependent pathways. **Endocr Rev** 24, 719-736.

Kaufmann WK, Heffernan TP, Beaulieu LM, Doherty S, Frank AR, Zhou Y, Bryant MF, Zhou T, Luche DD, Nikolaishvili-Feinberg N, Simpson DA and Cordeiro-Stone M **(2003)** Caffeine and human DNA metabolism: the magic and the mystery. **Mutat Res** 532, 85-102.

Kren BT, Wong PY and Steer CJ **(2003)** Short, single-stranded oligonucleotides mediate targeted nucleotide conversion using extracts from isolated liver mitochondria. **DNA Repair (Amst)** 2, 531-546.

Liu L, Rice MC, Drury M, Cheng S, Gamper H and Kmiec EB **(2002)** Strand bias in targeted gene repair is influenced by transcriptional activity. **Mol Cell Biol** 22, 3852-3863.

Lundin C, Schultz N, Arnaudeau C, Mohindra A, Hansen LT and Helleday T **(2003)** RAD51 is involved in repair of damage associated with DNA replication in mammalian cells. **J Mol Biol** 328, 521-535.

Munaron L, Antoniotti S and Lovisolo D **(2004)** Intracellular calcium signals and control of cell proliferation: how many mechanisms? **J Cell Mol Med** 8, 161-168.

Murnane JP, Byfield JE, Chen CT and Wang CH **(1981)** The structure of methylated xanthines in relation to their effects on DNA synthesis and cell lethality in nitrogen mustard-treated cells. **Biophys J** 35, 665-676.

Nur-E-Kamal, Li TK, Zhang A, Qi H, Hars ES and Liu LF **(2003)** Single-stranded DNA induces ataxia telangiectasia mutant (ATM)/p53-dependent DNA damage and apoptotic signals. **J Biol Chem** 278, 12475-12481.

Ochs K and Kaina B **(2000)** Apoptosis induced by DNA damage O6-methylguanine is Bcl-2 and caspase-9/3 regulated and Fas/caspase-8 independent. **Cancer Res** 60, 5815-5824.

Olsen PA, McKeen C and Krauss S **(2003)** Branched oligonucleotides induce in vivo gene conversion of a mutated EGFP reporter. **Gene Ther** 10, 1830-1840.

Olsen PA, Randol M and Krauss S **(2005)** Implications of cell cycle progression on functional sequence correction by short single-stranded DNA oligonucleotides. **Gene Ther** 12, 546-551.

Parekh-Olmedo H, Engstrom J and Kmiec EB **(2003)** The effect of hydroxyurea and trichostatin A on targeted nucleotide exchange in yeast and mammalian cells. **Annals of the New York Academy of Sciences** 1002, 43-56.

Parekh-Olmedo H, Ferrara L, Brachman E and Kmiec EB **(2005)** Gene therapy progress and prospects: targeted gene repair. **Gene Ther** 12, 639-646.

Pierce EA, Liu Q, Igoucheva O, Omarrudin R, Ma H, Diamond SL and Yoon K **(2003)** Oligonucleotide-directed single-base DNA alterations in mouse embryonic stem cells. **Gene Ther** 10, 24-33.

Rice MC, Bruner M, Czymmek K and Kmiec EB **(2001)** In vitro and in vivo nucleotide exchange directed by chimeric RNA/DNA oligonucleotides in Saccharomyces cerevisae. **Mol Microbiol** 40, 857-868.

Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K and Linn S **(2004)** Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. **Annu Rev Biochem** 73, 39-85.

Sarkaria JN, Busby EC, Tibbetts RS, Roos P, Taya Y, Karnitz LM and Abraham RT **(1999)** Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. **Cancer Res** 59, 4375-4382.

Shechter D, Costanzo V and Gautier J **(2004)** Regulation of DNA replication by ATR: signaling in response to DNA intermediates. **DNA Repair (Amst)** 3, 901-908.

Thorpe PH, Stevenson BJ and Porteous DJ **(2002)** Functional correction of episomal mutations with short DNA fragments and RNA-DNA oligonucleotides. **J Gene Med** 4, 195-204.

Wu XS, Xin L, Yin WX, Shang XY, Lu L, Watt RM, Cheah KS, Huang JD, Liu DP and Liang CC **(2005)** Increased efficiency of oligonucleotide-mediated gene repair through slowing replication fork progression. **Proc Natl Acad Sci U S A** 102, 2508-2513.

Zucchi R and Ronca-Testoni S **(1997)** The sarcoplasmic reticulum Ca^{2+} channel/ryanodine receptor: modulation by endogenous effectors, drugs and disease states. **Pharmacol Rev** 49, 1-51.