

ORIGINAL PAPERS

A single acute exposure to a chemotherapeutic agent induces hyper-recombination in distantly descendant cells and in their neighbors

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Homologous recombination can induce tumorigenic sequence rearrangements. Here, we show that persistent hyper-recombination can be induced following exposure to a bifunctional alkylating agent, mitomycin C (MMC), and that the progeny of exposed cells induce a hyper-recombination phenotype in unexposed neighboring cells. Residual damage cannot be the cause of delayed recombination events, since recombination is observed after drug and template damage are diluted over a million-fold. Furthermore, not only do progeny of MMC-exposed cells induce recombination in unexposed cells (bystanders), but these bystanders can in turn induce recombination in their unexposed neighbors. Thus, a signal to induce homologous recombination can be passed from cell to cell. Although the underlying molecular mechanism is not yet known, these studies reveal that cells suffer consequences of damage long after exposure, and that can signal unexposed neighboring cells to respond similarly. Thus, a single acute exposure to a chemotherapeutic agent can cause long-term changes in genomic stability. If the results of these studies of mouse embryonic stem (ES) cells are generally applicable to many cell types, these results suggest that a relatively small number of cells could potentially induce a tissue-wide increase in the risk of *de novo* homologous recombination events.

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Introduction

Our cells are constantly exposed to ionizing radiation and to chemicals that damage DNA. To prevent DNA damage from leading to permanent changes in sequence information, cells have evolved sophisticated DNA repair systems. When damage occurs to just one strand of the DNA duplex, the complementary strand is

generally used as a template during excision repair (Friedberg *et al.*, 1995). However, if both strands are damaged (e.g. by an interstrand crosslink or a double-strand break), another source of sequence information is required for accurate repair. Homologous recombination allows cells to use the undamaged sister chromatid or the homologous chromosome as a template for repair. In addition, DNA lesions can induce replication fork breakdown, which can be restored via homologous recombination (Kraus *et al.*, 2001; McGlynn and Lloyd, 2002; Helleday, 2003; West, 2003). Although homologous recombination is important for safeguarding genome integrity, transfer of genetic information carries with it a certain amount of risk. More than 10% of the mammalian genome is comprised of repetitive sequences (Schmid, 1996), and recombination between misaligned sequences can lead to insertions, inversions, translocations, and deletions (Bishop and Schiestl, 2000). In addition, crossovers between homologous chromosomes can cause loss of heterozygosity (LOH) (Morley *et al.*, 1990; Zhu *et al.*, 1992; Gupta *et al.*, 1997; Moynahan and Jasin, 1997; Shao *et al.*, 1999). Consequently, conditions that lead to hyper-recombination in people are associated with an increased risk of cancer (Aubrecht *et al.*, 1995; Thompson and Schild, 2002; Bishop and Schiestl, 2003).

A long-standing paradigm is that DNA damaging agents create lesions that directly induce mutations, chromosome aberrations, and recombination events via misrepair or when they are encountered by the replication fork. More recently, it has been demonstrated that exposure to DNA damage can cause cells to suffer from a persistent increased risk of sequence changes, many cell generations after the original exposure. For example, Kadhim and co-workers exposed cells to ionizing radiation, grew up clonal populations and measured chromosome aberrations. Surprisingly, cells within a clonal population had different types of aberrations, indicating that new aberrations had arisen during clonal expansion, long after the initial exposure (Kadhim *et al.*, 1992). Similar delayed chromosome aberrations were discovered in embryonic cells derived from irradiated zygotes (Pampfer and Streffer, 1989), and delayed point mutations were shown to occur in irradiated mammalian cells (Little *et al.*, 1990). These studies, together with many others (reviewed in Little, 2003; Lorimore and Wright, 2003), have clearly demonstrated that DNA

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damage can elicit persistent genetic instability that causes cells to suffer from delayed mutations and aberrations. Although the molecular basis for persistent genomic instability is not yet fully understood, there is evidence that progeny of irradiated cells suffer increased levels of reactive oxygen species (Clutton *et al.*, 1996; Limoli *et al.*, 1998) and increased levels of spontaneous double-strand breaks (Suzuki *et al.*, 2003).

Mitomycin C (MMC) is a chemotherapeutic bifunctional alkylating agent that forms recombinogenic interstrand crosslinks (Liu-Lee *et al.*, 1984; Tomasz *et al.*, 1987). While most studies of persistent effects have focused on the effects of ionizing radiation, it has long been known that alkylating agents induce delayed mutations in *Drosophila* (Auerbach, 1946). Furthermore, cells from rabbits and people who have been exposed to MMC have increased levels of sister chromatid exchanges (SCEs) months after exposure (Stetka *et al.*, 1978; Ohtsuru *et al.*, 1980). However, given the toxicity associated with these human and rabbit studies, one cannot distinguish between selection for cells that already had a high rate of recombination, and induction of a persistently increased rate of recombination. Recently, Brennan and Schiestl (2001) showed that exposure to an alkylating agent can increase the risk of delayed recombination events in *Saccharomyces cerevisiae*. Thus, we suspected that MMC might induce persistent hyper-recombination in mammalian cells.

In addition to persistent effects, ionizing radiation can also indirectly induce sequence changes via the bystander effect. Naive cells have been shown to have an increased susceptibility to mutations and chromosome aberrations simply by virtue of being in the vicinity of an irradiated cell or by exposure to media taken from an irradiated dish of cells (Kadhim *et al.*, 1992; Nagasawa and Little, 1999; Zhou *et al.*, 2000; Huo *et al.*, 2001). There is now substantial evidence that transmissible factors induce genetic instability (e.g. reactive oxygen species and cytokines) and can be passed from an irradiated cell to a bystander cell through the media or through gap junctions (Lehnert *et al.*, 1997; Narayanan *et al.*, 1997; Azzam *et al.*, 1998; Iyer *et al.*, 2000; Zhou *et al.*, 2000). Although the bystander effect was discovered by the observation that unirradiated cells cocultured with irradiated cells have an increased risk of SCEs (Nagasawa *et al.*, 1991; Nagasawa and Little, 1992), few studies have focused on the impact of the bystander effect on homologous recombination, and it was not known if the bystander effect is specific to ionizing radiation.

Although there are many reports describing direct induction of recombination by various DNA damaging agents, few studies have investigated the possibility that DNA damaging agents other than ionizing radiation induce persistent hyper-recombination or a bystander effect. Concurrent with the preparation of this manuscript, Huang *et al.* (2004) showed that ionizing radiation induces delayed hyper-recombination in mammalian cells. Here, we have shown the following: (1) persistent hyper-recombination is not confined to

ionizing radiation (more than 30 cell doublings after exposure to MMC, mammalian cells suffer an increased risk of recombination); (2) chemically induced hyper-recombination is not due to residual damage or inherited mutations; (3) unexposed naive cells can become hyper-rec merely by coculture with either cells descended from exposed populations or naive cells cocultured with such descendants.

Results

Creation of a fluorescence-based assay for quantifying recombinant cells

To create a system to study homologous recombination in mammalian cells, a direct repeat substrate was constructed that allows for expression of enhanced yellow fluorescent protein (EYFP) following recombination (Figure 1a). Essential sequences were deleted from either end of the *EYFP* coding sequence to create *5'eyfp* and *3'eyfp*, and mitotic homologous recombination events between these two nonfunctional cassettes can lead to a fluorescent phenotype. A direct repeat was made by placing *5'eyfp* and *3'eyfp* cassettes in tandem (as previously described in Hendricks *et al.*, 2003), and a positive selection marker that confers puromycin resistance and allows for expression of enhanced cyan fluorescent protein (*ECFP*) was inserted between the cassettes (detection of *EYFP* is not affected by *ECFP*). This positive selection marker was included to facilitate analysis of the underlying mechanisms of recombination in future studies.

The recombination substrate was subcloned into an *HPRT* targeting vector to allow for site-specific integration. Following electroporation into J1 mouse embryonic stem (ES) cells, 6-thioguanine and puromycin-resistant clones were selected, and correctly targeted clones were identified by Southern analysis (data not shown). We designated these clones FYDR-HPC, since they carried the FYDR recombination substrate (Hendricks *et al.*, 2003) at the *HPRT* locus and were Puromycin resistant and Cyan fluorescent (HPC).

Homologous recombination via any of several different mechanisms (e.g. gene conversion [with or without associated crossing over], single-strand annealing, and replication fork repair [Paques and Haber, 1999]) can reconstitute full-length *EYFP* sequence (Figure 1a), leading to a yellow fluorescent phenotype. Cells that express *EYFP* can be detected either by flow cytometry or fluorescence microscopy (Figure 1b). Regardless of the underlying mechanism, full-length *EYFP* coding sequence specifically results from homologous recombination events. To test for the presence of full-length *EYFP* sequences in the fluorescent FYDR-HPC cells, nonfluorescent and fluorescent cells were isolated by FACS and analysed by PCR. All of the fluorescent cells carried full-length coding sequences (Figure 1c, upper panel), whereas none of the nonfluorescent cells had full-length coding sequences (as expected, all cells carried at least a portion of the recombination substrate;

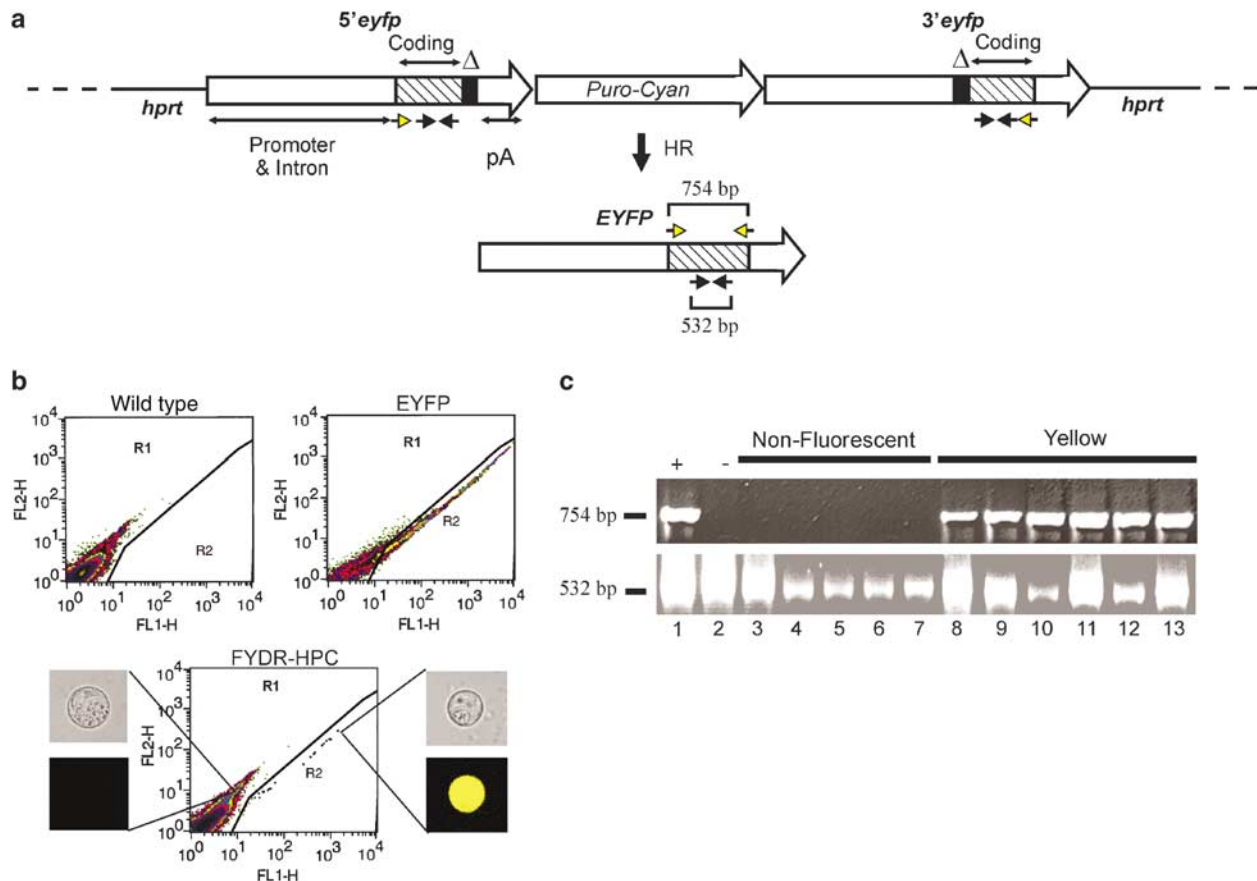


Figure 1 Fluorescence detection of mitotic homologous recombination. **(a)** Schematic showing the design of the FYDR-HPC recombination substrate integrated at the *HPRT* locus. Expression cassettes are represented by large arrows with emphasis on the coding sequences (hatched) and deleted regions (black). Expression is driven by the chicken β -actin promoter and cytomegalovirus enhancer (Okabe *et al.*, 1997). Expression of the puromycin-ECFP fusion protein (*Puro-Cyan*) is driven by the PGK promoter. Homologous recombination (HR) can restore full-length *EYFP* coding sequence (flanking sequences are variable depending on the mechanism of recombination, and have therefore been omitted). **(b)** Flow cytometry results for wild-type J1 ES cells, J1 ES cells expressing full-length *EYFP* and J1 FYDR-HPC cells. Relative fluorescence intensity is 515–545 nm (FL1) versus 562–588 nm (FL2). Yellow fluorescent cells fall within the R2 region, as confirmed by microscopic examination of cells isolated by FACS. **(c)** Nonfluorescent and yellow fluorescent cells were isolated by FACS and analysed by PCR using primers that exclusively amplify full-length coding sequences to yield a 754 bp product (upper panel). To test for the presence of the recombination substrate, PCR was performed using primers that amplify sequences present in *5'eyfp*, *3'eyfp* and *EYFP* (lower panel). Primer positions are indicated in **(a)**. Template DNA was from pCX-EYFP plasmid (lane 1), FYDR-HPC plasmid (lane 2), nonfluorescent clones (lanes 3–7) and fluorescent clones (lanes 8–13)

Figure 1c, lower panel). These results demonstrate that the frequency of recombination events can be estimated by quantifying recombinant fluorescent cells.

When the doubling times for recombinant fluorescent yellow cells and unrecombined nonfluorescent cells were measured, we found that there was no significant difference in their doubling times (11.89 versus 11.86 h, for fluorescent and nonfluorescent cells, respectively). In addition, *EYFP* was found to be stably expressed in recombinant yellow cells for more than 80 population doublings.

Direct induction of homologous recombination by MMC

MMC is a potent recombinogen that creates interstrand crosslinks that induce recombination at stalled replication forks (Niedernhofer *et al.*, 2004; Sasaki *et al.*, 2004). Cells were exposed to MMC at concentrations that

reduced relative growth up to 80% (Figure 2a). After waiting 48 h for maximal expression of *EYFP*, recombinant cell frequency was measured by flow cytometry. We observed a dose-dependent increase in the frequency of recombinant cells, and at the highest dose of MMC, there was an approximately five-fold increase in the frequency of recombinant cells (Figure 2b). Thus, MMC directly induces recombination events in FYDR-HPC cells within the first few cell divisions after exposure.

MMC induces delayed recombination events

To determine if MMC exposure induces an increase in the frequency of homologous recombination events that persists in the descendants of treated cells, we assayed for recombination events occurring many population doublings after exposure. FYDR-HPC cells were exposed to doses of MMC at concentrations that

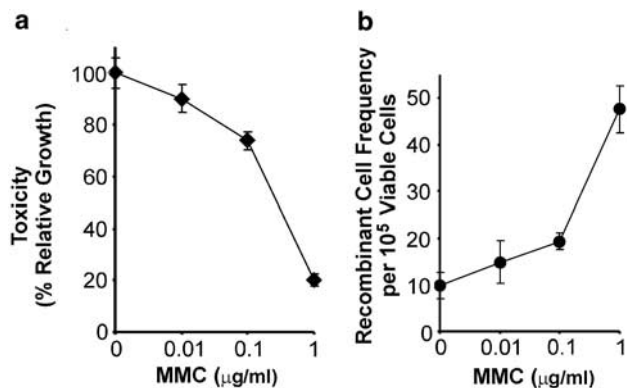


Figure 2 Direct induction of homologous recombination by MMC. (a) Relative cell growth as measured by trypan blue exclusion (normalized to controls). (b) Recombinant cell frequency as determined by flow cytometry 48 h after MMC exposure. (a and b) Representative data from one of three independent experiments is shown. Error bars indicate the standard deviations of three determinations

resulted in either ~50 or ~5% survival (0.01 μg/ml for 24 h and 1.0 μg/ml for 1 h, respectively; determined by a colony formation assay). After exposure, isolated cells were clonally expanded in drug-free media for ~20 population doublings, and the frequency of recombinant cells was evaluated by flow cytometry. Figure 3a shows the frequency of recombinant cells among clones expanded from either control or MMC-exposed cells. As expected, there was a range of recombination frequencies, which is consistent with recombination events occurring at different times during clonal expansion. Since recombination frequencies among independent cultures are not normally distributed, potential differences in the distributions of recombination frequencies were evaluated using the Mann–Whitney *U* test. Although the frequencies among the cultures exposed to 0.01 μg/ml MMC were not statistically significantly higher than controls, cultures descended from cells exposed to 1.0 μg/ml MMC were significantly higher than controls ($P < 0.05$).

The average recombination frequency among control cultures was $4/10^5$ cells. In contrast, the average recombination frequency in cultures descended from cells exposed to either 0.01 or 1.0 μg/ml MMC was $49/10^5$ and $18/10^5$ recombinant cells, respectively. In addition, the highest frequency observed among controls was 54 recombinants per 10^5 cells, whereas 11 cultures descended from MMC-exposed populations had even higher frequencies, ranging from 62 to 2536 recombinants per 10^5 cells (Figure 3a).

All of the cultures shown in Figure 3a originated from a single cell. If a recombination event had occurred during the first cell division, one would expect ~50% of the cells to be yellow fluorescent. If a recombination event had occurred during the second or third cell divisions, the expected frequency would be ~25 and ~12.5%, respectively, and so on. The MMC-treated culture with the highest observed frequency had ~2.5% recombinant cells. Assuming that all of the fluorescent

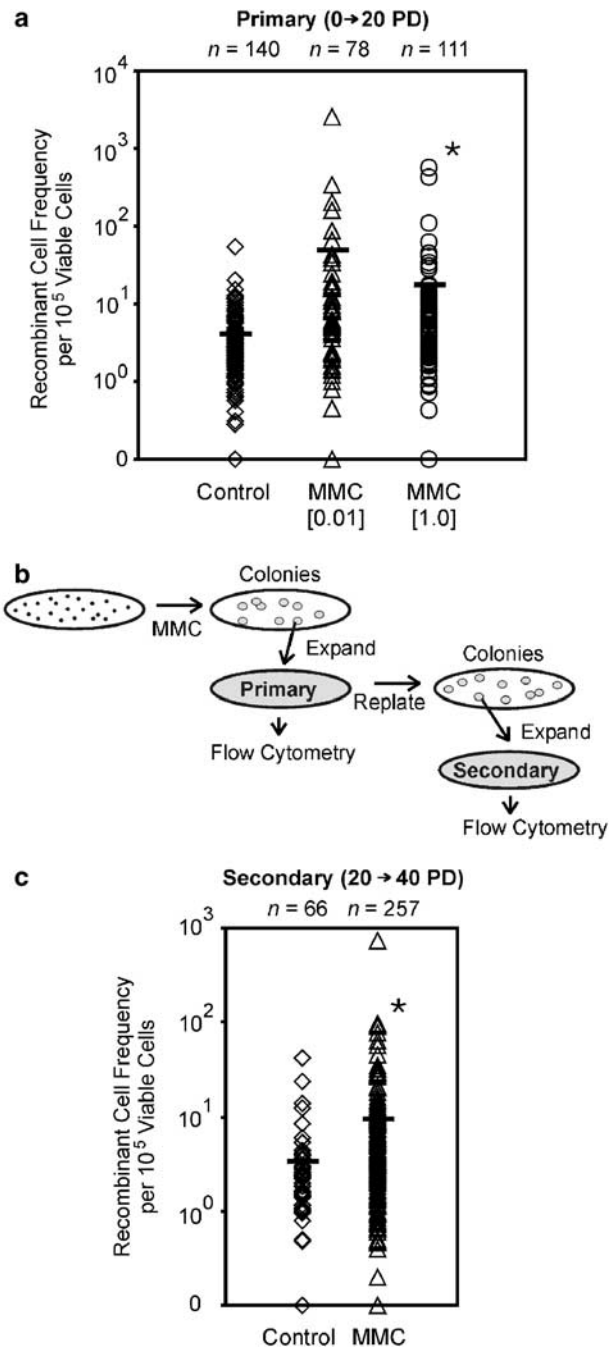


Figure 3 MMC-induced persistent hyper-recombination. (a) Recombinant cell frequency per 10^5 viable cells for clones expanded from control or MMC-exposed FYDR-HPC populations (primary clones). Results from six independent experiments are shown. (b) Schematic diagram indicating the source of primary and secondary clones. FYDR-HPC cells were either exposed to buffer or to MMC and were subsequently clonally expanded for 20 population doublings to create primary clones. Samples were then analysed by flow cytometry and replated at low density to create secondary clones. (c) Recombinant cell frequency per 10^5 viable cells among secondary cultures. Secondary clones were derived from six independent primary control clones, 14 primary clones derived from cells exposed to 0.01 μg/ml MMC and six primary clones derived from cells exposed to 1.0 μg/ml MMC. (a, c) The number of independent clones is indicated (*n*) and the means are shown as horizontal bars. Asterisks indicate a significant increase in recombination frequency relative to controls ($P < 0.05$, Mann–Whitney *U* test). PD, population doublings

cells descended from a single recombinant cell, then the earliest possible occurrence of the recombination event must have been ~ 5 population doublings after exposure. Similar analysis of all data found that most of the MMC-induced delayed recombination events occurred nine or more cell divisions after exposure.

One possible explanation for damage-induced delayed recombination events is that there is residual MMC present in some of the cells, or that template DNA damaged during the original MMC exposure persists long after exposure. To eliminate these possibilities, we initiated secondary cultures starting with cells derived from primary cultures that had undergone at least 20 cell divisions after exposure to MMC (Figure 3b). After 20 cell doublings, the expected residual concentration of MMC is diluted over a million-fold, to levels that are far below those that have been shown to directly induce recombination. Likewise, MMC-adducted template DNA is also diluted one million-fold, such that at most, one cell in a million would be at increased risk of damage-induced recombination due to a residual lesion located at the recombination substrate. Despite dilution of MMC and dilution of damaged template DNA, secondary cultures retained a significantly increased risk of recombination (Figure 3c). Among the secondary cultures, the average recombination frequency was $3/10^5$ among control cultures, and $9/10^5$ among the cultures descended from MMC-exposed cells, and overall, cultures descended from MMC-exposed populations had a higher frequency of recombinant cells ($P < 0.05$; Mann–Whitney U test). There were nine cultures among those derived from MMC-exposed populations that had higher frequencies than any of the control cultures (the highest being $42/10^5$), ranging from 45 to $747/10^5$ cells. These results show that MMC induces delayed recombination events more than 20 cell doublings after exposure, and that these events occur in the absence of residual MMC.

The recombinant cell frequency in secondary cultures is independent of their parental cultures

To determine if primary cultures that have high frequencies of recombinant cells consistently give rise to secondary cultures that have high frequencies of recombinant cells, data from primary and secondary cultures were further analysed. We did not find any correlation between the recombination frequency of the primary culture and that of its descendent cultures; a culture that started with a high frequency gave rise to cultures with a range of frequencies, from low to high. Thus, it is unlikely that the primary cultures with high recombination frequencies represent irreversibly hyper-rec clones.

MMC induces persistent SCEs

To determine if the increased frequency of homologous recombination events at the FYDR-HPC substrate is a measure of a persistent genome-wide hyper-recombination, the frequency of SCEs was assayed ~ 30 cell

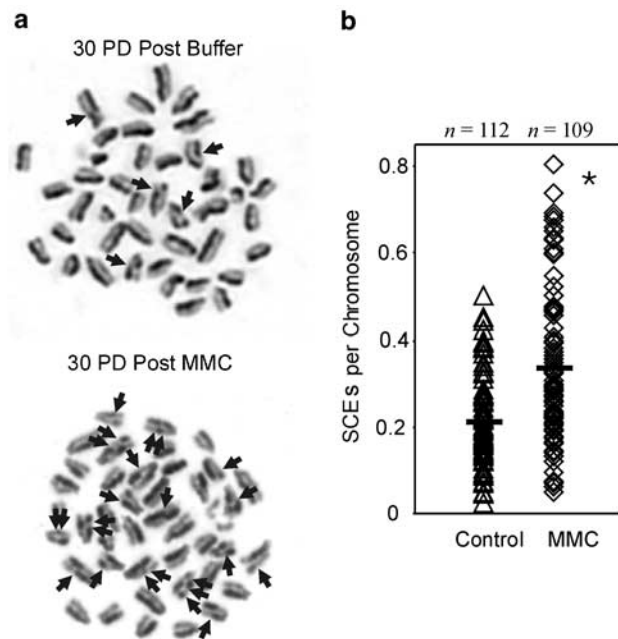


Figure 4 Persistent SCEs 30 population doublings after MMC exposure. (a) Images of metaphase spreads from a cell descended from an MMC-exposed cell and from a control cell 30 population doublings (PD) after exposure. Arrows indicate SCEs. (b) SCEs in descendants of primary cells derived from controls and from cells exposed to either 0.01 or 1.0 $\mu\text{g/ml}$ MMC. The total number of spreads examined in each group is indicated (n); each group was derived from four independent cultures. Means are indicated by horizontal bars and the asterisk indicates statistical significance ($P < 0.05$; Student's t -test)

divisions after MMC exposure. Figure 4a shows examples of metaphase spreads from progeny of MMC-exposed and control cells. The cell descended from an MMC-exposed cell has a significantly higher frequency of SCEs compared to the control cell. Given that sister chromatids are differentially stained by growing cells in the presence of BrdU for two cell divisions, only recombination events that occurred during the previous two replication cycles can be visualized in these metaphase spreads. Thus, these delayed MMC-induced recombination events must have occurred at least 30 doublings after exposure. Compared to control populations, cells descended from MMC-exposed cultures had significantly higher SCE frequencies ($P < 0.01$; Student's t -test). Indeed, fully 17% of the MMC-treated descendants had a higher frequency of SCEs compared to the highest level observed among control cells (Figure 4b). The increased levels of SCEs among cells descended from MMC-exposed populations was statistically significant, regardless of the initial dose ($P < 0.01$ for cultures descended either from 0.01 $\mu\text{g/ml}$ or 1.0 $\mu\text{g/ml}$ MMC-exposed populations; Student's t -test).

Descendants of MMC-exposed cells induce recombination in naive bystanders

The data presented above demonstrate that there is a heritable increased risk of *de novo* recombination events

in the progeny of MMC-exposed cells. In addition to effects that are directly inherited from the parental cell, cells may also affect each other through direct contact or through transmission of molecules between cells. We therefore designed an experiment to determine if MMC-exposed cells can induce recombination in naive bystanders.

There are many studies characterizing an ionizing-radiation-induced bystander effect that leads to genetic instability in naive unirradiated cells cocultured with irradiated cells. However, there are almost no studies that explore the potential of chemicals to induce changes in unexposed bystanders. One reason for the scarcity of such studies may be that it is difficult to differentiate between a bystander effect and direct transmission of a chemical from one cell to another. To prevent direct transmission, we continuously passaged cells for 3 weeks after exposure to MMC. During this time, the cells underwent over 30 doublings, so any residual MMC would have been diluted over a million-fold. These

progeny of MMC-exposed wild-type ES cells (as well as progeny from unexposed controls) were then mixed 1 : 1 with naive FYDR-HPC cells (Figure 5a). Following 2 weeks of coculture, puromycin was added to select for FYDR-HPC cells, and the FYDR-HPC cells were then plated at low density and clonally expanded in order to measure the frequency of recombinant cells. The average recombination frequency among clones expanded from cells cocultured with unexposed J1 cells was $2/10^5$. In contrast, the average recombination frequency was $6/10^5$ cells among clones that had been cocultured with descendants of MMC-exposed J1 cells. In addition, the highest recombinant cell frequency observed in control cultures was $10/10^5$, whereas nine cultures derived from bystanders of MMC-exposed cells had recombination frequencies that were greater, ranging from 12 to $50/10^5$ cells (Figure 5b). The increased susceptibility to homologous recombination events among the naive bystanders is statistically significant at the 90% confidence level ($P < 0.1$; Mann–Whitney U test). These are among

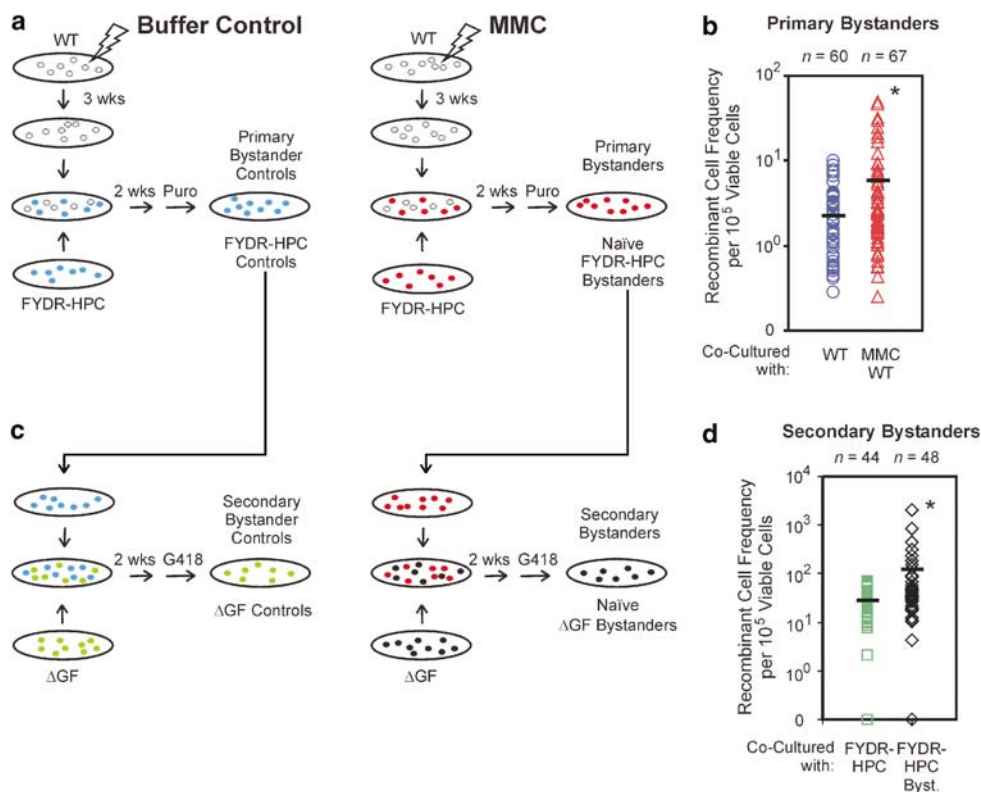


Figure 5 Increased homologous recombination is transmissible via a bystander mechanism. **(a, b)** Schematic diagrams showing the experimental design. **(a)** Wild-type (WT) ES cells were exposed to buffer or $1.0 \mu\text{g/ml}$ MMC for 1 h, rinsed and cultured for 3 weeks prior to cocultured with FYDR-HPC cells at a 1 : 1 ratio. Following 2 weeks of coculture, puromycin was added to the media to select for FYDR-HPC cells. Puromycin resistant FYDR-HPC cells were plated at low density and clonally expanded to determine the frequencies of recombinant cells among independent clones. **(b)** FYDR-HPC bystanders, which had been cocultured either with MMC-exposed WT cells or with control WT cells, were then cocultured with ΔGF cells at a 1 : 1 ratio for 2 weeks. G418 was added to select for ΔGF cells, and surviving cells were clonally expanded for frequency analysis. **(c)** FYDR-HPC bystanders that had been cocultured with MMC-exposed WT cells or with progeny of MMC-exposed WT cells. Asterisk indicates a significant increase in recombination frequency above controls ($P < 0.1$, Mann–Whitney U test). **(d)** Recombinant cell frequency per 10^5 viable cells in ΔGF cells that had been cocultured with control bystander FYDR-HPC cells (that had been cocultured with unexposed WT cells) or with MMC bystander FYDR-HPC cells (that had been cocultured with progeny of MMC-exposed WT cells). The numbers of independent clones are indicated (n) and the means are shown as horizontal bars. Asterisks indicates a significant increase in recombination frequency above controls ($P < 0.01$, Mann–Whitney U test)

the first studies to directly demonstrate that exposure to an alkylating agent causes cells to induce genetic instability in naive bystanders. Importantly, these studies also demonstrate that cells sustain the ability to induce a bystander effect for more than 3 weeks after exposure, during which time cells doubled over 30 times. These results are consistent with those of Lyng *et al.* (2002), who recently showed that 35 doublings after exposure to ionizing radiation, progeny of irradiated cells can induce genomic instability in naive bystanders.

The results of these studies suggest that a signal is transmitted from progeny of MMC-exposed cells to their neighbors. We next asked if cells that receive such a signal can in turn elicit a similar response in their neighbors. FYDR-HPC bystanders were cocultured with Δ GF ES cells that carry an analogous recombination detection system in which cells are neomycin resistant and fluoresce green following somatic homologous recombination (Jonnalagadda *et al.*, 2005). After 2 weeks of coculture, G418 was added to the media to select for Δ GF cells, and surviving cells were clonally expanded and analysed for recombination frequency (Figure 5c–d). The average recombination frequency among control clones was $3/10^5$. In contrast, the average among cultures expanded from cells cocultured with MMC FYDR-HPC bystander cells was $12/10^5$. Whereas the highest frequency observed in controls was $7/10^5$, 11 MMC bystander clones had recombination frequencies ranging from 9 to $210/10^5$ cells. The increased levels of homologous recombination among clones cocultured with FYDR-HPC bystanders as statistically significant ($P < 0.01$; Mann–Whitney *U* test). Thus, naive bystanders that have neither been exposed to MMC damage nor are descendants of such cells can acquire the ability to induce recombination in neighboring cells. These results demonstrate that (1) a persistent increase in susceptibility to *de novo* homologous recombination events can be passed from descendants of MMC-exposed populations to naive bystanders; and (2) bystanders are altered such that they can subsequently induce recombination in naive cells.

Discussion

Here, we have shown that MMC not only directly induces recombination but also induces persistent hyper-recombination in the progeny of exposed mammalian cells. Cells descended from MMC-exposed populations acquire a heritable increased risk of recombination that persists long after exposure. The magnitude of this effect is significant. For example, there was a significantly higher frequency of SCEs among cells descended from MMC-exposed populations more than 30 doublings after exposure. Indeed, $\sim 17\%$ of cells descended from MMC-exposed populations had higher SCE frequencies than the cell with the highest frequency among the controls. Thus, the biological impact of delayed recombination events may be significant.

Although most studies of persistent effects have focused on the effects of ionizing radiation (Morgan *et al.*, 1996), it has been shown that oxidizing agents induce a persistently increased risk of cell death (Mothersill *et al.*, 1998), and alkylating agents induce delayed homologous recombination events in *S. cerevisiae* (Brennan and Schiestl, 2001). Here, we have shown that an alkylating agent induces persistent hyper-recombination in mammalian cells. These results indicate that delayed homologous recombination events are an important facet of persistent genetic instability in mammalian cells, and that persistent hyper-recombination is inducible by a broader range of agents than was formerly appreciated. It has been argued that the spontaneous mutation rate is not sufficiently high in normal cells to account for the prevalence of cancer, and that a mutator phenotype is necessary to explain cancer incidence in people (Loeb, 1991). These experiments raise the possibility that a persistently increased risk of homologous recombination may in some cases contribute to a mutator phenotype that leads to cancer.

If a population of cells is maintained continuously for many cell doublings, cells that have a growth advantage can become over-represented relative to the initial population. If hyper-rec cells had such a growth advantage, then long-term maintenance of a culture would lead to an increased frequency of hyper-rec cells. In these experiments, the frequency of recombination did not increase in control cultures initiated 0 and 20 population doublings after exposure. Thus, selection for hyper-rec cells during long-term culture cannot explain a persistently increased recombination frequency. Furthermore, the observation that progeny of MMC-exposed cells can induce recombination in neighboring cells clearly shows that hyper-recombination cannot be caused by either inherited mutations or residual damage, and must therefore result from alternative mechanisms of heritability. Possible transmissible traits include epigenetic changes in methylation patterns (Wilson and Jones, 1983) and increased levels of reactive oxygen species (Clutton *et al.*, 1996).

What selective advantage might be conferred by persistent and bystander effects? One possibility is that those cells that have increased rates of recombination are protected against toxicity by a subsequent insult. It is well established that recombinational repair defends cells against DNA damage-induced cytotoxicity (Friedberg *et al.*, 1995). Thus, enhanced recombinational repair may be protective. On the other hand, enhanced levels of recombination are associated with an increased risk of deleterious genetic rearrangements. Thus, an optimal solution may be to enhance the levels of recombinational repair in a small percentage of cells, while maintaining normal recombination levels in the majority of cells. This way, if an organism encounters a subsequent insult, some of its cells have enhanced protection, which might allow the organism to survive; and if the organism does not encounter a subsequent insult, the risk of deleterious rearrangements has been minimized.

In addition to inducing persistent effects in the descendants of exposed cells, ionizing radiation also indirectly induces genetic instability in naive unirradiated mammalian cells that are adjacent to irradiated cells (Nagasawa *et al.*, 1991; Nagasawa and Little, 1992). Previous studies have shown that intercellular interactions via gap junctions and transmissible factors allow irradiated cells to trigger genomic instability in naive bystanders (Lehnert *et al.*, 1997; Zhou *et al.*, 2000; Nagasawa *et al.*, 2002), and these intercellular interactions may be an important facet of radiation-induced persistent genomic instability (reviewed in Little, 2003; Lorimore and Wright, 2003; Morgan, 2003; Mothersill and Seymour, 2004). Here, we have shown that cells descended from MMC-exposed populations induce recombination in unexposed neighboring cells, suggesting that the bystander effect may reflect a more general response to DNA damage than was previously appreciated. Furthermore, cells that receive signals to induce recombination can in turn signal their neighbors to induce recombination. Taken together, these results suggest that a signal can be amplified from one cell to a large number of surrounding cells. If these results in ES cells are applicable to normal somatic tissues, then such a transmissible signal may be advantageous to a tissue if the effect is to create cells that are resistant to a subsequent insult. Clearly, it will be important to learn the nature of this signal, and the extent to which these observations reflect a broadly used strategy among various tissues.

Taken together, these results expand on current paradigms of inheritance by showing that intercellular interactions can lead to a heritable increase in cellular susceptibility to recombination, and suggest that past insults can lead to long-term changes in the risk of *de novo* homologous recombination events. If these observations are generally true within normal human tissues, then a single acute exposure to a cancer chemotherapeutic could potentially increase the risk of tumorigenic sequence rearrangements long after the initial exposure.

Materials and methods

Construction of the recombination substrate

Coding sequences from pPUR (Clontech) were PCR amplified using primers carrying synthetic *BglII/KpnI* restriction sites. PCR products digested with *BglII/KpnI* were subcloned into similarly digested pECFP-N1 (Clontech) to create coding sequences for a fusion of puromycin^R and enhanced cyan fluorescent protein (ECFP). Coding sequences for the Puromycin^R-ECFP fusion protein (PC) were PCR amplified, Klenow filled, and subcloned into *SmaI*/Klenow digested pPGK-CAS (van der Lugt *et al.*, 1991) to create a PC expression cassette driven by the PGK promoter. The complete PC expression cassette was PCR amplified using primers carrying synthetic *SalI* sites. The *SalI* digested PCR product was subcloned into the fluorescent yellow direct repeat (FYDR) substrate (Hendricks *et al.*, 2003) at a unique *SalI* site located between the two truncated repeats. *NotI* digestion of the resulting plasmid released the FYDR-PC expression cassette.

A 6 kb fragment encompassing hypoxanthine phosphoribosyl transferase (*HPRT*) exons 6–7 and a 3 kb fragment encompassing *HPRT* exons 8–9 were PCR amplified from pHPT^{+1kb}LT20 (Camenisch *et al.*, 1996), using primers carrying synthetic *SacII/NotI* and *ClaI/XhoI* restriction sites, respectively. PCR products were digested and subcloned into the multiple cloning site of pBluescript II (Stratagene) preserving the *NotI* restriction site for subsequent insertion of the FYDR-PC expression cassette to create the targeting vector FYDR-HPC.

Targeting of ES cells

The FYDR-HPC targeting cassette was released via *SacI* digestion. The purified (Qiagen) cassette was electroporated into 1×10^7 J1 ES cells (gift from Dr R Jaenisch) as previously described (Engelward *et al.*, 1996). Cells containing stably integrated FYDR-HPC substrate were selected with 1 μ g/ml puromycin (Clontech). After 8 days, cells were replated and *hprt* mutant clones were selected with 25 μ g/ml 6-thioguanine (Sigma). After 8–10 days, FYDR-HPC colonies were isolated and expression of PC was verified by cyan fluorescence. ES cells were cultured as previously described (Engelward *et al.*, 1996), with the exception that feeder cells were omitted.

Quantification of doubling time

Yellow fluorescent FYDR-HPC cells were isolated from three independent primary clones (that had been exposed to MMC 2 weeks previously) by fluorescence-activated cell sorting (FACS) as previously described (Hendricks *et al.*, 2003). Pooled yellow fluorescent FYDR-HPC cells or nonyellow untreated FYDR-HPC cells were plated on eight 55-cm² dishes at a density of 500 cells per dish, and colonies were counted to estimate the initial cell number. The final cell number was estimated by Coulter Counting trypsinized dishes.

Assay for direct induction of homologous recombination by MMC

Non-yellow FYDR-HPC ES cells were sorted by FACS and plated at 2×10^5 , 5×10^5 , 7.5×10^5 and 1×10^6 cells per 55 cm² dish in triplicate, and after 48 h, dishes were exposed to 0.0, 0.01, 0.1 or 1.0 μ g/ml MMC (Sigma), respectively. After exposure to MMC for 1 h in unsupplemented DMEM, dishes were rinsed and media was replenished. After 48 h, recombinant yellow cell frequency was determined by flow cytometry (Hendricks *et al.*, 2003).

Assay for delayed homologous recombination events

FYDR-HPC cells were plated at low density (~ 25 cells per 1.9 cm² well) and exposed to MMC after 24 h. Cells were treated with either 1.0 μ g/ml MMC in DMEM for 1 h, or 0.01 μ g/ml MMC in ES cell medium for 24 h. Cells were rinsed with PBS and fresh medium added. Colonies were isolated after 1 week and expanded another 6–7 days to $\sim 80\%$ confluence in 9.4 cm² wells (for primary clones) or 4–5 days to $\sim 80\%$ confluence in 1.9 cm² wells (for secondary clones). Clones were analysed for recombinant cell frequency as previously described (Hendricks *et al.*, 2003).

SCE analysis

FYDR-HPC cells from control or MMC-exposed primary clones (0.01 μ g/ml) were analysed for SCE frequency ~ 30 population doublings after MMC exposure, as previously described (Sobol *et al.*, 2003).

Bystander induced homologous recombination

Wild-type J1 ES cells were treated for 1 h with 1.0 $\mu\text{g/ml}$ MMC in DMEM or DMEM alone. Following treatment, cells were cultured for 3 weeks, and then mixed 1:1 with FYDR-HPC ES cells. Mixed populations of cells were cocultured for 2 weeks. Puromycin (1 $\mu\text{g/ml}$) was then added for 3 days to select for FYDR-HPC ES cells, which were then replated at ~ 25 cells per 1.9 cm^2 well. After 1 week, colonies were isolated, expanded 4–5 days and analysed by flow cytometry. Similar conditions were used for the ΔGF bystanders, except that 400 $\mu\text{g/ml}$ G418 was used to select for ΔGF cells.

 ΔGF cells

ES cells were engineered to carry a direct repeat recombination substrate integrated at the Rosa26 locus. The design of this substrate is similar to that of the FYDR substrate, except that reconstitution of full-length coding sequences leads to

expression of enhanced green fluorescent protein (rather than yellow fluorescent protein), and *3'egfp* is upstream of *5'egfp*. This recombination detection system will be more fully described elsewhere (Jonnalagadda *et al.*, 2005).

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