DISSERTATION

DNA REPAIR AND SISTER CHROMATID EXCHANGE

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY R. TANNER HAGELSTROM ENTITLED DNA REPAIR AND SISTER CHROMATID EXCHANGE BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

DNA REPAIR AND SISTER CHROMATID EXCHANGE

Mitotic recombination that occurs between sister chromatids, known as sister chromatid exchange (SCE), is a common event in mammalian cells; yet very little is understood about SCE. Likewise, the biological relevance of SCE to humans is also unclear. It is generally thought that SCE represents no permanent alteration to genetic information, however, many cancer prone syndromes present elevated levels of SCE and it is not known whether they are a causal factor in cancer progression or simply a symptom of underlying genomic instability. It has also been purposed that SCE occurring in telomeres (T-SCE) may contribute to the aging phenotype seen in progeroid syndromes. Several accelerated aging syndromes, such as progeria, show highly elevated levels of SCE within telomeric regions. The role of DNA repair in SCE regulation and formation is also under investigation. While it has been shown that at least one of the DNA repair pathways, homologous recombination (HR), is likely to be involved in the formation of SCE, it is less clear whether other DNA repair pathways are also involved in either the formation or suppression of SCE.

Therefore, the goal of this research has been to better understand how DNA repair pathways can influence SCE frequency, and how SCE relates to cancer progression and aging. This research also examines how the physical location of SCE, whether it be in genomic (G-SCE) or telomeric (T-SCE) DNA, influence which DNA repair pathways are involved. I examined the role of HR by investigating the Werner (WRN), Bloom (BLM), and FANCD2 proteins. I also investigated the role of non-homologous end joining (NHEJ) by examining the

DNA-dependent protein kinase (DNA-PKcs), both the Ku70/80 heterodimer and the catalytic subunit (DNA-PKcs), and Artemis. ERCC1 is a representative member of the final DNA repair pathway examined, nucleotide excision repair (NER).

Lastly, I determined if/how DNA repair status can influence the ionizing radiation induced bystander effect (BSE). I was able to determine that at least some of the DNA repair proteins are critical in the generation of a bystander signal providing the first evidence that DNA repair can have an influence via an inter-cellular pathway.

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INTRODUCTION

Sister chromatid exchange (SCE) is the reciprocal exchange of genetic material between two sister chromatids in newly synthesized DNA (Figure 1). SCE has been used widely as a marker for genomic instability and is used in numerous mutagenic studies, yet surprisingly little about them is known. However, SCE are generally not thought to represent any permanent alteration in genetic information. This manuscript will attempt to better understand the biological relevance of SCE, and how DNA repair pathways can influence SCE frequency.

SCE are widely regarded as a response to DNA damage. The specific type of DNA damage that could ultimately result in a SCE is likely variable, as agents that produce base damage, interstrand crosslinks (ICL), and single-strand (SSB)/double-strand DNA breaks (DSB) can all lead to increases in SCE. Many cancer-prone syndromes, such as the chromosome breakage syndromes Blooms Syndrome (BS) and Werner Syndrome (WS), also show high levels of SCE. The most popular theory involves DNA damage, whatever type that may be, stalling progressing replication forks. SCE functions to bypass the stalled replication fork and continue replication. This theory has gained support, as it explains how extremely varied agents and diseases can all lead to an excess of SCE.

An interesting aspect of SCE is its relationship to DNA repair mechanisms. Because DNA damaging agents can give rise to SCE, it is intriguing that some DNA repair proteins seem to play an active role in the formation of SCE. It is intuitive that DNA repair pathways would be able to "fix" the DNA damage thereby bypassing the need for SCE. In fact, in some cases this is actually seen. However, it is also apparent that SCE may need an intact repair system to occur. Currently, there are no known mutations that can eliminate the formation of SCE

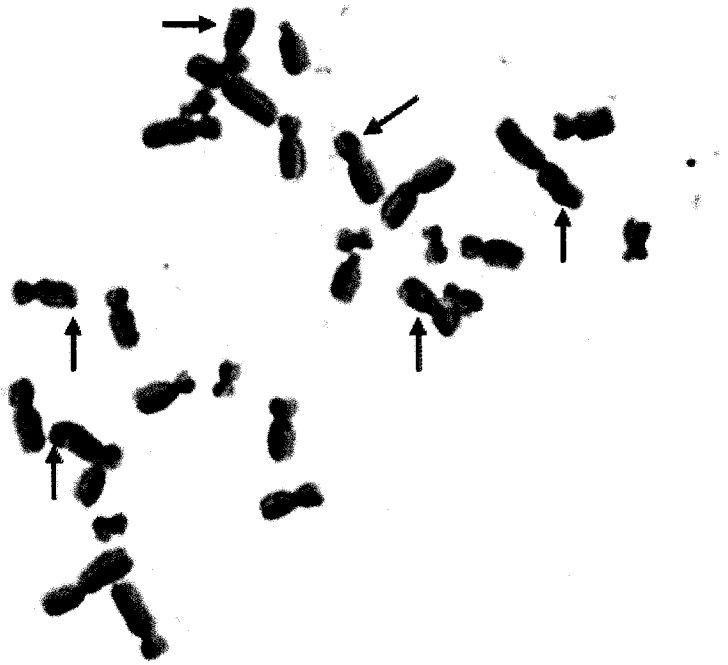

Figure 1: human metaphase chromosome illustrating differential "harlequin" staining pattern; arrows indicate SCE. Image courtesy of Abby Williams.

altogether. This may suggest that SCE are necessary for survival, or that the repair pathway that produces SCE is needed for survival. It is not clear what DNA repair pathways, or what specific proteins in these pathways, are actually involved in the formation of a SCE or what proteins are involved in the suppression of SCE. This research focuses on some of these issues. In particular, I show that certain members of the homologous recombination (HR), non-homologous end joining (NHEJ), and to some extent the nucleotide excision repair (NER) pathways can influence SCE frequencies. Also, I demonstrate differential regulation of SCE dependent on their physical location, i.e, whether genomic or telomeric. Finally I examine how ionizing radiation (IR) - induced bystander effect (BSE), a known SCE inducer, can be altered by a cell's DNA repair status.

Sister Chromatid Exchange

SCE has been used as a marker for genomic instability for a number of years (Jacobs, 1977; Kligerman, 1979; Nakanishi and Schneider, 1979). It is generally thought that the induction of chromosomal aberrations, along with an increase in SCE, indicate higher levels of genomic instability, which could ultimately lead to cancer. It has been shown that SCE can lead to deletions and reverse translocations (Oh et al., 2007). It has also been shown that the template strand itself is subject to deletions and loss of heterozygosity (LOH) (Abdulovic et al., 2006). These, in theory, could all lead to situations where cancer incidence would be increased. This would fit well with other studies of cytogenetic aberrations and cancer. For instance, Hagmar et al., analyzed lymphocytes from over 3500 patients and found that chromosomal aberrations indeed correlated to cancer incidence. However, when analyzing SCE frequencies in lymphocytes from over 2500 patients, no correlation between SCE frequency and cancer incidence was observed (Hagmar et al., 1998).

The lack of correlation between SCE's and cancer frequency is seen as especially interesting, as SCE's have been used in a number of mutagenic studies as an indicator of the potential cancer risk of particular agents. If SCE frequencies does not show a strong correlation to cancer incidence, that brings the validity of these studies into question and the importance of truly understanding SCE formation becomes of even greater importance. Therefore, it is necessary to understand both the mechanism of SCE formation and whether SCE's are detrimental, neutral, or perhaps even beneficial to a cell by allowing chromosome stability.

SCE was first described in the late 1950's using autoradiographic techniques. Using tritiated thymidine, Taylor and Hughes demonstrated a distinct recombination event between two

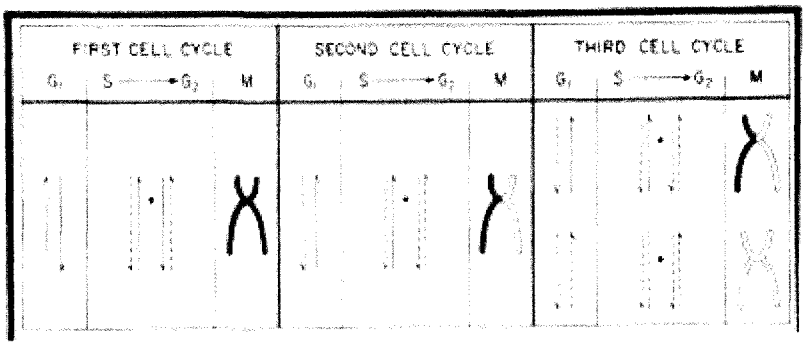

Figure 2. Diagram demonstrating BrdU incorporation dependent on cell cycle.

sister chromatids (Taylor, 1958). However, this technique was technically demanding, and resolution was poor; only very large SCEs could be detected. In the 1970's Samuel Latt developed the anti-BrdU staining technique, which was technically less demanding than autoradiographic techniques and afforded much better resolution, facilitating detection of much smaller SCEs (Latt, 1973). This was quickly followed up by the development of the fluorescence-plus-Giemsa (FPG) staining technique (Perry and Wolff, 1974). The FPG technique utilizes the thymidine analogue bromodeoxyuridine (BrdU); when the cells replicate, BrdU will be incorporated into the newly synthesized DNA. After a second round of replication, BrdU will again be incorporated into newly synthesized DNA, giving rise to two sister chromatids with differential BrdU substitution. The chromatid containing the original DNA

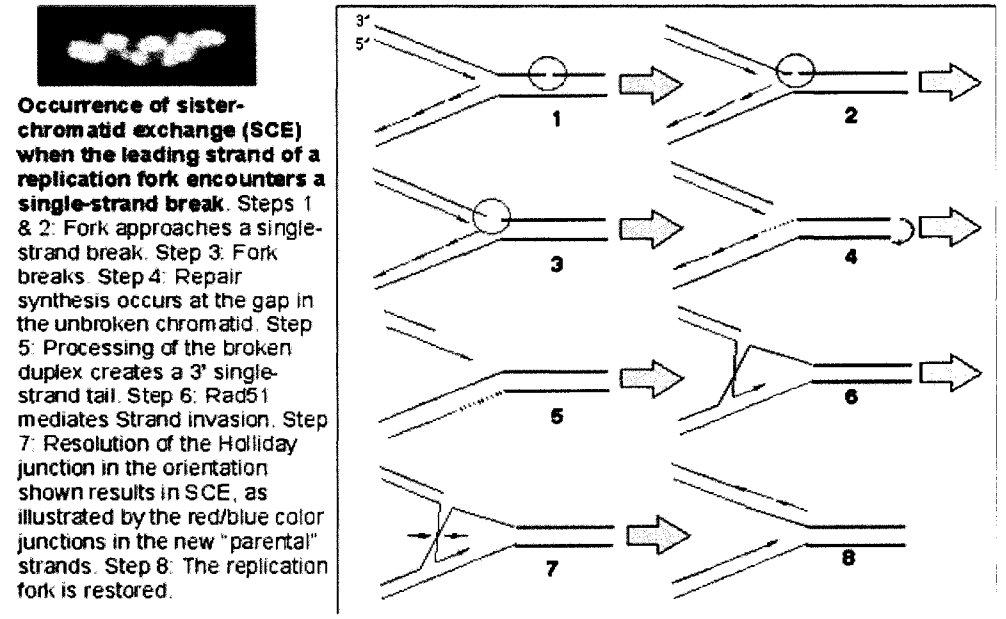

Figure 3. Diagram of SCE occurring at stalled replication fork. www.llnl.gov/bio/groups/dna_repair/scehtml.

strand will have one strand containing BrdU and one without BrdU. The other chromatid, the one made from the new strand created in the first round of replication, will have two strands containing BrdU (Figure 2). This can be detected visually using a fluorescence probe for BrdU, or in the case of FPG, the strands containing the BrdU are degraded, with UV and hot salt solutions, giving rise to "harlequin" stained chromosomes. In this way, the chromatid with the original DNA strand will have less BrdU incorporation, less degradation, and therefore more DNA which will stain darker upon exposure to Giemsa stain than the chromatid in which both strands contained BrdU that had more degradation and less DNA. In either case, a color "switch" between sister chromatids indicates a SCE.

The conventional model for SCE involves stalled replication forks (Figure 3). It is known that DNA can be damaged in normal metabolic activities leading to the creation of single

stranded "nicks"/breaks. During replication, replication forks will encounter these nicks and in the process, convert the single stranded break into a DSB. The DSB will cause the replication fork to stall and even potentially collapse. At this point, the resulting structure is a ready substrate for HR. There is one complete chromatid in close proximity to a DSB that has the same homologous sequence. It is assumed that Rad51-dependent strand invasion leads to the formation of a Holliday Junction (HJ). Depending on how the HJ is resolved will determine whether crossover, and ultimately a SCE, will occur. Considering that there are only two options, crossover and non-crossover, one could predict that a SCE would form approximately fifty percent of the time. However, it is becoming clearer that the proteins involved in SCE may push the frequency more one way or the other depending on the situation.

This model fits very well with much of the SCE experimental data. In this model there are three distinct steps; (1) generation of the DNA damage and stalling of the replication fork, (2) RAD51-dependent strand invasion forming the HJ, and (3) resolution of the HJ. The next sections will go over each step and give examples of what agents or proteins are known to be involved.

The Initiation

SCE are most likely a consequence of DNA damage. DNA can be damaged in several ways: base or nucleotide damage, ICL (interstrand crosslink), SSB (single-strand breaks), and DSB (double-strand breaks). All of these possess the potential of inducing SCE by causing replication forks to stall, explaining how a variety of different agents are capable of inducing SCE.

The classical SCE inducing agent is mitomycin C (MMC) (Abdel-Halim et al., 2005; Duydu et al., 2006; Wojcik et al., 2005). MMC is a potent inducer of SCE due to its mode of action. MMC is an ICL forming protein that also produces large amounts of reactive oxygen species (ROS). The ICL can easily stall replication forks by preventing DNA strand separation needed for progression of the replication fork. ROS can also damage DNA by direct oxidation of the DNA strand itself, or the oxidation of the hydration shell surrounding the DNA which, in turn, can oxidize the DNA.

MNNG (N-methyl-N'-Nitro-N-Nitrosguanidine) is an alkylating agent that is another common SCE inducer. This compound fits well with the proposed model, as MNNG's mode of action is via the induction of DNA SSB which readily stalls replication forks (Anderson et al., 1999). Another common agent that results in SCE induction is BrdU (Wojcik et al., 2003). When accompanied by 254 nm UV, this thymidine analogue causes a marked increase in SCE frequency. Interestingly, when 313 nm UV is used, no increase in SCE is seen. This is attributed to the probability that BrdU absorbs light energy at 254 nm but not at 313 nm. When BrdU is exposed to the 254 nm UV, it is very likely that the excitation could lead to the formation of SSB at the location of BrdU incorporation, leading to increased probability of stalled replication forks.

Hydroxyurea (HU) is an interesting inducer of SCE, as it's mode of action is different from the other classical agents. HU interferes with ribonucleotide reductase, thereby inhibiting nucleotide synthesis and ultimately stalling DNA replication (Matsuoka et al., 2004). The mode by which HU increases SCE strongly supports the model that stalled replication forks can lead to SCE.

There are also a number of therapeutic and environmental agents that increase SCE frequency (Kochhar et al., 1996; Marzano et al., 2004; Stanimirovic et al., 2005). The antineoplastic agent, cisplatin, is a known SCE inducer (Bergs et al., 2006). Agents that may be found in hazardous work conditions such as arsenic and platinum compounds, along with some pesticides have been shown to increase SCE levels (Feng et al., 2005; Miyachi and Tsutsui, 2005). Resveratrol, a compound typically found in red wine with radioprotective properties, has also been shown to increase SCE levels (Matsuoka et al., 2004). The hydroxyl group on resveratrol is essential for SCE induction, implying a possible role for ROS dependent DNA damage (Matsuoka et al., 2004). Along this line, people exposed to hyperbaric oxygen treatment display an increased sensitivity to MMC induced SCE (Duydu et al., 2006). Perhaps the most unusual inducer of SCE involves amino acids. Xing et al. demonstrated that an increase in amino acid concentration in media can lead to an increase in SCE (Xing and Na, 1996). While this was true for most amino acids, some seemed to be more potent inducers than others. There is no current hypothesis for the mechanism and if it has any biological relevance.

With the abundance of DNA damaging agents that can induce SCE, it is interesting that ionizing radiation (IR) does not affect SCE levels. Low LET (linear energy transfer) IR has been reported in the literature and shown in our lab to have little influence on SCE levels (Ardito et al., 1980). However, α particles and other higher LET types of radiation can induce genomic SCE (Nagasawa et al., 1990). This is probably due to both the greater amounts and the types of DNA damage that occur in the presence of high LET. High LET radiation would generate more ROS in the water surrounding the DNA, which could then lead to the specific type of damage that would lead to a SCE. It must also be understood that SCE frequency could be altered by the presence of "false" SCE. These are situations in which reciprocal translocations would look

identical to a SCE under cytogenetic evaluation. So it must always be a consideration that an increase in SCE frequency may actually be an increase in reverse translocations.

Proteins that are associated with initiating step in the SCE model include many DNA repair proteins. If the DNA damage is repaired before DNA replication occurs, the replication fork will not stall and necessitate a SCE. Along these lines, some of the first proteins implicated in SCE formation belong to the base excision repair (BER) pathway. One of these proteins, XRCC1 (x-ray repair cross complementing 1) was first identified in a mutant CHO (Chinese hamster ovary) cell line, EM9, that showed extreme hypersensitivity to ethyl methanesulfonate (EMS) leading to increased mutations and a seven fold increase in SCE (Thompson et al., 1982). It was later determined that the EM9 strain carried a defective copy of XRCC1 (Thompson et al., 1990; Thompson et al., 1985).

The BER pathway is very similar in function/mechanism to the nucleotide excision repair (NER) pathway which can also display elevated SCE levels. In each case the damaged base or nucleotide is removed by a specific glycosylase creating an abasic site. APE1 (apurinic/apyridinic endonuclease 1) can then cut the phosphodiester bond allowing DNA polymerase B to add the appropriate nucleotide to the strand. DNA ligase IIIα is then able to ligate the ends together repairing the break. XRCC1 is a non-enzymatic factor in BER and is associated with the nick ligation step most likely as a stabilizing factor for DNA ligase IIIα. XRCC1 may also play a scaffolding role in SSB repair (Fortini et al., 2003).

Poly (ADP-ribose) polymerase 1 (PARP-1) is involved in a number of different pathways including BER, SSB repair, and DSB repair. In BER, PARP-1 has been shown to interact with XRCC1 (Dantzer et al., 2006). It has also been shown to utilize its "PARP" function to transfer

ADP-ribose to other proteins upon exposure to SSBs. Finally PARP-1 has been shown to interact with the non-homologous end joining (NHEJ) protein DNA-PK (Audebert et al., 2006; Dominguez-Bendala et al., 2006), and may act as a switch between HR and NHEJ (Dominguez-Bendala et al., 2006; Wang et al., 2006).

Deficiencies of XRCC1, PARP-1, and also Ligase IIIα have all been shown to elevate levels of SCE (Fan et al., 2007; Yang et al., 2004). Given their roles in DNA repair, it is likely they are involved in the initiation step of SCE; in the absence of these proteins, unrepaired DNA damage is encountered by the replication fork.

Interactions at the stalled replication fork

In most circumstances, a cell will attempt to resolve a stalled replication fork and proceed through replication. It is important to keep the replication machinery at the site of the stalled replication fork, as the consequences of fork collapse are usually deleterious. As a result, a number of proteins are involved in the stabilization and restart of stalled replication forks.

ATR (ataxia telangectasia and Rad3-related kinase) is an important PI3KK that helps stabilize and restart stalled replication forks. ATR first helps stabilize the stalled fork by preventing the release of replication machinery, mainly Polymerases α and epsilon (Paulsen and Cimprich, 2007). ATR also assists in stabilization by signaling cell-cycle checkpoints via Chk1, thereby allowing the cell time to resolve the stalled replication fork (Zachos et al., 2005).

The RecQ helicases WRN and BLM act at stalled replication forks, via their helicase activity, helping to prevent aberrant structures from forming (Khakhar et al., 2003). WRN may help to resolve telomeric D-loop structures, facilitating replication (Opresko et al., 2004b). Several of the replication-associated polymerases require WRN and BLM for stabilization at

stalled replication forks (Bjergbaek et al., 2005). In addition, it appears that ATR and CHK1 are necessary for BLM stabilization at stalled forks, indicating another possible role for ATR involvement in re-starting stalled replication forks (Sengupta et al., 2004).

Strand Invasion

From very early on, SCE has been regarded as a RAD51-dependent HR event. The presence of two identical sister chromatids in close proximity makes HR possible. However, it has been difficult to examine the role of HR in SCE as mutants of this pathway are usually not viable, especially in higher eukaryotes. For this reason, much of the work has been done in yeast, or more recently, chicken cells.

In the yeast *Saccharomyces cerevisiae,* RAD51 mutants showed decreased levels of both spontaneous and damage-associated (via MMC) SCE (Fasullo et al., 2001). Interestingly, some of the RAD-related proteins, mainly RAD54, seem to be involved specifically in damage-associated SCE and not spontaneous SCE (Dronkert et al., 2000; Heyer et al., 2006). These proteins only show a difference when a cell is exposed to some sort of damaging agent, but are not involved in the resting background of SCE. Yeast has not only been useful in studying SCE because of their ability to grow in the absence of an intact HR pathway. However, results from yeast experiments do suggest another possible role for SCE. Gonzalez et al., utilizing recombination substrates ,were able to demonstrate that yeast can use SCE to repair exogenously induced DSB in artificial substrates in a RAD51-dependent manner (Gonzalez-Barrera et al., 2003). This is intriguing as it fits well with the stalled replication fork model, and it is dependent on the formation of a DSB before strand invasion can occur.

The immortalized B-cell DT-40 chicken cell line has also been used to study the role of RAD51 in SCE. Sonoda et al. generated HR defective knockouts in the DT-40 cell line, and thereby analyzed the role of both RAD51 and RAD54 in spontaneous and DNA damage-induced SCE (Sonoda et al., 1998; Sonoda et al., 1999). They concluded that RAD51 and RAD54 are important for both spontaneous and MMC- induced SCE, as the frequency of SCE was significantly reduced in the mutants. However Ku, the non-homologous end-joining protein, had no role on SCE in this system.

The results of Lambert et al. added additional support for a role of RAD51 in SCE formation in higher eukaryotes (Lambert and Lopez, 2001). Using CHO cell lines, they transfected in expression clones for either wild type RAD51 (MmRAD51) or a dominant-negative RAD51 (SMRAD51) and then analyzed the frequency of SCE. The wild type RAD51, MmRAD51, displayed normal levels of spontaneous SCE, while the SMRAD51 dominate-negative mutant showed slightly lower levels. Interestingly, no protein mutations have ever led to a zero SCE frequency. However, upon introduction of the alkylating agent (MNU), only the wild type RAD51 displayed increased levels of SCE (Lambert and Lopez, 2000; Lambert and Lopez, 2001). This suggested that for SCE formation after DNA damage, an intact HR system must be in place.

While it appears that RAD51 is necessary for SCE formation, the data for the involvement of RAD51 associated proteins seems contradictory. The breast/ovarian cancer susceptibility proteins BRCA1 and BRCA2 interact with RAD51 (Cousineau et al., 2005). BRCA2 in particular has been shown to directly associate with RAD51 (Powell et al., 2002). BRCA2 is believed to be important in nuclear transport of RAD51 and also in the formation of RAD51 filaments onto single-stranded DNA, which can then invade the duplex sequence

(Gudmundsdottir and Ashworth, 2006). BRCA1, on the other hand, seems to have a more indirect function as a scaffolding protein that interacts with DSB sensors such as ATM (ataxia telangectasia mutated), ATR, and the MRN (MRE11/RAD50/NBS1) complex (Cortez et al., 1999; Durant and Nickoloff, 2005; Kobayashi et al., 2004; Tibbetts et al., 2000). Considering their involvement with RAD51, especially BRCA2, one might assume that they too would influence SCE formation. In fact, Kim et al. demonstrated this by looking at SCE in lymphocytes from BRCA2 heterozygotes. They observed dramatic increases in spontaneous SCE frequency compared to the BRCA2 +/+ controls (Kim et al., 2004). Godthelp et al. reported slightly different results after examining the role of BRCA2 in both fibroblast and B-cell lines from BRCA patients. While they did not see an increase in spontaneous SCE, they did notice that these cell lines did not respond to MMC, a known SCE inducer (Godthelp et al., 2006). This suggests that BRCA2, like RAD51, is needed to form a SCE. On the other hand, BRCA1 may not have an essential role in SCE formation. Trenz et al. who were using cell lines from BRCA1-deficient patients saw no difference in SCE compared to controls upon exposure to cisplatin or bischloroethylnitosurea (BCNU) (Trenz et al., 2003). This would suggest that the damage sensing role of BRCA1 may not be necessary to form a SCE.

Resolution of the Holliday Junction

Following RAD51 strand invasion, a DNA polymerase uses the sister chromatid as a template to extend the invading single-stranded end. This extension helps the creation of two distinct structures. The first is the D-loop (displacement loop); the invading strand pairs with homologous sequences, the opposite strand is displaced creating an open loop. The second

structure involves a second strand invading a portion of the D-loop to form an "X-like" structure termed the Holliday Junction (HJ). The HJ was first described by Holliday in *Ustilago maydis* after exposure to MMC leading to recombination-based crossover (Holliday, 1964).

The proteins involved in HJ resolution have yet to be fully elucidated. A few proteins have been implicated, but their exact role is unclear. Perhaps the most prominent protein associated with HJ is the BLM helicase. BLM is an important member of a complex that is being termed the "resolvasome" or the "dissolvasome" which also contains many other member of the homologous recombination pathway such as the RAD52 group (Symington, 2002). BLM has been shown to branch migrate single HJ's. BLM has also been shown to be necessary to resolve double HJ's in vitro. This latter function is dependent on topoisomerase IIIα (TopoIIIα). It is likely that TopoIIIα relaxes the supercoiled DNA behind the double HJ which then allows BLM to resolve the DHJ by "colliding" the two separate HJ's. It is also possible that TopoIIIα can create nicks in front of the HJ progression allowing resolution. The BLM/TopoIIIα complex is held together by the BLM-Associated Polypeptide-75kD (BLAP75). This seems to be especially critical as siRNA experiments with BLAP75 show a marked increase in SCE similar to that seen in BLM-/- cells (Yin 2005). Another member of the resolvasome complex is replication protein A (RPA). RPA assists the BLM complex by assisting in converging branch migration.

A model has been proposed that BLM in solo can suppress SCE by migrating mobile D-loops thereby preventing full RAD51 strand invasion and promoting single strand annealing (SSA) (Cheok et al., 2005b). Also, BLM along with the so-called "guardian of the genome" p53 helps suppress RAD51 strand invasion in a BLM-dependent manner (Yang et al., 2002). BLM is necessary for p53 recruitment to stalled forks (Plank et al., 2006; Sengupta et al., 2003).

However, if RAD51 strand invasion does occur, then BLM, in conjunction with TopoIIIα, suppresses SCE by resolving the double HJ in a non-crossover manner (Plank et al., 2006). BLM can suppress SCE by migrating the HJ far enough to prevent outright collapse of the HJ (Karow 2000).

One might question why the resolvasome would be necessary. Crossover between two sister chromatids would not initially seem detrimental as they have identical sequences. However, as in the case of BLM cells, sister chromatid recombination with crossover can lead to deletions and translocations (Cheok et al., 2005a). Interestingly, it has also been reported that this is not strictly limited to the invading strand, but rather, the template strand itself can be subject to deletions (Schlitz 2006).

Once the resolvasome has resolved the HJ, the process is complete. Whether crossover occurred or not determines whether a SCE can be visualized. Given the number of proteins involved in the suppression of strand invasion, the stabilization of the HJ once formed, and the resolution of HJ into a non-crossover product, demonstrates the cell's attempt to prevent SCE's. This lends supports the idea that a SCE is, or can be, a deleterious manifestation of problems encountered during replication.

What are SCEs good for?

Almost from the beginning, SCE's have been used in mutagenic studies. Anthony Carrano was one of the first to suggest their usefulness in these types of studies. Given they occur with such high frequency and their apparent sensitivity to genotoxic stress, they seem ideal candidates for testing mutagenicity. In fact, one study demonstrated that SCE had a linear

response to mutagenic agents similar to that of *hprt* locus studies (Carrano et al., 1978).This sparked many studies utilizing SCE as a marker for toxicity of specific agents. Examples include, but are certainly not limited to, studies on specific agents such as formaldehyde and toluene (Hammer, 2002; Shaham et al., 2002), studies on specific medical treatments or medical devices (Baysal et al., 2003; Montanaro et al., 2006), and even to long-term studies involving professional workers who handled certain medications (Pilger et al., 2000). This is only a small sampling, as studies utilizing SCE as a marker ranges in the high thousands of articles.

In particular, SCE have been used in a variety of genomic instability studies. Genomic instability is the underlying instability that is often associated with cancer risk. For example, radiation exposure can cause long term instability which may account for the long term cancer risk associated with radiation exposure (Okayasu et al., 2000). The use of SCE as a marker of genomic instability is interesting as there is some debate as to whether SCE are even considered detrimental. While there have been some reports that do not find any correlation between SCE and cancer (Hagmar et al., 1998), it is now becoming the accepted theory that SCE are a result of underlying genomic instability and are therefore detrimental.

Some issues with SCE include that they are often a difficult marker to use in terms of experiments utilizing ionizing radiation (IR). Most forms of direct irradiation do not lead to any significant increase in SCE (Ardito et al., 1980; Morgan and Crossen, 1980). For awhile, many people thought that γ- irradiation to an increase in SCE and it was often cited in studies involving occupational workers that were incidentally exposed to radiation (Mrdjanovic et al., 2005). However, it was later determined that this was dependent on BrdU incorporation into the DNA before irradiation, and so was likely due to increased sensitivity from the BrdU incorporation and response to radiation (Morales-Ramirez et al., 1983). However, some types of radiation can lead

to increased SCE's, specifically high LET α particles. Aghamohammadi et al. demonstrated that plutonium-238 α-particles could induce SCE in human lymphocytes while X-ray irradiation could not (Aghamohammadi et al., 1988). This was also seen by other investigators and in other cells lines, including mouse cells (Nagasawa et al., 1990). These studies indicate that it may be the type or extent of damage that the high LET α-particles generate that is necessary to induce SCE. Interestingly, these studies eventually led to uncovering of the radiation-induced bystander effect (BSE), which will be discussed in more detail later.

While SCE make a good marker due to their extreme sensitivity to agents, it is important to keep in mind that using appropriate controls is absolutely critical. The percentage of oxygen cells are grown at, the age of the cells, and the even the media they are grown in all influence SCE frequencies. For that reason, it is often inappropriate to try and compare absolute numbers between studies.

Telomeres and SCE

The ability to study SCE at telomeres is a relatively new development due to recent technological advancements in molecular cytogenetics. Given telomeres relative small size ranging from about 5-15kb in humans, it would be difficult or impossible to visualize a SCE using solid staining techniques. Indeed, early studies on this issue state that SCE are more likely to occur at regions in the "middle" of the chromosome arm rather than the ends or the centromeres (Ladygina et al., 1991). However, with the development of Chromosome Orientation-Fluorescence *In Situ* Hybridization (CO-FISH) (Bailey et al., 1996), which will be

discussed later in much more detail, allowed for much improved detection of SCE within telomeric regions.

It was the development of CO-FISH that demonstrated that SCE occur in very high numbers in sub-telomeric regions (Cornforth and Eberle, 2001; Wang et al., 2005). It was later calculated using a different system that SCE occur in telomeres and sub-telomeric regions an astonishing 1,600 and 160 times, respectively, more frequently than other areas of the genome (Rudd et al., 2007). It now seems clear, at least with the current model, why SCE would occur in telomeric regions at such high frequencies. The telomere sequence, which contains a high percentage of guanine, is subject to complex secondary structures such as G-quadruplexes (Dai et al., 1995). These types of structures are able to stall replication machinery, the initiating event in our model. Also, the sequences allows for easy strand invasion.

The implications of SCE occurring within the telomeric DNA, termed T-SCE (telomeric SCE), is profound, especially in terms of cancer. Telomeres progressively shorten with each cell cycle, eventually leading to a critically short telomere that can trigger cellular senescence (Hayflick, 1965; Olovnikov, 1996). It is important for a cancerous cell to maintain its proliferative capabilities. Most cancers do this by reactivating telomerase, a reverse transcriptase that specializes in lengthening telomeres (Greider and Blackburn, 1989). However, a small subset of cancers use a telomerase-independent mechanism termed alternative lengthening of telomeres (ALT) (Bryan et al., 1995; Murnane et al., 1994). It is becoming more apparent that ALT uses T-SCE to make this exchange; however, the reverse is not necessarily true; the presence of a T-SCE does not mean that ALT is present. Further evidence that T-SCE is important in terms of cancer comes from a study by Cottliar et al. Studying the lymphocytes from patients suffering from ulcerative colitis they determined that the patients had a highly

unstable genome, particularly in the telomeres, leading to high frequencies of telomeric associations and T-SCE'(Cottliar et al., 2000). Recently, Glagoev and Goodwin demonstrated that asymmetric T-SCE could help bypass "short-telomere" induced senescence allowing for immortalization in ALT cell lines (Blagoev and Goodwin, 2008).

While SCE has been a valuable marker for mutagenic studies as mentioned earlier, the use of T-SCEs in those types of studies has not been utilized. It has generally been assumed that T-SCEs are regulated in a similar manner as their genomic counterparts. However, this appears not to be the case. Some recent studies have pointed out, that at least in several situations; SCE frequencies in the telomere and the genome are differentially controlled. An example of this is found in the chromosome breakage syndrome, Werner Syndrome (WS). Laud et al. created a mouse model that was null for both the Werner protein (Wrn) and telomerase (Terc). When aged for several generations, these mice displayed the typical accelerated aging phenotypes seen in WS humans (Laud et al., 2005). Interestingly, these mice displayed extremely high levels of T-SCE compared to controls, while there was no distinguishable difference in genomic SCE (G-SCE) (Laud et al., 2005).

SCE frequencies are influenced by a number of different proteins and pathways. The next few sections will focus on these pathways in general, and also on specific proteins that regulate SCE.

Homologous Recombination

It is vital for a cell to repair DSBs efficiently and correctly. A DSB that remains unrepaired can ultimately lead to cell death, while a DSB that is repaired incorrectly can lead to

mutagenesis (Hande, 2004). Homologous recombination (HR) is a main pathway for DSB repair, occurring mainly in late S phase/G_2 phase. HR is dependent on utilizing an unbroken strand of homologous sequence, usually a sister chromatid, in order to repair the DSB (Wilson and Thompson, 2007). While the homolog could be used for this process, it seldom is, mainly due to spatial reasons and potentially dangerous situations such as loss of heterozygosity (LOH) (Abdulovic et al., 2006).

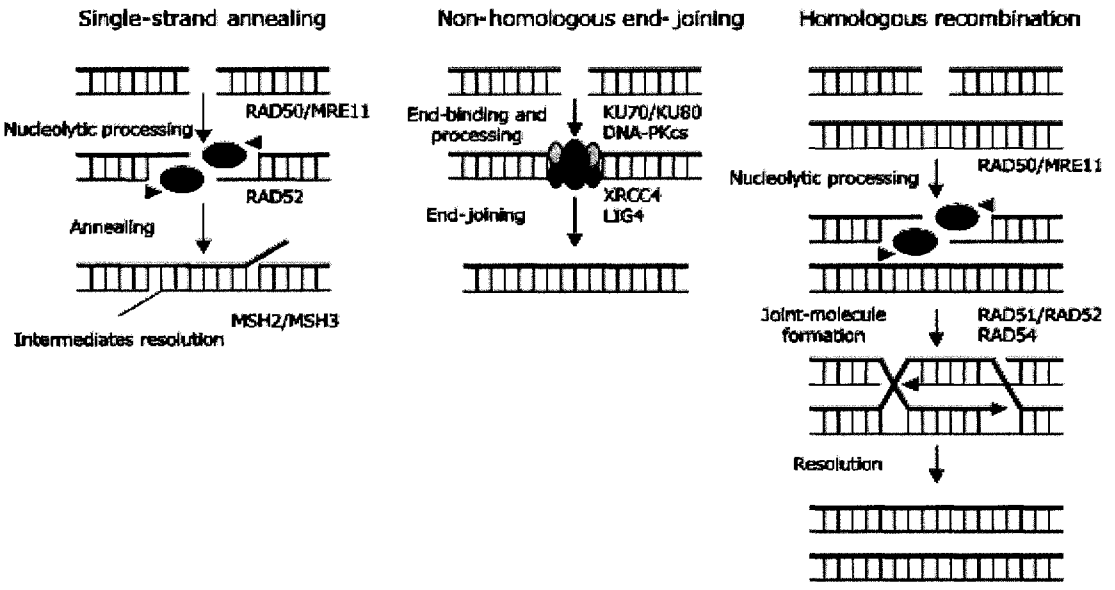

Figure 3. Diagrams of single-strand annealing, non-homologous end joining, and conventional homologous recombination. Predisposition to cancer and radiosensitivity. *P. Pichierri, A. Franchitto and F. Pali.* Genetics and Molecular Biology (2005).

There are actually several "sub-sets" of homology directed repair, of which HR is one (Figure 3). These include the classical double HJ model, synthesis-dependent strand annealing, and single-strand annealing. While they all have many commonalities, there are important differences between them.

The double HJ model is the "classical" version of homology directed repair. In it a two-ended DSB is repaired via crossover or non-crossover products. The beginning steps of all homology directed repair begin essentially the same way, the DSB must first be processed to form a 3' overhang in a MRN-dependent fashion (Paull and Gellert, 1998). Then RAD51 must

load onto the 3' single-stranded overhang, thereby displacing RPA which binds readily to single stranded DNA. The loading of RAD51 is accomplished with the help of the RAD51 paralogs (RAD51B, RAD51C, RAD51D, and XRCC2) (Sigurdsson et al., 2001) along with BRCA2 (Powell et al., 2002). RAD54 is also involved and may function in the loading of RAD51. After the initial strand invasion, the second end of the DSB can then invade the D-loop that was created by the initial strand invasion. What results is a "double X" structure, an essential element of the HJ model.

This was first described to explain gene conversion during meiosis, but was later adapted to fit normal DSB repair during mitosis (Szostak et al., 1983). However, crossover is not readily seen in normal HR in mitotic cells. This may be due to erroneously assuming that the model for meiosis and mitosis is the same, or more probably, that protein regulation (e.g. of BLM and TopoIIIα) specifically act to prevent crossover in mitosis but not meiosis.

Single strand annealing (SSA) is probably the least used of the homology directed repair mechanisms, most likely due to its error prone tendencies. As a matter of principle, SSA cannot occur without at least some deletion. SSA starts the same as many of the other homology directed repair pathways, with the ends of two DSB's being resected to yield two 3' single-stranded overhangs. These overhangs, if there is homologous sequence available, can then pair (Helleday et al., 2007). This will leave any sequence downstream of the pairing in a "flap" like structure that can be cleaved away, possibly by FEN-1. The nicks can then be ligated thereby fixing the DSB, albeit with a deletion. Haber has proposed that SSA is actually not a purposeful repair pathway, but rather a spandrel (unintended consequence) of the necessity of the other homology directed repair pathways to first process the ends of the DSB into 3' overhangs (Haber, 2006).

For our purposes, the most important feature of homology directed repair involves the repair of replication forks encountering a DSB, which has been called break-induced replication (Kraus et al., 2001). In this model, a replication fork encounters a SSB in one strand, and by the nature of the replication machinery converts the SSB into a DSB, while the other strand remains intact. This end is also processed in a MRN dependent fashion and RAD51 strand invasion occurs. However, given the presence of only one DSB end, only one HJ will be formed as compared to the double HJ model. Depending on how the HJ is resolved depends on whether a SCE occurs. It is for this situation that Helleday et al. has suggested that homology directed repair evolved (Helleday et al., 2007). Given the likely-hood that in every cell division there will be SSB converted into DSB, it is vital for a cell to efficiently deal with this situation. It is likely that for this reason RAD51 mutants are lethal (Sonoda et al., 1998).

WRN

A group of proteins closely associated with recombination, repair, and replication are the RecQ helicases, a conserved group that contains a specific 3' to 5' helicase domain. The two most studied of these include Werner (WRN) and Bloom (BLM). Both WRN and BLM have human progeria (accelerated aging) syndromes linked with their deficiency. Werner Syndrome (WS) patients experience many signs of premature aging, including graying and hair loss, atherosclerosis, osteoporosis, type II diabetes, and heart disease. They are also especially susceptible to mesenchymal sarcomas (Epstein et al., 1966). The estimated 10 million people with WS typically do not live past their forties (Driban and Bertranou, 1975). A commonality of the RecQ helicases is their helicase domain, (Gray et al., 1997) and their DNA binding domains.

RecQ helicases, while having several DNA interacting regions, all contain the RQC (RecQ C-terminal) domain, and many others also contain a HRDC (helicase and RNase D C-terminal) domain (Huber et al., 2006). WRN is the only member of the RecQ helicase family that also contains an N-terminal 3'-5' exonuclease activity (Shen et al., 1998). In culture, WRN cells senesce very rapidly, show an increased S-phase (Poot et al., 1992), and a decrease in the firing of replication forks; they also show an increase in translocation, rearrangements, and deletions (Martin, 2005; Salk et al., 1981). An interesting aspect of WS is that most of the WRN mutations result in truncations of the NLS (nuclear localization signal), thereby preventing WRN from entering the nucleus and making an effective null phenotype (Zhang et al., 2007b). However, small subsets of WS patients are actually defective in the lamin A/C gene, termed atypical WS (Mounkes and Stewart, 2004). This syndrome tends to be much more severe than typical WS.

WRN has proven to be a difficult protein when trying to ascertain its true biological function. WRN is known to interact with a large number of different proteins, thereby implicating it in a number of pathways including DNA mismatch repair, BER (base-excision repair) (Harrigan et al., 2006), NHEJ (Chen et al., 2003), ICL (interstrand crosslink) repair, stalled replication fork restart, and telomere stability (Laud et al., 2005). A small sampling of the proteins that have been shown to interact with WRN includes: DNA Pol γ, RPA, PCNA, FEN-1, DNA Topo I, Ku70/80, p53, BLM, PRAP-1/2, RAD52, and WRNIP1 (Dong et al., 2007).

Why WRN seems to interact with so many proteins is unclear. However, it may have more to do with WRN's DNA interactions than protein interactions. WRN is found at many complex DNA structures including short duplexes, D-Loops, G-quadruplexes, triplexes, and induced in branch migrating HJs (Opresko et al., 2004a; Opresko et al., 2004b). This suggests

that WRN is found at the site of many different types of damage and therefore interacts with many different proteins. Additional evidence for WRN being found at complex DNA structures comes from WRN co-localizing with γH2AX at DSBs (Cheng et al., 2005; Lan et al., 2005).

There is also evidence suggesting WRN is found at replication forks (Machwe et al., 2006; Otterlei et al., 2006). *In vitro* studies demonstrate that WS cells show significant replication fork asymmetry (Rodriguez-Lopez et al., 2007; Saintigny et al., 2002). Also, proliferation can be restored to WS cells after induction of a bacterial HJ resolvase. It has also been shown that WRN is needed for accurate replication at telomeres (Laud et al., 2005).

WRN's role at replication forks is most likely its primary role. It may be for this reason that WS cells tend to senesce so readily. Orren et al. suggests that it is the role of WRN at replication forks that may explain the presence of the 3'-to-5' exonuclease domain (Machwe et al., 2006; Machwe et al., 2007). DNA lesions can lead to stalled replication forks. If the lesion is on the lagging strand, a replication fork may use WRN to form what is termed a "chicken foot" structure. This is where the two newly replicated daughter strands are reversed and anneal to each other. This will allow extension of the daughter strands past where the lesion was located (the daughter strand with the lesion present using the normal daughter strand as a template). The daughter strands can then fold back to normal position and continue replication, the lesion having been bypassed and left to be repaired later in the cell cycle.

This model is different than the HJ model discussed earlier. While some mistakenly assume the two are the same, they are very much different. It may be that both models are accurate, and the location of the lesion may determine which model is utilized. If the lesion is on the leading strand, the lagging strand makes a good template that the leading strand (with its 3'

end) can easily invade. However, if the lesion is on the lagging strand, the lagging strand (a 5' end) cannot invade the leading strand. Therefore, it may resort to use of the chicken foot structure in order to bypass the lesion.

Another interesting aspect of WRN is its possible role in tumorigenesis. Opresko et al. has demonstrated a possible role of WRN as a tumor suppressor and a potential cancer therapy target (Opresko et al., 2004a). In this study they used short hairpin RNA (shRNA) to knock down WRN in 15 random tumor cell lines that were immortalized. Upon depletion of WRN, almost all of the tumor cell lines showed a very dramatic decrease in survival. This decrease was not due to p53 induced apoptosis as most of the tumor lines were p53 negative. Mortality was not due to aberrant telomere maintenance as most of the tumor lines were telomerase positive and displayed normal telomeres. The authors suggest that the increased cell killing is due to increased oxidative damage to the genome, ultimately leading to cell death. This overall scenario seems counterintuitive, as WS patients have elevated cancer rates, not decreased.

Considering WRN's role at replication forks, it is not surprising that WRN affects SCE frequency. What is surprising, is that its role seems to be predominately at the telomeres (Laud et al., 2005). In the absence of WRN and telomerase, mice displayed extremely high levels of T-SCE while no significant change in G-SCE was observed. This is striking, as many studies of WRN at replication forks did not report telomere dependence, suggesting multiple roles for WRN depending on the location of the replication fork.

Being that WRN is found at many different types of DNA structures, it is perhaps no wonder that WRN is involved in so many pathways. In fact, it is important when reading about WRN to analyze how the authors determine the connection. Often co-localization studies are utilized, and while useful, only suggests that any two proteins are in close proximity. Some

authors take this and inappropriately make the leap that the proteins interact functionally. In reality, some of the so-called "WRN interacting proteins" may merely in close proximity to WRN at some of the same structures.

BLM

Bloom (BLM) protein is another member of the RecQ helicase family that is involved in recombination, repair, and replication. It is closely related to WRN, but does not have an exonuclease domain. BS patients display many of the same features as WS, such as accelerated aging and increased cancer rates, particularly carcinomas (Hickson, 2003). BS cells display many cytogenetic phenotypes, such as deletions, insertions, loss of heterozygosity, telomere associations, quadriradials, and very high numbers of SCE (Chakraverty and Hickson, 1999). Surprisingly, even the template strand itself is subject to deletions in BS cells (Johnson-Schlitz and Engels, 2006). BLM also shows strong correlations to WRN in its preferred substrates, G quadruplexes and mobile D-loops (Bachrati et al., 2006).

It was the extremely elevated levels of SCE (generally about a 15 fold increase) that gave the first clue for BLM's function. It was suggested early on that BLM was needed to either prevent or re-start stalled replication forks. Findings since have helped confirm this hypothesis. BLM, which recruits p53, acts to suppress RAD51 recombination at stalled replication forks (Sengupta et al., 2003). BLM and p53 have a very intimate role at replication forks; together they lead to diminished apoptotic activity (Spillare et al., 1999), and p53 also helps HJ processing by BLM (Yang et al., 2002). BLM is also required for the recruitment of the MRN complex to stalled forks (Franchitto and Pichierri, 2002).

BLM is a member of the BASC (BRCA-associated complex) that contains BRCA, RAD51, PCNA, BLM, and MSH 2/6 (Futaki and Liu, 2001; Wang et al., 2000). BLM directly interacts with RAD51 (Wu et al., 2001). BLM is also found at PML bodies (promyelocytic leukemia), which may function as DNA damage stress sensors (Sanchez-Pulido et al., 2007).

At stalled replication forks, the BLM/TopoIIIα complex, along with BLAP75 (BLM-Associated Polypeptide-75kD), interact to suppress double HJ (Wu and Hickson, 2003). It also appears that RPA may be critical to this process (Plank et al., 2006). However, it is of note that BLM may play a dual role. In meiosis, BLM is actually responsible for the formation of double HJ in the absence of TopoIIIα (Cromie et al., 2006). Crossover is an important feature of meiosis, so why a protein that suppresses crossover in mitotic cells is involved in the formation of crossovers in meiotic cells is unknown. It may be due to BLM interaction with TopoIIIα in mitosis that is the key feature (Oh et al., 2007). BLM helps TopoIIIα to relax supercoiled DNA (Wu and Hickson, 2002), and this may allow a stalled replication fork to undergo HJ branch migration and ultimately converge with a replication fork in the opposite direction thereby resolving the HJ (Plank et al., 2006).

FANCD2

FANCD2 is a critical member of the Fanconi Anemia (FA) pathway. FA patients demonstrate increased genomic instability (Joenje and Oostra, 1983) leading to cancer (Tischkowitz and Hodgson, 2003), developmental defects, segmental progeroid affects, and an increased sensitivity to intercross linking agents such as MMC (Auerbach, 1993). The nuclear core complex for FA consists of FANCA/B/C/E/F/G/L/M (Garcia-Higuera et al., 2000). The

core complex monoubiquinates FANCD2 through FANCL, which can then travel to DNA damage (Garcia-Higuera et al., 2001). BRCA2, which is also a member of the FANC proteins, is also known as FANCD1 (Howlett et al., 2002). Mutations in BRCA2 predispose patients to breast and ovarian cancer (Wooster and Weber, 2003). It is also involved in HR DSB repair. It has been suggested that the nuclear core complex recruits FANCD2 and BRCA2 to the site of stalled replication forks possibly through FANCM (Niedernhofer, 2007). BRCA2 seems to be necessary for RAD51 strand invasion in HR (Wu et al., 2001).

Being that the FA pathway has direct involvement with the RAD51 dependent HR pathway, and that HR is thought to be a major pathway in the formation of SCE, it could be assumed that FA itself is important in SCE frequency. This seems true to some degree. The proteins FANCD2 (Yamamoto et al., 2005) and FANCC (Hirano et al., 2005) both show elevated SCE levels of spontaneous and induced SCE's. It has been shown that some of the proteins in the FA pathway do not influence SCE frequency (Godthelp et al., 2006). Why an increase in SCE is seen when FANCD2 and FANCC are mutated is unknown. It would be more reasonable if SCE frequency decreased in the absence of these proteins since they are vital for RAD51 dependent HR. It could be that they are more responsible in altering the resolution of HR; whether the end product results in crossover or non-crossover. This is yet another confusing piece of the SCE story; proteins that should be necessary for the formation of SCE given the current model actually result in an increase in SCE in their absence.

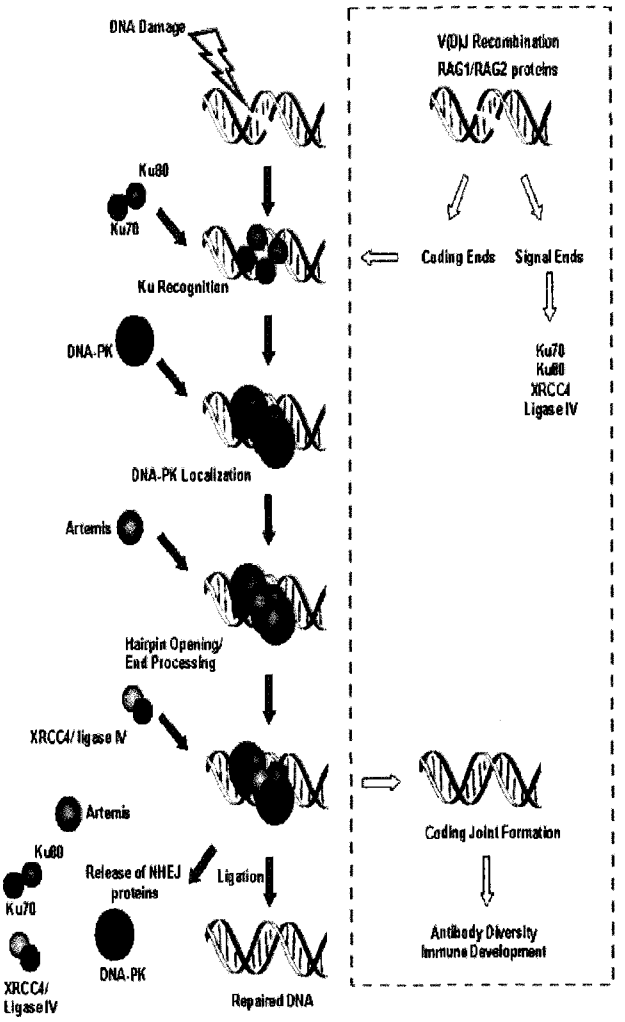

Figure 4. A schematic of Non-Homologous End Joining and interacting proteins. The life and death of DNA-PK. Spencer J Collis, Theodore L DeWeese, Penelope A Jeggo and Antony R Parker. Oncogene (2005)

Non-Homologous End Joining

The ability to repair a DSB is critical for a cell's survival. While accurate repair is obviously best, it is not always possible. For example, during the early stages of the cell cycle, G_1 and early S-phase, there is no sister chromatid present to be utilized by HR. In mammalian

systems, NHEJ is the primary pathway for repairing DSBs. While it is error prone due to its ability to ligate any two DNA ends together, it is still better than leaving them unrepaired. NHEJ also plays a critical role in immunity by facilitating V (D) J recombination in lymphocytes.

The key proteins involved in NHEJ include the Ku70/86 heterodimer, DNA-PKcs (DNA dependent protein kinase catalytic subunit), Artemis, XRCC4 (x-ray cross complementation group 4), Ligase IV, polymerases μ and ƛ, and XLF (Cernunnos) (Figure 4). The Ku heterodimer is the first member to respond to the DSB ends, and assists in recruiting many of the other components, such as DNA-PKcs/Artemis. Once the DNA-PKcs/Artemis complex has been recruited, the ends can be processed and XRCC4/Ligase IV can then ligate the two ends together. The polymerases μ and ƛ may be needed to process the ends before ligation. Cernunnos appears to stimulate XRCC4/Ligase IV dependent ligation, and deficiencies of Cernunnos leads to NBS (Nijmegen Breakage Syndrome 1) like symptoms (immunodeficiency), as well as a Lig4 similar defect in NHEJ (Buck et al., 2006). The recently discovered Apollo protein is related to Artemis and interacts with TRF2 and protects telomeres during S phase (van Overbeek and de Lange, 2006). The DNA-PK holoenzyme is central to NHEJ and is comprised of the Ku70/80 heterodimer and DNA-PKcs. The Ku70/80 heterodimer forms a basket structure with a central opening that can accommodate duplex DNA (Walker et al., 2001). Ku binds DNA ends, then has the ability to translocate inward from the break site, which may be important in recruiting DNA-PKcs (Walker et al., 2001). DNA-PKcs is a serine/threonine kinase that belongs to the PIKK (phosphoinositide 3-kinsase-related kinase) family of kinases (Jeggo et al., 1995). Other members of this family include ATM (Ataxia Telangectasia Mutated) and ATR (ATM- and RAD3- related protein kinase). DNA-PKcs has the ability to phosphorylate many substrates *in vitro*, yet *in vivo* targets have been much more difficult to identify. The ability of DNA-PKcs

to autophosphorylate itself is also of interest, as it contains multiple autophosphorylation sites that are essential for its function (Douglas et al., 2002). Two mouse strains, SCID (severe combined immunodeficiency) and BALB/c, illustrate the importance of DNA-PKcs. SCID mice contain a truncated version of DNA-PKcs (Bogue et al., 1998), and BALB/c has a mutated version containing two single-nucleotide polymorphisms (Yu et al., 2001); both mouse lines show increased sensitivity to ionizing radiation. BALB/c also has an elevated risk of developing radiation induced mammary cancer (Yu et al., 2001).

DNA-PKcs also interacts with Artemis (Ma et al., 2002). Artemis seems to be involved in processing of the DNA ends. When combined with DNA-PKcs, Artemis has both 5' and 3' endonuclease along with single stranded exonuclease activity (Ma et al., 2005).

While HR appears to play a leading player in SCE formation, NHEJ may also have a role, given the presence of DSB's during a SCE formation. We examined cells deficient in DNA-PKcs and/or Artemis. NHEJ may not be the primary pathway involved in SCE formation; however, there may be a subset of DSB's that require NHEJ in order to be resolved.

Nucleotide Excision Repair

We also investigated a role for the nucleotide excision repair (NER) pathway in SCE formation. This pathway is critical for repairing base lesions that result in abnormal DNA conformation, in particular DNA cross links (Dronkert and Kanaar, 2001; Legerski and Richie, 2002; McHugh et al., 2001; Park and Sancar, 1994). Members of this pathway were first described in patients who had increased sensitivity to the sun; this condition was later termed Xeroderma Pigmentosum (XP) (de Boer and Hoeijmakers, 2000; Lehmann, 2003).

While there are many participating proteins, NER is a fairly straightforward process. The lesion must first be detected, either by the XPC-HR23B complex or by RNA Polymerase II during replication (McHugh et al., 2001). The TFIIH complex then checks the lesion and marks the location for further processing (Lehmann, 2003). A pre-incision complex consisting of RPA, XPA, and XPG along with a DNA opening complex of XPB and XPD can then form at the lesion. Incision complexes consisting of ERCC1-XPF, which cuts on the 5' of the lesion and XPG, which cuts on the 3' of the lesion, can excise the oligomer with the lesion (Laczmanska et al., 2006). Polymerases can then refill the removed sequences, and lastly single-strand ligation occurs.

The ERCC1-XPF complex is a vital component of the NER pathway; the two proteins are dependent upon each other for stabilization. This complex has gained biological relevance of late, especially in terms of cancer therapies (Zhang et al., 2007a). Several studies suggest the fidelity of the NER pathway can determine the success of platinum based chemotherapy compounds. If a tumor cell has very active NER, then it may be more likely to overcome the inter-strand cross-links produced by the chemotherapeutic compounds, making them less effective. Conversely, if the normal tissue has a slightly defective NER, then they may be more sensitive to the compounds leading to unacceptable toxicity. Studies looking at the mRNA levels, protein levels, and single nucleotide polymorphisms (SNPs) in ERCC1 have suggested that this may be true.

Interestingly, mutations in ERCC1 have recently been found in humans. This was surprising, as ERCC1 was thought to be so critical to humans that they could not live without it. Even more intriguing, humans with ERCC1 mutations display progeroid type symptoms similar to WS and BS. Given the similarities in symptoms, we analyzed whether ERCC1 displayed

similar SCE characteristics to WS and BS. Previous studies, along with our own, have shown that ERCC1 cells do not show elevated G-SCE levels. We were the first to analyze T-SCE levels in these cells.

Bystander Effect and SCE

Radiation induced bystander effect (BSE) is a relatively new phenomena in the field of radiation biology. Cells that are directly "hit" by radiation generate a signal that is received by "non-hit", or bystander, cells and elicit responses. The BSE was first reported by Nagasawa et al. after irradiation of cells with extremely low fluences of α- particles (Nagasawa and Little, 1992). By using such a low fluence of α-particles, they could be assured only a very small percentage of cells would be directly hit by a particle. However, when they measured SCE levels, they observed a significant increase in approximately 30% of the cells. This is much higher than one would expect to see if only 1-2% of the cells were actually hit by the α particle.

Very little about the BSE, including the "signal", is known. Some evidence suggests that ROS (reactive oxygen species) or NOS (nitric oxide species) generated in the hit cell are responsible for the BSE (Maguire et al., 2007). However, other studies using antioxidants to suppress the BSE, report conflicting results, casting doubt on the role of ROS as the actual signal. There is also much controversy over how the bystander signal is conveyed/ transmitted. Some feel that the signal must travel through gap junctions (Azzam et al., 1998; Azzam et al., 2001), while there is also a lot of evidence suggesting that the bystander signal can move through the media (Lehnert et al., 1997; Mothersill and Seymour, 1998).

The BSE has been expressed using a number of endpoints including: micronuclei formation, clonogenic survival, apoptosis, and SCE (Grifalconi et al., 2007; Nagasawa et al., 2005; O'Neill-Mehlenbacher et al., 2007; Yang et al., 2007). The fact that SCE's can be used as an endpoint in the BSE is surprising, as most forms of direct IR do not lead to an increase in SCE. This would suggest that the bystander signal must be different from direct products of IR. In later chapters, we will examine how DNA repair pathways can alter the BSE and ultimately SCE. Understanding the BSE may not only give insight into radiation biology, but may also help increase understanding of some of the underlying mechanisms of SCE.

Purpose and Aims

The overall purpose of this research was to understand how DNA repair pathways can influence SCE frequency. By understanding how DNA repair influences SCE frequency, I may be able to shed light on the true biological relevance of SCE. Are SCE a manifestation of inappropriate DNA repair in response to DNA damage, or are they themselves a form of DNA repair?

To answer this question, I utilized a number of cell lines that are deficient in DNA repair. The DNA repair pathways that I analyzed were homologous recombination, non-homologous end joining, and nucleotide excision repair. In addition to using repair deficient lines, I utilized siRNA technology to specifically knock down expression of repair proteins in normal primary human fibroblasts. I utilized SCE frequency, either G or T-SCE, as the endpoints in all these experiments. Fluorescence plus Giemsa was utilized to measure G-SCE, while Chromosome Orientation- Fluorescence *In Situ* Hybridization to measure T-SCE.

Ultimately, I will attempt to demonstrate that different repair pathways can alter SCE frequency uniquely. Some repair proteins will affect on G or T-SCE while some may alter both or neither. It is my belief that this level of organization suggests that SCE is an active DNA repair pathway and not just a response to inappropriate repair.

Materials and Methods

Cell culture

Multiple cell lines were utilized in this research, each with their own media and care. In each section a more detailed section will cover the particular cell line being used. All cell culture was performed in a sterile hood.

Passage of Cells

To passage fibroblasts, media was aspirated off the cells using a vacuum. 3-4 ml of Trypsin-EDTA 0.25% was added to the cells and they were placed back into a 37°C incubator for several minutes, or until cells detached from the flask. 5 ml of the media was added to the flask and the entire cells/Trypsin-EDTA/media solution was placed into a 15 ml centrifuge tube. The cells were pelleted in a centrifuge at 1000 rpm for 5 minutes. The supernatant was aspirated and the cells were re-suspended in new media. Cells were counted using a Coulter Counter and seeded at a desired concentration. Media was added to flasks to bring to total volume of 12 ml for a T-75 flask or 6 ml for a T-25 flask.

Media

Fibroblasts: Most fibroblasts lines were grown in T-75's using αMEM media with 15% fetal bovine serume (FBS) and 2.5 ml of Pen/Strep for 500ml of media.

Lymphoblasts: Most lymphoblast lines were grown in T-25's using RPMI 1570 media with 15% FBS and 2.5 ml of Pen/Strep for 500 ml of media. They were not grown in an upright position

siRNA experiments: Cells were grown overnight to condition them in αMEM without any FBS or Pen/Strep. On the day of the experiment, cells were grown in 1 ml of the above mentioned αMEM and 1 ml of Eagle's OptiMEM.

Cytogenetic Techniques

Cell harvest

Fibroblasts: Add 10 µl of colcemid (0.1 to 0.2 µg/ml) for every 1 ml of media in the culture approximately 2-4 hours before intended harvest time. The longer the colcemid is in culture, the more metaphases will be present; however, the longer the colcemid is in culture, the quality of the metaphases will go down. At the time of harvest, aspirate the media off the cells being careful not to touch the pipette to the cells. Add 3 ml of trypsin-EDTA 0.25% in Hank's balanced salt solution (Hyclone) to the flask and let warm for several minutes in a 37° C incubator. Visualize the cells under a phase contrast microscope, after trypsinizing the cells should appear rounded up and detached from the flask. Pipette approximately 5 ml of media into flask and pipette up and down. Remove the media/trypsin/cell solution and place in a 15 ml conical tube.

Centrifuge the conical tubes for 5 minutes at 1000 rpm (rotations per minute). Aspirate the media/trypsin of the cells being careful not to remove the cell pellet. Re-suspend the pellet with 4 ml of 75mM KCl by adding the KCl drop-wise while vortexing the pellet at medium speed. Let the tubes sit for 15 minutes at room temperature. Add 1 ml of Carnoy's fixative aka "fix" (see solutions for fix preparation) to the tubes and vortex. Centrifuge the conical tubes for 5 minutes at 1000 rpm. Aspirate the supernatant and add 4 ml of fix drop-wise to the tubes while

vortexing. Let the solution sit at room temperature for 10 minutes and spin again. Wash by re-adding fix to the pellet while vortexing, let sit for 10 minutes, and centrifuge.

Harvest Solutions:

Hypotonic solution (0.075 KCl): Measure 5.59 g of KCl and add dH_2O to a final volume of 1 L. Filter-sterilize and store the solution at room temperature.

Fix (Carnoy's fixative): Fix is a 3:1 methanol/acetic acid solution. Using the plunger pipettes, pump 15 ml of methanol into an Erlenmeyer flask and follow with 5 ml of glacial acetic acid. Always use fresh fix! Fix more than a few hours old will have additional compounds that are not sought.

Slide Cleaning

Fischer Premium Slides were placed into slide racks and placed in glass chambers. A 1:1 methanol/ethanol solution was added, approximately 50 ml, or enough to fully cover the slides. A cover was placed on the chamber and left at room temperature for overnight. The next day, the methanol/ethanol solution was aspirated off, and the slides were rinsed 3x with dH_2O. After the final water rinse, the water was poured off and the slides were stored for later use in a -20°C freezer.

Dropping slides

Slides for cytogenetic analysis were all prepared using Fischer Premium Slides Frosted. Slides were stored at -20°C and rinsed 3x before use and placed on ice. After cell harvest, the cell pellets were washed using 3:1 methanol/acetic acid fix. After the wash, cells were centrifuged at 1000 rpm for 5 minutes and the supernatant was aspirated using a vacuum. Fix

was added to the pellet in an amount in order to make the solution "slightly milky". Approximately 3 drops of the cell solution was dropped onto a Fischer Premium Slide and steamed over a water bath for approximately 8 seconds. The slide was then dried on a warming tray until dry. The slides were visualized on a Zeiss phase microscope.

CO-FISH

Chromosome Orientation- Fluorescence *In Situ* Hybridization was done according to Bailey et al (Bailey et al., 1996). For descriptions of each solution, please see following sections.

Fixing steps:

Slides with metaphase spreads that were obtained from cells grown for one round of replication in the presence of 1 x 10^{-5} M 5'-bromo-2'-deoxyuridine (BrdU, Sigma) were used for these experiments. Slides were prepared by soaking in RNase A (100 µg/ml H_20) for 10 minutes in a 37° C water bath. Slides were then rinsed in PBS. Slides were fixed in 3% formaldehyde/PBS solution for 10 minutes at room temperature. Slides were then dehydrated in a cold ethanol series (75%, 85%, and 100%) ethanol for 2 minutes each. Then the slides were air dried.

Hybridization steps:

After the slides have dried, they are stained with Hoescht 33258 by placing 50 µl of the Hoescht 33258 into 50 ml of 2x SSC (0.5 µg/ml 2X SSC) for 15 minutes at room temperature. The slides are then flooded with 50 µl of 2x SSC and coverslip is placed on the slide. Using a Stratalinker 3000, expose the slides to 365 nm UV for 30 minutes. The slides are then removed and rinsed with H_2O and air dried. An Exonuclease III solution (Promega) is prepared while

drying (0.5 µl of enzyme, 45 µl of H_2O, and 5 µl buffer (50 mM Tris-HCl, 5 mM MgCl2, and 5 mM dithiothreitol, pH 8.0)) and applied at 50 µl per slide and a coverslip is mounted. This was allowed to sit at room temperature for 10 minutes. The slides are then rinsed with H_20 and air dried. After drying, the slides are placed in a coplin jar filled with 70% formamide/2x SSC solution heated in a H_2O bath at 70° C for 1 minute (be sure to heat the coplin jar in the H_2O bath at the same time, placing a cold Coplin jar in a hot water bath will cause breakage). After 1 minute, remove the slides from the hot solution and immediately place in a cold ethanol series. Let the slides soak in each ethanol solution (75%, 85%, and 100%) for 2 minutes each. Let the slides air dry. Apply 20 µl of hybridization solution to each slide and coverslip (see below for description of hybridization solution). Add 10 µl of H_2O to each well in the hybridization chambers and place slides in them. Let hybridize for approximately 2 hours at 37° C in the dark (times may vary depending on results).

Rinsing steps:

After hybridization, remove slides from hybridization chambers and place in 70% formamide/ 2x SSC at 29° with shaking for 20 minutes. Wash slides with PN buffer for 5 minutes at room temperature. Mount slides with 12 µl of Vectashield antifade and DAPI. Store the slides at 20° in the dark for future examination.

Alternative staining: It may be desired to incorporate anti-BrdU staining to the slides. After the 70° formamide/2x SSC at 29° wash, rinse for 1 minute in PN buffer. Add 20 µl of anti-BrdU probe (see below for description) to each slide and place in hybridization chambers. Incubate at 37° for 30 minutes. Remove from chamber and place in PN buffer for 5 minutes, and mount with Vectashield as above.

Image Analysis:

Slides were scored using a Zeiss Fluorescence microscope (Axioplaln 2ie MOT). For CO-FISH, the DAPI and Cy3 filters were used to visualize the chromosomes and telomere probes. A CCD camera (model CV-M4+CL, JAI PULNiX Inc., San Jose, CA, USA) was used to take the picture, and analyzed on a computer (Dell precision 360 workstation) with Isis FISH imaging software (Metasystems, Altussheim, Germany).

Solutions for CO-FISH:

RNAse A: Ribonuclease A was purchased from Sigma. Add 0.005 g of RNAse A to 50 ml of dH_2O, thoroughly dissolve.

3% formaldehyde solution: Use 4.05 ml of 37% Formalin (formamide) from Fischer Scientific in a Coplin jar. Bring the volume up to 50 ml total with PBS (see description of PBS below).

PBS (Phosphate buffered solution): Dissolve 8 g of NaCl (Fischer Scientific), 0.2 g of KCl, 1.44 g of Na_2HPO_4 (Merck), and 0.24 g of KH_2PO_4 (Fischer Scientific) in 800 ml of dH_20. Adjust the pH to 7.4 with HCl (Aldrich) using an Orion pH meter. Add dH2O to make final volume 1 L. Filter sterilize and store at room temperature.

Hoescht 33258: Make working solution by dissolving Hoescht 33258 to a final concentration of 500 μg/ml of H_2O. It is very light sensitive, so keep covered in aluminum foil.

70% formamide/2x SSC (for wash and denature steps): Add 5 ml of 2x SSC, 10 ml of dH_2O, and 35 ml of 37% formamide to a Coplin jar.

20x SSC (standard sodium citrate): For a final volume a concentration of 3 M NaCl and 0.3 M Na citrate (Fischer Scientific) is needed. Add 87.66 g NaCl and 44.12 g Na Citrate and dissolve in dH_2O. Final volume should be 500 ml. Filter sterilize and store at room temperature.

2x SSC: Take 100 ml of 20x SSC stock solution and add 900 ml of dH_20. Store at room temperature.

Working Probe solution: Take 1 µl of stock PNA-Cy3 probe (Applied Biosystems) and add to 99 µl of dH_20. Heat at 50° C for 30 minutes at store at -20° C.

Hybridization solution: For approximately 2 slides, add 35 µl of formamide, 12 µl Tris-HCl (12 µM), 2.5 µl KCl (5 µM), 0.5 µl MgCl2 (1 µM), and lastly, 3.3 µl of working probe solution. Denature probe right before use by heating for 5 minutes at 70° C and immediately placing on ice until use.

FPG

Fluorescence plus Giemsa (FPG) was performed according to (Perry and Wolff, 1974). Slides are initially stained with 50 µl of Hoescht 33258 () in 50 ml of 2x SSC (see above for description) for 15 minutes at room temperature. The slides are then rinsed with dH_2O and air dried. Mount the slides with 50 µl 2x SSC and coverslip (see above description for coverslips). Expose slides to 365 nm UV in a Stratalinker 3000 for 30 minutes. Remove coverslips and place in a Coplin jar of 2x SSC at 60° C, in a water bath, for 30 minutes. Do not let the slides dry in between. Take the slides out of the water bath and rinse with dH_2O very well, at least 5 rinses.

Stain the slides with 2% Giemsa for approximately 10 minutes. The amount of time will determine the coloration and contrast on the slides.

FPG solutions:

Hoescht 33258: Make working solution by dissolving Hoescht 33258 to a final concentration of 500 μg/ml of H_2O. It is very light sensitive, so keep covered in aluminum foil.

2x SSC: Take 100 ml of 20x SSC stock solution and add 900 ml of dH_20. Store at room temperature.

Giemsa 2%: Take 2 ml of Modified Giemsa Stain from Sigma, and place in a 50 ml conical tube. Add 48 ml of dH_2O, mix well.

siRNA

Pre-Treatment: This technique was communicated by Qingming Zhang in the Liber lab. All tubes and equipment must be RNase free. Normal human fibroblasts, 5C, were pre-treated by growing in αMEM overnight that contained only FBS but not any antibiotics.

Transfection: The day of the transfection, mix 10 μl of siRNA (Qiagen) + 600 μl of OptiMEM in a RNase free tube per sample. In a separate tube, mix approximately 25 μl of transfection reagent (Qiagen) + 600 μl of OptiMEM per sample (The amount of transfection reagent can vary, in general, any concentration between 1 to 4 times the siRNA works. For my experiments, I used a concentration of 2.5 times the transfection reagent/siRNA.). Invert gently five times and wait 5 minutes. Then add the transfection reagent mixture to the tube containing the siRNA. Invert the tubes 5 times and wait for 25 minutes. While waiting, aspirate all the media off the cells. Add 1 ml of αMEM without FBS or antibiotics. Add siRNA mixture to the flask

containing the cells, this should give a total volume of about 2.5 ml of media/siRNA transfection solution. Place cells back in a 37°C incubator and wait approximately 6 hours. This is long enough for any siRNA to transfect the cells. Add approximately 4 ml of αMEM with FBS and antibiotics to the flask for a total volume of 6 ml. The first time using a particular siRNA, plan the experiment so a collection can be made every day for five days. This will demonstrate the optimum time of knockdown.

Western Blots

Lysis Buffer: (Song et al., 1996)

500 µl of 1 M Tris-HCl, pH 7.5

1 ml of 1.5 M NaCl

40 µl 0f 500 mM EDTA

80 µl of 250 mM EGTA

500 µl of 500 mM NaF

1 ml of 250 mM beta-glycerophosphate

20 µl 0.2% Triton X-100

30 µl of 0.3% NP-40

100 ul of 0.1mM sodium ortho-vanadate

Prior to immediate use, add protease inhibitors:

1 µl of 0.1 mM PMSF

5 µl of 5 µg/ml leupepetin

2 µl of 5 µg/ml aprotinin

Collecting lysates:

Trypsinize cells and place in 15 ml conical tubes. Spin down at 1000 rpm for 5 min, and aspirate the supernatant. Re-suspend the pellet in cold PBS, vortex, and spin down again (It is important to keep all the steps from here as cold as possible to prevent protein degredation). Repeat this step. After second spin down, aspirate the supernatant and re-suspend the pellet in approximately 100 µl of lysis buffer (the amount of lysis buffer will vary. The more lysis buffer, the more lysate; however the more dilute the lysate). Place the solution into a 1.5 ml microcentrifuge tube. Place on ice and periodically flick the tubes to mix. Spin the tubes at high speed for 10 minutes at 4°C. Transfer the supernatant to a new centrifuge tube in 50 µl aliquots and store at -80°C.

Protein Quantification:

Protein quantification was done using a BIO RAD DC Protein Assay. The standard assay starts by preparation of the working reagent. Add 20 µl of reagent S to each ml of reagent A that will be used, this will now be termed reagent A'. Place 100 µl of sample into a clean test tube. Add 500 µl of reagent A' into each test tube and vortex. Add 4 ml of reagent B into the test tube and vortex immediately. After 15 minutes, the solution can be transferred to cuvettes and measurements can be obtained using a BIO RAD smart spec 3000 at absorbance of 750 nm. These measurements can then be used to calculate the total protein concentration.

Electrophoresis/Gel Transfer:

Solutions:

5x Running Buffer:

Tris base 15 g

Glycine 72 g

SDS 5g

Distilled H_2O to 1 Liter

1x Running Buffer:

Dilute the 5x Running buffer at a 1:5 buffer/H_2O mix.

2x Loading buffer:

0.10 M Tris-HCl pH 6.8

0.16 M DTT

2% SDS

0.2% bromophenol blue

20% Glycerol

5 µl beta-mercapthoethanol/ml

Gel Transfer:

Tris Base 4.5 g

Glycine 21.6 g

methanol 300 ml

distilled H_2O to 1.5 L

Electrophoresis:

Place the desired amount of lysate into a fresh 0.5 ml microcentrifuge tube (generally about 20 μg of total protein). Add 10 μl of 2x loading buffer and bring the total volume to 20 μl with dH_2O if necessary. Denature the samples at 100°C for 5 minutes using a heating block. Place the tubes on ice immediately afterwards. Set up the gen apparatus, using BioRad Precast Gels. Fill the upper chamber and bottom one third of the gel apparatus with 1x running buffer. Load the lysates into the wells using a pipette. Run the gel for approximately 2 hours at 125 volts (times and voltage are variable).

Gel Transfer:

Pour transfer buffer into a Pyrex tray. Cut four pieces of filter paper to match the size of a gel. Take out a Hybond ECL membrane. Assembling the cassette:

Place the black side of the cassette down in the Pyrex tray

Place a sponge pad on the black side of the cassette

Place two pieces of filter paper on the sponge

Place the gel on the two pieces of filter paper

Place the membrane on the gel

Place two more pieces of filter paper on the membrane

Place another sponge on the filter paper

Close the cassette

Place the cassette into the transfer apparatus. Add a stir rod and an ice pack to the apparatus and fill the apparatus with all of the transfer buffer. Place at 4°C and run overnight at 40 volts, make sure to turn on stir bar. (The colder all these steps can be carried out, the better.)

Immunoblotting:

5x TBST Buffer:

125 ml of 125 mM Tris base, pH 8.0

36.5 g of NaCl

1.25 ml of 0.125% Tween 20

Dilute the TBST buffer to 1x by diluting 200 ml of 5x buffer with 800 ml of water. Make a blocking buffer by dissolving 3 g of dried milk in TBST (from here on out 1x TBST will be referred to as TBST). Take the membrane out of the transfer apparatus being careful not to let any of the gel stick to it. Soak in TBST for 10 minutes. Place 20 ml of blocking buffer into a plastic tray, and place in membrane. Gently shake for 1 hour. Rinse the membrane twice with TBST, and wash 3 times with TBST in a shaker for 15 minutes each. Dilute primary antibody solution by making 1:1000 dilution of antibody/blocking buffer (list of primary antibodies is detailed below). Add primary antibody to membrane by placing both in a sealable plastic bag. Place in a shaker at room temperature for 1-2 hours. Remove the membrane and rinse 2 times and wash 3 times for 15 minutes in TBST (as above). Make the secondary antibody at a 1:1000 solution of antibody/blocking buffer. Place the membrane in a plastic bag with the antibody and shake for 2 hours at room temperature. Rinse and wash the membrane with TBST (similar to above). Detect by using the ECL kit and the Storm.

Cell Transfer Assay

Preparation of recipient cells: Recipient cells were grown up prior to the experiment and were in log phase growth. The day of the experiment, the cells were trypsinized and centrifuged at 1000 rpm for 5 minutes. They were then re-suspended in PBS and counted using a Coulter Counter. Approximately 400,000 recipient cells were then added to a T-75, and 10 ml of αMEM was added to the flask. Donor cells were then added (see below).

Preparation of donor cells: The donor cells were grown up in αMEM and were in log phase growth before use. The day of the experiment, the donor cells were irradiated using a Cs Mark VII irradiatior for a total dose of 1 Gy. After irradiation, cells were trypsinized and centrifuged down at 1000 rpm for 5 minutes. Cells were re-suspended in sterile PBS and counted using a Coulter Counter. Cells were then seeded into flasks containing the recipient cells at dilutions of either 1/100 or 1/1000 donor to recipients. BrdU was added to the culture at a concentration of 1 x 10^{-5}. The entire culture was allowed to grow for approximately two rounds of replication, harvested, and slides were prepared. FPG and cytogenetic analysis on the slides then allowed visualization of G-SCE.

Statistical Analyses

Metaphases were blinded and scored for either G or T-SCE. Standard deviations were calculated and used to determine standard error of the mean (SEM) for the error bars. Statistical analysis on SCE can be difficult as several assumptions must be made. The first is that the SCE's are

independent of each other, in that, one particular cell was not hypersensitive compared to the others. This also leads to the assumption that SCE follow a Poisson distribution. Finally, it must be assumed that DNA content scales similarly with chromosome number (unless it is confirmed using flow cytometry). Given the characteristics, it seems safe to make these assumptions. For most lines, SCE was calculated on a per chromosome basis as to avoid any issues with chromosome number variation between metaphases. For the bystander data, SCE per metaphase was used as only the normal human fibroblast cell line was used, and it has a fairly stable chromosome number. Also, with matching controls in every experiment, it is safe to analyze on a per metaphase basis.

The DNA homologous recombination repair proteins WRN, BLM, and FANCD2 differentially influence SCE frequency in genomic and telomeric DNA.

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Running Title: WRN, BLM, and FANCD2 influence G/T-SCE

Abstract

The formation and regulation of sister chromatid exchange (SCE) is thought to involve DNA repair pathways, and yet very little is known about exactly how DNA repair can influence SCE frequency. Here, we focus on three double strand break (DSB) repair proteins from the homologous recombination (HR) pathway, Werner (WRN), Blooms (BLM), and Fanconi Anemia complementation group 2 (FANCD2). WRN and BLM are members of the RecQ helicase family and have been shown to be involved in DNA recombination, repair, and replication. FANCD2 is also a member of the HR pathway, most likely interacting with BRCA1 and assisting in RAD51 dependent strand invasion. Interestingly, Bloom Syndrome (BS) and Werner Syndrome (WS) display accelerated aging, or progeroid phenotypes, as well as elevated cancer rates. Similarly, FANCD2 patients also display elevated cancer rates, particularly acute myeloid leukemia (AML). Here, we examine how these proteins influence SCE frequency within both genomic and telomeric DNA. Surprisingly, our results demonstrate that genomic SCE (G-SCE) and telomeric SCE (T-SCE) are influenced differentially. WRN suppresses T-SCE while having no affect on G-SCE, while BLM suppresses both G and T SCE. Our data suggests that FANCD2 can suppress G-SCE while it may also have a role in T-SCE. Understanding how these proteins differentially influence SCE within genomic and telomeric DNA may lead to insights into aging and cancer.

Introduction

Sister chromatid exchange (SCE) is the exchange of genetic material between two sister chromatids after DNA synthesis but before sister separation in anaphase. SCE has been demonstrated for over fifty years (Taylor, 1958), and has been used in many mutagenic studies, yet its biological relevance has yet to be fully understood or appreciated. The mutagenic studies suggest that SCE are a negative consequence of DNA damage (Jacobs, 1977; Kligerman, 1979; Nakanishi and Schneider, 1979). Indeed it has been shown that SCE can lead to large deletions, translocations, and loss of heterozygosity (Abdulovic et al., 2006; Martin, 2005; Salk et al., 1981); all possible precursors to genomic instability and possibly cancer. However some studies have also shown that SCE does not display a direct linearity in actual cancer patients (Hagmar et al., 1998). Here, we examine the role of the homologous recombination (HR) proteins Werner (WRN), Bloom's (BLM), and Fanconi Anemia complementation group 2 (FANCD2) in SCE frequencies.

Werner Syndrome (WS) is a progeroid syndrome that displays phenotypes of accelerated aging such as atherosclerosis, graying of the hair, diabetes, arthritis, and susceptibility to development of sarcomas (Epstein et al., 1966). The protein responsible for WS, WRN, is a member of the RecQ helicase family (a conserved group of helicases that are characterized by their DNA binding domains) that is implicated in the "3 R's" of DNA: repair, recombination, and replication. In addition to its helicase domain, WRN is the only member of the RecQ family to also possess a 3' to 5' exonuclease domain (Shen et al., 1998). The exact function of the exonuclease domain is unclear, but it has been proposed that it may assist in forming "reverse chicken foot structures" at stalled replication forks (Machwe et al., 2007).

Bloom's syndrome (BS) is also a progeroid syndrome with many similarities to WS. The protein mutated in BS, BLM, is a helicase in the same family of proteins as WRN, it also shares many of the same phenotypes such as accelerated aging and increased cancer incidence, mostly carcinomas (Hickson, 2003). BS cells show a high level of genomic instability characterized by an extreme elevation in sister chromatid exchange (SCE) frequency (Chakraverty and Hickson, 1999). BLM is especially sensitive to mitomycin C (MMC), an inter-strand cross-linking agent (Hirano et al., 2005). This suggests that BS patients suffer an inability to resolve complex DNA structures that may arise during DNA replication. It is thought that the helicase activities of both WRN and BLM are necessary to resolve inappropriate DNA structures, thus facilitating progression of the replication machinery. BLM seems especially adept at resolving Holliday Junctions (HJ), which are thought to form at both stalled replication forks and some DSBs (Plank et al., 2006; Wu and Hickson, 2002; Wu and Hickson, 2003).

An interesting aspect of the BLM helicase is its apparently differing roles in meiosis and mitosis. In meiosis BLM is necessary for homologous chromosome crossover during meiosis I (Cromie et al., 2006). However, during mitosis, BLM is involved in the suppression of crossover (Wu and Hickson, 2003). These seemingly contradictory roles for BLM may be dictated by BLM interacting proteins such as Topoisomerase III α (Topo IIIα) and BLM Associated Protein-75 kD (BLAP75) (Wu and Hickson, 2003). These proteins interact with BLM during mitosis, but are not associated during meiosis. It is thought that in either case, BLM is responsible for the migration of HJ (Cheok et al., 2005a; Plank et al., 2006). In the case of meiosis, branch migration leads to highly negative supercoiled DNA, which can then break and lead to crossover. In the case of mitosis, Topo IIIα and BLAP75 work together with BLM to reduce the amount of negative supercoiled DNA, thereby suppressing crossover.

FANCD2 is a critical member of the Fanconi Anemia (FA) pathway. The FA pathway has many players, with over 12 known proteins with FANCD2 being one of the newest, and perhaps the most important member. Most of the FA proteins act together to form a complex called the FA core complex (Garcia-Higuera et al., 2000). This core complex translocates from the cytoplasm into the nucleus upon DNA damage, where it can then monoubiquitonate FANCD2, thereby activating it (Garcia-Higuera et al., 2001). FANCD2, along with BRCA2, help recruit the proteins BRCA1, RAD51, and PCNA to DNA damage to initiate HR (Zhang et al., 2007d). While some of the FA pathways do not influence SCE formation (Godthelp et al., 2006), others such as FANCC and FANCD2 have been shown to suppress G-SCE formation (Hirano et al., 2005; Yamamoto et al., 2005).

In this paper, we will look at how these HR proteins influence SCE frequency. In particular, we will focus on how they influence SCE dependent on the location of the SCE in the genome. We demonstrate that these proteins differentially influence SCE frequency depending on whether the SCE occurs in genomic (G-SCE) or telomeric (T-SCE) DNA. Understanding the roles of these proteins at specific locations in the genome may help us better understand both the function and biological relevance of SCE in aging.

Materials and Methods

Cell Culture. All cell lines were grown at 37°C in 20% oxygen and 5% CO_2. Human BS and WS lymphoblasts were obtained from Coriell (WS-AG04103 and BS- GM16375). Mouse cell lines doubly deficient for WRN-/- and the RNA component of telomerase (Terc-/-) were obtained from the Sandy Chang laboratory. Human FANCD2 patient cell lines were obtained from the Markus Grompe laboratory. Mouse BLM-/- embryonic stem cells were obtained from the Paul Hasty laboratory. Lymphoblast cells lines were grown using HyClone RPMI 1640 media with 15% fetal bovine serum (FBS) and penicillin/streptomycin (2mg/ml). Mouse cell lines were grown in HyClone αMEM media with 15% FBS and penicillin/streptomycin (2mg/ml).

G-SCE Staining and Analysis. Slides were prepared using standard cytogenetic techniques and stained via Fluorescence Plus Giemsa (FPG) technique (Perry and Wolff, 1974). Briefly, slides were stained with Hoescht 33258 for 15 minutes at room temperature, rinsed with distilled water and exposed to UV light (365nm) for 25 minutes. Slides were then soaked in 2x SSC at 60°C for 30 minutes. Following thorough rinses with distilled water slides were allowed to air dry, then stained with 2% Giemsa for 10 mintues. Images were analyzed and captured using a Zeiss Axioskop2 Plus microscope equipped with a Photometrics Coolsnap ES2 camera and Metavue 7.1 software.

Chromosome Orientation- Fluorescence *In Situ* Hybridization (CO-FISH). CO-FISH analysis was done according to Bailey et al (Bailey et al., 1996). Briefly, cells were grown for one round of replication in the presence of 1×10^{-5} M 5'-bromo-2'-deoxyuridine (BrdU). Cells were harvested and slide preparations made. Slides were stained with Hoescht 33258 (0.5 μg

Hoescht 33258/ 2x SSC), and exposed to 365 nm UV in a Stratalinker 3000. Exonuclease III (Promega) buffered solution was used to degrade strands containing BrdU. A hybridization solution containing 0.2 μg/ml of telomere PNA probe (Applied Biosystems), 70% formamide (Fischer Scientific), 12mM Tris-HCl, 5mM KCl, and 1mM $MgCl_2$ was placed on the slides for approximately 2 hours. Slides were then washed with 2x SSC and PN buffer and counterstained with Vectashield antifade containing DAPI (Vector Laboratories). Slides were scored using a Zeiss Fluorescence microscope (Axioplaln 2ie MOT). For CO-FISH, the DAPI and Cy3 filters were used to visualize the chromosomes and telomere probes, respectively. Anti-BrdU along with a FITC channel was used to confirm the cells were first cycle. Images were captured with a CCD camera (model CV-M4+CL, JAI PULNiX Inc., San Jose, CA, USA), and a computer (Dell precision 360 workstation) running Isis FISH imaging software (Metasystems, Altussheim, Germany).

T-SCE Analysis. T-SCEs were scored after telomere CO-FISH. CO-FISH gives a distinct single-sided signal on each end of the chromosome, and any split in a signal was scored as 1 T-SCE (although it must be noted that two or more T-SCE that occur in the same telomere will give the same split signal, yet it will still only be scored as one). Results were categorized as # of T-SCE/chromosome. Error bars were calculated as standard error of the mean (SEM). Significance was calculated using a T-test analysis using software freely available online by GraphPad Quick Calcs T Test.

Western Blot Analysis. Protein expression levels were measured by Western Blot Analysis, as previously been described. WRN (C-19): sc-1956 primary antibody for WRN was purchased from Santa Cruz Biotechnology, Inc. ECL detection assay combined with STORM allowed visualization and quantification of protein levels.

siRNA. siRNA was purchased from Qiagen (hs_WRN_6_HP Validated siRNA), along with Qiagen transfection reagents. Briefly, cells were pre-treated by growing in media without any antibiotics (αMEM + 15% FBS) overnight. On the day of transfection, siRNA was prepared by adding 10μl of siRNA/flask in 1.2 ml OptiMEM, while 25 μl of transfection was added to another tube in 1.2 ml OptiMEM. After sitting for 5 min, the two mixtures are added together, mixed, and incubated for 25 minutes. The entire solution is then placed on cells for approximately 6 hours. Normal media with antibiotics is added and cells are incubated until harvest. Lysates for Western analysis were collected to confirm and quantify knockdown levels.

RESULTS

WRN and BLM Deficient Mice

To study the effects of WRN on of T-SCE frequency we first analyzed cells from knockout mice generously given by the Sandy Chang lab. Cells were obtained from early and late generation mice that were either heterozygous or homozygous null for Wrn. All cell lines were also null for the RNA component of telomerase, Terc-/-. Inactivation of telomerase is necessary in mice in order to produce a similar aging phenotype as seen in human WS patients due to their long telomeres (Pennisi, 1996).

Early generation (G3) WRN knockout mice did not display any abnormal elevation in T-SCE frequency; displaying an average frequency of ~ 0.10 T-SCE/chromosome (Figure 1). This may be explained by that early generation mice still display very long telomeres compared to humans and it takes multiple generations in the absence of telomerase to see enough of a decrease in telomere length to stimulate recombination. Similarly, a late generation (G5) mouse heterozygous for Wrn, did not show any elevation in T-SCE (0.08 T-SCE/chromosome; Figure 1) demonstrating that even with the shorter telomeres, Wrn can suppress T-SCE. Late generation (G5) mouse cells that were homozygous for both Wrn and Terc, displayed a significant increase in T-SCE was observed (0.65 T-SCE/chromosome; Figure 1) (Laud et al., 2005).

Spontaneously immortalized clones from the late generation double Wrn/Terc KO cell lines were isolated and analyzed. At passage 5, the immortalized cell lines displayed a very high level of T-SCE (0.75 T-SCE/chromosome; figure 1), which remained elevated even until passage 54 (0.65 T-SCE/chromosome; figure 1). These immortalized Wrn-/- Terc-/- cells were

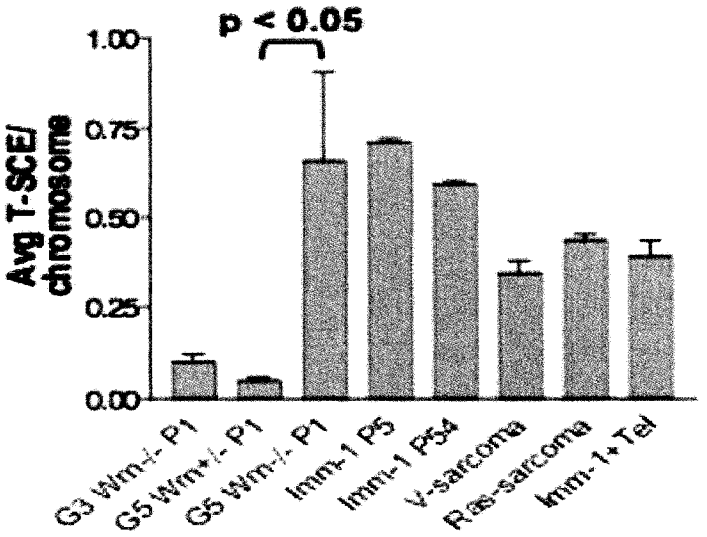

Figure 1. Mouse double knockout for WRN and Terc. G3= generation 3, G5= generation 5, P= passage number, Imm= immortalized, V-sarcoma and Ras-sarcoma= tumors developed from immortalized lines in SCID mice, Tel= the addition of active telomerase.

transplanted into SCID mice and very quickly formed tumors. These tumors retained the very high levels of T-SCE (V-sarcoma had a frequency of 0.40 T-SCE/chromosome, Ras-sarcoma had a frequency of 0.45 T-SCE/chromosome; Figure 1) (Laud et al., 2005). Interestingly, when telomerase was re-introduced into the immortalized cell line, the levels of T-SCE remained high (0.45 T-SCE/chromosome; Figure1) suggesting that once activated, the pathway generating the T-SCE was not shut off by telomerase. On the other hand, it was shown that the WRN helicase can lower T-SCE levels after re-introduction (Laud et al., 2005). Interestingly, the levels of G-

SCE in all mouse lines remained at background levels, suggesting that WRN is involved only in the suppression of T-SCE and not G-SCE.

Considering WRN's role in regulating T-SCE but not G-SCE frequencies, we examined the RecQ helicase BLM to determine its role in SCE frequency. It is well established that BS cells display extremely elevated SCE frequencies; in fact, BS is often diagnosed by the very high levels of SCE (Bartram et al., 1976). Therefore, with BLM's known role in the suppression of G-SCE, we hypothesized a "reciprocal" role in T-SCE; WRN may be the RecQ helicase operating at telomeres, while BLM may be the helicase operating in the rest of the genome.

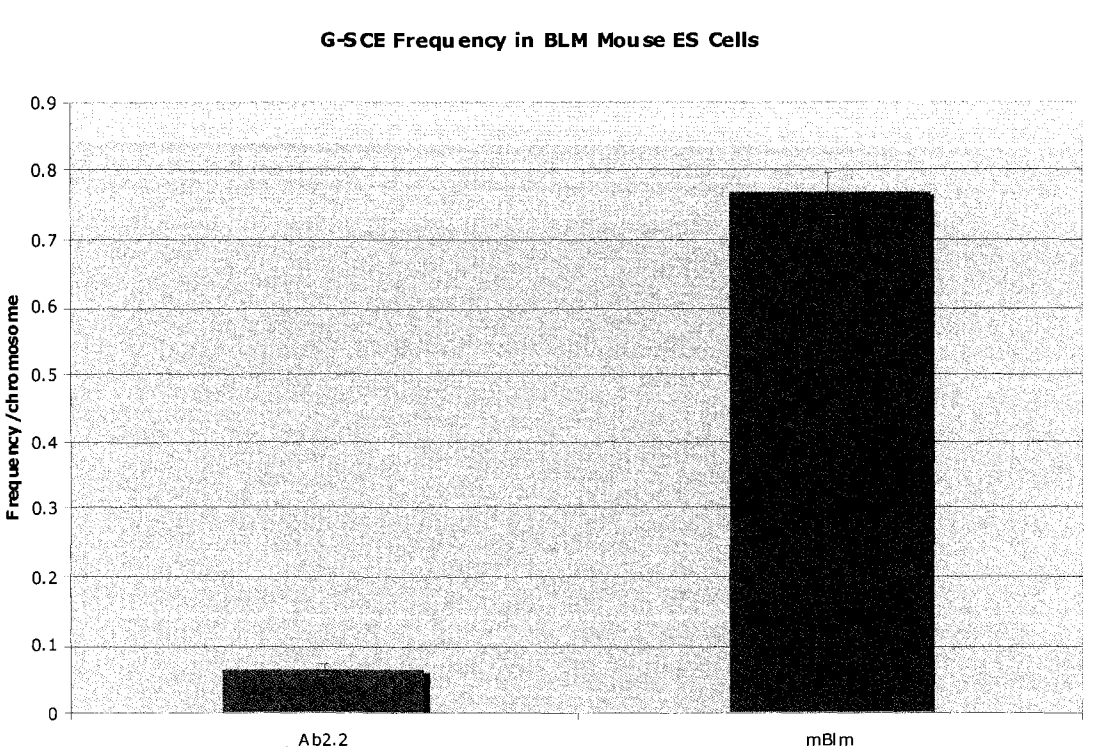

Figure 2. Mouse Embryonic Stem Cells Displaying Elevated Levels of G-SCE in BLM-/-. Ab2.2 is the control cell line, mBlm is deficient in the BLM helicase.

Mouse BLM-/- embryonic stem (ES) cells were grown for two rounds of replication in the presence of BrdU, and subsequent FPG analysis allowed detection of G-SCE. The BS mouse ES, similar to normal BS cells, displayed very high levels of G-SCE. The average G-SCE frequency was 0.75 G-SCE/chromosome, while the control was 0.08 G-SCE/chromosome (Figure 2). Although determination of T-SCE frequencies in the BLM-/- mouse ES cells was attempted, it was not successful. There was also concern that the telomerase positive status of these cells would mask a T-SCE phenotype, therefore, we pursued human cell strategies.

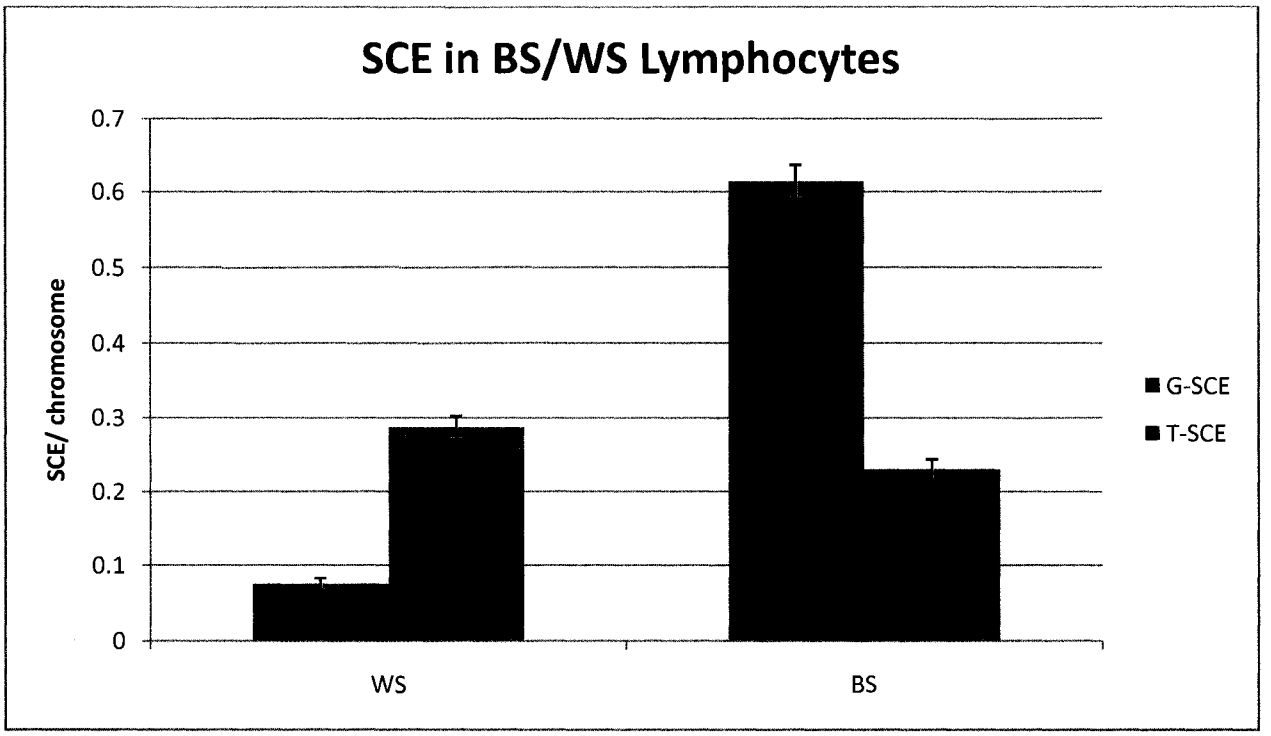

Figure 3. Werner Syndrome and Bloom Syndrome lymphoblast lines displaying G-SCE frequencies (blue) and T-SCE frequencies (red).

WS/BS Lymphoblasts

To further understand how WRN and BLM influence SCE frequencies specifically within telomeres, we obtained human immortalized lymphoblast lines established from patients. These cells were EBV immortalized, therefore, had active telomerase.

Similar to the WRN knockout mouse lines, the WS human lymphoblasts did not display an elevation in G-SCE with a frequency of 0.075 G-SCE/ chromosome (Figure 3). Consistent with other reports, the BS human lymphoblasts displayed very high levels of G-SCE with a frequency of 0.61 G-SCE/ chromosome (Figure 3). Interestingly, neither the WS nor BS lymphoblasts displayed an increase in T-SCE as measured by CO-FISH; with frequencies of 0.29 T-SCE/chromosome and 0.23 T-SCE/ chromosome, respectively (Figure 3.) The fact that even the WS lymphoblasts did not show any elevation in T-SCE lends support to the idea that the

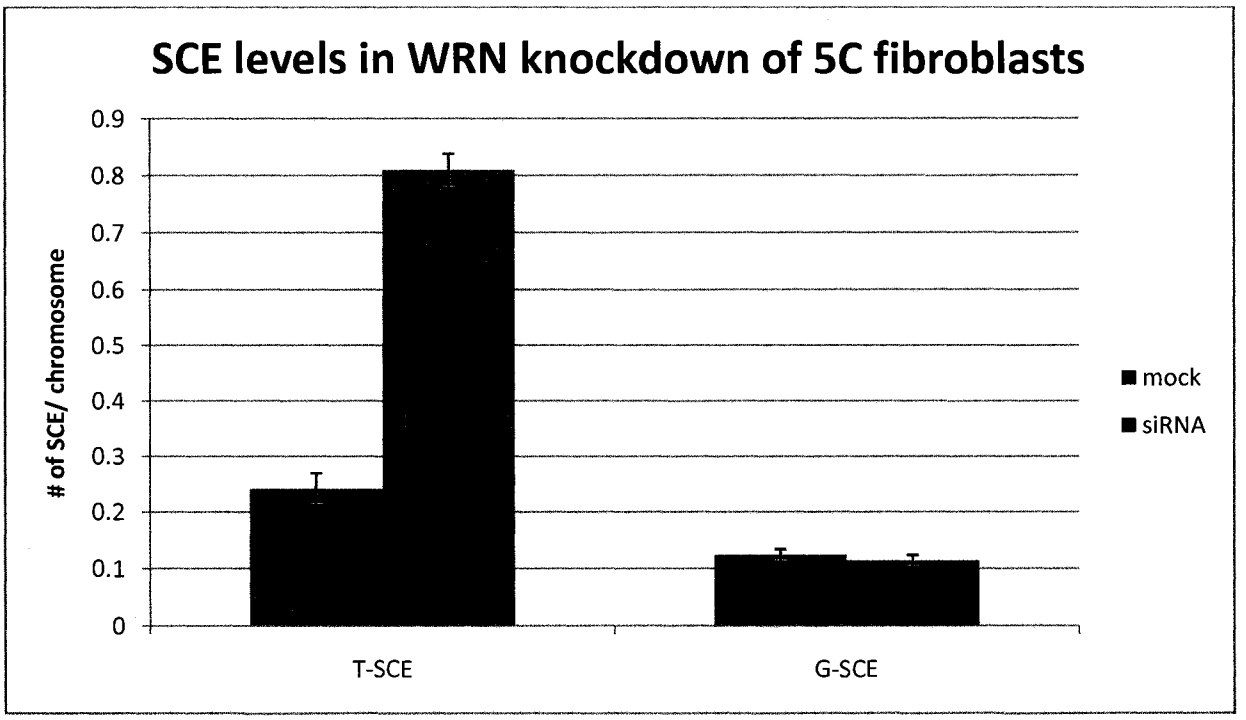

Figure 4. siRNA knockdown of WRN in normal human fibroblasts (5C). Knockdown level was 93% and 99% respectively. Mock frequencies are shown in blue, siRNA frequencies are shown in red.

action of telomerase inhibits T-SCE. This would be a similar situation to that observed in the WRN-/- Terc-/- mouse, where an elevated T-SCE phenotype was seen.

WRN/BLM Knockdown

The apparent ability of telomerase to "mask" or inhibit the phenotype of elevated T-SCE led us to utilize siRNA (small interfering RNA) technology to knockdown WRN and BLM in normal primary human fibroblasts (5C HDF), which are telomerase negative. siRNA from Santa Cruz Biotechnology was purchased for both the WRN and BLM proteins. Fibroblasts were seeded into 8 T-25 flasks at a density of ~ 200,000 cells per flask. The following schematic demonstrates each flask for each knockdown.

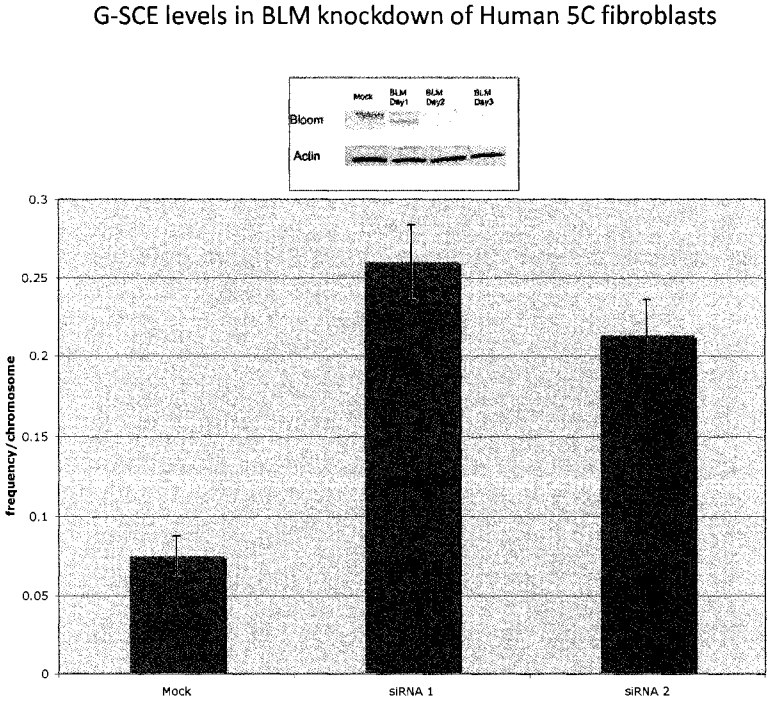

Figure 5. siRNA knockdown of BLM in normal human fibroblasts (5C) displaying elevated levels of G-SCE with two separate siRNA's. Knockdown level was 95% compared to the mock.

Mock	siRNA (WRN or BLM)
1) +Bu(1X) for telomere CO-FISH (T-SCE)	2) +Bu(1X) for T-SCE
3) Lysates for (1X), Westerns	4) Lysates for (1X), Westerns
5) +Bu(2X) for telomere CO-FISH (T-SCE)	6) +Bu(2X) for T-SCE
7) Lysates for (2X), Westerns	8) Lysates for (2X), Westerns

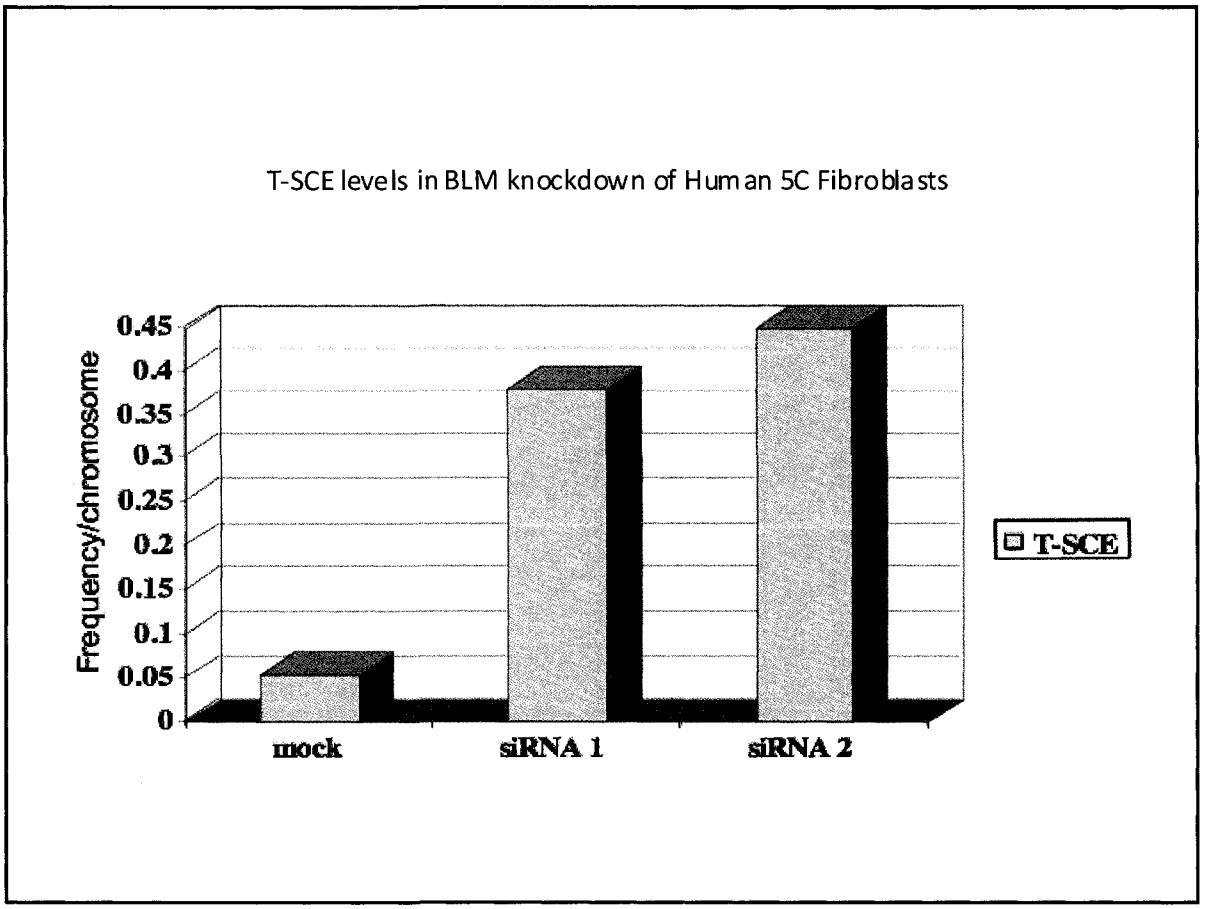

Figure 6. siRNA knockdown of BLM in normal human fibroblasts (5C) displaying T-SCE levels using two separate siRNA's. Knockdown expression level was 68% compared to the mock.

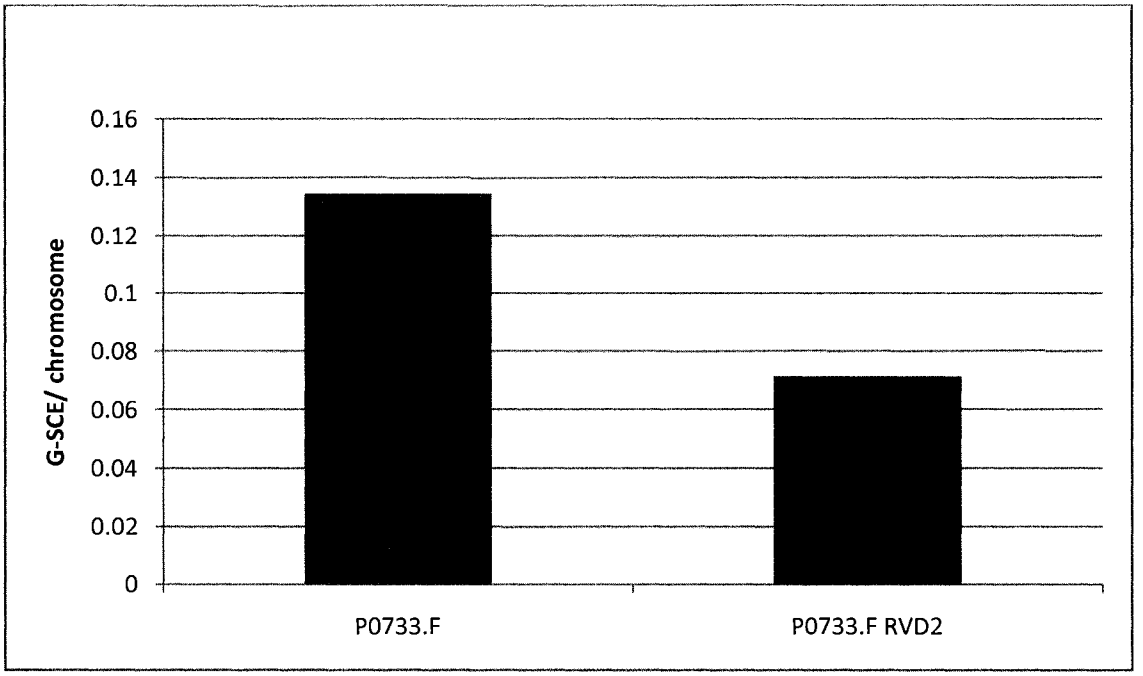

Figure 7. FANCD2 deficient primary human cell line (PO733.F) showing elevated G-SCE levels compared to the rescued PO733.F RVD2.

The +Bu (1x) and +Bu (2x) samples indicate the rounds of replication grown in the presence of BrdU. Western blot analysis confirmed the knockdown of the target proteins and how effectively it was. The WRN protein was knocked down for both days, 93% and 99% respectively (data not shown). G-SCE were not increased in the 5C HDF cells with the WRN siRNA +Bu (2X) slides as measured by FPG (Figure 4). The average frequency was 0.11 G-SCE/chromosome, in accordance with the background level of G-SCE seen in normal 5C HDF cells, 0.12 G-SCE/ chromosome. This indicates that WRN, similar to the mouse and the human lymphoblasts, does not play a role in regulating G-SCE frequency. The T-SCE levels were significantly elevated compared to the control (0.81 T-SCE/chromosome and 0.24 T-SCE/ chromosome, respectively; Figure 4), demonstrating WRN's role in suppression of T-SCE in the absence of telomerase in human cells.

We also utilized siRNA to knockdown the expression of BLM protein in telomerase negative normal human fibroblasts. Two separate siRNA's were used. Figure 5 demonstrates that the siRNA was effective at knocking down the BLM protein for both day 2 and day 3 of collection, 95% and 68% respectively. On day 2, +Bu (1X) cells were collected for CO-FISH analysis, while on day 3, +Bu (2X) cells were collected for FPG analysis. The siRNA's knockdown of BLM caused a dramatic increase in G-SCE, with an average frequency of 0.26 G-SCE/ chromosome and 0.22 G-SCE/ chromosome compared to the mock of 0.08 G-SCE/chromosome (Figure 5). This increase is not as elevated as observed in the BS lymphoblasts, likely due to the fact that siRNA does not totally ablate the BLM protein. The T-SCE frequencies, as measured by CO-FISH, were also elevated with an average frequency of 0.37 T-SCE/chromosome and 0.45 T-SCE/chromosome compared to the mock of 0.06 T-SCE/ chromosome (Figure 6). These studies demonstrate that unlike WRN, the BLM helicase has suppressive roles in both the genome and at telomeres. Similarly to WRN, telomerase appears to suppress the elevated T-SCE phenotype of BS.

FANCD2

To investigate the role of FANCD2 on G and T-SCE frequencies, we analyzed cells established from a FANCD2 patient. PO733.F is the primary FANCD2 cell line. These cell lines displayed abnormally high G-SCE with a frequency of 0.13 G-SCE/ chromosome (Figure 7). The PO733.F RVD2 cell line represents the patient cell line that was then rescued by re-introduction of a normal FANCD2 gene. The rescued cell line had a G-SCE frequency of 0.07 G-SCE/ chromosome (Figure7), demonstrating that FANCD2 is responsible for the G-SCE phenotype seen in the patient cell line P0733.F.

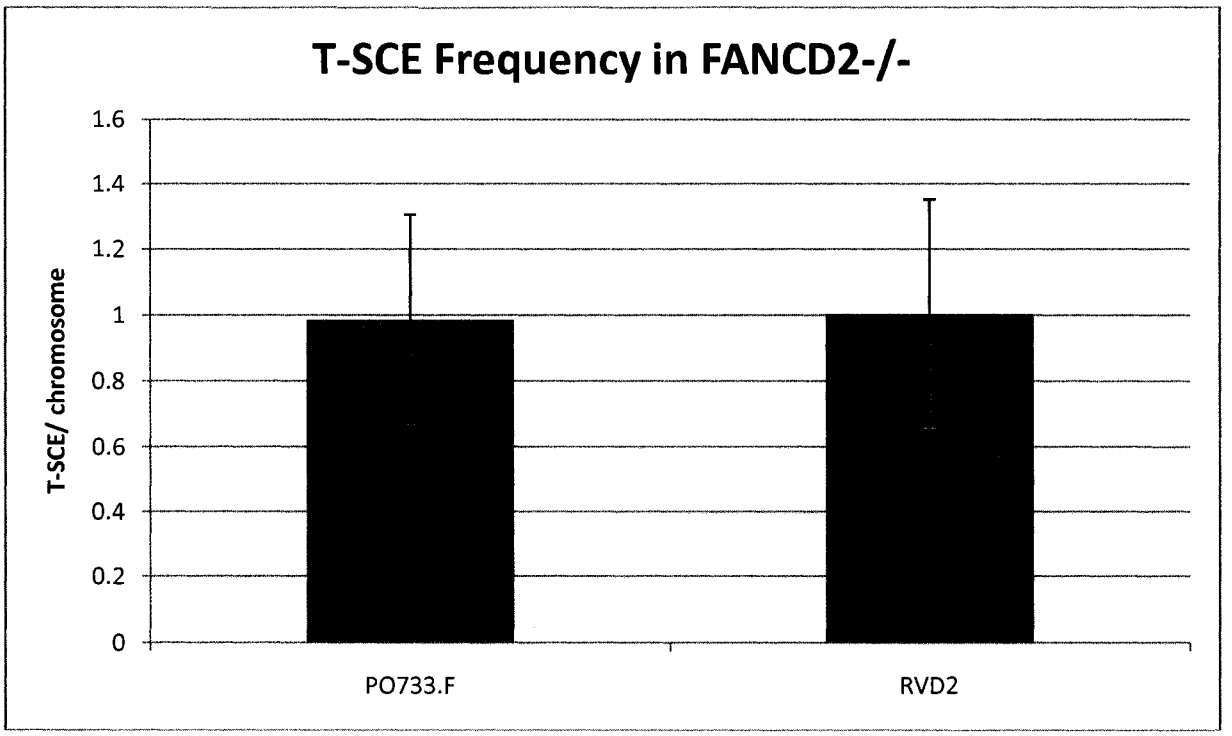

Figure 8. Both FANCD2 deficient cell line PO733.F and rescued RVD2 display extremely high levels of T-SCE.

CO-FISH was done on both cell lines to examine FANCD2's role on T-SCE frequencies. The patient PO733.F cell line displayed an extremely high level of T-SCE, with a frequency of 0.99 T-SCE/ chromosome (Figure 8). Interestingly, unlike what was seen with G-SCE levels, re-introduction of FANCD2 did not rescue the elevated T-SCE levels perhaps indicating that the T-SCE phenotype may not be due to the absence of FANCD2. The PO733.F RVD2 still had extremely elevated levels of T-SCE with a frequency of 1.00 T-SCE/ chromosome (Figure 8). Therefore, it is our conclusion that FANCD2 is responsible for suppressing G-SCE, and may possibly be responsible for T-SCE suppression.

Discussion

It is clear that HR plays a role in influencing SCE frequencies. Our results demonstrate that the simplistic model of a stalled replication fork being bypassed via a HR mechanism cannot be completely accurate. Studies in yeast and the chicken DT40 cells clearly demonstrate a possible role for RAD51 in SCE formation, as RAD51 mutants in these cell lines display much lower SCE levels than controls although not absent (Fasullo et al., 2001; Sonoda et al., 1998; Sonoda et al., 1999). Also, RAD51 interacting proteins such as BRCA and some of the FA proteins also seem to influence SCE levels, supporting the possibility that HR is involved in SCE (Hirano et al., 2005; Kim et al., 2004; Yamamoto et al., 2005).

However, the data presented here, suggest that SCE's are not the same. A stalled replication fork in the genome may present different structures, and is therefore regulated differently than a stalled fork in the telomeres. It is also likely that the location of the lesion that stalls the replication fork, whether it's on the leading or lagging strand, determines what proteins are involved in the SCE. A lesion that occurs on the lagging strand will contain a 3' end that suits itself perfectly for strand invasion. Therefore it may easily be resolved via a RAD51-dependent HR mechanism. However, a lesion that occurs on the leading strand will create a 5' end. This 5' end would not be conducive to strand invasion, therefore another mechanism must exist in order to resolve this structure, and i.e., perhaps a reverse chicken foot structure or single strand annealing.

Here, we also describe another possible level of control based on chromosomal location. Our data clearly suggests a role for WRN in the suppression of SCE specifically within telomeric DNA. This suppressive role seems dependent on the status of telomerase, as only cells lacking

telomerase display the extreme elevations in T-SCE frequencies in the absence of WRN. Compare this to the BLM protein, which despite many of it commonalities with WRN, displays a suppressive role in regards to SCE throughout the entire genome. In all experiments (BLM-/- mouse ES cells, BS human lymphoblasts, and the BLM knockdown in humans), BLM was necessary for suppression of G-SCE. Additionally, in the absence of telomerase, BLM was necessary for the suppression of T-SCE similar to that of WRN. Finally, we demonstrated that while restoring FANCD2 into a patient cell line deficient for the protein was sufficient to rescue the elevated G-SCE phenotype, it was not able to rescue the elevated T-SCE phenotype, suggesting more is involved.

SCE are used in a variety of mutagenic and genomic instability studies. Without clear mechanistic knowledge of how SCE are formed and regulated, it would be difficult to tell if its mutagenic or not. Therefore, further investigation is need to understand the mechanisms that can regulate SCE; not only between the genome and the telomeres but also possibly the leading and lagging strand. This would allow for a better understanding of a possible mutagenic agent that is being studied.

Non-homologous End Joining influences Genomic Sister Chromatid Exchange but not Telomeric Sister Chromatid Exchange.

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Keywords: NHEJ, SCE, DNA-PKcs, Artemis

Running Title: NHEJ influences G-SCE.

Abstract

The non-homologous end joining (NHEJ) pathway is crucial for repair of DNA double strand breaks (DSBs), and is especially important for DSB repair following exposure to ionizing radiation (IR). NHEJ predominates during G1 and early S-phase of the cell cycle before DNA replication and creation of a sister chromatid that can serve as a substrate for homologous recombination (HR) in G2. Sister Chromatid Exchange (SCE) is the exchange of genetic material between two sister chromatids. Considering SCE occurs during or after DNA replication, and that it involves exchange between two nearly identical sequences, HR is thought to be the primary pathway involved in SCE formation. Indeed, various studies have reported that many HR proteins can influence SCE frequency (Fasullo et al., 2001; Thompson et al., 1982). Here, we demonstrate that NHEJ may also play a role in SCE frequency. Using mouse cell lines deficient in the catalytic subunit of the DNA dependent kinase (DNA-PKcs), we demonstrate a decrease in SCE that occur within the genome (G-SCE), and an increase in SCE that occur within the telomeres (T-SCE). In addition, we examined the role of the NHEJ proteins DNA-PKcs and Artemis in T-SCE formation in a human lymphoblastoid cell line (WTK1). Using siRNA to knockdown the expression of the protein Artemis, no significant change in T-SCE frequency was observed. However, when combined with a specific inhibitor of DNA-PKcs, a significant increase in T-SCE was observed. Together, these results suggest a role for DNA-PKcs in influencing G-SCE and T-SCE frequencies.

Introduction

Sister chromatid exchange (SCE), the exchange, by breakage and re-union, of DNA sequences between sister chromatids at apparently homologous sites, was first reported over fifty years ago (Taylor, 1958). With the development of the fluorescence plus giemsa (FPG) staining technique, SCE became easily identifiable (Latt, 1973; Perry and Wolff, 1974)(Figure 1). Analysis of SCE is a sensitive indicator for detecting DNA damage induced by mutagens and carcinogens (Jacobs, 1977; Kligerman, 1979; Nakanishi and Schneider, 1979), however they are not sensitive to most types of ionizing radiation. Remarkably, however, little about their regulation or formation is known. It is generally believed and accepted that SCE occurs as a consequence of stalled replication forks and that they are a RAD51-dependent homologous recombination (HR) event (Saleh-Gohari et al., 2005). Indeed, it has been shown that RAD51 deficient yeast and chicken DT40 cells have lower spontaneous and induced SCE levels (Lambert and Lopez, 2000; Sonoda et al., 1999). However, absence of many of the HR proteins actually results in elevated levels of SCE, indicative of a suppressive function in terms of SCE formation (Hirano et al., 2005; Laud et al., 2005; Wu and Hickson, 2003; Yamamoto et al., 2005).

The non-homologous end joining (NHEJ) pathway is a well characterized DNA double strand break (DSB) repair pathway. Its central component is the DNA-PK holoenzyme, comprised of the catalytic subunit DNA-PKcs and the Ku 70/80 heterodimer (Meek et al., 2004). The basic steps for repair of DSBs by NHEJ is as follows reviewed in (Collis et al., 2005). Ku 70/80 recognizes the DSB ends and binds, translocating inward along the DNA, facilitating recruitment and docking of DNA-PKcs, forming the holoenzyme DNA-PK. This holoenzyme can then bridge the two free DNA ends together, which allows XRCC4/LigIV to join them.

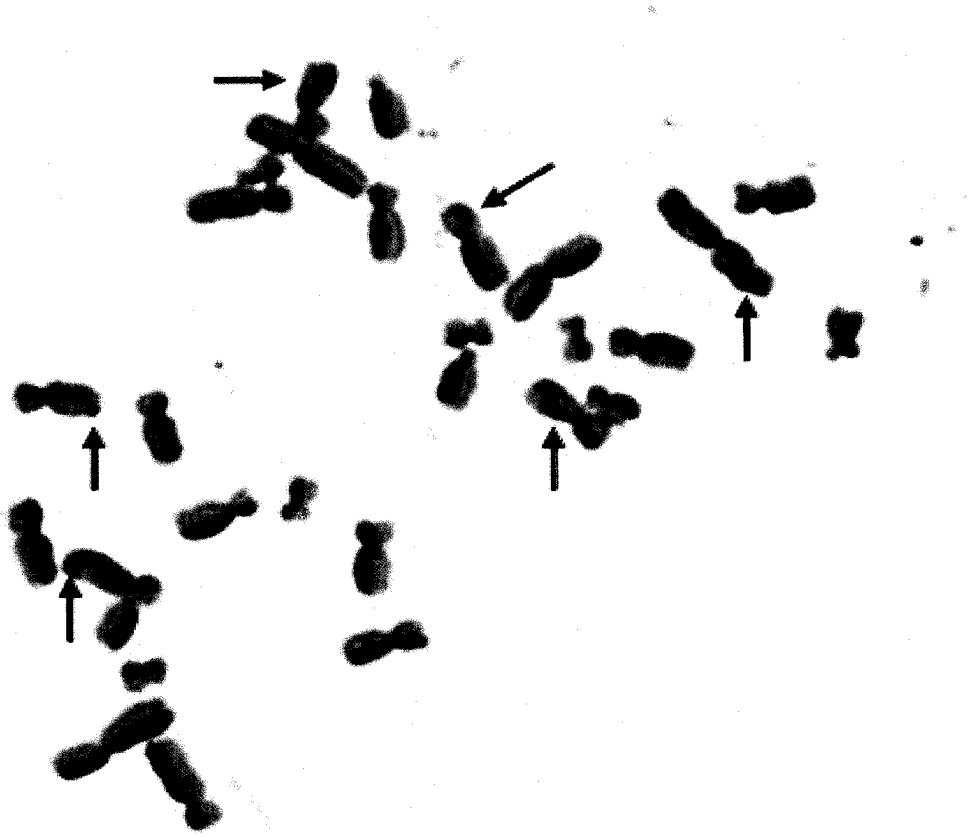


Figure 1: human metaphase chromosome illustrating differential "harlequin" staining pattern; arrows indicate SCE. Image courtesy of Abby Williams.

Artemis, an endo/exonuclease, is associated with DNA-PKcs and assists in modifying the DNA ends before ligation (Rooney et al., 2003). NHEJ is regarded as error prone since it joins any two ends it can result in inappropriate ligation and often is accompanied by loss of sequence (Jackson, 2002).

To date, there is little evidence suggesting NHEJ playing a role in either regulation or formation of SCE. It has been reported that Ku70/ TRF2 deficient cell lines display elevated T-SCE levels (Celli et al., 2006). Also, DNA damage in Ku-/- yeast results in elevated rates of SCE over controls (Fasullo et al., 2005). Here we demonstrate that DNA-PKcs deficient mouse lines have lower genomic SCE (G-SCE) levels than wild type mice. The mouse lines C57/Bl6,

BALB/c, and SCID (severe combined immunodeficiency) were used based on their DNA-PKcs status. C57/Bl6 is wild type and has normal DNA-PKcs activity. BALB/c contains two single-nucleotide polymorphisms in the *Prkdc* gene that encodes for DNA-PKcs and has reduced expression and activity (Ponnaiya et al., 1997; Yu et al., 2001). SCID contains a truncated/ rapidly degarded version of DNA-PKcs and is essentially a null phenotype (Fulop and Phillips, 1990). Interestingly, G-SCE levels decreased in accordance with DNA-PKcs function, while T-SCE (SCE's that occur specifically within telomeric DNA) levels between the mouse lines were slightly elevated, suggesting that DNA-PKcs may act differentially in genomic and telomeric SCE. To further examine the role of NHEJ on T-SCE, we utilized siRNA technology to knockdown Artemis, an endo/exonuclease that associates with DNA-PKcs, in the human lymphoblastoid cell line WTK1. In addition, the inhibitor Nu2076 was used to abolish the kinase activity of DNA-PKcs. While knockdown of Artemis did not influence T-SCE frequency, the addition of the DNA-PKcs inhibitor caused a significant increase in T-SCE further implicating DNA-PKcs in SCE.

Materials and Methods

Mouse Primary Kidney Fibroblasts. Kidneys from 8–12 week old female mice (C57/Bl6, BALB/c, and SCID) were minced, and digested in 199 medium containing collagenase (Worthington Type III; 200 units/ml) at 37°C for 3–5 h with gentle agitation. Disaggregated cells were washed 6x in phosphate buffered saline (PBS) containing 0.5% fetal bovine serum (FBS) and cultured in α-MEM medium (15% FBS, pennicillin/streptomycin). Media was changed after 3 days of incubation.

Cell Culture. Mouse cells were grown in αMEM (Hyclone) supplemented with 15% fetal bovine serum (FBS) and antibiotics. Cells were seeded into T-75 flasks and placed in 37°C incubators with 5% CO_2. WTK1 cells were grown in RPMI 1640 (Hyclone) media supplemented with 15% FBS and antibiotics.

G-SCE Staining and Analysis. Slides were prepared using standard cytogenetic techniques, and then stained via the Fluorescence Plus Giemsa technique (Latt, 1973; Perry and Wolff, 1974). Briefly, slides are stained with Hoescht 33258 for 15 minutes at room temperature, rinsed with distilled water and exposed to UV light (365nm) for 25 minutes. Slides are then soaked in 2x SSC at 60°C for 30 minutes. Following thorough rinsing with distilled water, slides are allowed to air dry, then stained with 2% Giemsa for 10 mintues. Images were analyzed and captured using a Zeiss Axioskop2 Plus microscope equipped with a Photometrics Coolsnap ES2 camera and Metavue 7.1 software.

Chromosome Orientation- Fluorescence *In Situ* Hybridization (CO-FISH). CO-FISH analysis was done according to Bailey et al (Bailey et al., 1996). Briefly, cells were grown for one round of replication in the presence of 1 x 10^{-5} M 5'-bromo-2'-deoxyuridine (BrdU). Cells

were harvested and slide preps were made. Slides were stained with Hoescht 33258 (0.5 μg Hoescht 33258/ 2x SSC), and exposed to 365 nm UV in a Stratalinker 3000. Exonuclease III (Promega) buffered solution was used to degrade strands containing BrdU. A hybridization solution containing 0.2 μg/ml of telomere PNA probe (Applied Biosystems), 70% formamide (Fischer Scientific), 12mM Tris-HCl, 5mM KCl, and 1mM $MgCl_2$ was placed on the slides for approximately 2 hours. Slides were then washed with 2x SSC and PN buffer and counterstained with Vectashield antifade containing DAPI. Slides were scored using a Zeiss Fluorescence microscope (Axioplaln 2ie MOT). For CO-FISH, the DAPI and Cy3 filters were used to visualize the chromosomes and telomere probes. Anti-BrdU, along with a FITC filter, were used to verify the cell cycle number. A CCD camera (model CV-M4+CL, JAI PULNiX Inc., San Jose, CA, USA) was used to take the picture. A computer (Dell precision 360 workstation) with Isis FISH imaging software (Metasystems, Altussheim, Germany).

T-SCE analysis. T-SCEs were scored after CO-FISH with a telomere probe. CO-FISH gives a distinct single-sided signal on each end of the chromosome, and any split in the signal between sister chromatids was scored as 1 T-SCE. Results were categorized as # of T-SCE/chromosome. Error bars were calculated as standard error of the mean (SEM). Significance was calculated using a T-test analysis using software freely available online by GraphPad Quick Calcs T Test.

siRNA. siRNA was purchased from Qiagen (hs_WRN_6_HP Validated siRNA), along with Qiagen transfection reagents. Briefly, cells were pre-treated by growing in media without any antibiotics (αMEM + 15% FBS) overnight. The day of transfection, the siRNA was prepared by adding 10μl of siRNA/flask in 1.2 ml OptiMEM, while 25 μl of transfection was added to another tube in 1.2 ml OptiMEM. After sitting for 5 min, the two mixtures are added together, mixed, and incubated for 25 minutes. The entire solution is then placed on cells for

approximately 6 hours. Normal media with antibiotics is added and cells are incubated until harvest.

DNA-PKcs inhibitor. The DNA-PKcs inhibitor NU7026 was purchased from Sigma. It was prepared by mixing 5 mg with 2 ml of DMSO. A concentration 10 μM of the inhibitor was added to the cells for the entire time of culture.

Western Blot Analysis. Protein expression levels were measured by Western Blot Analysis, as previously been described. WRN (C-19): sc-1956 primary antibody for WRN was purchased from Santa Cruz Biotechnology, Inc. ECL detection assay combined with STORM allowed visualization and quantification of protein levels.

Results

DNA-PKcs and G-SCE

Mouse C57/Bl6, BALB/c, and SCID kidney fibroblasts were grown for either one or two rounds of replication in BrdU (1X or 2X respectively). The C57/Bl6 mouse represents wild type *Prkdc,* the gene that encodes the catalytic subunit of DNA-PK (DNA-PKcs). The BALB/c mouse contains alleles of DNA-PKcs that possess two single-nucleotide polymorphisms that result in overall decreased function (Okayasu et al., 2000; Yu et al., 2001). The SCID mouse carries a truncated version of DNA-PKcs that leads to a functionally null phenotype and severe immunodeficiency (Blunt et al., 1996).

CO-FISH analysis was performed on the BrdU (1X) slides to measure T-SCE frequencies, while FPG analysis was done on the 2X slides to measure G-SCE frequencies. The cell lines displayed progressively decreasing levels of G-SCE from C57/Bl6 to BALB/c and SCID (Figure 2). All G-SCE frequencies represent ratios relevant to the control C57/Bl6. The C57/Bl6 cells displayed an average G-SCE frequency of 0.19 SCE/ chromosome +/- 0.011; this value was set to 1. BALB/c had a slightly depressed G-SCE phenotype with a frequency of about 80% (0.15 G-SCE/ chromosome) compared to the control C57/Bl6. The SCID cells showed a much more severe depression of G-SCE with only 40% (0.09 G-SCE/ chromosome) compared to the WT C57/Bl6. Thus, DNA-PKcs deficiency results in decreased frequencies of G-SCE, suggestive of a role for NHEJ in SCE formation.

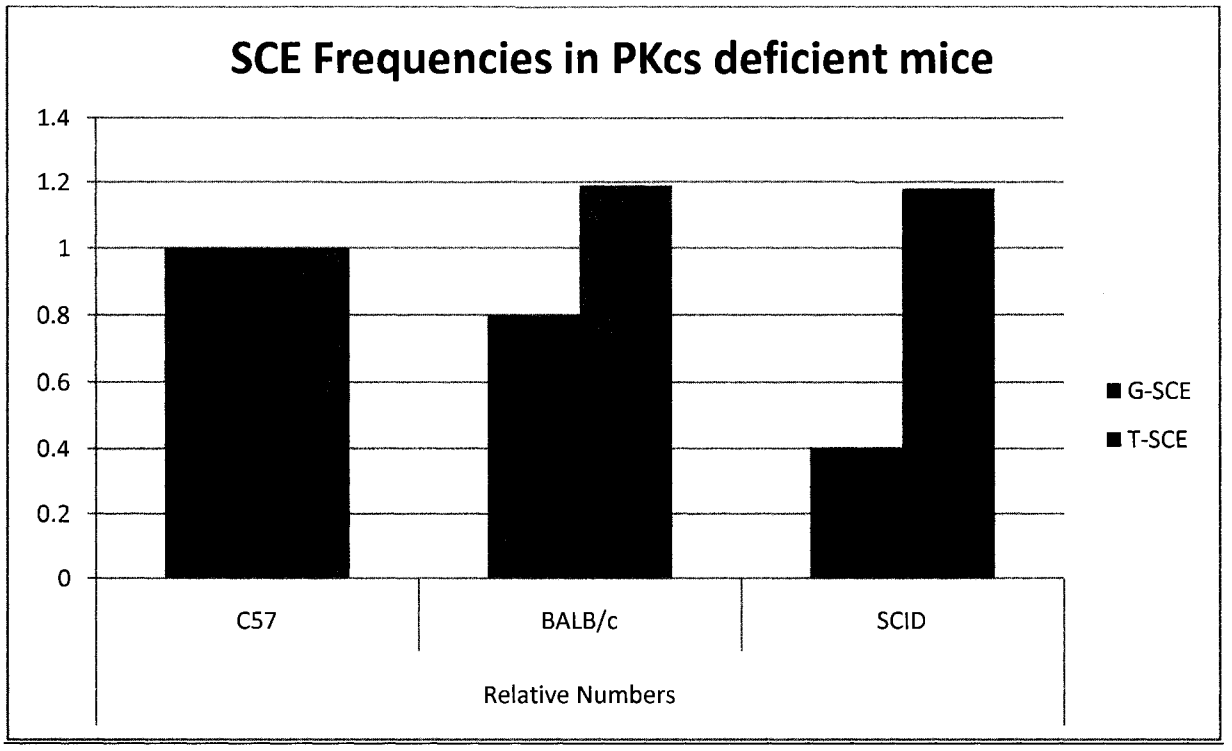


Figure 2. G-SCE levels in C57/Bl6 WT, BALB/c, and SCID mouse lines. Numbers are relative compared to C57/Bl6.

DNA-PKcs and T-SCE

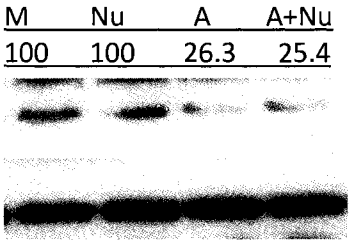
We examined the role of DNA-PKcs in terms of regulating SCE within telomeric DNA. CO-FISH selectively degrades the newly synthesized strand of DNA in cells that were grown in BrdU for one round of replication. Hybridization of a single stranded telomere probe, selectively targets either the leading- or lagging-strand telomere (Bailey et al., 2001). This approach gives rise to a chromosome that has one telomere signal on each end, often diagonal from one another (Figure 3). If a T-SCE occurred, the single signal will split between the two sister chromatids. However, as opposed to standard FISH signals, CO-FISH T-SCE signals are often of unequal intensity. Each "double" signal visualized with CO-FISH is scored as one T-SCE.

Figure 3. CO-FISH with yellow arrows demonstrating T-SCE

Analysis with telomeric CO-FISH on C57/Bl6, BALB/c, and SCID revealed a slight elevation in T-SCE frequency (Figure 2). The C57/Bl6 control frequency was set to one and the other two strains were compared to it (ratios relative to control). Both the BALB/c and the SCID had slight increases compared to the wild type C57/Bl6, displaying an average increase in T-SCE of about 20% for both. Thus, DNA-PKcs deficiency caused a decrease in T-SCE frequency.

Artemis, DNA-PKcs, and Telomeres

To determine the role of DNA-PKcs and its interacting partner Artemis in telomere function in human cells, we utilized siRNA to knockdown expression of Artemis. In addition, some of the samples were exposed to a specific DNA-PKcs inhibitor (NU7026). The treatments are as follows:



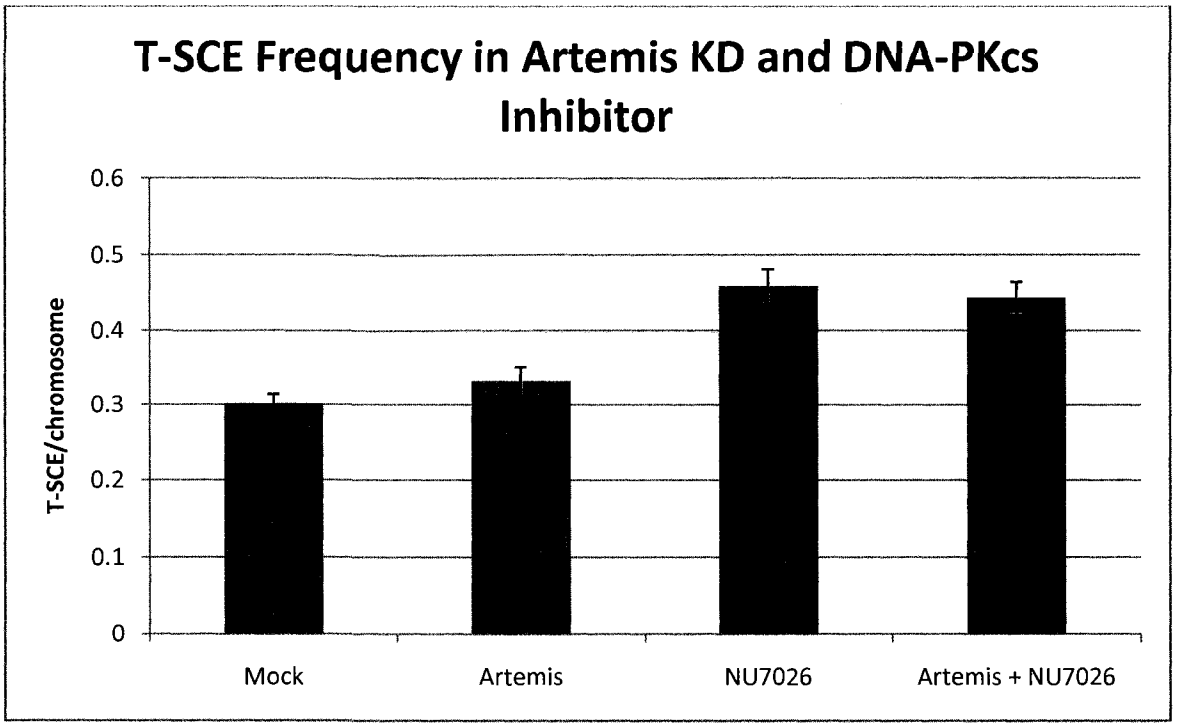


Figure 4. Western showing knockdown levels of Artemis, showing ~75% knockdown. T-SCE levels in the knockdown and in conjunction with a DNA-PKcs inhibitor.

Sample #1- WTK1 mock

Sample #2- WTK1 Artemis siRNA

Sample #3- WTK1 mock + NU7026

Sample #4- WTK1 Artemis siRNA + NU7026

All samples were grown in BrdU for one round of replication, then harvested for metaphases and slides made. Telomere CO-FISH analysis facilitated visualization of T-SCE, as well as screening of other chromosome and chromatid type aberrations. The mock knockdown displayed an average frequency of 0.30 T-SCE/ chromosome, while the siRNA Artemis reduction resulted in a similar frequency of 0.33 T-SCE/ chromosome (Figure 5). This demonstrates that Artemis is not involved in either suppression or formation of SCE at the telomeres. Interestingly, treatment with the DNA-PKcs inhibitor NU7026 produced an average T-SCE frequency of 0.46 T-SCE/ chromosome, and the combined treatment with inhibitor plus Artemis knockdown produced a frequency of 0.44 T-SCE/ chromosome (Figure 5). Thus again, the Artemis knockdown did not influence T-SCE levels, however, the DNA-PKcs inhibitor did significantly raise the T-SCE frequency. This suggests that DNA-PKcs may function as a suppressor of SCE at telomeres.

Discussion

Although SCE has been recognized for many years, a satisfactory mechanistic understanding of SCE has remained elusive. The widespread use of SCE as a marker in both mutagenic and genomic instability studies necessitates the need to know more about SCE regulation and formation. Such knowledge would allow an investigator to say not only that a specific compound causes increased mutagenesis, but also that the compound does so by interacting or interfering with specific proteins or pathways.

It does appear clear from the literature that SCE is influenced by the HR pathway, as numerous papers report alteration of a specific protein in the HR pathway influences the overall frequency of SCE (Helleday, 2003; Nagasawa et al., 2005; Sonoda et al., 1999). However, in many of these cases, alteration of these proteins often leads to an increase in either spontaneous or induced SCE. This suggests more of a suppressive role of SCE for these proteins normally, rather than a role in SCE formation.

It is believed that SCE form in response to stalled replication forks in a RAD51-dependent manner. Studies that support this view utilize either a yeast model, or in some cases the chicken cell line DT40 (Lambert and Lopez, 2001; Sonoda et al., 1999). These studies demonstrate that in the absence of RAD51 both spontaneous and induced SCE frequencies diminish. This suggests that RAD51 is necessary for SCE formation, thereby implicating HR. However, the reason RAD51 studies are done in yeast and chicken cells are that RAD51 loss is lethal in most higher eukaryotic organisms. This naturally brings the validity of these studies, in terms of biological relevance to mammalian cells, into question. If RAD51 is essential in higher organisms, how can yeast and the DT40 cell lines live so readily without it? Does that imply that

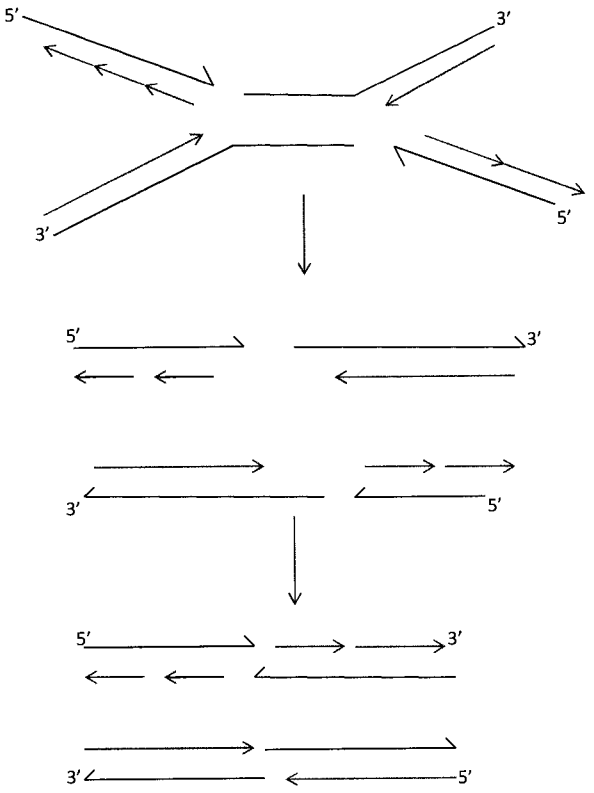


Figure 5. Working model for NHEJ on SCE. Colliding replication forks collapse at two lesions on opposing strands leading to two DSBs. Inappropriate DSB repair, approximately 50% of the time, leads to exchange. Upon a second round of replication, the resulting structures look like a SCE.

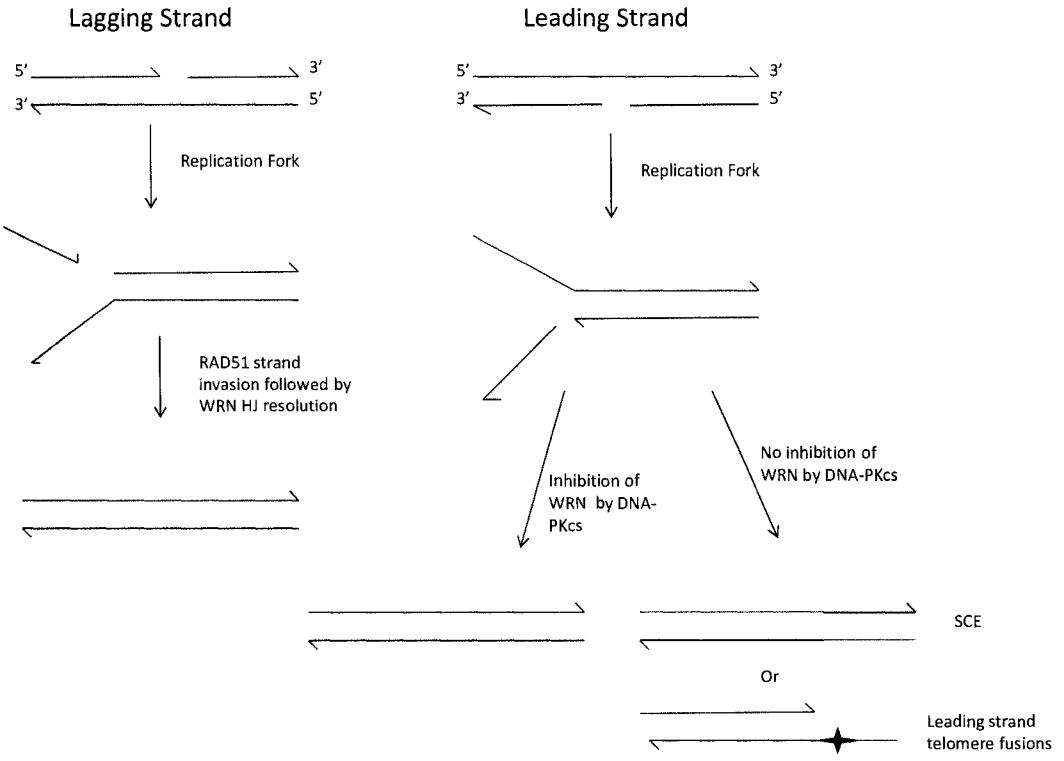
RAD51 has different functions in these cell lines, or is there some sort of backup system that can attenuate the RAD51-/- phenotype? Another issue with some of these systems, especially the DT40 cell line, is that they display hyper recombination phenotypes naturally. This suggests that these cells display elevated spontaneous levels of instability, and how this may affect SCE frequencies, is not known.

We felt it valuable to investigate other potential pathways for involvement in regulation of SCE frequencies. Further, we investigated differential regulation of SCE frequencies based on chromosomal location, i.e., genomic versus telomeric SCE. The NHEJ pathway was an

attractive candidate, as many models of stalled replication forks involve at least one free double-stranded end. It seems reasonable that NHEJ may act on this double strand end, especially given how rapidly Ku has been shown to respond to break ends (Baumann and West, 1998). In fact, it has been suggested that Ku70 may have a suppressive role in T-SCE in a TRF2 dependent manner (Celli et al., 2006).

Our data suggest that DNA-PKcs plays a role in regulating SCE frequency. The fact that G-SCE frequencies decreased in DNA-PKcs deficient mice suggests a possible role for DNA-PKcs in the formation of SCE. In support of this view, the level of SCE depression correlated well with the status of DNA-PKcs. The BALB/c mouse, with its two polymorphisms in the *Prkdc* gene, gives it a reduced activity but not completely absent phenotype. The SCID contains a truncated version of the proteins and has almost no DNA-PKcs activity. The G-SCE data followed this trend, with the BALB/c showing depressed levels but still fairly close to the wild type C57/Bl6, while the SCID displayed a much more significant decrease in G-SCE.

How NHEJ might be involved in G-SCE formation is not readily apparent. It is possible that NHEJ is involved in only a subset of SCE. If there are sufficient lesions, it is possible that two single stranded "nicks"/breaks could be in close enough proximity that they would be converted into DSBs which NHEJ could then resolve. For example, if a nick on the leading strand is encountered by a replication fork, the fork will stall and a single DSB end will be created. If there is a nick in close enough proximity on the lagging strand, a replication fork from the opposite direction could then create another single DSB. It would then be possible for NHEJ to inappropriately join these two ends leading to a SCE (Figure 5).



Figrure 6. NHEJ and WRN resolving stalled replication forks. When the lesion occurs on the lagging strand stalling a replication fork, a cell can resolve the lesion via RAD51 strand invasion followed by Holliday Junction (HJ) resolution by WRN in a non-crossover manner. When the lesion occurs on the leading strand, DNA-PKcs normally inhibits WRN to prevent inappropriate resolution of the stalled fork. Without DNA-PKcs, increases in T-SCE and leading strand fusions are seen.

While this may be possible, a more plausible mechanism for DNA-PKcs involvement in G-SCE might invoke a more "competitive" balance. In the absence of NHEJ, cells have been demonstrated to have a heightened HR response (Pierce et al., 2001). This heightened response may then be more readily available to act on stalled replication forks and resolve them in a non-crossover manner more efficiently; and for a SCE to be visualized, they must be resolved with crossover. While it is often assumed that a stalled fork may resolve itself, either with a crossover or not, at a rate of approximately fifty percent, this may not be the case. Indeed, it seems when a

fully functioning HR response is intact, non-crossover resolution may be favored (Raynard et al., 2006; Wu and Hickson, 2003). Therefore when NHEJ is absent, the HR response is elevated and ultimately SCE are reduced.

A satisfactory explanation for the increase in T-SCE frequency in cells deficient in DNA-PKcs is more difficult, especially since the trend is opposite of that seen in G-SCE. Our data consistently point to a role for DNA-PKcs in suppression of T-SCE. This is consistent with earlier reports (Bailey et al., 2004a). The most simplistic reasoning for this inconsistency may lie in the interacting proteins found at telomeres. There are a variety of proteins found at telomeres that are not found elsewhere in the genome. For example, TRF2 (telomere binding factors 2) is found exclusively at telomeres (Williams et al., 2007) and evidence suggests that TRF1 and 2 are needed for efficient movement of a replication fork through telomeres (Miller et al., 2006).

Another possibility for DNA-PKcs differential regulation of G versus T-SCE may lie in the telomere itself. The telomere is comprised of tandem arrays of the TTAGGG repeat, in the 5' to 3' direction, forming the lagging strand telomere. The leading strand telomere consists of the complementary sequence, AATCCC, in a 3' to 5' direction. The lagging strand also forms a long single-stranded 3' overhang, giving the telomere a very unique "strand specific" structure, in which the two strands may actually behave differently, at least at or shortly after replication.

Secondary structures or lesions may be differentially regulated depending on whether they originate in the leading or lagging strands. For example, if a single strand break occurs in the lagging strand, a replication fork may transform this nick into a free double strand end. Due to the polarity of the lagging strand (5'- to - 3'), RAD51 strand invasion is possible. Then with

the assistance of interacting proteins, such as the WRN helicase, the HJ that results from the strand invasion can be resolved in a non-crossover manner leading to a suppression of SCE.

A single strand break on the leading strand would by necessity have to be handled differently. The double strand end created by the replication fork would, due to its polarity (3' to 5'); not be capable of strand invasion. Therefore an as yet unknown mechanism to resolve this situation must be present. In fact, there are some lines of evidence to suggest this. It has been demonstrated that Ku and DNA-PKcs, when combined, inhibit the WRN helicase (Karmakar et al., 2002).

The strand specific nature of the initiating lesion that stalls a replication fork may explain our observations. We have previously shown that WRN specifically suppresses T-SCE (manuscript in preparation). It has also been shown that loss of WRN can lead to spontaneous loss of the leading strand telomere (Crabbe et al., 2004). However, it is important to note that that in this article the number of sister telomere loss events reported in this paper was so high that the cultures would have senesced before the experiment could be set up; bringing the validity of this report into question. Together with our data demonstrating an increase in T-SCE a new model is emerging. In the absence of WRN, lesions that occur on the lagging strand, result in strand invasion and resolve them in a non-specific manner leading to crossover, and ultimately SCE. Lesions that occur on the leading strand are not able to strand invade, so DNA-PKcs is needed to inhibit WRN (Karmakar et al., 2002). Without this inhibition, inappropriate resolution of the stalled fork occurs, leading to an increase in T-SCE and perhaps also a DSB in the leading strand telomere. This fits well with previously reported data, that DNA-PKcs is needed to prevent inappropriate telomere fusions involving the leading strand (Zhang et al.,

2007c). This may help explain how DNA-PKcs, a DSB repair enzyme, prevents telomeric uncapping and subsequent fusions.

The Nucleotide Excision Repair Protein, Ercc1-XPF, is not involved in G-SCE but may be in T-SCE.

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Keywords: Ercc1, NER, SCE

Running Title: Ercc1's influence on SCE

Abstract

Ercc1-XPF is an attractive candidate for playing a role in regulating T-SCE (telomeric-sister chromatid exchange) frequencies, as an ERCC1-XPF patient has recently been identified, and this individual displayed premature aging. Nucleotide excision repair (NER) is a DNA repair pathway critical for repair of base lesions that occur during normal DNA metabolism and a variety of exogenous agents, including UV-light from the sun. Members of this pathway have been implicated in disease. Xeroderma Pigmentosum (XP) is a disease classified by extreme sensitivity to the sun, and is resultant from mutations in one or more of the members of the NER pathway (de Boer and Hoeijmakers, 2000). The protein complex, Ercc1-XPF, is a critical member of the NER pathway acting as the nuclease that cleaves on the 5' side of the lesion. Recently, Ercc1-XPF has also been demonstrated to display a progeroid type syndrome in humans (Jaspers et al., 2007). Here, we analyze Ercc1-XPF's role in regulating SCE frequency. In previous work, we have demonstrated that other progeroid syndromes, such as those deficient in WRN, BLM, and FANCD2, have altered SCE frequencies. SCE's that occur in the telomeres, T-SCE, may be a symptom of telomere dysfunction that can lead to premature senescence or perhaps aging. Our results demonstrate that Ercc1-XPF does not play a role in G-SCE; however, it may have a role in influencing T-SCE frequency.

Introduction

The nucleotide excision repair (NER) pathway is one of several DNA repair mechanisms that exist in order to repair lesions that occur normally during DNA metabolism. NER is specifically good at repairing base pair lesions and interstrand crosslinks (Dronkert and Kanaar, 2001; Legerski and Richie, 2002; McHugh et al., 2001; Park and Sancar, 1994). The NER pathway seems to specifically be involved in repair of interstrand crosslinks (ICLs) generated by UV exposure from the sun. In fact, many members of this pathway display extreme sensitivity to UV and are characterized by the condition Xeroderma Pigmentosum (XP) (de Boer and Hoeijmakers, 2000; Lehmann, 2003). XP patients tend to develop basaliomas and other skin malignancies at early ages.

NER is similar to the base excision repair (BER) pathway in its approach to repair. In NER, lesions are first detected by the XPC-HR23B complex or by RNA Polymerase II during replication (McHugh et al., 2001). The lesion is then marked by the TFIIH complex so further processing can occur (Lehmann, 2003). A complex consisting of replication protein A (RPA), XPA, and XPG comprise the first incision complex, combines with the opening complex of XPB and XPD. Together they are able to make an incision on the 3' end of the lesion. Ercc1-XPF can then make an incision on the 5' end of the lesion, thereby removing the lesion (Laczmanska et al., 2006). After removal, polymerases refill the removed sequences, and ligation occurs.

Recently, ERCC1 was found mutated in a human patient (Jaspers et al., 2007). This was a surprising find, as ERCC1 was believed to be so essential that humans could not live without it. Perhaps even more surprising was that the ERCC1 patient displayed a progeroid, or accelerated

aging, phenotype that was similar to Werner Syndrome (WS) and Bloom Syndrome (BS) patients (Jaspers et al., 2007; Niedernhofer et al., 2006).

In earlier reports, we analyzed the role of WRN (Werner protein) and BLM (Bloom protein) in regulating SCE frequencies. Using mouse cell lines, human patient lines and siRNA knockdown strategies, we found that WRN specifically suppresses SCE in telomeres (T-SCE) while BLM was found to suppress both T-SCE and SCE's that occur within the genome (G-SCE). The fact that both WS and BS are progeroid syndromes, we hypothesized that the accelerated aging phenotype seen in these patients may be due to the apparent telomere dysfunction characterized by elevated T-SCE levels. Similarly, we demonstrated that the homologous recombination (HR) protein, FANCD2, also was involved in suppressing both G-SCE and T-SCE. Fanconi Anemia (FA) patients also display a progeroid type syndrome (Neveling et al., 2007). Recently reports have further suggested a possible link between FA and Ercc1 (Niedernhofer, 2007).

To further examine this hypothesis, we analyzed Ercc1's role in regulating SCE frequencies utilizing an Ercc1 knockout mouse embryonic fibroblast (MEFs). While most of the embryos did not display any change in G-SCE frequency, one Ercc1-/- did show a slight elevation in T-SCE levels. The fact that this phenotype was not very pronounced may be due to the telomerase status of the mouse. In our earlier reports, the WRN mouse had to be null for both WRN and telomerase before the T-SCE phenotype could be seen. This may further strengthen the case that the accelerated aging phenotypes contain dysfunctional telomeres that can be visualized by T-SCE.

Materials and Methods

Cell lines. MEFs were graciously donated by Laura Niedernhofer, and were generated from four littermates; two were genotyped as Ercc1-/- while the other two were Ercc1+/- controls.

Cell Culture. Low passage MEFs were grown in αMEM (Hyclone) supplemented with 15% fetal bovine serum (FBS) and antibiotics. Cells were seeded into T-75 flasks and placed in 37°C incubators with 5% CO_2. WTK1 cells were grown in RPMI 1640 (Hyclone) media supplemented with 15% FBS and antibiotics.

G-SCE analysis. Slides were prepared using standard cytogenetic techniques and then stained via the Fluorescence Plus Giemsa technique, producing differential staining necessary for SCE detection (Latt, 1973; Perry and Wolff, 1974). Briefly, slides were stained with Hoescht 33258 for 15 minutes at room temperature, rinsed with distilled water and exposed to UV light (365nm) for 25 minutes. Slides were then soaked in 2x SSC at 60°C for 30 minutes. Following thorough rinsing with distilled water, slides were allowed to air dry, then stained with 2% Giemsa for 10 mintues. Images were analyzed and captured using a Zeiss Axioskop2 Plus microscope equipped with a Photometrics Coolsnap ES2 camera and Metavue 7.1 software.

Chromosome Orientation- Fluorescence In Situ Hybridization (CO-FISH). CO-FISH analysis was done according to Bailey et al (Bailey et al., 1996). Briefly, cells were grown for one round of replication in the presence of 1×10^{-5} M 5'-bromo-2'-deoxyuridine (BrdU). Cells were harvested and slides prepared. Slides were stained with Hoescht 33258 (0.5 μg Hoescht 33258/ 2x SSC), and exposed to 365 nm UV in a Stratalinker 3000. Exonuclease III (Promega) buffered solution was used to degrade strands containing BrdU. A hybridization solution containing 0.2 μg/ml of telomere PNA probe (Applied Biosystems), 70% formamide (Fischer Scientific),

12mM Tris-HCl, 5mM KCl, and 1mM $MgCl_2$ was placed on the slides for approximately 2 hours. Slides were washed with 2x SSC and PN buffer and counterstained with Vectashield antifade containing DAPI. Slides were scored using a Zeiss Fluorescence microscope (Axioplaln 2ie MOT). For CO-FISH, the DAPI and Cy3 filters were used to visualize the chromosomes and telomere probes. Images were capture using a CCD camera (model CV-M4+CL, JAI PULNiX Inc., San Jose, CA, USA) and a Dell precision 360 workstation running Isis FISH imaging software (Metasystems, Altussheim, Germany).

T-SCE analysis. T-SCEs were scored following CO-FISH with a telomere probe, which produces distinct and characteristic single-sided signal on each end of the chromosome, therefore any split in the signal was scored as 1 T-SCE. Results were categorized as # of T-SCE/chromosome. Error bars were calculated as standard error of the mean (SEM). Significance was calculated using a T-test analysis using software freely available online by GraphPad Quick Calcs T Test.

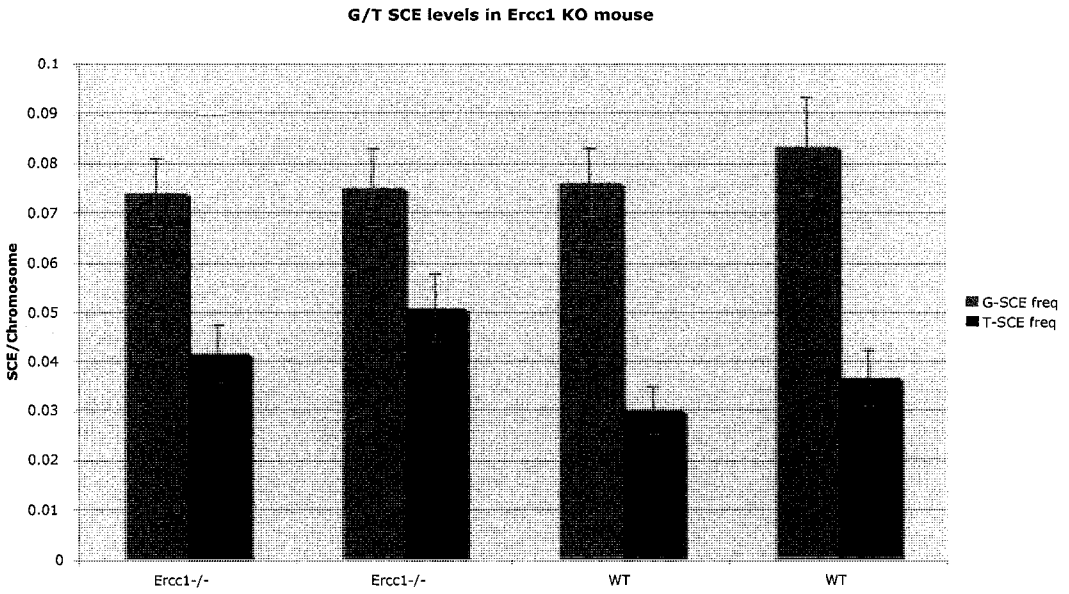

Figure 1. Ercc1-/- mice display normal levels of G-SCE compared to WT controls. One Ercc1-/- mouse showed a significant rise in T-SCE levels compared to one of the controls suggesting a possible role in T-SCE suppression.

Results

The four MEFs littermate were analyzed for G-SCE using fluorescence plus Giemsa (FPG) staining. The two control cell lines (Ercc1+/-) displayed frequencies of 0.076 and 0.083 G-SCE/chromosome (Figure 1). The Ercc1-/- MEFs displayed G-SCE frequencies of 0.072 and 0.074 G-SCE/chromosome (Figure 1). These data suggest that Ercc1 does not play a role in regulating SCE frequencies that occur within the genome.

The same MEFs were also analyzed for T-SCE frequency using CO-FISH. The two control MEFs displayed levels of 0.030 and 0.037 T-SCE/ chromosome (Figure 1). The two Ercc1-/- MEFs displayed levels of 0.041 and 0.051 T-SCE/ chromosome (Figure 1). The 0.051 T-SCE/ chromosome represented a significant elevation in T-SCE compared to one control. This suggests the possibility that Ercc1 plays a role in regulating T-SCE frequencies.

Discussion

The progeroid syndromes are useful genetic tools in that although they are not exactly the same as normal aging, they do provide useful insights into how an organism ages. What is astonishing is the interplay between different repair pathways and similar progeroid phenotypes. This suggests that there must be some underlying factor that links these different pathways. It is reasonable to assume that telomere maintenance may be the common link. Telomeres have long been tied to aging, and this is supported by the fact that short telomeres can trigger cellular senescence. Telomeres are also implicated in cancer, as cancerous cells must come up with a way to keep telomeres at normal lengths in order to achieve immortalization. Most accomplish this by reactivating telomerase, the enzyme that can add telomeric sequence de novo (Blackburn et al., 1989; Greider and Blackburn, 1987). A small percentage of tumors utilize alternative lengthening of telomeres (ALT), a recombination based mechanism to maintain telomere lengths (Bryan et al., 1995; Murnane et al., 1994).

Interestingly, ALT cells display an elevated T-SCE phenotype. However, it is important to remember that while ALT displays elevated T-SCE levels, the mere presence of T-SCE does not imply that ALT is activated. In fact, during ALT the formation of PML bodies is seen. ALT-associated PML bodies (APB's) are donut shaped structures that specifically associate with telomeres (Lamond and Earnshaw, 1998; Yeager et al., 1999). They comprise of several proteins such as the PML (promyelocytic) protein, RAD51, RAD52, RPA, the telomeric proteins TRF1/2 in addition to telomeric DNA. APB's have become a standard method for testing for ALT in tumor cell lines; what their function in maintaining replicative capacity could be is still unclear.

So the question arises, that if ALT appears to use T-SCE in order to lengthen telomeres, then why do the progeroid syndromes seem to have elevated T-SCE? The answer may lie in the fact that there is more to ALT than T-SCE. The presence of PML bodies in ALT suggests a higher order of complexity than just unequal T-SCE. An interesting aspect of ALT is how can it achieve its ultimate goal of unending replicative potential? It is easy to envision that if unequal exchange between two telomeres occurs after replication, one telomere can become substantially longer while the other shorter (Bailey et al., 2004a). The longer telomere would then be able to divide for a longer amount of time, while the short one would likely senesce. The issue arises in terms of probability. Even if ALT is able to lengthen the telomere, what would be the odds that the two dividing cells would receive either all of the long telomeres or all of the short telomeres? With 46 chromosomes in the human, it would seem highly unlikely that one daughter cell would receive all the long telomeres therefore extending its lifespan.

Therefore, ALT must have a mechanism to deal with this in order to be successful. Perhaps, the presence of the PML bodies helps regulate which daughter cell receives the long telomeres and which daughter receives the short telomeres. This may then be why in the absence of ALT, T-SCE lead to accelerated aging. Without this coordinated effort to make sure that one daughter receives all of the long telomeres, both daughters end up with a mix. It has been demonstrated that only one or a few short telomeres are enough to trigger senescence (Ref). So in the absence of ALT, T-SCE may lead to a situation where both daughters are likely to senesce much faster. It has also been suggested that APB's may be staging areas that assist with the shorter telomeres (Yeager et al., 1999).

However, at this point, much of this is still speculative. The T-SCE phenotype we see in the Ercc1-/- mice is not very dramatic, and occurred only in one mouse, although there may be

an explanation for this. Given that mice have active telomerase, the T-SCE phenotype may be masked. In the WRN mouse (Laud et al., 2005), it was only in the absence of telomerase that the full WRN/T-SCE phenotype become obvious. The same was also true with the WS and BS patient lymphoblasts. Therefore further experiments in normal human cells that do not have telomerase need to be done to truly access Ercc1's role in T-SCE.

DNA-PKcs and ATM influence generation of ionizing radiation-induced bystander signals

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Keywords: DNA-PKcs, ATM, Ionizing Radiation, Bystander Effect

Running Title: DNA-PKcs and ATM influence bystander signaling

Abstract. The phenomenon by which irradiated cells influence non-irradiated neighboring cells, referred to as the bystander effect (BSE), is not well understood in terms of the underlying pathways involved. We sought to enlighten connections between DNA damage repair and the BSE. Utilizing sister chromatid exchange (SCE) frequencies as a marker of the BSE, we designed cell transfer strategies that enabled us to distinguish between generation versus reception of a bystander signal. We found that DNA-dependent Protein Kinase catalytic subunit (DNA-PKcs) and Ataxia Telangectasia Mutated (ATM) are necessary for the generation of such a bystander signal in normal human cells following gamma (γ)-ray exposure, but are not required for its receipt. Importantly, we also show that irradiated human cells are refractory to receipt of a bystander signal, helping to explain why the BSE is a low dose phenomenon. These studies provide the first evidence for a role of the DNA damage response proteins DNA-PKcs and ATM specifically in the generation of a bystander signal and inter-cellular signaling.

Introduction

Current research supports the concept that the effects of low-dose radiation are considerably more complex than one might imagine based on linear no-threshold extrapolations from the high-dose radiation received by Japanese atomic-bomb survivors. The observation of a low dose IR-induced bystander effect (BSE), i.e., irradiated cells signaling their distress to non-irradiated neighbors and inducing an effect, is a case in point.

The BSE occurs when a directly irradiated cell generates and transmits a signal, such as reactive oxygen species (ROS) (Kashino et al., 2007a), nitric oxide species (NOS) (Shao et al., 2006), or cytokines (Banaz-Yasar et al., 2007); either through gap junctions (Azzam et al., 1998; Azzam et al., 2001) or through media (Lehnert et al., 1997; Mothersill and Seymour, 1998), to a neighboring non-irradiated cell that exhibits an effect. Given that the multiple markers that have been used to study the BSE, i.e., micronuclei formation (Yang et al., 2007), clonogenic survival (O'Neill-Mehlenbacher et al., 2007), apoptosis (Grifalconi et al., 2007), and sister chromatid exchange (SCE) (Nagasawa et al., 2005) (Figure 1), are themselves considered to be detrimental; it has been assumed that the BSE is harmful to neighboring cells. However, it has also been proposed that the BSE may actually be beneficial at a tissue level; cells exposed to a bystander signal are more radioresistant to subsequent IR induced damage indicative of an adaptive response (Iyer and Lehnert, 2002). It is also worthy note that of these markers, only SCE frequency is not significantly influenced by direct low LET (e.g., gamma-rays) radiation exposure (Ardito et al., 1980), making SCE an ideal marker of the BSE.

Given numerous studies demonstrating the importance of DNA repair proteins in directly irradiated cells, we sought to examine what, if any, role they might play in the BSE. We focused on the repair proteins DNA-PKcs (DNA-dependent Protein Kinase catalytic subunit) and ATM

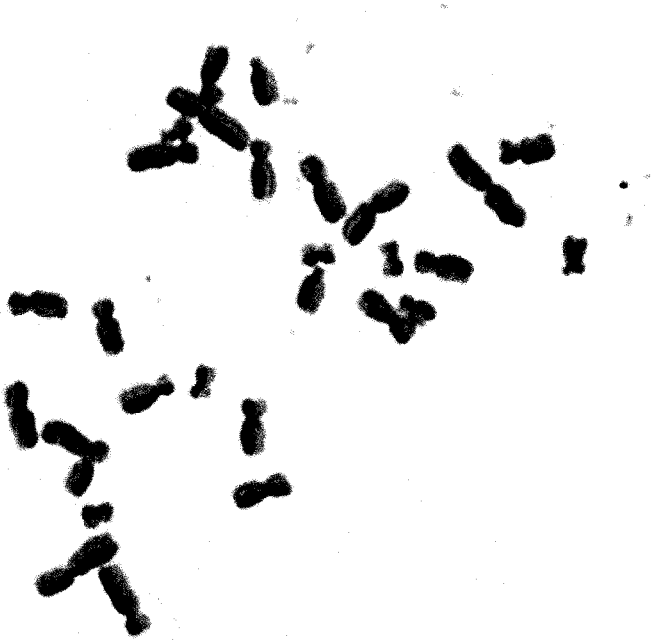


Figure 1. Sister Chromatid Exchange (SCE). Human fibroblast (5C HDF) metaphase chromosome spread illustrating FPG harlequin staining (100x). A "color switch" (arrows) indicates an SCE has occurred.

(Ataxia Telangectasia Mutated). DNA-PK is a primary component of the Non-Homologous End-Joining (NHEJ) DSB repair pathway and consists of the Ku 70/80 hetero-dimer and the catalytic subunit DNA-PKcs (Collis et al., 2005). DNA-PKcs is critical for DSB repair and for V(D)J recombination (Jackson and Jeggo, 1995). It has also been shown that DNA-PKcs is important for the protection of mammalian telomeres by helping to maintain effective end-capping and preventing inappropriate fusions (Bailey et al., 2004b; Bailey et al., 2001; Bailey et al., 1999). Like DNA-PKcs, ATM is a member of the phosphoinositide 3-kinase-like kinase (PIKK) family. ATM plays a role in the early detection of IR-induced DSBs (Barzilai et al.,

2002) and is responsible for phosphorylation of a numerous proteins involved in cell cycle control, apoptosis and DNA repair (Lavin and Kozlov, 2007).

Previous studies investigating how DNA repair status influences the BSE include several by Nagasawa et al., who demonstrated that cells deficient in DNA repair proteins tend to exhibit large bystander responses following alpha-particle irradiation (Nagasawa et al., 2005). These authors speculated that cells experiencing defective repair of DNA damage induced by direct irradiation, display an increased bystander response likely due to increased production of ROS (Nagasawa and Little, 2002). These early experiments were not capable of determining whether the role of these proteins was in the generation or in the reception of the bystander signal. Later, media transfer experiments revisited the role of several DNA repair proteins in generation of the bystander signal; here it was concluded that these proteins played no role in the BSE (Mothersill et al., 2004). However, media transfer experiments inherently limit the role of ROS, which many believe to be a crucial contributor to the BSE.

In the current study, we designed cell transfer strategies to assess the role of DNA-PKcs and ATM in the generation and/or reception of the IR-induced BSE following γ-ray exposure. Cells were divided into two groups, donors (irradiated) and recipients (non-irradiated). The donor cells were either un-irradiated (control) or exposed to 1 Gy of ^{137}Cesium γ-rays (treated), rinsed and then co-cultured with the recipient cells at a dilution of either 1:100 or 1:1000. Cells were harvested after two cell cycles in the presence of bromodeoxyuridine (BrdU) in order to facilitate visualization and analyses of SCE frequencies in unirradiated recipient cells as a marker of the BSE. Utilizing both mouse and human cell lines deficient in either DNA-PKcs or ATM and normal human fibroblasts, and by altering which was the donor, we assessed how DNA-PKcs and ATM influence the generation and/or reception of bystander signals.

Materials and Methods

Cell Lines and Cell Culture. Kidney tissue from 8–12 week old female C57 BL/6, BALB/c, or congenic mice were minced, and digested in 199 medium containing collagenase (Worthington Type III; 200 units/ml) at 37°C for 3–5 h with gentle agitation. Disaggregated cells were washed 6x in phosphate buffered saline (PBS) containing 0.5% fetal bovine serum (FBS) and cultured in α-MEM medium (15% FBS, penicillin/streptomycin). Media was changed after 3 days of incubation. Low passage neonatal Human Dermal Fibroblasts (HDF C-004-5C; Cascade Biologics) were grown in α-MEM supplemented with 10% fetal bovine serum and antibiotics. Cells were counted using a Coulter Counter (Coulter Beckman, Fullerton, CA) and 4 X 10^5 human fibroblasts were plated into T-75 flasks, then co-cultured with 1:100 dilutions of either 0 Gy or 1 Gy γ-irradiated, exponentially growing donor cells. Donor cells were not allowed to near confluency and included human ATM-/- (AG04450), DNA-PKcs deficient (BALB/c mouse), wild-type DNA-PKcs (C57BL/6 mouse), or congenic DNA-PKcs (manuscript in preparation). Irradiations were performed using a sealed-source Mark I ^{137}Cs γ-irradiator (J.L. Shepherd and Associates). 5'-bromo-2'-deoxyuridine (BrdU; Sigma) was added to cultures at a final concentration of 2 X 10^{-5}M and cells were allowed to grow for two rounds of cell division. Colcemid (Invitrogen) was added at a final concentration of 0.2 μg/ml and cells were harvested approximately 2-3 hours later. Cells were trypsinized, centrifuged, then resuspended in 0.075M KCl for 15 minutes at room temperature and then fixed with 3:1 methanol: acetic acid.

C.B6-Prkdc and B6.C-Prkdc^{BALB} congenic mouse strains. Two strains congenic for the common allele (C57BL/6) and BALB/c variant allele of Prkdc were generated (manuscript in

preparation) using the parental strains C57BL/6J (B6) and BALB/cByJ (C) (both obtained from Jackson Laboratory). For the congenic strain **C.B6-Prkdc**, B6CByF1 females were mated with C.B6 males to produce the N2 generation. Subsequent generations N2 – N10 were repeatedly backcrossed to BALB/cByJ mice. For congenic strain **B6.C-Prkdc^{BALB}**, CByB6F1 females were mated with B6 males to produce the N2 generation. Subsequent generations N2 – N10 were repeatedly backcrossed to C57BL/6J mice. In both congenic strains, progeny were selected for backcross mating if they carried donor Prkdc sequence as determined by PCR/RFPL (Yu et al., 2001). Additionally, a marker-directed breeding strategy (speed congenics) was adopted at backcross generations N8 – N12 which selected against progeny carrying background donor genome (Weil et al., 1997). Microsatellite markers polymorphic between B6.C and C.B6 were used to select backcross progeny whose genome contained the least donor sequence at loci other than Prkdc. Mice at backcross N10 or later were intercrossed and progeny homozygous for the donor Prkdc allele were selected for inbreeding. Mouse colonies were maintained at the Colorado State University Painter Center.

SCE Staining and Analysis. Slides of metaphase chromosomes were prepared using standard cytogenetic techniques, then stained using the Fluorescence Plus Giemsa technique (Wolff and Perry, 1975) in order to obtain harlequin staining and to visualize SCE. Briefly, slides were stained with Hoescht 33258 (Thermo Sci Acros Organics) for 15 minutes at room temperature, rinsed with distilled water and exposed to UV light (365nm; Stratalinker) for 25 minutes. Slides are then soaked in 2x SSC at 60°C for 30 minutes. Following thorough rinses with distilled water, slides are allowed to air dry, and then stained with 2% Giemsa for 10 minutes. Images

were analyzed and captured using a Zeiss Axioskop2 Plus microscope equipped with a Photometrics Coolsnap ES2 camera and Metavue 7.1 software.

Statistical Analysis. Slides were blinded and scored by independent investigators for SCE. Standard deviations were calculated and used to determine the standard error of the mean (SEM) to generate error bars. A student's T-test was calculated to determine statistical significance. All conditions were repeated at least twice, and each experiment was scored by at least two individuals. If results were not significantly different, data was pooled.

Results

SCE frequencies in primary normal human dermal fibroblasts (5C HDF), both with and without exposure to 1Gy of direct γ-irradiation (^{137}Cs), were determined. It has been reported previously that direct low LET IR exposure does not enhance SCE frequency (Ardito et al., 1980), which we confirmed here; 5C HDF's did not display elevated SCE frequencies subsequent to direct γ-irradiation as compared to the 0 Gy controls. SCE frequencies were 3.76 SCE/ metaphase (0 Gy) and 3.4 SCE/ metaphase (1 Gy) (Figure 2A), with no statistically significant difference between sample means.

We designed a cell transfer approach that utilizes SCE frequencies as a marker of the IR-induced BSE, and importantly facilitates discrimination between generation versus receipt of bystander signals. A small number of irradiated cells (donors) were added to a non-irradiated cell population (recipients). Immediately following IR exposure (1 Gy ^{137}Cs γ-rays), human fibroblast (5C HDF) donor cells were pelleted and rinsed in PBS to remove any remaining media. Donor cells were then diluted either 1:100 or 1:1000 and added to non-irradiated recipient cells (5C HDF). The co-culture was collected following two rounds of replication in the presence of BrdU and scored for SCE (vast majority were non-irradiated recipient cells). Our results revealed a significant elevation in SCE frequency in the samples whose donor cells were irradiated compared to the control samples whose donors were not irradiated (Figure 2A). The 1:100 dilutions displayed a frequency of 3.14 SCE/ metaphase (0 Gy) and 5.28 SCE/ metaphase (1 Gy). The 1:1000 dilutions displayed a frequency of 3.68 SCE/ metaphase (0 Gy) and 5.48 SCE/ metaphase (1 Gy). Distribution graphs (Figure 2B) illustrate that this is an overall increase, rather than a few cells with many SCE skewing the data. It is also interesting to note that there was a similar increase in SCE frequency for both the 1:100 and 1:1000 dilutions,

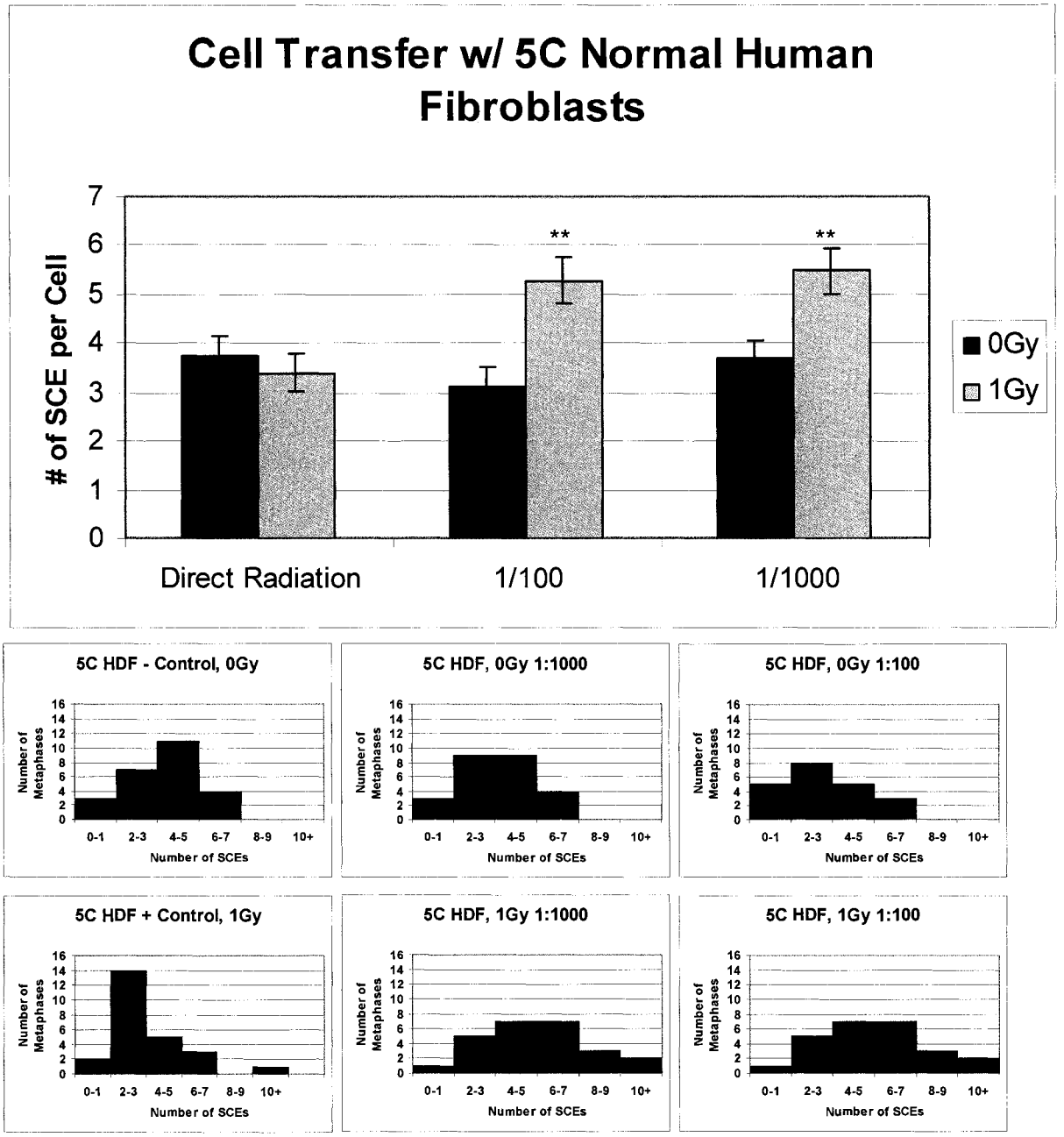
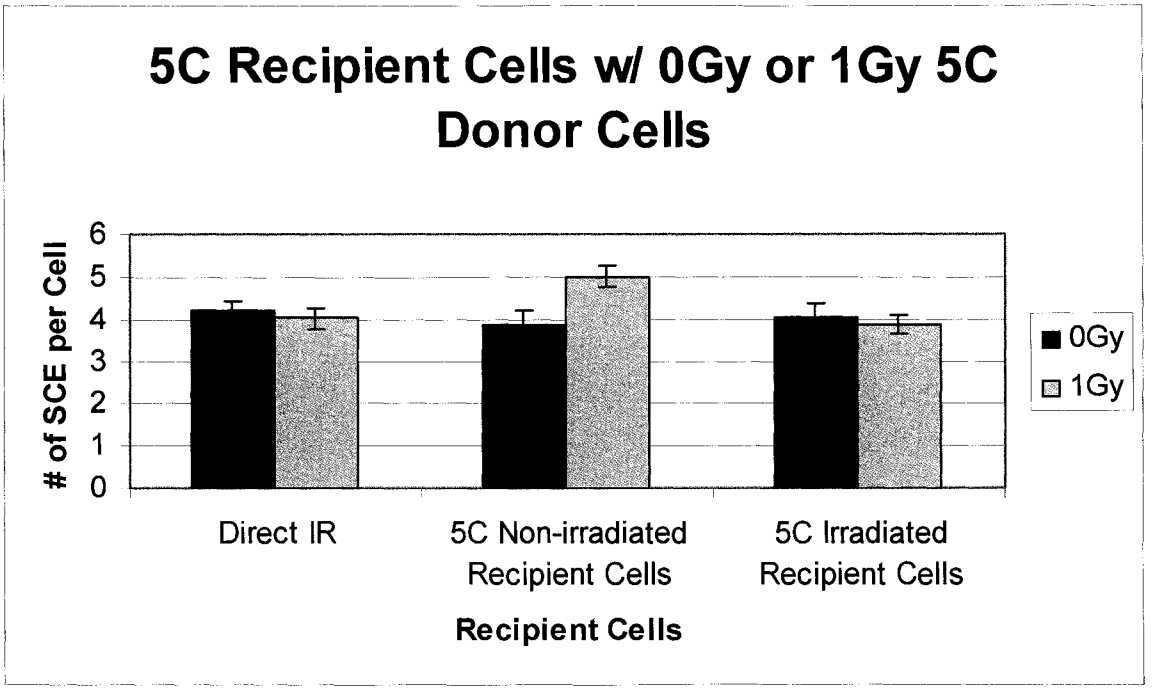


Figure 2A. Direct Irradiation versus BSE. Following □-ray direct irradiation (no cell transfer), human fibroblasts (5C HDF) show no significant increase in SCE frequency. Using 5C HDFs as both donor (1 Gy), and recipient (0 Gy) bystander cells at 1:100 and 1:1000 dilutions, significant, and similar, increases in SCE levels were observed; p-values of 0.0059 and 0.0018. **B.** The distributions of SCE number per metaphase illustrate an overall increase in SCE levels. * ≥ 95% confidence, ** ≥ 99% confidence.

which is consistent with previous reports that the BSE appears to operate by an "on/off" mechanism (Deshpande et al., 1996; Hu et al., 2006; Nagasawa and Little, 1992). Therefore, only the 1:100 dilution cell transfer method was utilized in subsequent experiments.

We confirmed that direct IR (γ-ray) exposure did not elevate SCE frequencies in our system. These results imply that directly irradiated cells are refractory to the bystander signal, i.e., there is no increase in SCE. To test this hypothesis, we repeated the experiments outlined above, with addition of irradiated recipient cells (5C HDF) to the protocol (Figure 3). As expected, SCE frequencies did not increase in directly irradiated cells; 4.2 SCE/ metaphase (0 Gy) and 4.03 SCE/ metaphase (1 Gy) (Figure 3A). Also as expected, an increase in SCE frequency was observed when irradiated donor cells were added to non-irradiated recipient cells; 3.9 SCE/ metaphase (0 Gy) and 5.03 SCE/ metaphase (1 Gy) (Figure 3A.) However, there was no significant increase in SCE when irradiated donor cells were added to irradiated recipient cells; 4.08 SCE/ metaphase (0 Gy) and 3.88 SCE/ metaphase (1 Gy). This result supports the hypothesis that directly irradiated cells are unable to receive a bystander signal once they have activated the mechanism to generate bystander signals.

We recognized the unavoidable reality that some, although very few, directly irradiated cells were scored as bystander cells in our cell transfer approach. Although we repeatedly determined that SCE frequencies do not increase in directly irradiated 5C HDFs (Fig 2A and 3A), we sought to further ensure that only bystander, non-hit cells were scored for SCE. Therefore, mouse cells, whose chromosome morphology is clearly distinguishable from human, were used as the irradiated donor cells and 5C HDFs were used as the non-irradiated recipient cells. Wild-type C57BL/6 mouse donor cells were irradiated and added to and cultured with non-irradiated 5C



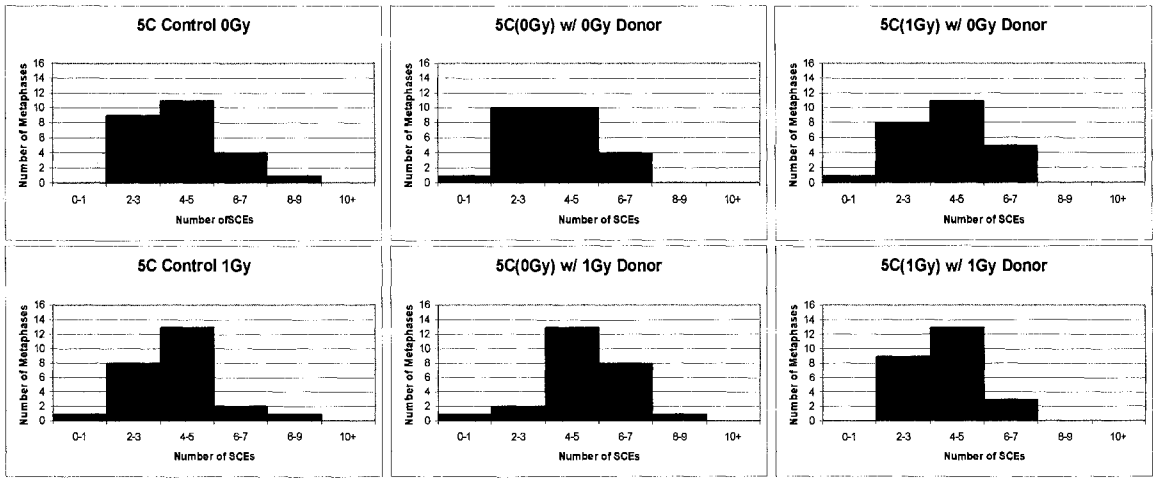
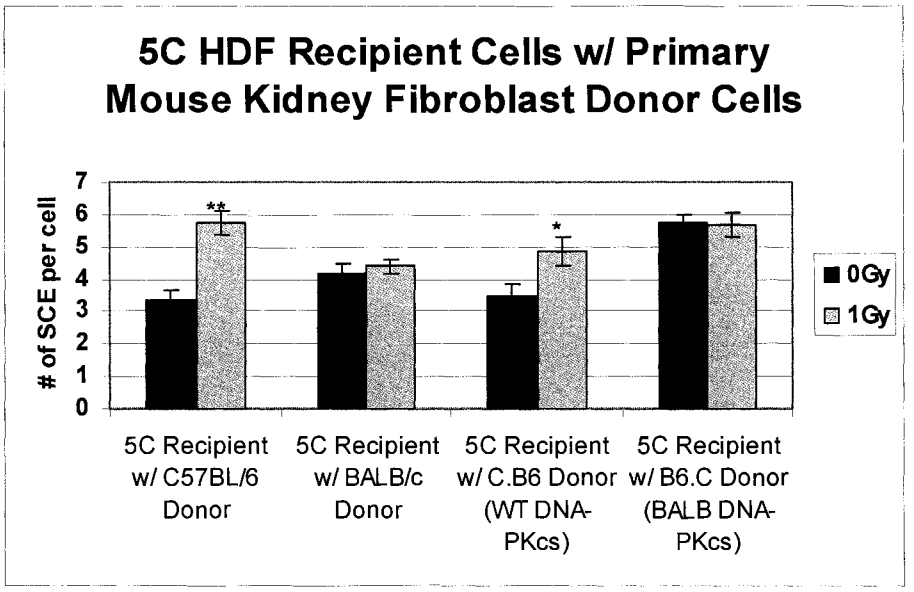


Figure 3A. Irradiated cells are refractory to bystander signals. Direct irradiation does not increase SCE levels; 4.2 SCE/cell (0 Gy), 4.03 SCE/cell (1 Gy). Irradiated donor cells (5C HDF) induce an increase in SCE frequency in non-irradiated recipient cells (5C HDF); 3.9 SCE/cell (0 Gy), 5.03 SCE/cell (1 Gy). When recipient cells were irradiated (1 Gy), they were no longer able to respond to the bystander signal; 4.08 SCE/cell (0 Gy), 3.88 SCE/cell (1 Gy). **B.** Distributions of SCE number per metaphase illustrate the average frequency.

HDF recipient cells. A significant increase in SCE frequency was observed in the 5C HDF recipients; 3.32 SCE/ metaphase (0 Gy) and 5.72 SCE/ metaphase (1Gy) ($p < 0.05$) (Figure 4A). The distribution of SCE again showed a general increase in SCE numbers (Figure 4B).

To examine the role of DNA-PKcs in the generation and/or reception of bystander signals in our system, we utilized BALB/c primary mouse kidney fibroblasts, which contain a hypomorphic variant of DNA-PKcs that results in reduced expression and kinase activity (Okayasu et al., 2000). Irradiated BALB/c donor cells were added to non-irradiated 5C HDF recipient cells. No significant increase in SCE frequency was observed in the 5C HDF recipient cells; 4.19 SCE/ metaphase (0Gy) and 4.39 SCE/ metaphase (1Gy) (Figure 4A), suggesting that DNA-PKcs is required for generation of the bystander signal. Most experiments were repeated at least twice and each experiment was scored by at least two independent blinded individuals. While background numbers varied slightly (common with SCE evaluation), all trends were consistent. Reverse experiments were also preformed so that irradiated or non-irradiated 5C HDF's were added to non-irradiated mouse cells. Both the recipient C57BL/6 and BALB/c mouse cells displayed significant increases in SCE frequencies after the addition of irradiated human donor cells (5C HDF). The C57BL/6 mouse cells displayed a SCE frequency of 0.106 SCE/chromosome (0 Gy) and 0.148 SCE/ chromosome (1 Gy) (Figure 4B). The BALB/c mouse cells displayed frequencies of 0.108 SCE/chromosome (0 Gy) and 0.154 SCE/chromosome (1 Gy) (Figure 4B). Note that SCE frequencies for mouse cells must be calculated on a per chromosome basis as they do not have stable karyotypes. Taken together, these results demonstrate that while DNA-PKcs is needed for the generation of bystander signals, it is not necessary for the receipt of such signals.



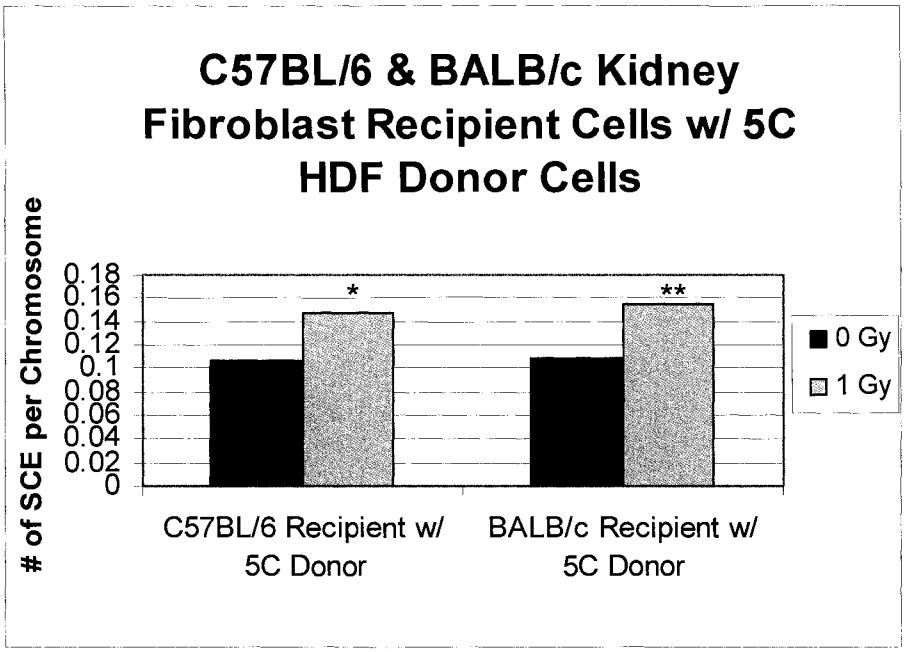
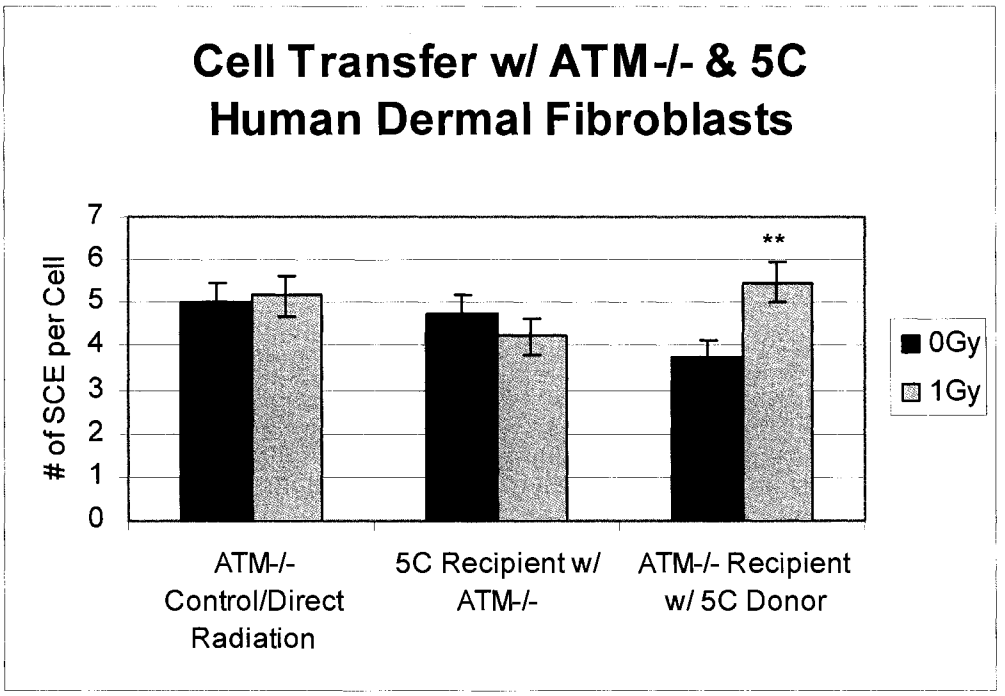


Figure 4A. Role of DNA-PKcs in generation, but not reception of bystander signals. Gamma-ray irradiation of both mouse C57BL/6 and congenic C.B6 (wild type Prkdc) cells produced a significant increase in SCE frequencies in bystander cells (5C HDF); p-values of 0.0004 and 0.011 respectively, while BALB/c and B6.C ($Prkdc^{BALB}$) did not. **B.** Reverse experiments demonstrate that DNA-PKcs is not necessary for the receipt of bystander signals. Both C57BL/6 and BALB/c show significant increase in SCE when irradiated 5C HDF are added; p-values < .05 and .005 respectively.

To further confirm that DNA-PKcs is necessary for generation of bystander signals, additional transfer experiments were performed using unique congenic mice recently created in our laboratory (manuscript in preparation). B6.C-Prkdc^{BALB} mice have a C57BL/6 wild type genetic background with the BALB/c variant allele of the Prkdc gene, while C.B6-Prkdc mice have a BALB/c genetic background with the C57BL/6 wild type Prkdc gene. Consistent with our C57BL/6 results, SCE frequencies were significantly increased when irradiated C.B6 donor cells (wild type Prkdc) were added to the 5C HDF recipient cells; 3.48 SCE/ metaphase (0Gy) and 4.84 SCE/ metaphase (1Gy) (Figure 4A). The distribution plots (Figure 4C) confirmed a general increase in SCE frequencies. SCE frequencies were also evaluated in 5C HDF recipient cells using the B6.C strain (Prkdc^{BALB}) as the irradiated donor cells. Consistent with our BALB/c results, no significant increase in SCE frequency was observed; 5.72 SCE/ metaphase (0 Gy) and 5.68 SCE/ metaphase (1 Gy). However, we do note that both the 0 Gy and 1 Gy B6.C samples were slightly higher than in the C.B6 strain. Why this particular strain has a higher background of SCE is not readily obvious, but SCE frequencies between cell lines often show variability. Our results utilizing these unique congenic mouse strains add additional mechanistic support for DNA-PKcs being involved in generation of bystander signals.

Our focus then turned to ATM, another DNA repair and signaling protein in the same PI3K family as DNA-PKcs. A human dermal fibroblast line (AG04450) derived from an Ataxia Telangectasia patient was used to determine if ATM also plays a role in the bystander response. Similar to the 5C HDF controls, ATM -/- cells did not show an increase in SCE frequency when directly exposed to γ-radiation; 5.0 SCE/ metaphase (0 Gy) and 5.16 SCE/ metaphase (1 Gy) (Figure 5A). There was also no significant increase in SCE frequency when ATM-/- donor cells were irradiated and added to the non-irradiated 5C HDF recipient cells; 4.76 SCE/ metaphase (0



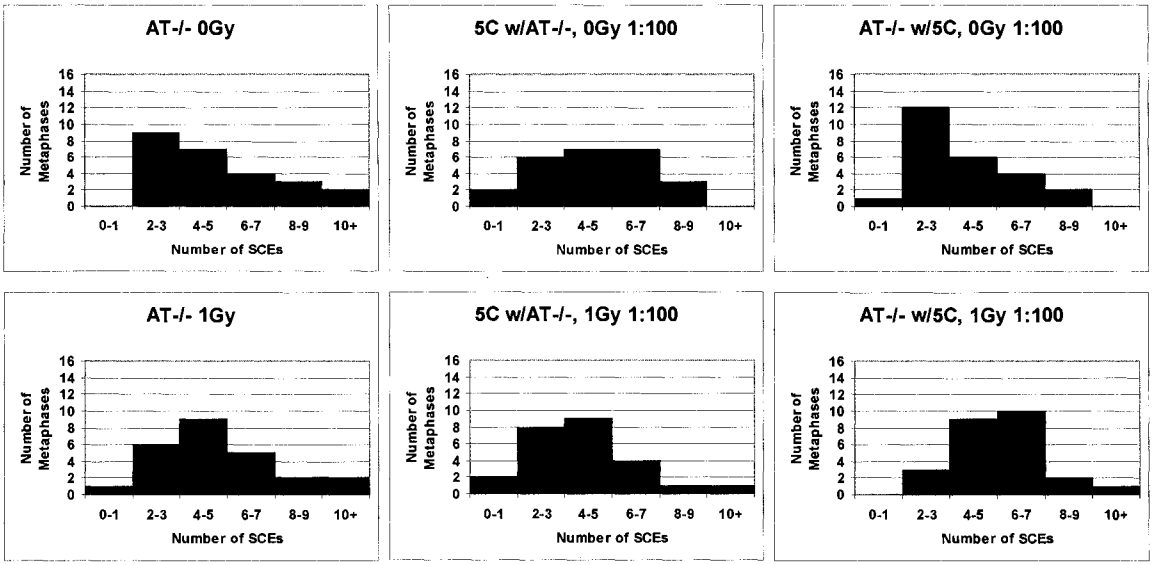


Figure 5A. Role of ATM in generation, but not reception of bystander signals. No increase in SCE frequency was observed following direct irradiation of ATM-/- human fibroblasts. Irradiated donor ATM-/- cells added to non-irradiated 5C HDF recipient cells, produced no significant change in SCE frequency. However, the reverse experiment revealed a significant increase in SCE ($p = 0.0018$) in non-irradiated ATM-/- (recipients) when irradiated 5C HDF donor cells were added. **B.** Distributions of SCE number per metaphase further support these results. (* $\geq$ 95% confidence, ** $\geq$ 99% confidence).

Summary Table

Cell Sample	Mean Frequency/Cell		p-value		Cells Scored	
	0Gy	1Gy			0 Gy	1 Gy
5C HDF Control	3.76	3.4	.532	NS	25	25
1:100	3.14	5.28	.0054	**	21	25
1:1000	3.68	5.48	.0018	**	25	25
Direct IR	4.2	4.03	.641	NS	40	40
Non-IR Recipients	3.9	5.03	.009	*	40	40
IR Recipients	4.08	3.88	.614	NS	25	50
5C HDF with C57BL/6 MPF	3.32	5.72	.0004	**	50	50
5C HDF with BALB/c MPF	4.19	4.39	.4013	NS	47	50
5C HDF with C.B6 MPF	3.48	4.84	.017	*	25	25
5C HDF with B6.C MPF	5.72	5.68	.933	NS	50	50
C57Bl/6 with 5C [a]	0.106	0.148	< 0.05	*	20	10
BALB/c with 5C [a]	0.108	0.154	< 0.005	**	30	20
AT-/- HDF Control	5.0	5.16	.741	NS	25	25
5C HDF with AT-/-HDF	4.76	4.24	.412	NS	25	25
AT-/- HDF with 5C HDF	3.76	5.48	.0018	**	25	25

NS: Non-significant
*: >95% confidence level
**: >99% confidence level
[a]: SCE/chromosome

Table 1. Summary of SCE frequencies and statistical outcomes for all cell transfer experiments. (* ≥ 95% confidence, ** ≥ 99% confidence).

Gy) and 4.24 SCE/ metaphase (1 Gy) (Figure 5A). The reverse experiment, adding irradiated 5C HDF donor cells to ATM-/- non-irradiated recipient cells, revealed a highly significant increase in SCE frequencies; 3.76 SCE/ metaphase (0 Gy) and 5.48 SCE/ metaphase (1 Gy) (Figure 5A). Distribution diagrams (Figure 5B) confirmed a general increase in SCE frequencies. These data suggest that ATM, like DNA-PKcs, is necessary for generation of the bystander signal, but is not required for receiving such signals.

Discussion

DNA-PKcs and ATM are members of the PI3K family and each participates in multiple cellular processes. DNA-PKcs, the catalytic subunit of DNA-PK, orchestrates NHEJ in response to DSBs. It is also critical in V(D)J recombination and is essential for effective mammalian telomeric end-capping function (Bailey et al., 1999; Dudley et al., 2005; Lieber, 1999; Meek et al., 2004; Weinstock and Jasin, 2006; Zhang et al., 2007c). Activation of ATM is an early event in response to IR-induced DSBs, and once activated ATM mediates downstream damage response pathways that include DNA repair, cell cycle control, and apoptosis (Lavin and Kozlov, 2007). ATM is reported to play a role in telomere maintenance as well (Denchi and de Lange, 2007; Pandita, 2002). In addition to DNA-PKcs and ATM's well-established repair and intra-cellular roles (Collis et al., 2005; Lavin and Kozlov, 2007; Nagasawa et al., 2003; Nagasawa et al., 2005), we propose a possible role for these proteins in the BSE and inter-cellular signaling.

We designed a cell transfer strategy that enables us to differentiate between the generation versus the reception of bystander signals. In our system, donor cells are irradiated (1 Gy γ-rays) and seeded at a very low concentration (1:100 or 1:1000) into non-irradiated normal human fibroblast recipient cells. Using a low concentration of donor cells and ensuring that recipient cells were at low confluency, we reduced and/or eliminated a bystander response transmitted via gap junctions. We then measured SCE frequencies in the normal human fibroblast recipient cells as an indicator of IR-induced BSE.

To validate our approach, we tested 5C HDF as both the donor and recipient cells to be assured that they were able to both generate and receive a bystander signal. Human fibroblast cultures were at low-passage (non-transformed) to circumvent any problem of decreased BSE

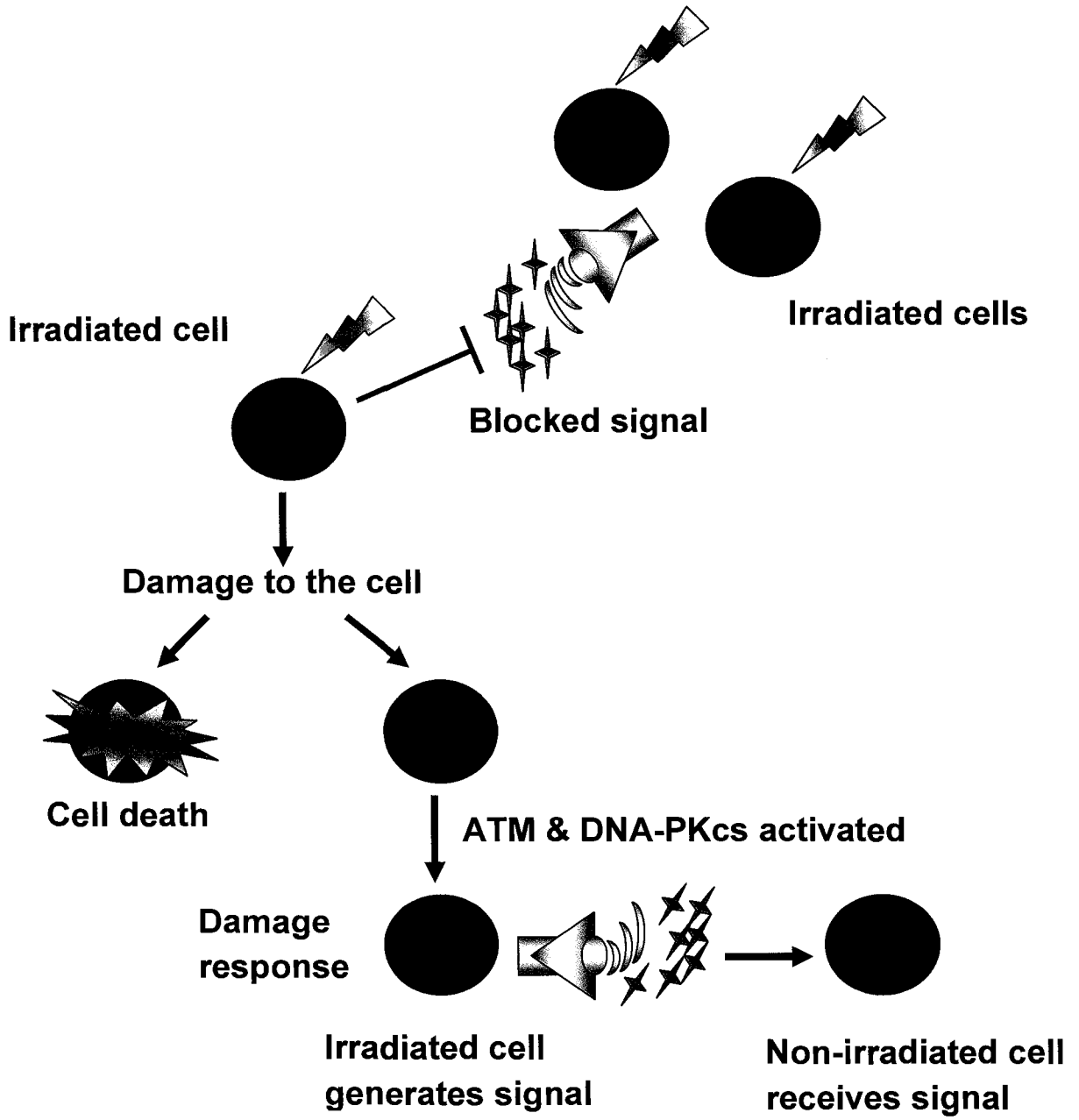


Figure 6. Proposed model for the function of DNA-PKcs and ATM in the generation of radiation-induced bystander signals.

with increasing passage. When directly irradiated, 5C HDFs do not display an increase in SCE frequency, in agreement with previous reports showing that direct low LET IR does not influence SCE levels (Ardito et al., 1980). Our cell transfer strategy also demonstrated that 5C HDFs can generate a bystander signal, inducing significant increases in SCE frequencies in recipient cells. The observation that the irradiated donor cells at both dilutions were able to increase SCE levels in the non-irradiated recipient cells by approximately the same amount is consistent with previous data suggesting that the BSE operates through an "on/off" switch mechanism (Deshpande et al., 1996).

The data demonstrating that directly irradiated cells do not display elevated SCE frequencies suggests that directly irradiated cells are themselves refractory to bystander signals. To test this, we used our cell transfer assay to again show that directly irradiated cells do not show elevated levels of SCE (Figure 3A). Also in agreement with our other results, we show again that by seeding irradiated donor cells with non-irradiated recipient cells, an elevation in SCE frequency in the non-irradiated recipient cells occurs (Figure 2A). However, when the reverse is done and *recipient* cells are irradiated (1 Gy γ-rays) before irradiated donor cells are added, there is no elevation in SCE frequency observed in the recipient population. This supports the hypothesis that once irradiated, "hit" cells become refractory to *receiving* a bystander signal, perhaps providing an explanation as to why the BSE is considered a low dose phenomenon. Only when non-irradiated recipient cells are present, such as with low doses, is a BSE observed.

We sought to determine whether the repair protein DNA-PKcs plays a role in the BSE. The BALB/c mouse strain contains two single nucleotide polymorphisms in the Prkdc gene, which produces a hypomorphic version of DNA-PKcs (Yu et al., 2001). We compared the wild

type C57BL/6 mouse strain to the BALB/c strain for the ability to generate and/or receive bystander signals. Our results show that wild type C57BL/6 mouse cells are able to generate a bystander signal in response to IR. The irradiated C57BL/6 donor cells increased SCE frequency in the 5C HDF recipient cells by over 40% compared to the 0 Gy controls when seeded at a 1:100 dilution (Figure 4A). Similar results were observed when the C57BL/6 donor cells were seeded at 1:1000 (data not shown). However, irradiated BALB/c donor cells were not able to significantly influence SCE frequencies in the 5C HDF recipient cells, demonstrating that DNA-PKcs deficient BALB/c mouse cells are unable to generate a bystander signal following γ-ray exposure. The reverse experiments revealed that DNA-PKcs is not necessary for receipt of bystander signals (Figure 4B). We conclude that DNA-PKcs is necessary for the generation, but not the reception of bystander signals.

To confirm that DNA-PKcs deficiency, rather than a coincidental mutation in BALB/c mice, is responsible for abolishing the bystander response, we utilized congenic mouse strains generated in our laboratory. The B6.C-Prkdc^{BALB} strain has a C57BL/6 background with the BALB/c variant of the Prkdc gene, while the C.B6-Prkdc has the BALB/c background with the C57BL/6 Prkdc gene. Interestingly, the C.B6-Prkdc showed a significant increase in SCE frequency, thus was able to generate a bystander response; however, the B6.C-Prkdc^{BALB} was not able to significantly influence SCE levels. These results provide additional support for DNA-PKcs playing a critical role in generation of a bystander response.

It is important to note that both the unirradiated BALB/c and the B6.C-Prkdc^{BALB} donor samples produced higher levels of SCEs in 5C HDF recipients than the other cell lines. While the BALB/c results were still within normal limits in terms of variation between samples, the B6.C-Prkdc^{BALB} results were significantly higher than the C57 BL/6 and the C.B6. One possible

explanation is that cells with the BALB/c Prkdc allele naturally generate higher levels of ROS due to increased instability, which are then able to activate a bystander response regardless of irradiation status. Given that these experiments were carried out multiple times, each time producing no increase in SCE, and that the background levels in the B6.C do not represent an absolute ceiling of SCE levels (higher levels of SCE have been seen in different cell lines), we believe that the absence of a difference between the 0 and 1 Gy samples represents a real failure to produce a bystander signal without sufficient DNA-PKcs.

Next we examined the role of the closely related protein ATM, in generating and/or receiving bystander signals. Again, we found no significant increase in SCE levels following direct irradiation of human ATM-/- cells. By irradiating ATM-/- cells (donors) and using our cell transfer approach, we found no significant increase in SCE frequencies in the 5C HDF recipient cells. However, when the reverse cell transfer was performed, the irradiated 5C HDF donor cells were in fact able to generate a response in the ATM-/- cells, implying that ATM -/- recipient cells can receive a bystander signal, but they cannot generate one. Therefore, like DNA-PKcs, ATM is necessary for the generation, not the reception of bystander signals.

Why DNA repair proteins would be involved in the generation of a bystander signal is not clear. It is possible that because cells lacking DNA-PKcs and ATM are more radiosensitive than normal cells, these cells die more readily upon exposure to radiation and are therefore, not able to generate a signal. However, given that it is highly unlikely that every cell lacking DNA-PKcs or ATM would die upon exposure to 1 Gy γ-rays, and that the BSE can be seen with just a few irradiated cells, this explanation seems insufficient.

A much more satisfactory explanation for a role for DNA repair proteins in the generation of bystander signals may involve DNA-PKcs and ATM's capabilities as DNA damage sensors in signaling pathways. Such a damage response may initiate as yet undefined pathways that ultimately lead to the generation of a BSE in non-irradiated cells, and hints at a tissue-level response to radiation injury moderated by some of the same proteins that orchestrate the inter-cellular response to DNA damage. While an intra-cellular IR-induced signaling response has been demonstrated (Wu et al., 1999), it has also been shown that ATM and DNA-PKcs signaling activates NF-kB via the p53-independent MEK/ERK/p90rsk/IKK signaling pathway in an anti-apoptotic response to DNA damage (Panta et al., 2004). In addition, DNA-PKcs is required for the activation of the stress kinases SAPK/JNK (Fritz and Kaina, 2006). Taken together, these data support the idea that ATM and DNA-PKcs may regulate other kinds of signaling events, such as the BSE (Figure 6). This model would suggest that the BSE is an active process in response to IR, rather than a passive response to DNA damage.

While this model is currently speculative, our data do suggest previously unrecognized roles for the repair proteins DNA-PKcs and ATM in generation, but not receipt, of bystander signals. It should be noted that a study by Mothersill et al. concluded that DNA repair proteins were not involved in generation of the bystander signal (Mothersill et al., 2004). Our conflicting results may reflect differences in experimental design including: cells used (primary fibroblasts versus various cell lines), endpoints examined (SCE versus clonogenic survival), and methods used (cell transfer versus media transfer). For example, the media transfer experiments limit the role of ROS, whereas in our cell transfer approach, the likelihood for continued ROS generation and interaction remains.

Although the bystander signal itself is not yet known, a better understanding of the underlying pathways involved in its generation and reception is an essential step to better understanding of the BSE. Moreover, because predominately low biologically relevant doses of radiation elicit a bystander response (Morgan, 2003; Nagasawa and Little, 1992; Seymour and Mothersill, 2000), increased knowledge about this phenomena holds important implications for individual susceptibility and radiosensitivity caused by inadequate DNA repair capacity, a condition relevant to human populations and health.

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DISCUSSION

The purpose of this dissertation project was to clarify how DNA repair can influence SCE. While much was learned the question may now be even more complex, as so often is the case. Understanding SCE's is important given their widespread use in scientific literature. A quick internet search reveals literally thousands of peer reviewed journal articles that use SCE as a measurement. However, many of these papers are not focused on understanding SCE, but rather, using SCE as a measurement for some type of instability.

SCE assays have become a mainstay as a biological endpoint for mutagenic studies. Everything from chemicals to dental materials has used SCE to determine their potential ability to negatively alter a cells genome (Hammer, 2002; Montanaro et al., 2006; Stanimirovic et al., 2005). A general increase in SCE is usually interpreted as a negative response for the particular chemical or material. SCE assays have also been used in a number of genomic instability studies (Bozsakyova et al., 2005; Cefle et al., 2006; Kim et al., 2004).

The reason for the widespread use of SCE is twofold. First, due to the development of the FPG method (Perry and Wolff, 1974), SCE are an easy method to perform and score microscopically. The entire method can be done in a relatively short amount of time; the longest period being the growing of the cells in BrdU for approximately two cell cycles. Secondly, the SCE method yields very high numbers of events compared to the total number of cells scored. SCE occur on the order of more than a thousand times more often as compared to other cytogenetic endpoints such as chromatid breaks or dicentrics in non-treated cells (Wilson and Thompson, 2007). This not only saves time in scoring, but allows reliable statistic analysis while using a relatively small number of cells.

SCE do have drawbacks, however. The "absolute" number of SCE in a particular cell line in a particular situation tends to fluctuate. This raises concerns as it may be difficult to know if the particular agent or situation is causing the differences in SCE or perhaps the natural variability of the SCE is to blame. The tendency of basal levels of SCE to fluctuate may be in part due to the levels of oxidation during cell growth, cell cycle times, levels of confluency, and even age of the cells (some even say the phase of the moon!). Therefore it is necessary not to rely on historical numbers of SCE frequencies but rather to always have matched controls for each experiment.

Just the fact that there seem to be so many ways SCE frequency can be altered, dictates a more in depth understanding of SCE mechanism if they are going to be continued as a marker for mutagenic and genomic instability studies. Therefore, we have attempted to increase understanding of SCE by studying DNA repair pathways and proteins that may be involved in either the formation or suppression of SCE. Throughout the manuscript, several models have been proposed as viable mechanisms for the formation of a SCE. Here, we will briefly discuss each model and how the different repair pathways may be involved in each one.

Holliday Junction

The classical model for SCE involves a structure termed Holliday Junction (HJ). HJ's were first described as possible mechanism for homology driven DSB repair (Holliday, 1964). After a DSB end is formed, the 3' end can invade a homologous sequence, usually located on a sister chromatid formed after DNA replication forming an "X" like structure termed the HJ. The invading strand can then use the homologous sequence to extend its 3' end (Figure 1). The invasion causes displacement of one of the strands forming a displacement loop (D-loop). The

Sister Chromatid Exchange (SCE)

Occurrence of sister-chromatid exchange (SCE) when the leading strand of a replication fork encounters a single-strand break. Steps 1 & 2: Fork approaches a single-strand break. Step 3: Fork breaks. Step 4: Repair synthesis occurs at the gap in the unbroken chromatid. Step 5: Processing of the broken duplex creates a 3' single-strand tail. Step 6: Rad51 mediates Strand invasion. Step 7: Resolution of the Holliday junction in the orientation shown results in SCE, as illustrated by the red/blue color junctions in the new "parental" strands. Step 8: The replication fork is restored.

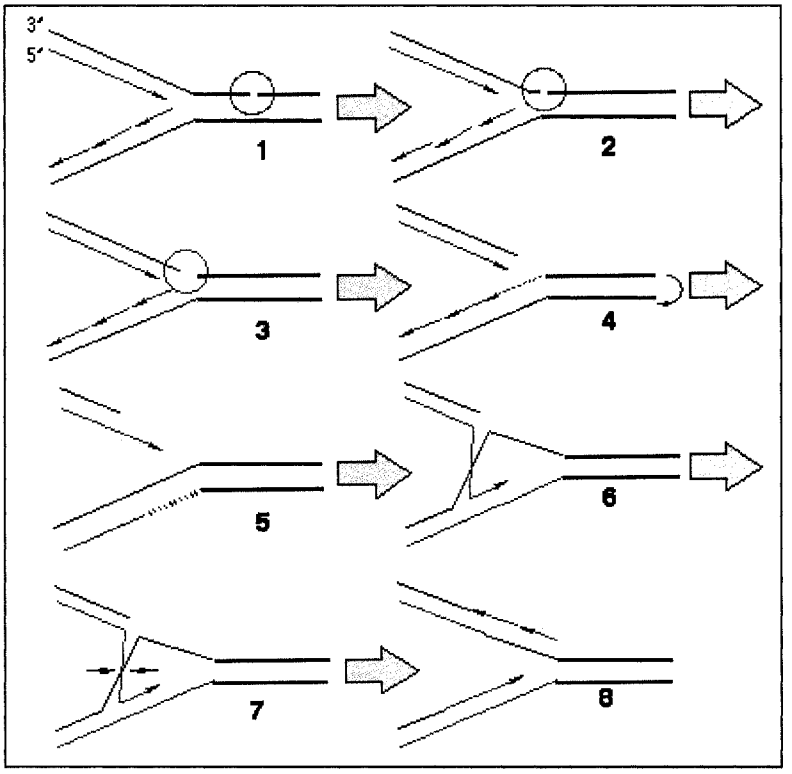


Figure 1. Schematic of possible SCE formation after encountering a stalled replication fork. Thompson, 2007

other end of the DSB can utilize the D-Loop as a template to extend itself. Finally, the HJ is resolved by cleavage in either two places. If the cleavage occurs at the middle of the "X" (Figure 1, #7), crossover arises leading to a SCE. If the cleavage occurs at the top of the "X"; no crossover will arise leading to gene conversion (only part of the sequence is switched compared to a complete crossover), rather than SCE.

The HJ model for SCE is the most used model for explaining SCE. This stems from the involvement of HR proteins on SCE. RAD51 appears vital for SCE formation, as absence of it results in a dramatic decrease in both spontaneous and damage-induced SCE (Fasullo et al., 2005). Many other proteins involved in HR also seem to be involved in SCE, including RAD54, WRN, BLM, BRCA, and several of the FA proteins

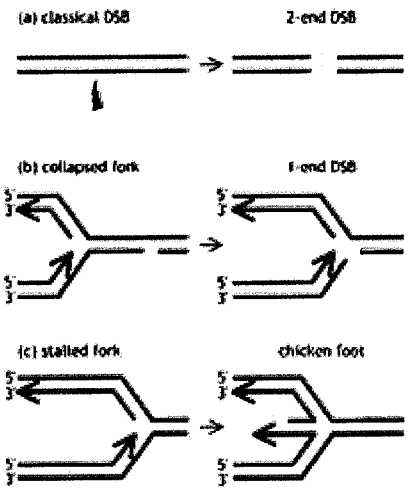


Figure 2. Reverse chicken foot structure. Helleday, 2003

(Dronkert et al., 2000; Kim et al., 2004; Laud et al., 2005; Yamamoto et al., 2005). Interestingly, many of these proteins seem to have elevated levels of SCE actually demonstrating more of a suppressive role in SCE.

Reverse Chicken Foot

The reverse chicken foot model is often mistaken or inappropriately used in conjunction with the HJ model (Figure 2). The key difference is that the reverse chicken foot model does not involve strand invasion. The reverse chicken foot model in terms of SCE still involves a stalled replication fork. However, instead of strand invasion after encountering the lesion, the leading daughter strand reverses back into the replication bubble. Once reversed, the lagging daughter can also reverse and use the daughter strand from the leading strand as a template of replication.

This model relies on that replication is not perfectly symmetrical. It has been demonstrated that, in certain cases, either the leading or lagging strand can replicate "ahead" of the other strand. Therefore, the strand that does not encounter the lesion would be able to be the template for the other strand upon reversal. After further replication, the chicken foot could then "un-reverse" and re-anneal effectively bypassing the lesion without actually having to fix the lesion.

The interesting aspect of the reverse chicken foot model is that a SCE would not be a consequence under normal circumstances. In fact, the reverse chicken foot model would assume almost no noticeable effects. The lesion would still be present and there should not be any change in sequence, nor would any type of exchange be present.

Colliding Replication Fork

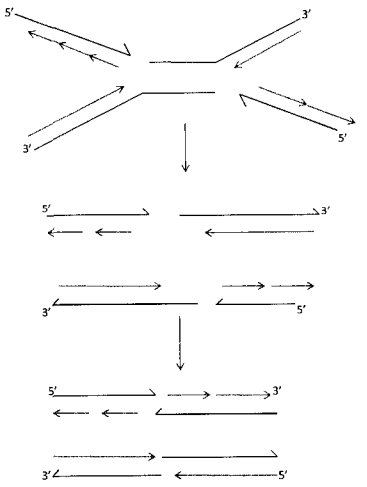


Figure 3. Model for NHEJ on SCE. Colliding replication forks collapse at two lesions on opposing strands leading to two DSBs. Inappropriate fusions, approximately 50% of the time, leads to exchange. Upon a second round of replication, the resulting structures look like a SCE.

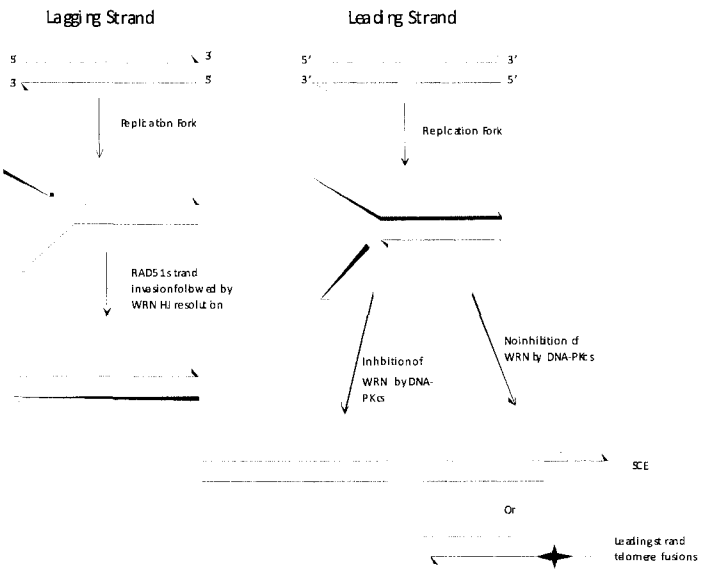
The colliding replication fork model represents one idea of how NHEJ may be involved in the formation of a SCE (Figure 3). The data mention in chapter 3, suggests that NHEJ is involved in the formation of G-SCE but not T-SCE. What could account for NHEJ only influencing G-SCE? One possible explanation is that in the genome, there are multiple replication forks. If one fork stalls due to a lesion such as a single strand break, it may be converted into a full DSB when a replication fork from the other direction "runs into" the stalled fork. Once a DSB is created, either HR or NHEJ may be employed to fix the break.

However, there is little evidence to support this model. It would seem highly unlikely that there would be two single strand breaks in close enough proximity that two separate replication forks could convert them into a DSB. However if this could occur, it would seem reasonable that NHEJ would be the predominate pathway for fixing the DSB, especially in mammalian cells. So the involvement of NHEJ in SCE would be one of special circumstances. HR may predominate the formation of a SCE, however, when a DSB is created either by an agent or by colliding replication forks, NHEJ may be able to form a SCE. This may also explain why, in some cases, SCE are seen to have deletions or reverse translocations in the "template" strand. NHEJ is rarely perfect, and often requires end processing resulting in the loss of sequence. This would especially be true if the two single stand breaks had some distance between them.

However, a much more likely scenario is that of a competitive nature between HR and NHEJ. HR has been shown to be up-regulated in the absence of NHEJ (Pierce et al., 2001). Therefore, it is possible that an up-regulated HR pathway would be much more efficient at recognizing and repairing stalled replication forks leading to an overall decrease in SCE. This does not account for the differences in G and T-SCE. In either case, the role of NHEJ in SCE is very surprising and needs further investigation to understand its true role.

Strand Dependent Model

The strand dependent model was created to explain how T-SCE's may be formed, and how the apparently very different pathways may actually be involved. It is a model that is really



Figrure 4. NHEJ and WRN resolving stalled replication forks. When the lesion occurs on the lagging strand stalling a replication fork, a cell can resolve the lesion via RAD51 strand invasion followed by Holliday Junction (HJ) resolution by WRN in a non-crossover manner. When the lesion occurs on the leading strand, DNA-PKcs normally inhibits WRN to prevent inappropriate resolution of the stalled fork. Without DNA-PKcs, increases in T-SCE and leading strand fusions are seen.

incorporating the first two models. In chapter 3, we demonstrated a possible suppressive role for NHEJ in T-SCE. We also demonstrated a role for HR proteins WRN, BLM, FANCD2, and a possible role for the NER protein, Ercc1 in T-SCE. So at the telomeres, there seem to be at least three distinct pathways involved in T-SCE, confusing matters even worse.

The answer may lie in the unique structure of telomeres. The strand made by lagging strand synthesis (which we will refer to as the lagging strand), is made up of the tandem array

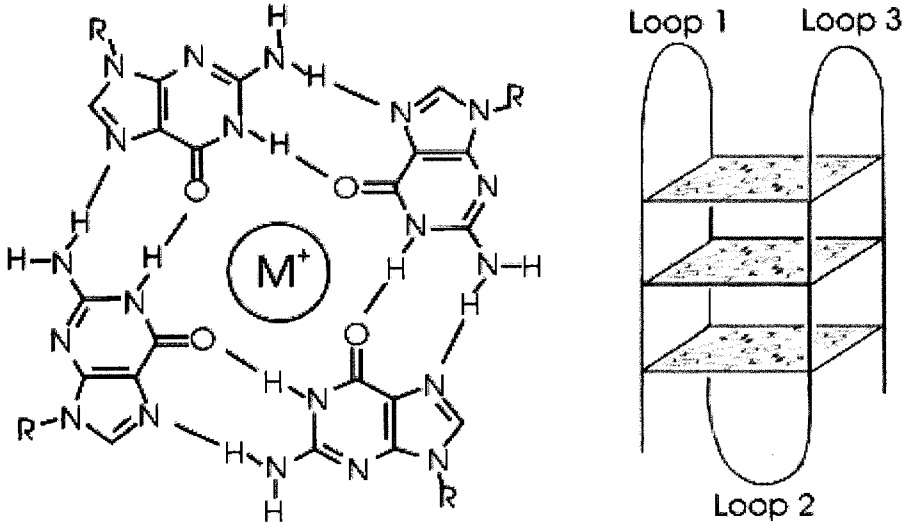


Figure 5. G-quadruplex's are secondary structures that can form in G-rich lagging strand telomeres and can stall replication forks. Image from Wikipedia.com

repeated sequence of TTAGGG in the 5' to 3' orientation (interior to end of the chromosome). By necessity, this means the strand made by leading strand synthesis (which will be referred to as the leading strand), is made up of the tandem array repeated sequence of AATCCC in the reverse 3' to 5' orientation. This makes the two strands distinct from each other. One strand is G-rich and possesses a 5' to 3' orientation, while the other strand is C-rich with a 3' to 5' orientation.

With its G-rich sequence, the lagging strand is likely going to have more difficulties during replication. G-rich sequences are highly susceptible to oxidative damage, in particular the formation of 8-oxo-G which has been demonstrated to inhibit replication (Krahn et al., 2003). G-rich sequences are also more likely to form secondary structures such as G-quadruplexes (figure 5) which have also been demonstrated to stall replication forks (Kan et al., 2007).

The fact that the lagging strand is going to have more difficulties during replication is not the only difference between the two strands. If the lagging strand replication encounters a single strand break and coverts it to a double strand end, that end contains a 3' end which can then be used for strand invasion. This event would most likely be a RAD51 dependent event and therefore many of the HR pathway proteins such as WRN and BLM would also be involved. While it may be assumed that RAD51 strand invasion leads to a structure where approximately 50% of the time crossover will occur, it is probably not correct. The WRN and BLM helicases are thought to be involved in suppression of crossover by acting at the HJ in manner that favors non-crossover (Plank et al., 2006).

But what happens when the single strand nick or break occurs on the leading strand? Due to its orientation, the leading strand will contain a 5' end which would not be suitable for strand invasion. Therefore, another mechanism must be in place in order to resolve the stalled replication fork. This is perhaps where the reverse chicken foot structure may come in. With its 5' end, the leading strand would be able to be "reversed" and replication could continue for a small stretch. Then, the lagging strand could also be reversed and using the leading strand as a template and continue its replication. After, the ends have been extended, they can invert back to their normal position, thereby passing the lesion in a non-crossover manner.

In this model, the RecQ helicases, WRN and BLM, are likely to play a major role. To first reverse the fork, and then to invert it back after replication, requires helicase activity. WRN has also been shown to use its exonuclease domain to degrade the leading strand by a few bases (Machwe et al., 2007) assisting in the reversal of the fork. Yet, in this model, the use of the classical HR proteins is not likely as there is no strand invasion.

If both models are used depending on which situation presents itself, how do the WRN and BLM proteins which are key proteins in each model, know how to react? This may be where NHEJ can assist in SCE. One of the key components of NHEJ is the protein kinase DNA-PK. It consists of a Ku70/86 heterodimer and the catalytic subunit, DNA-PKcs. Both DNA-PKcs and Ku have been shown to have direct interactions with WRN. Ku, by itself, has a stimulatory effect on both the WRN helicase and exonuclease domain (Orren et al., 2001). However, when Ku is combined with DNA-PKcs, they act together to actually suppress the WRN helicase and exonuclease domains (Karmakar et al., 2002). This suggests multiple layers of control. It may be that when the lesion occurs on the leading strand, HR is utilized to perform strand invasion and non-crossover resolution of the HJ. This would necessitate an active RAD51 pathway and an active WRN protein. However, if the lesion occurs on the leading strand, RAD51 strand invasion would be inappropriate, therefore, DNA-PKcs acts together with Ku to suppress WRN and that particular pathway.

The interesting aspect of this model is that it would help explain some data seen in ours and others laboratories. It has been demonstrated that DNA-PKcs is located at telomeres, and is necessary for their protection (Bailey et al., 2004b). This is unusual, as why would a protein that is involved in DSB repair be required to protect what is essentially a DSB end? Perhaps its main role is to regulate WRN, and possible HR, in the resolution of stalled replication forks that occur on the leading strand. And in the absence of DNA-PK, suppression of that pathway does not occur, and inappropriate handling of the stalled fork via WRN occurs. This inappropriate resolution of a stalled replication fork with the lesion on the leading strand could manifest itself in several ways. The first may be to form a T-SCE, and while this may be a way to by-pass the lesion it is likely not the safest or best way due to possible loss of sequence. Also, in the

absence of DNA-PKcs an elevation in leading strand telomere fusions was seen (Bailey et al., 1999). All of these things might arise if WRN is inappropriately trying to resolve a stalled replication fork on the leading strand, ultimately leading to a dysfunctional telomere.

An interesting aspect of this model is that it corresponds to the "levels" of the phenotypes seen in the different conditions. WS cells display exceptionally high levels of T-SCE, while the DNA-PKcs-/- cells displayed elevated levels of T-SCE but not to the same degree. This corresponds well with the earlier statements that the lagging strand is going to be more susceptible to damage. If WRN is involved in repairing stalled forks specifically in the lagging strand, than it would be expected that its phenotype would be more severe than the DNA-PKcs phenotype which would be involved in the less damaged leading strand.

Bystander Response

Finally, we studied what role the DNA repair proteins, DNA-PKcs and ATM had in generating an IR-induced BSE. Some work has been done on DNA repair's involvement in the reception of the bystander signal (Kashino et al., 2007b). Our work was surprising, as our data suggest that both DNA-PKcs and ATM are needed in order to generate the BSE. In general, donor cells were irradiated with 1 Gy, and then placed onto normal human fibroblasts. The donor cells had varied DNA-PKcs and ATM status. Only the donor cells that contained normal DNA-PKcs and ATM were able to cause an increase in the recipient normal human fibroblasts. Cells that were deficient for either DNA-PKcs or ATM were not able to increase SCE levels compared to matched controls.

Understanding the mechanisms behind BSE generation is important. It is assumed that if the BSE is also found at the organism level, it would most likely be a detrimental effect. This would impact the use of radiation therapy. As treatment therapies are continually trying to decrease dosage while maintaining an effective treatment, they are getting ever closer to the doses that activate the BSE seen in tissue culture (Seymour and Mothersill, 2000). Therefore, by trying to limit side effects from the radiation therapy, physicians may actually be doing more damage by dropping in to the dose range of BSE. However, much of this is still speculative as the BSE has not been definitively been proven at the organism level. Further, it has not been definitively proven that the BSE is negative consequence. It has been suggested that the BSE may actually be protective in that it makes the bystander cells more radioresistant (Maguire et al., 2007).

DNA repair being involved in the generation of a bystander signal is startling at first observation. However, it corresponds well with a variety of published data. First, it is important to remember that we are specifically speaking about DNA-PKcs and ATM. Both proteins are kinases and their involvement does not necessitate the involvement of the entire DNA repair pathways. It has been shown that both proteins can interact with a variety of other pathways, including the MAP kinase signaling pathway (Hamada et al., 2007).

There are a variety of data that give insights into the generation of the bystander signal. At least in some tissue culture systems, the bystander signal does seem entirely dependent on space. This may contradict the thought that the bystander signal is the generation of ROS (reactive oxygen species) or NOS (nitric oxide species) through the direct ionizations in the irradiated cell that are then released into the media or through gap junctions. If that was true, one would expect a concentration dependency on both dose and on distance. Several studies

demonstrate that ROS and NOS inhibitors can inhibit the BSE (Kashino et al., 2007a; Shao et al., 2006). However, it is possible that the DNA repair proteins, DNA-PKcs and ATM, are able to sense the IR and therefore communicate it to pathways that specifically generate the ROS or NOS. While this seems similar to the present theory of ROS/NOS being the bystander signal, the difference lies in their generation. In the current model, the ROS/NOS would be generated as a byproduct of the ionizations, while in our model the ROS/NOS are actively made by the cell in response to IR.

This model is currently speculative, and would need much more investigation before it can gain any credibility. However, our data demonstrates that NHEJ may be involved in the generation of the bystander effect, as measure by SCE. Understanding the pathways involved in the generation of the BSE may lead to further insights into the bystander signal itself and its biological relevance.

Overall conclusions

SCE can be a powerful tool in studying genomic instability and mutagenic studies. However, appropriately matched controls (cell lines, treatments, and date of experiment) must be in place in order to compensate for their high level of variability. Furthermore, much more research is needed to truly understand the mechanism of SCE. Here, we have demonstrated that there is indeed differential regulation of SCE depending on the location, i.e., either in the genome or the telomere. Whereas WRN was able to suppress T-SCE but not G-SCE, BLM was able to suppress both. FANCD2 was able to rescue the G-SCE phenotype seen in FANCD2-/- cell line, but seemed unable to do so in telomeres. The NHEJ protein DNA-PKcs seemed to be

necessary for the formation of G-SCE, as levels were lower without it, yet seemed to display a suppressive role for T-SCE. The status of DNA-PKcs and ATM were both also necessary for the generation of the bystander response when measured by SCE. Finally, the Ercc1-/- mouse lines suggested a possible role for Ercc1 in suppressing T-SCE but not G-SCE.

The reason that more investigation into the underlying mechanisms of SCE is necessary is quite simple. How can a person say a compound is mutagenic, when the endpoint being used is still a mystery? The fact that T-SCE and G-SCE are also different suggests that these mutagenic studies need to start considering T-SCE as well instead of only focusing on G-SCE. Perhaps a particular agent is only influencing T-SCE but not G-SCE, in a manner similar to the WRN-/- cells. In that case, the standard mutagenic assay would miss any difference and suggest that a particular compound is safe when it might not be. If more knowledge is gained, it might be possible to determine to a higher standard if a particular agent will lead to increased genomic instability. It may be found that compounds that only increase one or the other or perhaps both, are really detrimental to the cell. If that is the case, some previously classified "dangerous" compounds may actually be re-classified as safe; and the reverse is also true. In either case, if researchers are going to continue to use this powerful tool, we need to learn much more about SCE, and then it may be possible to expand their use even further.

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APPENDIX

List of Abbreviations

ALT	Alternative Lengthening of Telomeres
APE1	Apurinic/Apyridinic Endonuclease 1
ATM	Ataxia Telangectasia Mutated
ATR	Ataxia Telangectasia and Rad3-related Kinase
BASC	BRCA-associated Complex
BCNU	bischloroethylnitrosurea
BER	Base Excision Repair
BLAP75	BLM-Associated Polypeptide-75 kD
BLM	Bloom's Protein
BS	Bloom's Syndrome
BSE	Bystander Effect
CHO	Chinese Hamster Ovary
CO-FISH	Chromosome Orientation – Fluorescence *In Situ* Hybridization
D-loop	Displacement loop
DNA-PKcs	DNA-dependent Kinase catalytic subunit
DSB	Double Strand Break
EMS	Ethyl Methanesulfonate
FA	Fanconi Anemia
FANCD2	Fanconi Anemia- complementation group D2
FPG	Fluorescence plus Giemsa
G-SCE	Genomic- Sister Chromatid Exchange
HJ	Holliday Junction

HRDC	Helicase and RNase D C-terminal
HU	Hydroxyurea
ICL	Interstrand Crosslink
IR	Ionizing Radiation
LET	Linear Energy Transfer
LOH	Loss of Heterozygosity
MMC	Mitomycin C
MNNG	N-methy-N'-Nitro'-N-Nitrosguanidine
MRN	MRE11/RAD50/NBS1
NER	Nucleotide Excision Repair
NHEJ	Non-homologous End Joining
NLS	Nuclear Localization Signal
NOS	Nitric Oxide Species
PARP-1	Poly (ADP-ribose) Polymerase
PIKK	Phosphoinositide 3-kinase-related Kinase
PML Bodies	Promyelocytic Leukemia Bodies
ROS	Reactive Oxygen Species
RPA	Replication Protein A
RQC	RecQ C-terminal Domain
SCE	Sister Chromatid Exchange
SCID	Severe Combined Immunodeficiency
siRNA	small interfering RNA
SNP	Single Nucleotide Polymorphism
SSA	Single Stranded Annealing
SSB	Single Strand Break

T-SCE	Telomeric- Sister Chromatid Exchange
WRN	Werner Protein
WS	Werner's Syndrome
XP	Xeroderma Pigmentosum
XRCC1	X-ray Repair Cross Complementing 1