DISSERTATION

INVESTIGATIONS OF RADIATION-INDUCED AND SPONTANEOUS CHROMOSOMAL INVERSION FORMATION AND CHARACTERISTICS

Submitted by

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ABSTRACT

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In contrast to small "point" mutations involving only one or a few DNA base changes, chromosomal mutations are large, of several types, and figure prominently in many human diseases, including cancer. One important type of chromosomal mutation, sometimes referred to as a rearrangement, can involve an exchange of material between chromosomes (*inter*change or translocation) or it can involve the inversion of a segment (*intra*change) within a chromosome. Unless they grossly alter the position of the centromere or result in an obvious change in various chromosomal banding patterns, these inversions are very difficult to detect. Sometimes they are referred to as cryptic (unseen) aberrations. These chromosomal abnormalities or aberrations can occur spontaneously or after treatment with so-called clastogenic or chromosome-breaking agents like radiation.

Little is known regarding possible differences in the way *inter*changes and *intra*changes form either spontaneously or as a result of various kinds of radiations. The difficulty in studying the differences arises from the fact that only a very small portion of the induced *intra*changes or inversions are observable by most measurement methods applied to date. Considerable data from many studies point to double strand breaks (DSB) in DNA as the initiating lesions leading to the formation of these aberrations, while the development of the aberrations results from faulty biochemical processing normally operating to rejoin such DNA breaks. Most breaks are rejoined correctly but a few are mis-rejoined to cause the exchange aberration. Two main processes

known to operate for this purpose are non-homologous end joining (NHEJ) and homologous recombinational repair (HRR).

Because both sparsely ionizing radiations, like X- or gamma rays, and densely ionizing radiations, like high energy heavy charged particles (HZE), both produce all types of aberrations and that these aberrations are also known to occur spontaneously, it seemed reasonable to suggest at the outset of this research that, first, perhaps NHEJ and HRR may be involved in the formation of the different aberrations to different extents depending on the kind of radiations used, and second, that the kind of spontaneous aberrations may also depend, to different extents, on the operation or lack of operation of the different repair systems. There are two possibilities underlying the suggestion that the relative production of *intra- vs inter*- changes may depend on radiation ionization density. One is that because *inter*changes involve breakage and misrejoining *between* two or more different chromosomes occupying different chromosome domains while for *intra*changes two or more breaks *within* the same chromosome are required, the spatial distribution of the same number of ionizations in the nucleus (same dose) may thus favor the *intra*changes for densely ionizing radiations. Further, this should differ greatly depending on the degree of condensation of chromatin at the time of irradiation.

This dissertation aims to address the broad central hypothesis that different DNA repair/damage response pathways are involved in the formation of radiation-induced inversion as opposed to the development of spontaneous inversions and that there will also be radiation quality dependence and a chromatin structural dependence on the relative yields of radiation induced inversions. Three specific aims were devised and tested using several newer methodologies developed to better observe the previously unseen (cryptic) inversions. Aim 1 was designed to test the hypothesis that radiation quality and chromatin structure affect the

characteristics of radiation-induced inversions. Aim 2 utilized cells bearing mutations affecting NHEJ and/or HRR pathways and a modified differential fluorescence staining technique to identify the most important pathway contributing to the induction of radiation-induced. Finally, aim 3was designed to test the hypothesis that an intact HRR and specifically the Fanconi Anemia (FA) pathway is involved in the formation of spontaneous inversions.

Aim 1investigate the effects of both radiation quality and chromatin structure on the formation of radiation-induced chromosomal inversions by utilizing both G1 and M phase synchronized CHO10B2 cells and exposing them to 0 or 2 Gy of either X-rays or Fe ions. A BrdU based differential stain allowed us to observe inversions as small as a few Mb; this was determined by evaluation of pixel density. It was shown that densely ionizing or high linear energy transfer (LET) radiation not only induced more inversions, roughly twice as many inversions as low LET per unit dose, but also produced much smaller inversions than low LET radiation; the average size of a high LET radiation induced inversion was ~9.2 Mb as compared to the average size of a low LET induced inversion, which was ~12.7 Mb. In addition, it was also shown that chromatin structure only affected the formation of radiation-induced inversions when exposures involved high LET radiation. High LET exposed G1 cells contained more, 1.17 inversions per cell, and larger inversions, 13.34 Mb, than high LET exposed M phase cells, 0.85 inversions per cell with an average size of 12.02 Mb.

Aim 2 investigated the role of various DNA repair pathways in the formation of radiation-induced inversions. Various Chinese Hamster Ovary (CHO) wild type (CHO10B2), NHEJ deficient cells (XRS5, V3, XR-1), HR (51D1, irs1SF), and FA mutant (KO40) deficient cells were synchronized into the G1 stage of the cell cycle and exposed to 0 or 2 Gy of gamma radiation. A single cell cycle incubation period in EdU (a thymidine analogue), followed by a

differential stain protocol was utilized to identify true radiation-induced inversions. Data for frequencies followed a Poisson distribution. It was seen that inhibition of the NHEJ pathway resulted in decreased number of radiation-induced inversions, roughly a 50% decrease when compared to the CHO wild type. Interestingly, inhibition of the FA pathway resulted in an increase in both the number of spontaneous inversions observed at 0 Gy, but also the number of radiation-induced inversions observed after exposure to 2 Gy of ionizing radiation. It was observed that FA deficient cells contained roughly 330% (1.24 inversions per cell) more spontaneous inversions and 20% (0.4 inversions per cell) more radiation-induced inversions then the wild-type CHO cell lines.

Lastly, aim 3 investigated the formation of spontaneous inversions in human FA mutants. The use of a directionally orientated chromatid probe allowed for the study of spontaneous chromosomal inversions that occurred at any point in the life of the cell, not just chromosomal inversions formed in the immediate cell cycle prior to collection. We show that only human FA mutant cell lines display an increased frequency of spontaneous inversions as compared to normal human fibroblasts, FA positive cancer cell lines, or other DNA repair deficient cancer cells. Approximately 4% of chromosome 3's observed in FA mutants contained a spontaneous inversion. These inversions could be isolated and expanded clonally, indicating that what we were observing are in fact transmissible inversions and not "false" inversions caused by double sister chromatid exchange events.

Presented here are novel findings on the formation and characteristics of both radiation-induced and spontaneous chromosomal inversions. First, high LET radiation produced smaller inversions than low LET radiation. Additionally, condensed chromatin structure at the time of irradiation resulted in smaller inversions. These findings support the assumption that radiation-

induced inversions form as a result of two DSBs. Second, consistent with other radiation-induced structural rearrangements, inversions also require the NHEJ pathway for their formation. Further, loss of the FA core complex resulted in increased frequencies of both radiation-induced and spontaneous inversions. These findings highlight the importance of DNA damage response/repair pathways in the formation of inversions, and for the first time identifies a difference in inversion pathway choice dependent on when and where the damage is encountered.

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Dedicated to my wife and son, I love you

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CHAPTER 1

INTRODUCTION

Chromosome Inversion

Formation and Function

Chromosome inversions are symmetrical chromosomal rearrangements that cause a rearrangement of gene sequence within a chromosome without resulting in the loss of a significant amount of genomic information. There are two types of inversions; they either involve the centromere, pericentric, or are located on a single arm, paracentric (Davisson et al., 1981; de la Chapelle et al., 1974). It is believed that most inversions are formed by radiation and are the results of the non-homologous endjoining (NHEJ) pathway rejoining the ends, in the reverse orientation (Richardson and Jasin, 2000; Simsek and Jasin, 2010). This pathway was highlighted by Drs. Muhlmann-Diaz and Bedford, who showed that inversions and centric and acentric rings formed at the same rate after exposure of G0/G1 human fibroblasts to ionizing radiation. This led to the presentation of a model that showed that inversions arose from a broken fragment of DNA formed by two DSBs occurring within a sufficiently close proximity to one another on a single chromosome so that mis-rejoining of the broken ends, rather than restitution of the original breaks, caused the inversion. As addressed in chapter 3 inversion formation appears to be far more complex than the two double strand break model put forth by Drs. Muhlmann-Diaz and Bedford (Muhlmann-Diaz and Bedford, 1995).

History and Significance

When inversions were first observed in the early 20th century in *Drosophila* polytene chromosomes the vast majority of inversions were not associated with a phenotypical change in the *Drosophila* (Bridges, 1936; Hughes, 1939; Painter, 1931; Painter, 1934). Prior work done in

by Dr. Alfred Sturtevant noted that large heterozygous inversions suppressed recombination in the inverted segments; however, he also noted that the *Drosophila* experienced no other detrimental effects at the time of the study (Sturtevant, 1921). After extensive research with *Drosophila* genetics, Dabzhansky was the first to identify that several inversions led to changes in the *Drosophila* phenotype (Dobzhansky, 1970). With the advancements being made in the field of cytogenetic staining protocols there was a shift from the utilization of *Drosophila* to mammalian cells in the study of chromosome inversions.

Eventually, it was observed that pericentric inversions reduced the fertility of organisms carrying them; this work was originally conducted in plants, but was soon repeated in mammalian cells (Coyne et al., 1991; Davisson et al., 1981; Laufs et al., 1999). During meiosis a heterozygous pericentric or paracentric inversion can create unbalanced gametes by preventing the correct segregation of the chromosomes carrying the inversion (Coyne et al., 1991). Additionally, pericentric inversions can lead to inhibition of recombination in meiotic cells, suggesting that an inversion can potentially inhibit homologous recombination (HR) in somatic cells (Kirkpatrick, 2010). In plants, pericentric inversions are negatively selected for and eventually are bred out, supplying evidence that inversions are potentially detrimental to the cell and organism carrying them (Hoffmann and Rieseberg, 2008). The decreased fertility rates associated with pericentric inversions and inhibition of recombination may be two factors why large inversions are fairly uncommon in the evolution from ape to humans and are rarely observed. It is interesting to note that of the 1576 inversions that differ between humans and chimpanzees, only 66 are greater the 25 Kb in size with the largest being 4.3 Mb in size (Feuk et al., 2005).

Observation of Chromosome Inversions

Chromosome inversions are notoriously difficult to observe due to the fact that many inversions do not create a microscopically visible structural change in the chromosome; in fact, only pericentric inversions with unequal exchange across the centromere can create a microscopically visible change to the chromosome. Due to the fact that inversions very often do not cause a visible structural change in the chromosome, they are extremely difficult to study (Savage, 1976; Trask, 2002). The most applied techniques to study inversions in mammalian cells are outlined in Table 1. The first studies that investigated inversions in mammalian cells utilized Q/G/R Banding techniques. These approaches allowed researchers to see the organization of a chromosome expressed in the form of dark and light bands and would show when a segment is inverted if it is large enough to produce a visible change in the light-dark pattern of the bands (de la Chapelle et al., 1974; Holmquist et al., 1982). mBANDing greatly improved the sensitivity of inversion identification, however it is still limited to the observation of inversions of 20 or more megabases (Mb) in size and on a single chromosome (Hada et al., 2007; Hande et al., 2003). The next step in observing inversions was the modified Giemsa-plus fluorescence technique that utilized a single cycle BrdU/C incorporation followed by irradiation, as compared to the two cycle BrdU/C following irradiation approach used to study sister chromatid exchanges (SCE), to create a chromosome that had a single strand of BrdU/C incorporated DNA that allowed for differential staining (Muhlmann-Diaz and Bedford, 1995). The two main issues with this technique are first, that it only can measure inversions produced in

Table 1.1: Comparison of Differential and Banding Staining Techniques

	Q-Band	R-Band	mBAND	BrdU/EdU	Chromatid Paint
Range	Whole Genome	Whole Genome	Single Chromosome	Whole Genome	Single Chromosome
Sensitivity	Limited to band size	Limited to band size	Limited by band size	~1 Mb	~1 Mb
Stain Quality (Reproducibility of stain)	Dependent of technique and chromatin compaction	Dependent of technique and chromatin compaction	Excellent	Excellent	Excellent
Detection of Pre-existing Inversions	No	No	Yes	No	Yes
Affected by SCE	No	No	No	Yes	Yes
Cost Effectiveness	Inexpensive	Inexpensive	Very Costly, requires multiple fluorescent filters, specific imaging software, and costly probe set	Inexpensive	Moderate, requires z-stage for best results

the previous cell cycle and that it cannot detect pre-existing ones, and second there is no way to differentiate between a true inversion and a false inversion, formed by two SCE occurring on a single chromosome. However, this technique can be reliable for observing radiation-induced chromosomal inversions because relatively few SCEs are produced by x or gamma rays, allowing for the observed chromosomal inversions above the background to be considered true x- or gamma ray induced inversions (Muhlmann-Diaz and Bedford, 1995). This may not be entirely the case of high LET radiation, which has been shown to induced SCE (Nagasawa and Little, 1992). So care must be exercised in determining both the spontaneous and radiation-induced SCEs in all cases.

Role in Human Health and Disease

Despite the lack of research into how inversions form and their role in carcinogenesis, there has been extensive research into the study of how inversions have influenced evolution over time. One study that compared *Homo sapiens* and their near relatives the chimpanzee identified a total of 1,576 putative regions of inverted orientation, covering more than 154 Mb of DNA differing between the two species (Feuk et al., 2005). With many of these inversions there was nearly no difference in the genetic content of the inverted and uninverted segments other than the linear order of the DNA through the break point regions (Feuk et al., 2005). It has also been noted in several studies that a vast majority of inversions inherited over time in both mammalian and *drosophilae* were extremely small, less than 5 Kb in size (Furuta et al., 2011; Kirkpatrick, 2010). It is also believed that one of the main reasons the Y chromosome in mammalians is unable to recombine with the X chromosome is due to inversion repeats that formed early on in the evolution of the mammalian species (Lahn and Page, 1999). An inversions ability to change the location of a gene on a single chromosome without changing the

genetic information contained in that gene allows the inversion to be a unique force in evolution. It has been shown that inversions can select for recessive traits by inhibiting recombination and preventing loss of heterozygosity (Kirkpatrick, 2010).

Despite the fact that inversions can be extremely difficult to detect, there have been several reported incidences of inversions leading to mutagenic changes in a cell eventually leading to the formation of a cancer. It has been documented in previous research that a type of radiation-induced thyroid cancer is caused by an inversion that creates a RET/PTC1 fusion protein (Nikiforova et al., 2000). The two break points are roughly 30 Mb apart and separated by an average of 1-3 microns in the interphase nuclei of cells in G1 (Nikiforova et al., 2000). This fusion protein is the result of the tyrosine kinase domain of the RET gene being fused to the promoter region of the H4 gene (Santoro et al., 1992). In normal cells the RET protein is part of Glial cell line-derived neurotropic factor family of proteins and is associated with extracellular signaling molecules and requires the fusing of two RET proteins to become activated. The RET protein is a membrane bound protein and activation is highly regulated (Baloh et al., 2000). Once fused, however, the chimeric protein becomes constituently activated due to the fact that the tyrosine kinase domain is no longer membrane bound and can be easily bound to a second tyrosine kinase domain. It has been shown that the tyrosine kinase activity triggers mitogenic signaling, specifically the VegF and Ras-MAPK pathway (Xing et al., 1998). Up-regulation of VegF and Ras/MAPK has been shown to increase cellular survival, migration, and proliferation. VegF and Ras/MAPK expression is often seen in highly metastatic cancers (Giles, 2001).

The RET/PTC1 fusion protein is a great example of how an inversion can result in the formation of a cancerous change in a cell through the truncation of a gene; however. Inversions can also cause potential carcinogenesis by creating additional genomic instability. Prior studies

have shown that an inversion can form a fragile site, highly repetitive DNA sequences rich in AT or CGG repeats, in the genome that could lead to further breaks/translocations leading to the formation of a specific form of acute myeloid leukemia (Prebet et al., 2009; de Kok et al., 1995). This inversion induced instability is also seen in individuals suffering from Williams-Beuren syndrome. It was shown in a recent study that there is an 1.5 Mb inversion on chromosome 7 and that the inversion are highly associated with microdeletions not observed on the chromosome lacking the inversions (Osborne et al., 2001).

Ionizing Radiation

Introduction and Overview

Ionizing radiation (IR) is one of the most damaging agents to DNA, producing complex and multifaceted damages, and has been shown to be able to produce inversions (Bedford, 1991; Bedford and Dewey, 2002; Muhlmann-Diaz and Bedford, 1995). IR possesses enough energy to eject an electron from its orbit. This compared to non-ionizing radiation, which only contains enough energy to raise an electron to a higher energy state (Hall et al., 1988). The energy in a single ionizing event, roughly 33 electron volts, is enough energy to break a covalent bond, i.e. the chemical bond between DNA bases to create a strand break (Hall, 2006). The most commonly used form of ionizing radiation utilized in research labs are X-rays and γ-rays, both of which are electromagnetic radiation, the only difference being X-rays are produced extranuclearly while γ-rays are produced intranuclearly (Turner, 2007). IR also comes in the form of particulate IR, this includes α-particles, electrons or β-particles, protons, neutrons, or larger heavy charged particles (HZE) (Turner, 2007).

The most important type of damage caused by IR is damage to the DNA itself. DNA damage can be caused either indirectly or directly. As Figure 1 depicts, indirect damage is when a free radical created by the interaction of the radiation with water molecules located in the nucleus damages the DNA. Compared to direct damage that is when the DNA is damaged directly by the ionizing radiation (Hall et al., 1988). IR can produce base damages, single strand breaks (SSB), and DSB. These damages must be repaired in order for cells to continue to grow and divide. Inappropriate repair of these damages can result in chromosome aberrations, deletions, translocations, inversions, and various other types of mutations (Hall, 2006).

Formation of Chromosomal Aberrations

The DNA damage caused by IR has the potential to form a chromosome aberration if the damage is repaired incorrectly by the DNA repair machinery. Dr. John R. K. Savage eloquently described the vast number of chromosomal aberrations and how they were potentially formed using basic Giemsa staining in his 1976 journal article (Savage, 1976). Based on Savage's work we can accurately predict at what stage in the cell cycle the cell was damaged, and what type of damage was experienced, single DSB or several. Aberrations can be separated into two basic types, chromosome or chromatid type aberrations. Chromosome type aberrations are formed during the G1 phase of the cell cycle resulting in an aberration involving both chromatids at the same locus when observed in M phase immediately following their formation. These types of aberrations include asymmetric types such as dicentrics, centric/acentric rings, and small interstitial deletions that appear like double minutes, and symmetric types such as balanced translocations or paracentric/pericentric inversions. On the other hand, chromatid type aberrations are formed during late S or G2. Chromatid type aberrations involve an abnormality in one chromatid, with the other being normal. These types of aberrations include breaks/gaps,

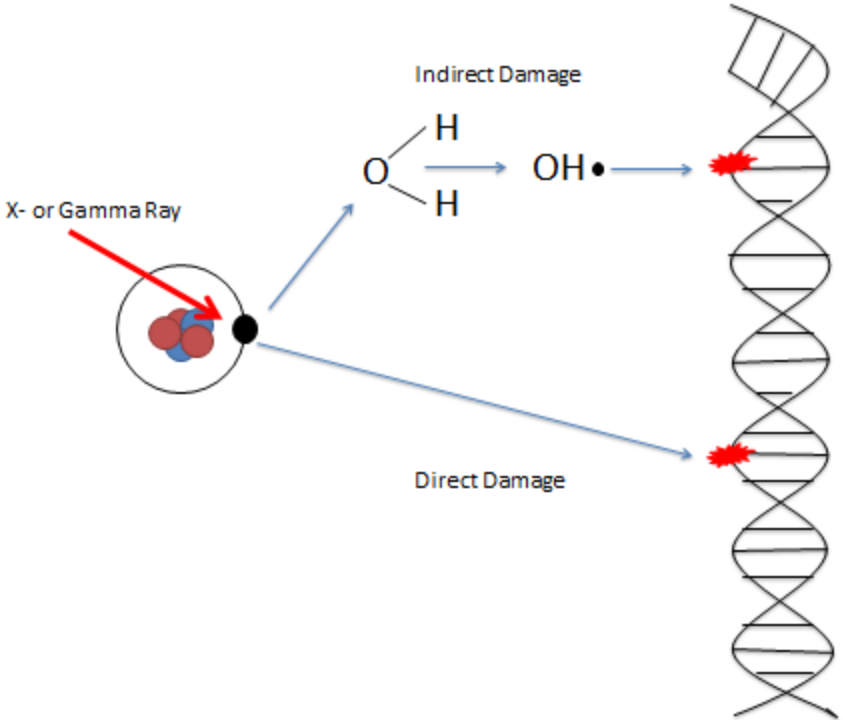

Figure 1.1: This figure depicts the difference between indirect and direct damage as caused by low LET X- or Gamma rays.

symmetrical and asymmetrical translocations, radials, isodeletions, and chromatid type interstitial or terminal deletions. Savage described how different aberrations were caused by 1 or 2 DSB. Gaps/breaks and terminal deletions are the two most common chromosome aberration that can result from a single DSB. Translocations, dicentrics, and rings are exchange aberrations that all involve at least two DSBs in order to form (Savage, 1976).

Chromosome aberrations are an excellent tool for analyzing radiation exposure due to the fact that for any given radiation quality and dose rate they form at very specific dose-related frequencies (Awa et al., 1978; Bender and Gooch, 1962; Finnon et al., 1999; Lucas et al., 1992). Cytogenetic analysis of dicentric and centric ring formation is one of the most reliable biomarkers for assessing the radiation dose delivered to an individual who has been exposed to ionizing radiation when no physical dose estimate is available. However, dicentrics are not the only aberration that can be analyzed to estimate dose exposure, a recent paper by Dr. Ray et al. has shown that inversions can be used to effectively estimate dose exposure (Ray et al., 2014). For dose estimation with dicentric analysis, peripheral blood lymphocytes are collected from exposed individuals and the number of dicentrics are observed and compared to a standard dose–response curve obtained after an in vitro irradiation (Hayata et al., 2001; Sasaki et al., 2001). Peripheral blood lymphocytes are typically utilized because they are non-dividing and will remain circulating in the blood for several weeks after exposure, allowing for easy collection (Bender and Gooch, 1962). With the advent of novel centromere and telomere PNA/DNA probes, which allowed for effective FISH staining, Giemsa staining analysis for dicentric analysis is being replaced with the use of fluorescence based analysis. Since the fluorescence based analysis relies on the identification of two easy to see centromere signals on a single chromosome there is the potential for a high throughout system to identify dicentrics (Schmid et

al., 1995; Vaurijoux et al., 2009). One of the shortcomings of the fluorescence based analysis is the length of time that is required to complete the staining protocol (Durm et al., 1997; Garcia-Sagredo, 2008; Paulasova and Pellestor, 2004). In the recent few years the FISH protocol has been shortened to a few hours, but still involves hybridization at different temperatures for very specific times (Kitayama et al., 2000; Shi et al., 2012). Our recently published paper has shown that the classic FISH protocol can be shortened even further, yet still maintains the sensitivity of the traditional protocol (Cartwright et al., 2013; Genet et al., 2013).

LET Effect on Chromosomal Aberration Formation

All radiation is not created equal, the amount of damage caused to a cell is directly dependent not only on the dose received, but also the properties of the radiation the cell was exposed to. One such property is the Linear Energy Transfer (LET) of the radiation, LET is a term used to describe how ionizing radiation deposits its energy per unit track length and the density of the energy deposition (Ward, 1994). The LET of IR is typically measured in keV/μm in water. Ionizing radiation is typically classified as being either low or high LET. X-rays and γ-rays are both considered low LET radiation and deposit their energy sporadically throughout the cell in discrete ionizing events (Hada and Georgakilas, 2008b; Pastwa et al., 2003; Ward, 1994). The two most commonly utilized γ-rays sources utilized in research are Cs^{137} and Co^{60} which have respective energies of 662 keV and 1173/1332 keV (Chen, 2004). In chapter 3 we utilized Fe ion radiation as a high LET source. The Fe ions used in chapter 3 had 500 MeV/nucleon of initial energy and 200 keV/μm LET (Cartwright et al., 2014).

High LET is considered much more "effective" than low-LET per unit dose at causing DNA damage (Hall, 2006). This effectiveness can be attributed to the nature of how high LET radiation deposits its energy. High LET radiation deposits its energy in the form of ionizations

that occur very densely along discrete tracks (Hall, 2006). This dense energy deposition leads to an increase in complex DNA damage sites, requiring more time and a variety of DNA repair proteins to correct the damage. Additionally, the energy deposition of high LET radiation causes the vast majority of DNA damage to be caused by direct damage, compared to low LET radiation, for which ~2/3 of the damage is caused by indirect damage (Hall, 2006).

Given the fact that DNA damage caused by high LET radiation is more complex and takes more time to repair then DNA damage induced by low LET radiation it is easy to see why high LET radiation induces more and more complex chromosome aberrations then low LET radiation (Hada and Georgakilas, 2008a; Nagasawa et al., 1990). In fact, not only does high LET induce more chromosomal aberrations per unit dose then low LET radiation, high LET radiation has also been shown to induce delayed genomic instability to a greater extent, as well (Little et al., 1997b; Ritter et al., 2000).

This increase in both the overall number of chromosomal aberrations and their complexity is expected when the relative biological effectiveness (RBE) of high LET radiation is taken into account. The term RBE refers to the ratio of the biological effectiveness of one type of radiation to another given the same amount of absorbed energy The higher the RBE of a type of radiation, the more damage that type of radiation can produce per unit dose. (Storer et al., 1957). High LET radiation has a much higher RBE, 20 for alpha particles and 3-5 for Fe ions, than low LET x- and gamma rays, both have a RBE of 1 (Asaithamby et al., 2008; Bedford and Goodhead, 1989).

Role in Human Health and Disease

Due to the fact that ionizing radiation has the ability to induce a massive amount of DNA damage, depending on the dose and dose rate, ionizing radiation is an extremely potent

carcinogen (Hall, 2006). On average after exposure to 1 Grey (Gy) of γ-rays a cell will experience ~3000 nucleotide/deoxyribose damages, ~1000 SSB, and ~20-50 DSB (Sutherland et al., 2000a; Sutherland et al., 2000b). If multiple types of damage occur within a few base pairs of each other, it's considered a complex damage site (Thompson, 2012). It is important to note that a DSB is simply two SSBs occurring within 10-20 base pairs of each other on opposite DNA strands (Hall, 2006). This DNA damage lends itself to mutations and potential cancerous changes to a cell. There have been numerous epidemiological studies, Hiroshima and Nagasaki being the largest and longest, which have furthered our understanding of the cancer risk associated with IR exposure and support the mutagenic properties of IR observed in cellular studies (Gilbert, 2009).

DNA Repair

Introduction and Overview

Eukaryotic cells have developed efficient repair mechanisms to combat the daily DNA damage insult each cell experiences, but these repair machineries are not ideally suited to deal with large number of DSBs (Jackson and Bartek, 2009). Base excision, nucleotide excision, and mismatch repair pathways handle single-stranded DNA damage quickly and with high fidelity. These pathways are highly conserved and utilize the opposite DNA strand as a template for repair (Barnes et al., 1993; Satoh et al., 1993). Typically, agents that induce single-stranded DNA damage are very ineffective at causing cell death, unless the cell is exposed to extremely high concentrations of the agent. DSBs are much more likely to be lethal to the cell and have to be repaired very quickly by the cell (Thompson, 2012). DSBs have been shown to cause mutations in cells at higher rates than SSBs (Hall, 2006). There are two primary repair pathways, outlined in Table 2 along with the FA pathway, that are responsible for the repair of DSB, the

Table 1.2: Comparison of DNA Repair/Damage Response Pathways

	NHEJ	HR	FA
Function	DNA Repair	DNA Repair	Damage Response/Signaling
Primary Lesion	DSB	DSB	ICL, potentially DSB
Major Proteins Associated	Ku 70/80, DNA-PKcs, XRCC4	MRN complex, Rad51, Rad51 paralogs, BRCA1/2, XRCC3	FA core complex (FANC A,B,C,E,F,G,L,M), FANCD2
Cell Cycle Stage Active	G1/Go, S, G2	Late S, G2	G1/G0, S, G2

NHEJ and HR repair pathway (Thompson, 2012). Each of these pathways is highly controlled and regulated and repairs DNA in a unique way (Hinz et al., 2005; Ira et al., 2004; Scully et al., 1997; Takata et al., 1998; Willers et al., 2000).

Non-homologous End-Joining Pathway

NHEJ is the primary DNA DSB repair pathway in mammalian cells. The NHEJ pathway is active in the G1, S, and G2 phase of the cell cycle, but does not utilize homologous DNA as a template for repair (Thompson, 2012). Due to this fact, NHEJ is often referred to as an "error-prone" DNA repair pathway and is believed to be the driving force of mutations and carcinogenesis after radiation exposure (Little, 1998).

After the initial identification of the DSB by various damage reporting proteins such as the MRN complex (Mre11-Rad50-Nbs1), 53BP1, MDC1; the heterodimers Ku70/80 are recruited to and bind to the free DSB end (Gatei et al., 2000a; Gatei et al., 2000b; Iwabuchi et al., 2003; Kysela et al., 2003; Paillard and Strauss, 1991; Smith et al., 2003). The binding of Ku70/80 to the DNA results in the recruitment of the DNA-PK catalytic subunit (DNA-PKcs). DNA-PKcs binds to the Ku70/80 dimer and initiates the annealing of the broken DNA strands utilizing next to no sequences of homology and thus the repair of the DSB. XRCC4/LigIV finalizes the repair process, XRCC4/LigIV are recruited by the DNA-PK complex (Roth and Wilson, 1986; Rothkamm et al., 2003; Valerie and Povirk, 2003).

Homologous Recombination

HR is typically referred to as an "error-free" DNA repair pathway due to the use of extensive homologous sequences during the repair process. The HR pathway is often active in late S and G2 that allows for the use of sister chromatid as a source of homologous DNA sequences (Hall, 2006). Despite the fact that extensive homologous DNA sequences are available

in G1, the homologous chromosome and repetitive DNA sequence, HR is inhibited by the NHEJ proteins and thus inhibits potential genomic instability and illegitimate recombination (Thompson, 2012).

Upon the detection of a DSB by damage reporting proteins in specific stages of the cell cycle, the MRN complex and BRCA1 interact to promote 5'-3' end resection and production of a 3' single-stranded overhang (Thompson, 2012). The single-stranded DNA is coated with replication protein A (RPA). BRCA1/2 along with Rad51 paralogs (XRCC2, XRCC3, Rad51B, Rad51C, and Rad51D) load Rad51 onto the RPA coated single-stranded DNA (Thompson, 2012; Thompson and Schild, 2001). The Rad51 coated DNA recruits both Rad52 and Rad54 which trigger a Tp53-dependent search for homologous templates. These homologous templates are often found in the closely associated sister chromatid (Linke et al., 2003). Through a process not completely understood, the homologous DNA strand is opened to form a D-loop that allows for the Rad51 coated single-stranded DNA to invade and bind the homologous sequences (Liang et al., 1998; Susse et al., 2000). Eventually HR is completed with the resolution of the Holliday junction that can potentially result in the formation of a crossover event, SCE. These crossover events can be observed by BrdU labeling and fluorescence-plus-Giemsa or harlequin staining techniques (Bhattacharyya and Wolff, 1974; Liang et al., 1998; Nagasawa and Little, 1992).

Fanconi Anemia Pathway

The FA complex of proteins has been shown to be essential for repair of interstrand DNA crosslink repair (ICR) (Kee and D'Andrea, 2010). It has also been shown that the FA proteins also co-localize and associate with repair proteins from the nucleotide excision repair, HR, and the translesion synthesis pathways (Bogliolo et al., 2007; Garcia-Higuera et al., 2001; Hussain et al., 2004; Kook, 2005; Nakanishi et al., 2002). It has been noted that loss of FA only results in a

mildly sensitive phenotype to ionizing radiation, less severe than HR mutants and far less severe than the sensitization observed in NHEJ mutants (Bogliolo et al., 2007; Kook, 2005). FA mutants typically present with the phenotype of elevated chromatid breaks, gaps, truncations, and the formation of radials (Kee and D'Andrea, 2010; Moldovan and D'Andrea, 2009). Additionally, both human and rodent FA mutants do not show elevated levels of spontaneous SCE, they do however experience greatly elevated levels of induced SCE (Hayashi and Schmid, 1975; Niedzwiedz et al., 2004; Weinberger et al., 1974).

The FA pathway is driven by one of two protein complexes; 1). FA core complex is composed of the FANC paralogs A, B, C, E, F, G, L, M and 2). The FANCD2 complex is composed of FANCD2 and I. The FA core complex is responsible for ubiquitinating the FANCD2 complex. The ubiquitination of the FANCD2 complex allows for the interaction of FANCD2 with the BRCA1 proteins and the HR pathway (Kee and D'Andrea, 2010; Moldovan and D'Andrea, 2009).

The specifics of how the FA family drives the repair of ICL is relatively unknown, but it is believed that the FA family of proteins interacts with elements from NER and HR. Moldovan and D'Andrea outlined very eloquently how ICL's are repair. The FA core complex is recruited to replication forks stalled at ICL. Mus81-Eme1 and Ercc1-XPF cleave on either side of the ICL. Once cleaved, Rev1 and Polζ interact to allow bypass of the ICL. Undetermined NER proteins fully remove the ICL and it is believed that the FA family ultimately leads to the recruitment of HR proteins that complete repair of the ICL. The lack of FA leads to unregulated HR repair and an over activation of NHEJ (Kook, 2005; Mosedale et al., 2005; Wang et al., 2004). This over activation of NHEJ is the reason why FA mutants typically present with elevated numbers of

spontaneous radials, which are the result of the incorrect repair of two double strand breaks by the NHEJ pathway (Kee and D'Andrea, 2010; Moldovan and D'Andrea, 2009).

FA individuals experience a high rate of various types of cancer. In addition to Acute Myeloid Leukemia, FA individuals also experience elevated rates of squamous cell carcinomas of the head and neck (400-500x higher than the general population). The typical onset of these cancers is 27 years of age (Rosenberg et al., 2003). When FA mutant cells are analyzed for spontaneous chromosome aberrations the most common aberration observed are chromatid gap/break and radials, it has also been observed that FA mutants have a slight elevation in spontaneous symmetrical and asymmetrical translocations as well (Joenje and Patel, 2001).

Genomic Instability

Introduction and Overview

Genomic instability refers to a cell line or lineage accumulating new mutations at an elevated rate compared to spontaneous mutations. Nearly all cancers display some form of genomic instability. This instability is one of the main reasons why cancers can be extremely difficult to treat; genomic instability can cause a tumor to contain several heterogeneous cell populations. Within these subpopulations, a random mutation can lead to the cell being able to resist apoptosis, excrete a chemotherapeutic or even become more radioresistant (Bertagnolli et al., 2009; Gryfe et al., 2000). Genomic instability can range from a single change in a nucleic acid to translocation of a whole arm of a chromosome (Lengauer et al., 1998; Schmitt et al., 2012). The genomic instability displayed in Hereditary non-polyposis colorectal cancer is an excellent example of genomic instability causing elevated levels of single nucleotide substitutions, deletions, and/or insertions (Kinzler and Vogelstein, 1996). As compared with the genomic instability displayed by Fanconi Anemia (FA) mutants in which the vast majority of aberrations are radials and gap/breaks (Joenje and Patel, 2001).

Chromosomal instability is seen in cancers more often than nucleotide level instability. Chromosomal instability results in changes in ploidy, translocations, deletions, amplifications, and inversions (Kinzler and Vogelstein, 1996). The human cancer cell lines U2OS and M059K, osteosarcoma and glial blastoma cell lines respectively, highlight this spontaneous genomic instability. As discussed in detail in chapter 4 of this dissertation both of these cell lines were analyzed for spontaneous chromosome aberrations. They both display changes in chromosome number, M059K has on average 115 chromosomes and U2OS has 63. The majority of the aberrations observed in M095K and U2OS were acentric fragments and deletions.

One of the most common forms of chromosomal instability observed in FA mutants are chromatid gaps/breaks. As described by Dr. Savage chromatid gaps/breaks form from unrepaired DSB occurring after DNA replication in late S or G2 (Harvey et al., 1997). As described in more detail below, DSBs can often arise from the faulty repair of interstrand crosslinks (ICL), which cells experience spontaneously every day. Despite the common occurrence of chromatid breaks/gaps, this form of instability will does result in a high rate of mutation. This is due to the fact that the daughter cell inheriting the gap/break will most often not survive due to the fact that the gap/break often results in a terminal deletion (Harvey et al., 1997; Savage, 1976). If two of these ICL occur on two neighboring chromosomes or on a single chromosome several different types of aberrations may arise, including radials, chromatid type deletions, and symmetrical/asymmetrical translocations, all of which are exchange type aberrations. Both radials and asymmetrical translocations will be lethal to one of the daughter cells and no mutation is inherited.

Genomic instability can occur spontaneously or can be induced by DNA damaging agents such as IR. Spontaneous genomic instability is often the result of a deficient DNA repair

pathway that results in the incorrect repair of normal cellular damage. The main difference between IR induced genomic instability as compared to spontaneous instability is that IR can induce instability in normal cells and does not require a faulty DNA repair pathway (Giles, 2001).

Radiation-induced Genomic Instability

Carcinogenesis is a multi-step process that often takes decades to result in a tumor. IR is a potent carcinogen because of its ability to not only create a large amount of damage in a single exposure, but also due to its ability to induce genomic instability in the progeny of exposed cells (Little, 2000; Little et al., 1997a). The phenomenon of IR induced genomic stability has been observed in both mouse and tissue culture models, after exposer to both high LET alpha and low LET X-ray radiation (Little et al., 1997a). Genomic instability observed after exposure to IR can vary from simple deaminations to the more lethal DSBs (Hoeijmakers, 2001; Suzuki et al., 2003; Ward, 1988). It's extremely interesting to note that it appears that DSBs alone do not appear to be enough to induce genomic instability (Morgan et al., 1998; Wojcik et al., 1996). IR has the ability to not only cause damage to the DNA but also alter gene expression and ultimately cellular homeostasis (Barcellos-Hoff and Brooks, 2001; Baverstock, 2000; Paquette and Little, 1994).

Spontaneous Genomic Instability

Genomic instability can be a naturally occurring process, most often in DNA repair deficient cells and cells lacking cell cycle regulation (Aguilera and Garcia-Muse, 2013). There are numerous inheritable cancer disorders that have been linked to elevated spontaneous genomic instability and nearly all are linked to DNA repair deficiencies include, for example, Breast Cancer susceptibility (BRCA1/2), Nijmegen breakage syndrome (NBS), Werner syndrome,

Bloom syndrome, and Fanconi Anemia (FA) (Kennedy and D'Andrea, 2006; Ripperger et al., 2009). In these syndromes, the cells fail to correctly repair naturally occurring DNA damage. This results in the accumulation of both SSBs and DSB, which then go on to form deletions, translocations and various other recombination events. In addition to DNA repair deficiencies resulting in genomic instability, it has also been reported that mutations in cell cycle regulation and caretaker genes also result in genomic instability. Two examples are mutations in the TP53 and ATM (Ataxia Telangiectasia mutated) genes, mutations in either one of the genes can result in the early onset of cancer in affected individuals (Cahill et al., 1998; Kinzler and Vogelstein, 1997).

As discussed prior, genomic instability is a major component in tumorgenesis. Individuals suffering from a genetic predisposition for genomic instability highlight this. In FA individuals the average age of cancer onset is 27 years, this is much older than the average onset of cancer in NBS/Bloom patients which is 15 years of age (German, 1997).

Objectives of Dissertation

The main objective of this dissertation is to increase understanding of the mechanisms and characteristics of spontaneous and radiation-induced inversions.

Chapter 2 of this dissertation details our efforts to understand how chromosomal inversions are effected by chromatin structure and radiation quality. CHO10B2 cells were synchronized in either G1 or M phase before being exposed to X-rays or Fe ions. The cells were then collected in the immediate M-phase post irradiation and the chromosomes harvested. In order to observe radiation-induced chromosomal inversions we modified the fluorescence plus Giemsa protocol utilized in earlier studies (Muhlmann-Diaz and Bedford, 1995). We cultured the CHO10B2 cells in the presence of BrdU for a single cell cycle before being exposed to radiation. By culturing the cells for a single cycle after irradiation without BrdU we were able achieve a

differential stain that allows us to identify chromosomal inversions formed after IR, during the cell cycle immediately prior to collection.

Chapter 3 of this dissertation details our studies investigating the role of various DNA repair pathways in the formation of radiation-induced chromosomal inversions. We utilized various CHO DNA repair mutants to investigate the role of the NHEJ, HR, and FA pathway in the formation of radiation-induced chromosomal inversions. The CHO cell lines where synchronized in G1 and exposed to either 0 or 2 Gy of γ-rays. Radiation-induced inversions and rings were observed in each cell line. Results demonstrated that when the NHEJ repair pathway is inhibited, there is a significant decrease in radiation-induced inversions, indicating that the NHEJ repair pathway is responsible for the formation of radiation-induced inversions. Additionally, it was observed that when the FA pathway is inhibited there is an increase in the number of radiation-induced inversions. This result leads us to believe that the FA pathway, when functioning properly, inhibits the formation of inversions following IR exposure.

Chapters 4 of this dissertation details our effort to show that chromosomal inversions occur spontaneously and are an important form of genomic instability in human FA mutants. In this study we utilized both primary and immortalized human FA mutant fibroblasts along with several human cancer lines that contain a mutation in a FA protein. As controls we used both primary and immortalized normal fibroblasts along with several FA positive human cancer lines and DNA repair mutants. This chapter deals with the investigation of spontaneously occurring chromosomal inversions so there was a need to use a staining technique that allowed us to visualize inversions formed at any point in the "life" of the cell line. To achieve this we used a single stranded probe that allowed for the visualization of a single chromatid, and more importantly the formation of an inversion at any point in the life of the cell line. These

chromosomal inversions could have been formed in the patient before the cell line was created or in the cell cycle immediately before collection. The cells were held in G1 with the use of isoleucine before being released and cultured in the presence of BrdU and BrdC for a single cycle. This chapter shows that FA mutants experience an elevation in spontaneous chromosomal inversions, relative to apparently normal human cells. This lower level seen in the "normal" control cells was also similarly low in all of the human cancer lines investigated. Lastly, we successfully clone a chromosomal inversion in a FA mutant cell line proving that the some if not all inversions observed in the FA were pre-existing and not formed immediately prior to chromosome collection.

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CHAPTER 2

ROLE OF LET AND CHROMATIN STRUCTURE ON CHROMOSOMAL INVERSION IN CHO10B2 CELLS

Introduction

Exposure to radiation often results in the formation of microscopically visible chromosome aberrations, the type of chromosome aberration observed often changes with the type of radiation the cell was exposed to and at what point in the cell cycle the cell was exposed. Here, we evaluated the effect of linear energy transfer (LET) and chromatin structure on the induction of chromosomal inversion in CHO10B2 cells. High LET radiation causes more complex DNA damage than low LET radiation, a type of damage more difficult to repair that may result in increased inversion formation. CHO10B2 cells were synchronized in either G1 or M phase and were exposed to 0 or 2 Gy of X-rays or 500 MeV/nucleon of initial energy and 200 keV/μ m Fe ion radiation. We modified the conventional Giemsa plus fluorescence technique so that cells were only allowed to incorporate BrdU for a single cycle verses 2 cycles prior to irradiation. The cells were then cycled for a single cycle without BrdU before being harvested. Each chromosome contains a single BrdU incorporated DNA strand and three strands without BrdU. The BrdU incorporated DNA strand was labeled using a BrdU antibody and an Alexa Fluor 488 probe. This modified technique allowed us to observe inversions smaller than 0.6 megabases (Mb) across all chromosomes. We have shown in this study that high LET radiation produced more small inversions then low LET radiation and that chromatin structure influences the size of inversions only when the cell is exposed to high LET radiation. It was predicted that 80% of observed chromosomes in G1 cells exposure to 2 Gy high LET radiation contained a true radiation-induced inversions as compared to only 31% of chromosomes in low LET exposed G1 cells. High LET exposed M phase cells were predicted to have a true radiation

induced inversion on 49% of the observed chromosomes, low LET exposed M phase cells were only predicted to have a true radiation-induced inversion on 16% of observed chromosomes. In addition to causing more inversions, high LET also produced smaller inversions then low LET radiation. High LET exposed G1 and M phase cells contained inversions that averaged 9.9 and 8.7 Mb in size respectively; whereas low LET exposure G1 and M phase cells had inversions with an average size of 13.34 and 12.02 Mb, respectively.

Background

Chromosome inversions are symmetrical chromosome aberrations that are an aberration that cause a rearrangement of the chromosome in which a segment of DNA is inverted and reinserted into the original chromosome. There are two types of chromosomal inversions: a pericentric inversion, one that involves the centromere, and a paracentric inversion, one that is located on a single arm of the chromatid. Since pericentric inversions involve the centromere they can often be detected with simple karyotyping with Giemsa staining if the breaks occur asymmetrically across the centromere (Davisson et al., 1981; de la Chapelle et al., 1974). Paracentric inversions, however, do not cause a visual change in the chromosome, causing them to be extremely difficult to detect with banding or differential staining techniques. The most current banding technique, mBAND, is effective at identifying both pre-existing and induced inversions, however this technique is prohibitively costly and limited to detecting only one chromosome at a time (de la Chapelle et al., 1974; Hada et al., 2007; Hande et al., 2003; Holmquist et al., 1982; Mitchell et al., 2004). It has been shown that high LET radiation, such as charged particle radiation, creates more complex DNA damage than low LET x-ray and gamma radiation. Such complex damage lends itself to an increase in chromosomal aberrations, including inversions (Hada et al., 2007; Hada and Georgakilas, 2008b; Pastwa et al., 2003; Ward,

1994). Inversions are a potentially important class of chromosome aberration because the cell undergoes a symmetrical recombination event with little loses of genomic material; this damage is easily transmissible leading to a potential mutation. Radiation-associated papillary thyroid cancer has been shown to result from a rearrangement of the RET gene due to an inversion; the common RET/PTC1 rearrangement is an inversion on chromosome 10 where RET and H4 are juxtaposed, two genes, normally 30 Mb apart and roughly separated by 1–3 μm. It was shown that following a single track of x-ray radiation the two loci were brought together (Nikiforova et al., 2000). Finally, it has been shown that inversions can cause genomic instability by causing a fragile site in the DNA that could lead to future DSBs or translocation (de Kok et al., 1995). This leads us to hypothesize that despite the fact little to no DNA information is lost; inversions have the potential to result in mutagenesis of the irradiated cells, whether through direct rearrangement of regulatory elements or creation of fragile sites. Aside from their association with cancer, inversions are thought to have played a key role in evolution of the primate genome, as 1,576 putative regions of inverted orientation, covering more than 154 Mb of DNA, have been identified (Feuk et al., 2005). Of these inversions, paracentric inversions represent the majority, highlighting their importance in speciation and evolution (Nickerson and Nelson, 1998).

In 2013 Bailey et al. utilized a directionally orientated single stranded probe to identify radiation-induced inversion on human chromosomes 3 and 10, an approach that facilitated visualization of inversions as small as 1 Mb (Ray et al., 2013). Here we altered the modified Giemsa plus Fluorescence (GPF) approach used by Muhlmann-Diaz and Bedford by incorporating BrdU for a single cell cycle then labeling the BrdU incorporated DNA strand with a BrdU antibody conjugated with Alexa Fluor 488, to observe inversions as small as 0.6 Mb, over all 21 chromosomes of the CHO10B2 genome. (Muhlmann-Diaz and Bedford, 1995). The

increased sensitivity allowed us to better appreciate the extent of radiation-induced inversions. We also have shown that both radiation quality and chromatin structure influence the number of inversions formed, as well as their size.

Materials and Methods

Cell lines

Chinese Hamster Ovary 10B2 (CHO10B2) cells were kindly supplied by Dr. Joel Bedford at Colorado State University (Fort Collins, CO). Cells were cultured in MEM-alpha (Gibco, Indianapolis, IN) supplemented with 10% fetal bovine serum (FBS, Sigma, St Louis, MO) and 1% antibiotics and antimycotics (Gibco), and maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. The CHO10B2 cells were cultured for one cell cycle (12 hours) with 1 μM BrdU (Sigma) to ensure uniform incorporation into newly synthesized DNA and then harvested either in the G1 or M phase of the cell cycle by mitotic shake off (Bailey and Bedford, 2006; Littlefield et al., 1979; Wolff et al., 1974). CHO10B2 cells were chosen due to their short division time and ease of synchronizing populations into either G1 or M phase.

Synchronization

Cells were synchronized into either G1 or M phase via mitotic shake-off; only cells with a mitotic index of 90% or higher were used (Fox et al., 1987; Sinclair and Morton, 1965; Terasima and Tolmach, 1961). For collection of G1 synchronized cells, mitotic cells were incubated for 2 hours at 37°C to allow cells to proceed from M phase to G1. For collection of M phase synchronized cells, mitotic cells were collected immediately prior to irradiation and transferred into pre-warmed T25 flasks and irradiated.

Irradiation sources

Cells were irradiated with X-rays using a TITAN x-ray generator (Shimadzu, Tokyo, Japan) outfitted with 5 mm Al and Cu filters at 200 kVp and 20 mA. The dose rate was approximately 1 Gy/min for X-ray. Cells were also irradiated with accelerated iron-ions at HIMAC (Heavy Ion Medical Accelerator in Chiba), the National Institute of Radiological

Sciences in Chiba, Japan, which had 500 MeV/nucleon of initial energy and an LET of 200 keV/μm.

Metaphase chromosome preparation

Cells were sub-cultured immediately after irradiation and 0.1 μg/ml of colcemid was added to the flask of cells for 10 hours later to block mitosis. Cells were harvested during the first post-irradiated metaphase. Cells were trypsinized and suspended in 6 ml of a 75 mM KCl solution warmed to 37°C and placed in a 37°C water bath for 20 minutes. Carnoy's solution (3:1 methanol to acetic acid) was added to the samples according to the standard protocol. The fixed cells were dropped onto slides. These were set aside and allowed to dry until the Carnoy's solution had evaporated, roughly 4–5 minutes (Cartwright et al., 2013).

Staining

Chromosomes where denatured for 3 minutes in an 80°Celsius 70% formamide in 2x saline-sodium citrate (SSC) solution than washed in 2x SSC for 10 minutes (Muhlmann-Diaz and Bedford, 1995). The chromosomes where stained with 1/1000 anti-BrdU antibody (BD Biosciences, San Jose, CA) for 2 hours and then a secondary Alexa Fluor 488 (Invitrogen, Washington, D.C.) antibody was applied for 2 hours. Chromosomes were counter stained with Prolong Gold Antifade containing 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen).

Image analysis

An Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a Q-imaging Aqua cooled CCD camera (Q-imaging, Surrey, BC, Canada) was used for image capture. DAPI and anti-BrdU signals where merged using ImageJ software (National Institute of Health, Maryland, USA).

Measurements of inversions

The size of individual inversions was determined using Velocity (PerkinElmer, Waltham, MA) image analysis software. The illuminated pixels of each inversion and the total illuminated

pixels in all 21 chromosomes in each cell were measured using Velocity as well. To determine the size of the inversion we compared the total number of illuminated pixels to the CHO genome size, roughly 4.9 Gb, to determine the number of basepairs per pixel (Xu et al., 2011). Using the number of basepairs per pixel we then estimated the number of basepairs in each inversion using the total number of pixels the inversion contained.

Statistical analysis

Statistical comparison of mean values was performed using a two tailed t-test. Differences with a P-value of <0.05 were considered statistically significant. Error bars indicate the standard error of the means. Confidence interval values were calculated by Prism 5™ software (GraphPad, La Jolla, CA, USA). Induction rates were considered statistically similar if the slope fell within the 95% confidence interval of compared slope.

Classification of aberrations

Inversions were categorized into two groups, total inversions and micro inversions. Micro inversions are a subset of the total inversions. Any inversion smaller than the width of a chromatid, roughly 15 Mb in size, was considered a micro inversion.

Results

Validation of BrdU staining protocol

Initially, we had to differentiate between true and false inversion. As seen in panel A of Figure 1, true inversions are created during the G1 phase of the cell cycle when cells were irradiated. Panel B of Figure 1 shows how two sister chromatid exchange (SCE) events can cause a false inversion; these look exactly the same as a true inversion when imaged. One way to discriminate between true and false inversions is to calculate the predicted number of false inversions caused by background levels of SCE. It was noted that the background level of total inversions, both true and false, was roughly two exchanges per cell. We attempted to account for the background level of false inversion by calculating the likelihood of a chromosome

Table 2.1: The Predicted False and Spontaneous True Inversions at 0 Gy

	G1 X-Ray	**M X-Ray**	**G1 Fe**	**M Fe**
Average Exchanges per Cell	10.07 ±0.59	10.78 ±0.54	9.13 ±0.47	8.87 ±0.67
1 SCE per Chromosome	0.48 ±0.03	0.51 ±0.03	0.43 ±0.02	0.42 ±0.02
Predicted 2 SCE per Cell	1.49 ±0.23	1.66 ±0.24	1.29 ±0.15	1.23 ±0.16
Observed 2 SCE per Cell	2.21 ±0.08	2.04 ±0.15	1. 82 ±0.08	1.77 ±0.10
Predicted 2 SCE per Cell within 15Mb	0.19 ±0.02	0.21 ±0.05	0.17 ±0.03	0.16 ±0.02
Observed 2 SCE per Cell within 15Mb	0.40 ±0.60	0.48 ±0.05	0.51 ±0.20	0.49 ±0.8
Predicted True Inversions within 15 Mb	0.21 ±0.08	0.27 ±0.05	0.37 ±0.08	0.36 ±0.15

having one SCE within a chromosome, two SCE within a chromosome, or two SCE events within 15 Mb of each other on a single chromatid. The R-value is the average number of SCE events per chromosome. Using this we calculated the cumulative frequency, 0.102, and calculated the predicted number of SCE events per 21 chromosomes. To calculate the predicted number of two SCE events within 15 Mb, we averaged the chromosome size, 234 Mb, and determined there was a likelihood, 30/232 chance, of having a second SCE event within 15 Mb of the first. We multiplied these odds against the odds of two SCE occurring on a single chromatid to estimate the predicted value of two SCE within 15 Mb. It was noted that the predicted number of false inversions was slightly lower than the observed values; this indicates that we are underestimating false inversions leading to an over estimate of true inversions. We believe that this finding is negligible due to the fact that false inversions were equally underestimated for each group. Based on these calculations, we have shown that the use of a Poisson calculation can accurately predict the number of false total false inversions, but also the false micro inversions by the use of the average SCE events per cell. As seen in Figure 2 the average number of SCE per cell increased when cells were exposed to 2 Gy or both X-rays and Fe ions. Due to this increase in SCE we had to calculate the predicted number of false inversions, this is shown in table 2. The values from table 2 allowed us to quantify the true number of inversions and micro inversions.

Effect of radiation quality and chromatin on Induction of chromosomal inversion

To assess any influence of radiation quality or chromatin on induction of inversions, we analyzed radiation-induced inversions following 2 Gy of Fe ion or X-ray exposure in both G1 and M phase cells. In Figure 1C and D, we see a 21 chromosome CHO10B2 spread stained using our single cycle BrdU and antibody approach. The arrows indicate inversions, which we

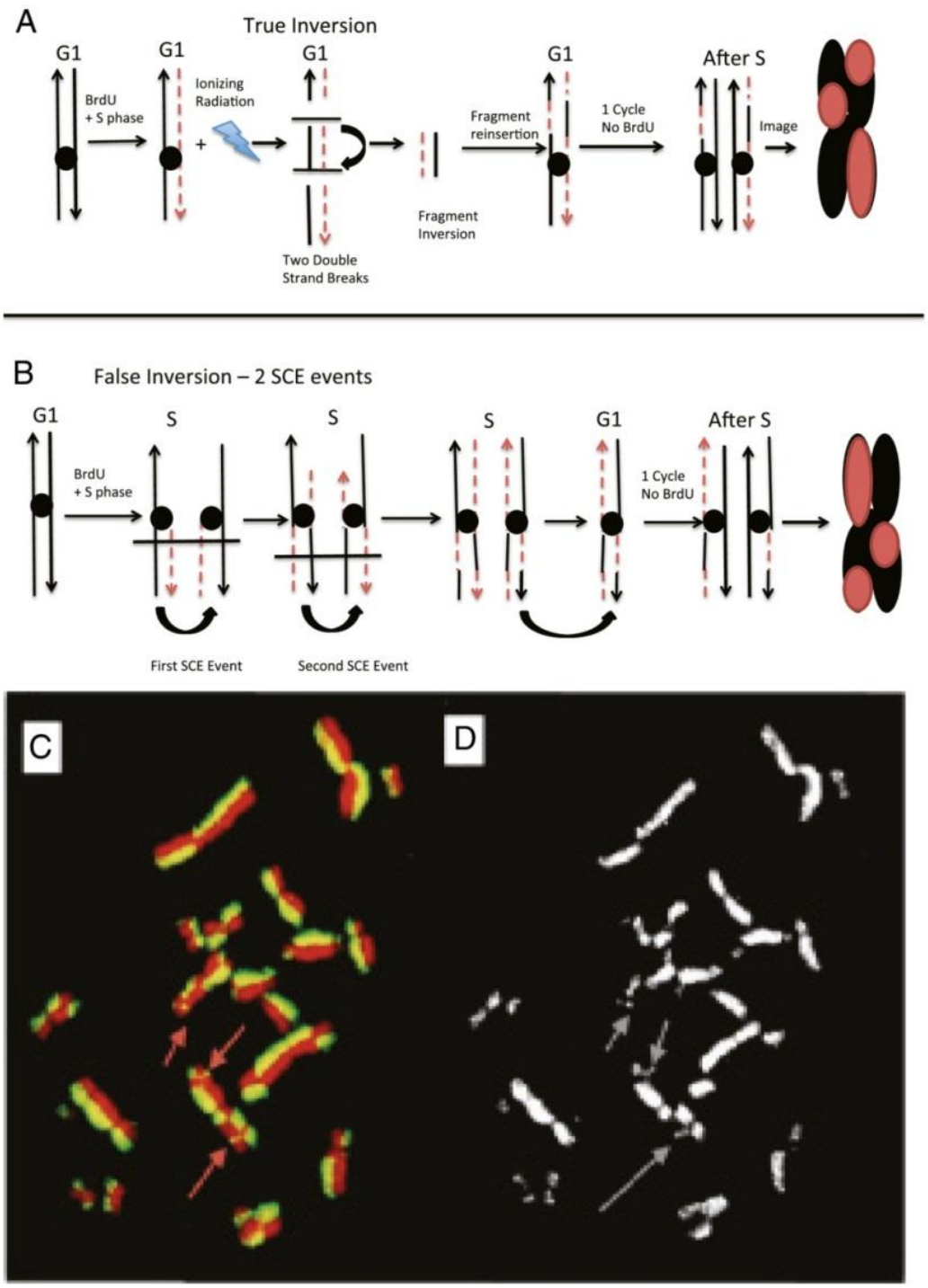

Figure 2.1: *Panel **A** depicts how a true inversion is formed. Panel **B** depicts how 2 SCE events can cause a false inversion. Panel **C**. depicts a 21 chromosome CHO10B2 spread exposed to 2 Gy Fe ion radiation and stain with DAPI (pseudo-colored red) and Anti-BrdU (green). The arrows indicate micro inversions, which are considered true inversions; roughly 1–10 Mb. Panel **D**. is the black and white image of the Anti-BrdU image used to measure inversions pixel intensity.*

classified as micro inversions. To determine the radiation-induced inversions at 2 Gy we simply subtracted the false inversions calculated in Table 2 from the overall radiation-induced inversions, this was done for both total and micro inversions. As seen in Figure 3A, the total number of radiation-induced inversions was statistically similar for both G1 and M phase cells following Fe ions and X-ray. The difference between Fe ions and X-rays becomes apparent when observing the number of radiation-induced micro inversions. As seen in Figure 3B, Fe ions induced more micro inversion in G1 cells than X-rays both M phase and G1 cells, a p-value of 0.0001 and 0.0001 respectively. G1 cells exposed to Fe ions also produced statistically more micro inversions than M phase cells at the same dose, a p-value of 0.0064. Additionally, we observed that the M phase Fe ion exposed cells produced statistically more micro inversion than M phase cells exposed to X-rays, a p-value of 0.0079. Both G1 and M phase cells exposed to X-rays had statistically similar induced inversions, a p-value of 0.381.

Analysis of inverted fragment sizes

In an effort to better characterize the induced inversions we utilized Volocity software to quantify the size of the inversions. Inversions were observed as small as 0.6 Mb and as large as 90 Mb in cells exposed to 2 Gy of radiation. As seen in Figure 4 radiation-induced inversions in the Fe ion exposed cells appeared to be smaller in size then the radiation-induced inversions in the X-ray exposed cells. To better understand the role of LET on the size of induced inversions, inversions smaller than 30 Mb were analyzed. As seen in Figure 5 the differences between Fe ion exposed and X-ray exposed cells becomes more evident. Induced inversions in both G1 and M phase Fe ion exposed cells were shown to be statistically smaller than the inversions formed in X-ray exposed cells (P value of 0.0036 and 0.0027 for G1 and M phase respectively). The average size of inversions smaller than 30 Mb for X-ray G1 exposed cells was 13.34 Mb and for

M phase cells it was 12.02. When compared to Fe exposed cells the observed inversions averaged 9.9 and 8.7 Mb for G1 and M phase cells respectively. Upon analyzing the inversion sizes of both Fe ion exposed G1 and M phase cells we found the largest variation between the cell cycle phases was seen in fragments smaller than 15 Mb. We observed that M phase exposed cells produced statistically smaller inversions than G1 exposed cells.

Discussion

It can be seen in our study that both the LET of the radiation and chromatin structure play a role not only in the induction of chromosomal inversion, but also in the size of the induced inversions. Our modified staining protocol has allowed inversions to be observed on a level not seen in previous studies. To account for inversions observed at 0 Gy we used Poisson Distribution to show that most of these inversions were actually two SCE events occurring on a single chromatid within a close distance to one another. As seen in Table 1 the Poisson distribution was unable to account for all observed inversions at 0 Gy, to ensure that this underestimate was taken into account we subtracted the predicted true micro inversions at 0 Gy from the predicted radiation-induced true micro inversions at 2 Gy, as seen in Table 2, and we still see the same results as we do when we just address the predicted radiation-induced true micro inversions. Our data strongly correlates with prior observations, with the exception of one prior study. In this paper, it was noted that radiation-induced interstitial exchanges were formed primarily by true SCE events (Wojcik et al., 1999). Based on our observation of micro inversions, aberrations that were undetectable in this earlier study, and results from several other studies we believe that the majority of radiation-induced inversions are in fact true inversions

Table 2.2: Expected and Observed Number of Inversions in Cells Exposed to 2Gy

	G1 X-Ray	**M X-Ray**	**G1 Fe**	**M Fe**
Average Exchanges per Cell	14.67 ±0.48	14.16 ±0.52	15.06 ±0.49	13.54 ±0.45
1 SCE per Chromosome	0.07 ±0.02	0.67 ±0.02	0.72 ±0.02	0.64 ±0.02
Predicted 2 SCE per Cell	2.55	2.43	2.64	2.29
Observed 2 SCE per Cell	4.21 ±0.45	3.81 ±0.41	4.36 ±0.17	3.85 ±0.21
True Total Inversions	1.66 ±0.45	1.37 ±0.41	1.72 ±0.17	1.56 ±0.21
Predicted 2 SCE per Cell within 15Mb	0.33	0.31	0.34	0.30
Observed 2 SCE per Cell within 15Mb	0.85 ±0.09	0.74 ±0.06	1.51 ±0.22	1.15 ±0.10
True Micro Inversions	0.52 ±0.09	0.43 ±0.06	1.17 ±0.22	0.85 ±0.01
Background Corrected True Micro Inversions	0.31 ±0.08	0.16 ±0.05	0.80 ±0.05	0.49 ±0.08

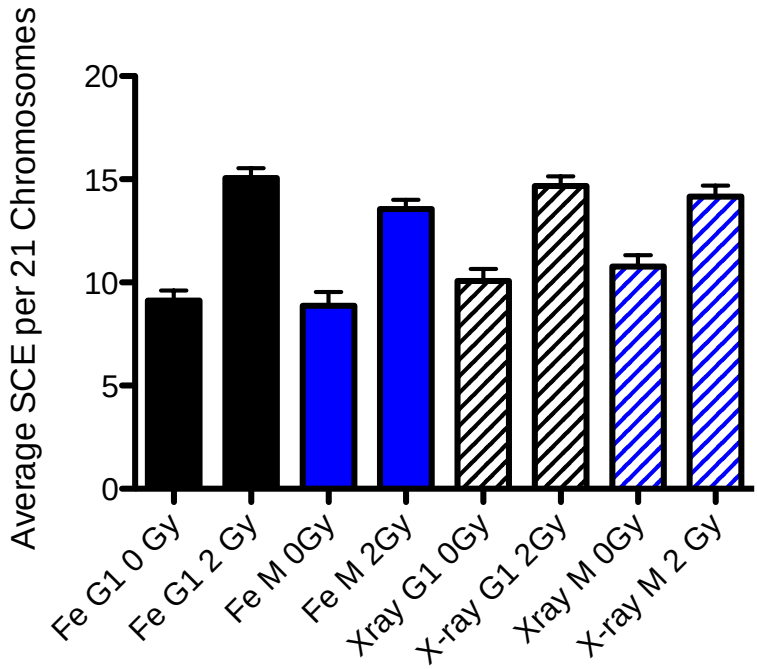

Figure 2.2: *This figure depicts the average number of SCE events observed in both G1 and M phase cells exposed to 0 or* 2 Gy of *200 keV/µm Fe ions or 200 kVp X-ray. The error bars are the standard error of the mean.*

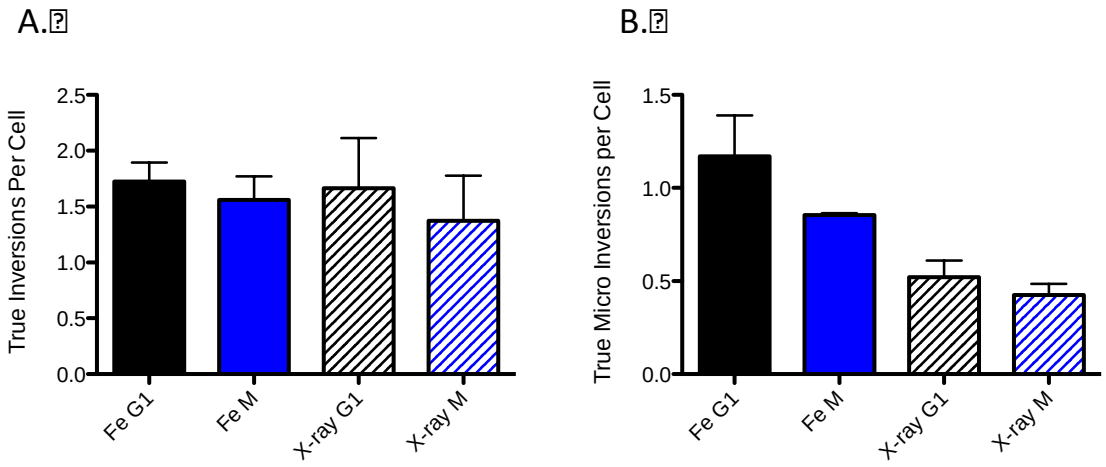

Figure 2.3: *This figure depicts the average true inversions in panel **A** and the average true micro inversions in panel **B** observed in both G1 and M phase cells exposed to 2 Gy of 200 keV/µm Fe ions or 200 kVp X-ray. The error bars are the standard error of the mean.*

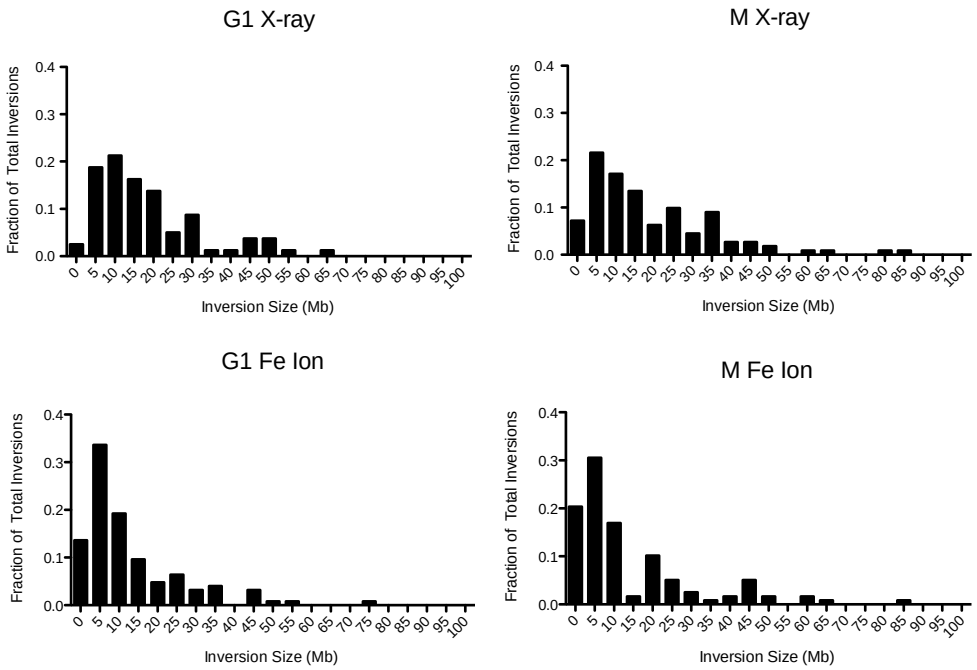

Figure 2.4: *This figure depicts G1/M phase cells exposed to 2 Gy of either X-ray or Fe ions. The value of 0 indicts values 0–5 Mb.*

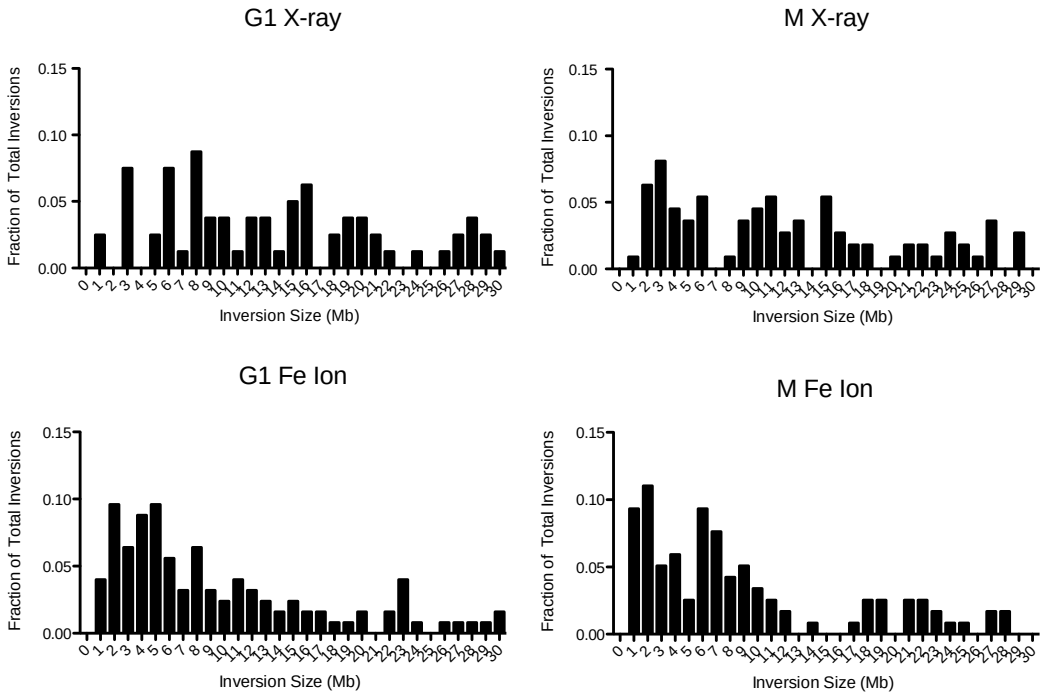

Figure 2.5: *This figure depicts inversions smaller than 30 Mb in size in G1/M phase cells exposed to 2 Gy of either X-ray or Fe ions. The value of 0 indicts values 0–1 Mb.*

and not caused by 2 SCE events. These findings highlight the importance of these micro inversions and additionally support the idea that ionizing radiation do in fact produce inversions.

This study has shown that the size and number of radiation-induced inversions are affected by both the LET of the radiation and the chromatin structure of the DNA. It appears that high LET radiation, specifically Fe ions, results in inversions whose size and number are directly dependent on chromatin structure; this observation was not seen in cells exposed to low LET X-rays. Additionally, we found that high LET radiation was more effective at inducing inversions than the low LET radiation. Finally, the staining protocol utilized in this study enabled observations of inversions smaller than previously reported, allowing accurate recording of the number of induced inversions and to avoid the background level of false inversions.

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CHAPTER 3

ROLE OF VARIOUS DNA REPAIR PATHWAYS IN CHROMOSOMAL INVERSION FORMATION IN CH0 MUTANTS

Introduction

Cells have evolved extremely intricate and complex cellular pathways to sense and repair DNA damage, there are unique pathways for specific forms of DNA damage. Here, we evaluated the role of the two main DNA double strand break repair pathways, the non-homologous end joining (NHEJ) and homologous recombination (HR) pathway, and the Fanconi Anemia (FA) pathway, responsible for the repair of interstrand crosslinks, on the formation of radiation-induced chromosomal inversions. Various Chinese Hamster Ovary (CHO) wild type (CHO10B2), NHEJ (XRS5, V3, XR-1), HR (51D1, irs1SF), and FA mutant (KO40) cells were synchronized into the G1 stage of the cell cycle and exposed to 0 or 2 Gy of gamma radiation. A single cycle 5-ethynyl-2'-deoxyuridine (EdU), a thymidine analogue, differential stain protocol and Poisson distribution were utilized to identify true radiation-induced inversions. The background number of expected true inversions per cell for CHO wild type was 0.35. At 2 Gy of gamma-rays, 1.81 inversions per cell were formed in CHO wild type. The NHEJ mutants displayed roughly a 50% decrease in radiation-induced inversions when compared to the controls, whereas the FA mutants had an approximately 20% increase in radiation-induced inversions. KO40 showed the highest number of spontaneous inversion among all of the mutant cell lines. The HR mutants showed similar number of radiation-induced inversion as the wild type cells. Gene restored V3 mutants showed increased radiation-induced inversions and gene restored KO40 decreased radiation-induced inversions compared to their gene mutated cells. We concluded that NHEJ repair contributes to radiation-induced inversion formation by the

misrejoining of two DNA double stand breaks. Additionally, the FA damage response pathway, which is responsible for the repair of DNA interstrand crosslinking, prevents the formation of spontaneous and radiation-induced inversions through an unknown mechanism.

Background

Eukaryotic cells have developed advanced repair mechanisms to combat the daily damage each cell experiences, these repair machinery is suited to handle the limited number of double strand breaks (DSB) formed during S-phase, these DNA repair systems are not ideally suited to handle the massive number of DSBs incurred after exposure to IR (Jackson and Bartek, 2009). One Gy of IR can produce roughly 20-40 DSB per cell. Eukaryotic cells possess two major pathways to repair DSBs, the non-homologous end joining (NHEJ) and the homologous recombination (HR) repair pathway. NHEJ repairs DSBs extremely quickly and works in both the G1 and S/G2 phase of the cell cycle; it does, however, lack a proof reading mechanism to ensure that it is connecting the correct broken ends together. The HR pathway utilizes the homologous chromatid and in certain situations the homologous chromosomes as a template to repair DSBs, however, this process can take extended time to complete repair and only functions in S/G2 (Hinz et al., 2005; Ira et al., 2004; Scully et al., 1997; Takata et al., 1998; Willers et al., 2000). The Fanconi Anemia (FA) complex of proteins has been shown to be essential for repair of interstrand crosslinks (ICL) (German et al., 1987; Rogatko and Auerbach, 1988). It has also been shown that FA proteins also co-localize and associate with repair proteins from the nucleotide excision repair (NER), HR, and the translesion synthesis. It has been noted that loss of any FA protein in the core complex or FANCD2 results in a mild radiosensitivity (Bogliolo et al., 2007; Garcia-Higuera et al., 2001; Hussain et al., 2004; Kook, 2005; Mosedale et al., 2005; Nakanishi et al., 2002; Taniguchi et al., 2002; Wang et al., 2004). Misrepair of DNA by both the

NHEJ and HR pathway result in the formation of chromosomal aberrations. As described by Savage et. al there is a huge range of structure aberrations resulting from one or several DSBs. When two DSBs occur on a single chromosome at relatively the same time a broken fragment can be created. These fragments have several fates, they can be reinserted in to the DNA resulting in a symmetrical rearrangement, an inversion, or they can remain outside of the chromosome and form a true centric/acentric ring (Savage, 1976). It is believed that most inversions are formed after G1 irradiation and are simply the result of the NHEJ pathway rejoining the ends incorrectly, in the reverse orientation (Richardson and Jasin, 2000; Simsek and Jasin, 2010).

Inversions, like many other symmetrical chromosomal rearrangements, lead to a recombination event in which a segment of DNA several Kb to Mb in size is inverted and reinserted into the originating chromosome. In germ cells undergoing meiosis, a pericentric inversion can cause a structural change between homologous chromosomes and this can inhibit both correct segregation of the homologous chromosomes and recombination (Kirkpatrick, 2010). Additionally, pericentric inversions can lead to inhibition of recombination in non-germ cells as well (Kirkpatrick, 2010). Due to the fact that only pericentric chromosomal inversions cause a visible change in chromosome morphology, they have been extremely difficult to study without the use of banding or differential stains (Savage, 1976; Trask, 2002). Despite the fact that most inversions appear to be benign do to the fact that no genetic information is lost, it has been documented in previous research that a type of radiation-induced thyroid cancer is caused by an inversion that creates a RET/PTC fusion protein. The two break points are roughly 30 megabases (Mb) apart and separated by 1-3 microns in G1 (Nikiforova et al., 2000). It has also been shown that inversions can form a fragile site in the genome that could lead to further

breaks/translocations and that the presence of repetitive DNA sequences may suggest that the HR pathway is involved in the formation of an inversion (de Kok et al., 1995).

Traditionally, inversions could only be observed using a Giemsa banding, a differential Giemsa stain or an advanced fluorescence mBAND technique (Hada et al., 2007; Muhlmann-Diaz and Bedford, 1995). The pitfalls with these techniques include low resolution and high cost and difficulty. Giemsa and fluorescence BANDing have a minimal detection of inversions roughly 20 Mb or larger based on the size of the bands. In addition to limited sensitivity, a differential Giemsa staining cannot visually differentiate between an inversion and two sister chromatid exchanges (SCE). The combination of these difficulties has limited the study of inversions. Here, we utilized a 5-ethynyl-2'-deoxyuridine (EdU) based differential fluorescent staining technique to identify radiation-induced inversions in the first mitosis post irradiation. In our previous study, we demonstrated that a BrdU differential fluorescent staining technique could reliably identify radiation-induced inversions as 1 Mb (Cartwright et al., 2014). Our EdU based approach does not require denaturing of the DNA, allowing the chromosome to maintain its structure for better visualization of inversions. Repair pathways responsible for the formation of radiation-induced inversions were investigated and it was shown that inhibition of the NHEJ pathway resulted in a decrease in overall radiation-induced inversions and that inhibition of the FA pathway resulted in an overall increase in spontaneous and radiation-induced inversions.

Material and Methods:

Cell Lines:

CHO10B2, V3, XR-1, XRS5, irs1SF, 51D1, KO40, XRS5 corrected, 51D1 corrected, and KO40 corrected cells were kindly supplied by Dr. Joel Bedford at Colorado State University (Fort Collins, CO) and Dr. Larry Thompson at Lawrence Livermore National Laboratory

(Livermore, CA) (Fuller and Painter, 1988; Hinz et al., 2006; Jeggo and Kemp, 1983; Rubin and Whitmore, 1987; Stamato et al., 1983; Tebbs et al., 2005). Specific gene defects and affected pathways for each cell line is outlined in Table 1. Cells were cultured in α-MEM (Gibco, Indianapolis, IN) supplemented with 10% fetal bovine serum (FBS, Sigma, St Louis, MO) and 1% antibiotics and antimycotics (Gibco), and they were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. The wild type and mutant CHO cells were cultured for one cycle with 1 μM EdU (Sigma) for 12 hours to ensure uniform incorporation into newly synthesized DNA and then harvested in the G1 phase of the cell cycle by mitotic shake off (Fox et al., 1987; Sinclair and Morton, 1965; Terasima and Tolmach, 1961).

Synchronization:

To achieve cell cycle synchronization, cells were manually shaken off to harvest loosely attached mitotic cells (Fox et al., 1987; Sinclair and Morton, 1965; Terasima and Tolmach, 1961). Once a mitotic index of at least 90% was achieved, the cells were plated into pre-warmed T12.5 flasks and incubated for 2 hours before they were irradiated.

Irradiation Sources:

Cells were irradiated with gamma-ray radiation using a J. L. Shepherd Model Mark I-68 ~1500Ci Cs-137 source at a dose rate of 2.5 Gy per min at room temperature at Colorado State University.

Metaphase Chromosome Preparation:

Cells were cultured for 12-16 hours after irradiation before being cultured in the presence of 0.1μg/ml of colcemid (Gibco) for 2 hours before being collected in the first mitotic phase. Cells were trypsinized and suspended in 6mL 75mM KCl solution warmed to 37°C and placed in

Table 1: The characteristics of CHO mutant cell lines.

Cell lines	Gene mutations	Mutated Proteins	Affected pathway
CHO10B2	wild type	wild type	wild type
V3	*prkdc*	DNA-PKcs	Non homologous end joining
XR-1	*xrcc4*	XRCC4	Non homologous end joining
XRS5	*xrcc5*	Ku80	Non homologous end joining
51D1	*rad51d*	Rad51D	Homologous Recombination
irs1SF	*xrcc3*	XRCC3	Homologous Recombination
KO40	*fancg*	FANCG	Fanconi Anemia Signaling
V3 restored	a null mutant complimented with human *PRKDC*		
51D1 restored	a null mutant complimented with hamster *rad51d*		
KO40 restored	a null mutant complimented with hamster *fancg*		

a 37°C water bath for 20 minutes. Carnoy's solution (3:1 methanol to acetic acid) was added to the samples according to the standard protocol. Slides were placed in ice water and allowed to chill. The cell solution was dropped onto the cold slides. These were set aside and allowed to dry until the Carnoy's solution had evaporated, roughly 4-5 minutes (Cartwright et al., 2014).

Staining:

Chromosomes where fixed for 15 minutes in a 4% paraformaldehyde. The chromosomes where then submerged in 0.2% triton in phosphate buffered saline (PBS) for 10 minutes and briefly washed in PBS. EdU labeled cells were stained utilizing a Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Invitrogen, Carlsbad, CA). The chromosomes where counter stained with ProLong® Gold Antifade containing 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen).

Image Analysis:

Images were captured with a Zeiss Axioskop microscope outfitted with a motorized Z-stage, Metamorph system (Carl Zeiss AG, Jena, Germany), and a CoolSNAP HQ2 camera (Photometrics, Tucson AZ). Images were combined using ImageJ software (National Institute of Health, Maryland, USA). At least 50 metaphase spreads were analyzed for each data point in triplicate.

Statistical analysis:

Statistical comparison of mean values was performed using a *t*-test or a one way ANOVA when comparing groups. Differences with a p-value of <0.05 were considered to indicate a statistically significant. Error bars indicate standard error of the means. 95% confidence interval values were calculated with Prism 5™ software (GraphPad, La Jolla, CA, USA).

Results

Differentiating True and False Inversions

Figure 1 depicts a CHO10B2 metaphase chromosome spread with EdU differential staining. Cells were cultured with EdU for one cell cycle before exposure to 2 Gy gamma-rays at G1 and cultured without EdU until the first mitosis. It is not standard post radiation exposure two cycle EdU incorporated metaphase spread for standard SCE analysis. Arrows indicate two SCEs of several different sizes. These could be either a true or false inversion. A false inversion is simple 2 SCE events on a single chromosome. Yields of false inversions were predicted using a Poisson distribution, $p(x) = \frac{\lambda^x e^{-\lambda}}{x!}$, where λ = the SCE per chromosome and x = the variable. Table 2 shows the predicted likelihood of having at least two SCE within a chromosome from observed SCE per chromosome and observed 2 SCE per chromosome. We calculated the likelihood of at least two SCE within a chromosome to account for the total inversions (true and false inversions). As seen in Table 2 the observed 2 SCE values for all cell lines except KO40 and V3 restored cells were statistically similar for the predicted 2 SCE values. Therefore, we assume spontaneous SCE formation is randomly occurring on the chromosomes. FA signal pathway mutant showed spontaneously higher true inversion formation than other cells and this phenotype was rescued by gene restoration.

Figure 2A shows both the spontaneous and radiation-induced SCE in all cell lines. It was shown that both the control CHO10B2 cells and the NHEJ mutants all had a statistically significant ($p<0.05$) but small increase in observed SCE events at 2 Gy. The HR mutants showed low background SCE and no increase in radiation-induced SCE

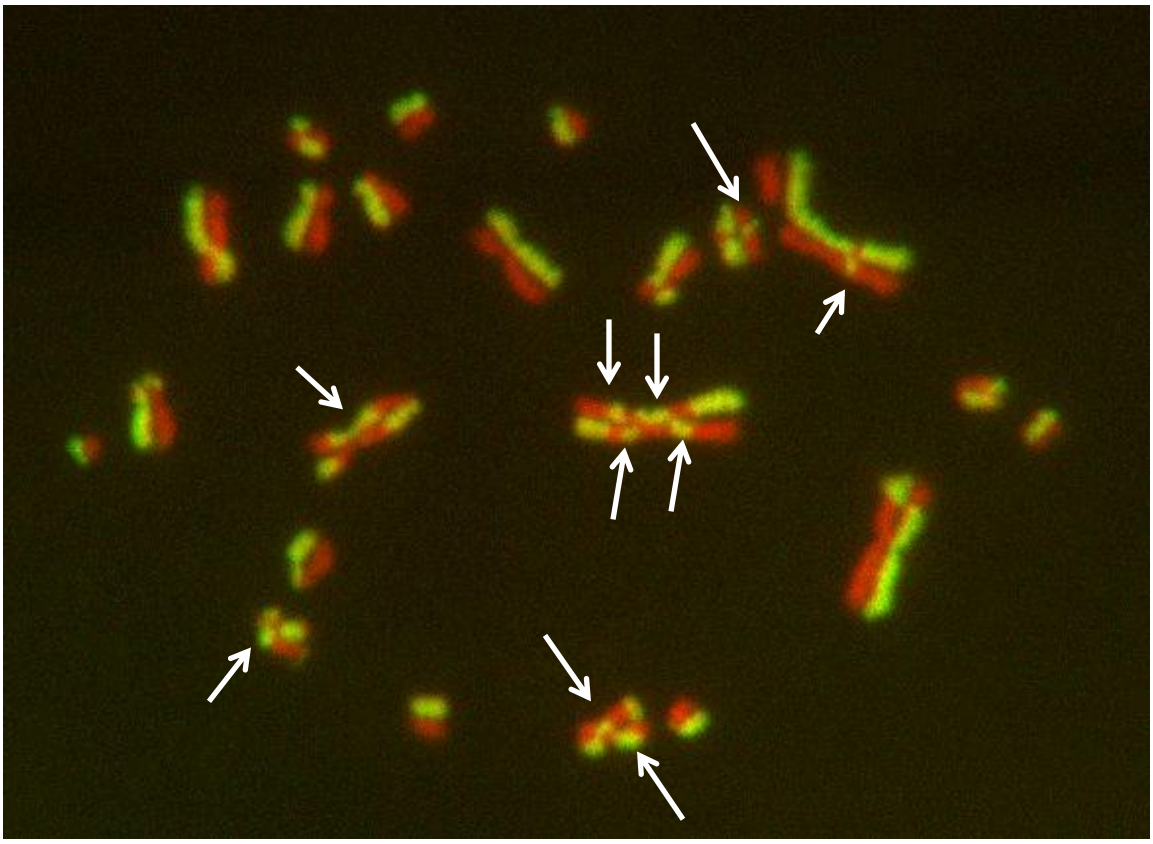

Figure 1: Differential staining for radiation induced inversion detection.
The figure depicts a CHO10B2 spread after exposure to 2 Gy stained with an EdU differential stain. EdU incorporated chromatids are visualized with yellow color and non-EdU chromatids were visualized with red color (pseudo-color). Arrows indicate two SCE per chromosome. They can be true inversions or false inversions from two SCEs.

events between the unirradiated and irradiated cells. FA mutants showed high background SCE and no increase in SCE events between the unirradiated and irradiated cells.

Figure 2B shows the observed spontaneous and radiation-induced two SCEs per chromosome. This number is a sum of true inversions and false inversions from two SCEs. All cells showed increased in two SCE per chromosome after exposure to 2 Gy of gamma-rays. The same Poisson distribution calculations were applied using the SCE values from cells exposed to 2 Gy, these values are shown in Table 3. True inversions were inversions above the predicted false inversion levels. Radiation-induced inversions were obtained from the number of 2 Gy exposed true inversions subtracted from the number of spontaneous true inversions.

Effects of DNA Repair Inhibition on Inversion Formation

Figure 3A shows the true radiation-induced inversions in each cell line. CHO wild type cells showed 1.8 true inversions after exposure of 2 Gy. It was observed that NHEJ mutants experienced an approximately 50% decrease in true radiation-induced inversions, P values < 0.05. There was no statistical change in radiation-induced true inversions in HR mutants, P values > 0.05. Finally, it was observed that there was an approximately 20% increase in radiation-induced true inversions in the FA mutant KO40 cells.

Restoration of Wild Type Phenotype

To confirm that the mutated DNA repair/damage response protein was responsible for the changes in radiation-induced inversion formation we utilized restored V3, 51D1, and KO40 cell lines. The gene complemented cell lines were exposed to 0 or 2 Gy of IR and the number of radiation-induced inversions was observed for each cell line. Figure 3A shows that when cell lines were restored the number of radiation-induced true inversions returned toward the CHO10B2 WT. V3 restored cells showed increased in radiation-induced inversions.

Table 2: Expected and Observed Number of Background Inversions. +/- is the SEM.

Cell Line	CHO10B2	V3	XR-1	XRS5	51D1	irs1SF	KO40	V3 Restored	51D1 Restored	KO40 Restored
Observed SCE per cell	13.62 ±0.68	14.24 ±0.71	9.10 ±0.51	11.46 ±0.50	10.87 ±0.48	8.35 ±0.75	14.62 ±0.90	15.21 ±0.99	13.19 ±0.74	12.16 ±0.47
Observed SCE per chromosome	0.65 ±0.03	0.68 ±0.03	0.43 ±0.02	0.55 ±0.02	0.52 ±0.02	0.40 ±0.04	0.70 ±0.04	0.72 ±0.05	0.63 ±0.04	0.58 ±0.02
Predicted 2 SCE within a chromosome per cell	2.9 ±0.23	3.12 ±0.24	1.49 ±0.15	2.20 ±0.16	2.01 ±0.15	1.28 ±0.20	3.25 ±0.32	3.46 ±0.35	2.76 ±0.25	2.42 ±0.15
Observed 2 SCE within a chromosome per cell	3.25 ±0.29	3.45 ±0.29	1. 7 ±0.21	2.37 ±0.22	2.00 ±0.18	2.15 ±0.18	4.76 ±0.54	4.58 ±0.36	3.49 ±0.27	2.70 ±0.19
Expected true inversions per cell	0.35 ±0.23	0.33 ±0.45	0.21 ±0.12	0.17 ±0.04	-0.01 ±0.03	0.87 ±0.29	1.51 ±0.76	1.12 ±0.53	0.43 ±0.10	0.32 ±0.21

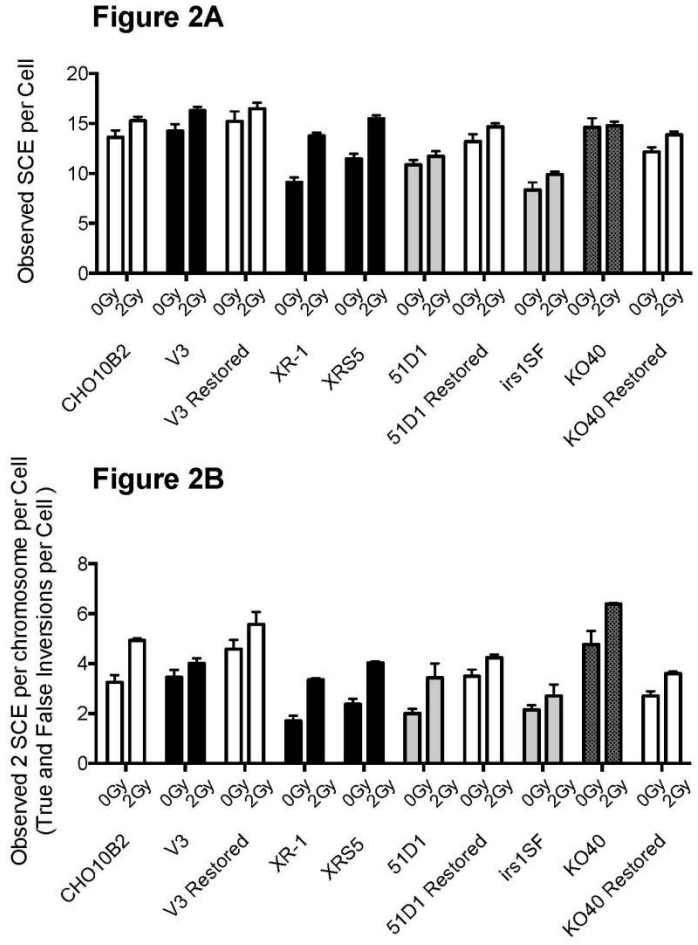

Figure 2: Radiation induced one and two SCEs in CHO wild type and mutant cells*. A) The number of spontaneous and radiation induced SCE per cell.*

B) The number of spontaneous and radiation induced two SCE per chromosome per cell. It contains true (inversions) and false (two SCEs) events. 50 cells in three independent experiments, for a total of 150 cells, were analyzed. Error bars indicate the standard error of the means of three independent experiments.

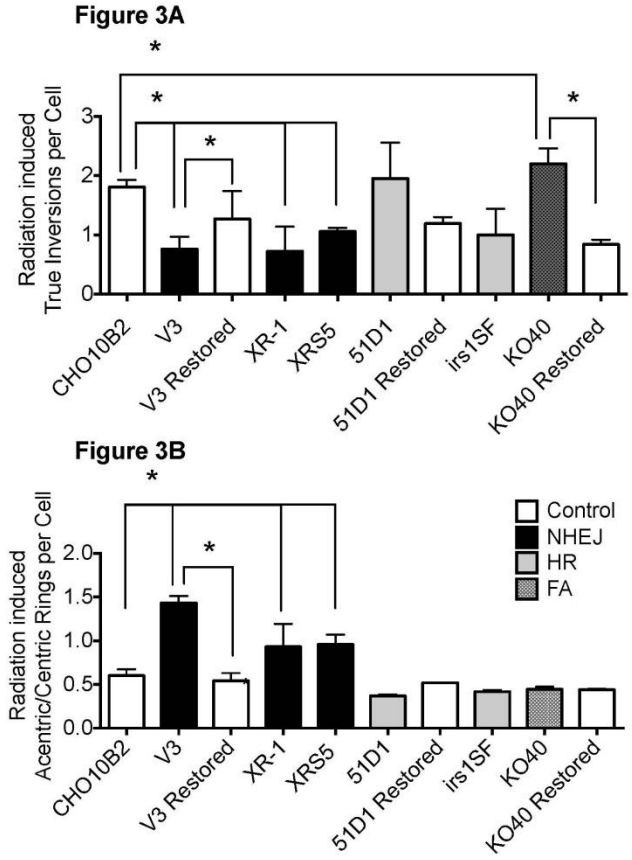

Figure 3: Radiation induced Inversions and Rings in CHO wild type and mutant cells.

A) The true radiation induced inversions after exposure to 2 Gy of gamma-rays were plotted data obtained in Table 3.

B) The number of radiation induced acentric and centric rings after exposure to 2 Gy of gamma-rays.

Error bars indicate the standard error of the means.

** Indicate a statistical difference from the control.*

Additionally, as seen in Figure 2A and 2B the number of SCEs and two SCEs at both 0 and 2 Gy were statistically similar to the CHO10B2 WT cells as well.

Effects of DNA Repair Inhibition on the formation of Centric and acentric Rings

In an effort to better understand how inversions are formed we analyzed three DNA repair/damage response pathway mutants for the formation of both acentric and centric rings. Figure 3B shows the radiation-induced rings in both CHO10B2 and all of the DNA repair mutants along with gene restored mutants. The NHEJ mutants a statistically significant two times increase in rings formed, P value = 0.04 when compared as a group. The HR mutants and KO40 had statistically no differences in ring formation following 2 Gy of gamma radiation compared to wild type, P value =0.096 and 0.1488 respectively.

Discussion:

In this study we evaluated the role of several DNA repair/damage response pathways in the formation of radiation-induced inversions. By utilizing CHO mutant cells we were able to investigate the NHEJ, HR, and FA signal pathways. CHO wild type and CHO mutants were cultured in the presence of EdU for 12 hours before being synchronized in G1 and irradiated with 0 or 2 Gy of gamma rays. Cells were collected in the first post-IR mitosis and the number of induced inversions evaluated. In this study we utilized EdU as the thymidine analogue since it does not require heat denaturing in formamide (Vogel et al., 1986). Formamide heat denaturing removes proteins from the DNA strand resulting in the relaxation of the chromatin structure (Heng and Tsui, 1993; Singh et al., 1977). By limiting the denaturation during staining we are able to maintain the chromosome structure and potentially allowing for a more accurate analysis of the radiation-induced inversions.

Table 3.3: Expected and Observed Number of Inversions in Cells Exposed to 2Gy. +/- is the SEM

Cell Line	CHO10B2	V3	XR-1	XRS5	51D1	irs1SF	KO40	V3 Restored	51D1 Restored	KO40 Restored
Observed SCE per cell	15.25 ±0.40	16.29 ±0.36	13.75 ±0.32	15.48 ±0.33	11.72 ±0.50	9.90 ±0.29	14.78 ±0.42	16.48 ±0.60	14.67 ±0.33	13.88 ±0.32
Observed SCE per chromosome	0.73 ±0.02	0.78 ±0.02	0.65 ±0.02	0.74 ±0.02	0.56 ±0.02	0.47 ±0.01	0.70 ±0.02	0.78 ±0.03	0.70 ±0.02	0.66 ±0.02
Predicted 2 SCE within a chromosome per cell	2.77 ±0.09	2.99 ±0.08	2.34 ±0.07	2.73 ±0.07	1.87 ±0.12	1.11 ±0.07	2.57 ±0.09	2.95 ±0.13	2.55 ±0.07	2.37 ±0.07
Observed 2 SCE within a chromosome per cell	4.93 ±0.07	4.01 ±0.20	3.35 ±0.07	4.03 ±0.05	3.43 ±0.57	2.70 ±0.46	6.38 ±0.05	5.57 ±0.50	4.24 ±0.12	3.60 ±0.08
Expected true inversions per cell	2.16 ±0.07	1.02 ±0.20	1.01 ±0.07	1.30 ±0.05	1.56 ±0.07	1.59 ±0.53	3.81 ±0.05	2.62 ±0.05	1.69 ±0.12	1.23 ±0.08
Radiation-induced inversions per Cell	1.81 ±0.12	0.76 ±0.21	0.72 ±0.42	1.06 ±0.06	1.95 ±0.61	1.00 ±0.44	2.20 ±0.26	1.27 ±0.47	1.19 ±0.11	0.84 ±0.08

We showed that both the NHEJ and FA pathways influenced the formation of inversions (Figure 3A). In the NHEJ mutants there was a decrease in the number of observed true radiation-induced inversions. Additionally, the NHEJ mutants were observed having an increase in radiation-induced acentric/centric rings, this was not seen in the control or HR/FA cell lines (Figure 3B). Therefore, most likely in NHEJ mutants, chromosomal fragments that could be a chromosomal inversion, being left and forming of ring chromosomes. NHEJ plays a role for chromosome fragment rejoining even in different orientation to form chromosome inversions. It is unknown how fragment of chromosome becomes ring without NHEJ. Whereas it was observed the FA mutants experienced an increase in true spontaneous and radiation-induced inversions. This indicates that the FA pathway may play a role in inhibiting inversion formation during DNA repair.

Recent research has shown that there is extensive interaction between the Fanconi Core Complex and FANCD2 with numerous DNA repair pathways, including HR (Moldovan and D'Andrea, 2009; Niedzwiedz et al., 2004). Most notably, the FA family of proteins appear to be crucial for the correct repair of interstrand DNA crosslinking (ICL) (German et al., 1987; Rogatko and Auerbach, 1988; Sasaki, 1975). During the repair of ICL the FA family of proteins has been shown to interact with both the nucleotide excision repair and HR pathway. It has been shown in several reports that FANCD2 mutants have decreases in homologous recombination (Nakanishi et al., 2005; Niedzwiedz et al., 2004; Sung and Klein, 2006; Taniguchi et al., 2002). FA proteins have been shown to associate with several HR proteins such as Rad51 and BRCA1/2. This co-locolization, but lack of complete inhibition of the HR pathway indicates that the FA proteins may serve as a binding structure for the HR proteins (Bogliolo et al., 2007; Garcia-Higuera et al., 2001; Hussain et al., 2004; Nakanishi et al., 2002; Taniguchi et al., 2002).

The lack of FA may potentially lead to unregulated HR repair and an over activation of NHEJ (Kook, 2005; Mosedale et al., 2005; Wang et al., 2004). The over activation of NHEJ could allow for the formation of an inversion if several of these ICL were to occur close together.

In conclusion, this showed that the NHEJ pathway appears to be the DNA repair pathway responsible for the formation of true radiation-induced inversions. Radiation-induced chromosomal inversion is a unique exception in that the loss of a DNA repair pathway actually contributes to a decrease in the yield of a specific chromosomal aberrations. Additionally, it was observed that the FA pathway appears to be responsible for preventing the formation of true spontaneous and radiation-induced inversions. Most importantly, this study suggests that loss of FA signal pathway may accumulate spontaneous inversions in the genome.

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CHAPTER 4

CHROMOSOMAL INVERSIONS ARE SPECIFIC SPONTANEOUS CHROMOSOMAL ABERRATIONS ASSOCIATED WITH FANCONI ANEMIA MUANTS

Introduction

It has been shown that the Fanconi Anemia (FA) pathway plays a critical role in facilitating the correct repair of interstrand DNA cross-link (ICL) damages through the homologous recombination (HR) pathway. It has also been shown that cells lacking the FA pathway appear to repair ICLs through the Nonhomologous end joining pathway, often resulting in the formation of chromatid type aberrations. In this study we investigated the formation of spontaneous chromosomal inversions in human FA mutant cell lines. Human normal and cancer cell lines with normal DNA repair, DNA repair deficiency, and FA mutations were observed for formation of spontaneous inversions utilizing a strand specific chromosome 3 probe. Importantly, this strategy also enabled high-resolution visualization of inversions that occurred at any point in the life of the cell line. We show that only human FA mutant cell lines have an increase in spontaneous inversions not seen in normal human fibroblasts, FA positive cancer cell lines, or DNA repair deficient cancer cells. It was shown that roughly 4% of chromosome 3's observed in FA mutants contained a true spontaneous inversion. These true inversions could be isolated and expanded clonally. Additionally, the vast majority of the spontaneous chromosomal aberrations observed in FA mutants, DNA repair deficient cancer cells, and several cancer cell lines were chromatid-type gaps, breaks, deletions, and radials. We concluded that inversions are a unique spontaneous chromosomal aberration specific to FA deficient cells.

Background

FA is an inheritable genetic blood disorder that results in aplastic anemia, extreme sensitivity to ICL agents, and a slight radiosensitivity in affected individuals (German et al.,

1987; Rogatko and Auerbach, 1988). The FA family of proteins are found in one of two protein complexes; 1) the FA core complex composed of the paralogs FANC A, B, C, E, F, G, L, M and 2) the FANCD2 complex composed of the FANCD2 and FANCI proteins. A mutation in any one of the FANC proteins in either complex results in the inhibition of the FA ICL repair pathway. This is due to the fact that the FA core complex is responsible for ubiquitinating the FANCD2 complex and that the ubiquitination of the FANCD2 complex allows for the interaction of FANCD2 with the BRCA1 protein and the HR pathway (Garcia-Higuera et al., 2001; Wang et al., 2004).

An ICL can be formed several ways; along with various chemicals, ionizing radiation (IR) is very effective at forming ICLs. Additionally, ICL can occur spontaneously in cells due to natural cellular metabolism (Dronkert and Kanaar, 2001). ICLs are one of the more toxic forms of DNA damage to a cell. In normal cells ICL have been shown to cause various chromosome aberrations and eventually mutations (Scott et al., 1976). In DNA repair mutants as few as 40 ICLs have been shown to kill the cell, this has also been shown in human FA patients (Lawley and Phillips, 1996).

Sensitivity to ICL-inducing agents is believed to be due to the shift from HR repair to NHEJ. Interaction with the BRCA2 proteins and HR is crucial for the complete and correct repair of ICLs (Joenje and Patel, 2001). When a FA protein is mutated, repair of ICL is completed by the NHEJ pathway and can result in misrepair. This is observed as the formation of radials and chromatid gaps/breaks in FA mutants (Joenje and Patel, 2001). It appears that the FA family of proteins is more likely involved as a singling molecule or damage reporter protein (Joenje and Patel, 2001). It is noted that mutations in either the FA core complex or FAND2 complex result in a fairly mild phenotype when compared to mutations in essential proteins for

NHEJ or HR (Myers et al., 2012). Like most HR mutants, FA mutants are extremely sensitive to ICL agents, however unlike HR mutants FA mutants display only slight radiosensitivity (Joenje and Patel, 2001).

In addition to sensitivity to ICL-inducing agents, FA mutants experience genomic instability, evidenced as elevated levels of chromatid-type gaps/breaks and radials, aberrations shown to be lethal to the daughter cells inheriting them (Karon et al., 1972; Meyer et al., 2012). Radials result in the formation of dicentrics that cause mitotic death in cells containing them. Additionally, breaks/gaps typically result in the loss of the terminal portion of the affected chromosome, often leading to loss of genomic information or formation of a dicentric (Savage, 1976). To a lesser extent FA mutants have been reported to experience elevated levels of symmetrical and asymmetrical translocations. Symmetrical translocations are relatively non-lethal to the daughter cell inheriting the aberration, unless the translocation truncates an essential gene (Meyer et al., 2012; Myers et al., 2012).

Like various other DNA repair deficient syndromes, like BRCA1/2 heterozygous mutations, Nijimegan Breakage Syndrome, Werner syndrome, and Bloom syndrome, FA individuals experience earlier on set cancers, acute myeloid leukemia being the most common (Al-Tassan et al., 2002; Fishel et al., 1993; Kennedy and D'Andrea, 2006; Leach et al., 1993; Ripperger et al., 2009). FA individuals also have an elevated rate of squamous cell carcinomas of the head and neck, the median age of diagnosis being 27 years and often in individuals who are non-smokers and non-drinkers (Rosenberg et al., 2003). Additionally, like various other DNA repair mutants, the cancers that develop in FA individuals are due to the inherent genomic instability observed in FA cells. Here, we utilized a chromosome 3 chromatid paint to evaluate spontaneous inversions in various human FA mutants and various human cell lines with

proficient and deficient DNA repair pathways in order to investigate the role of DNA repair and damage response pathways in the formation of spontaneous inversions.

Materials and Methods

Cell Lines:

The cell lines utilized in this study are outlined in Table 1 and were kindly supplied by Dr. Joel Bedford at Colorado State University (Fort Collins, CO, USA), the Oregon Health and Science University Cell Repository (Portland, OR, USA), or obtained from ATCC (Manassas, VA, USA). Cells were cultured in α-MEM (Gibco, Indianapolis, IN, USA) supplemented with 15% fetal bovine serum (FBS, Sigma, St Louis, MO, USA) and 1% antibiotics and antimycotics (Gibco), and were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

Synchronization:

When the cultures approached ~80% confluence in T-25 tissue culture flasks the normal growth medium was replaced twice with isoleucine-deficient α-MEM containing 5% 3× dialyzed FBS for 24 hours to synchronize the cells in G1 phase (Tobey and Ley, 1971). G1 synchronized cells were released and placed into three T-25 tissue culture flasks with normal growth media containing 5 μM BrdU and 1 μM BrdC (Sigma) for 36 hours to ensure uniform incorporation into the newly synthesized DNA.

Metaphase Chromosome Preparation:

Cells were cultured in the presence of 0.1μg/ml of colcemid for ~4 hours to block mitosis, 24 hours after BrdU and BrdC was added. Cells were trypsinized and were suspended in 6ml 75mM KCl warmed to 37°C and placed in a 37°C water bath for 20 minutes. Carnoy's solution (3:1 methanol to acetic acid) was added to the samples according to the standard protocol. Slides were placed in ice water and allowed to chill. The cell solution was dropped onto

the cold slides. These were set aside and allowed to dry until the Carnoy's solution had evaporated, roughly 4-5 minutes (Cartwright et al., 2014).

Chromatid Painting:

Slides with metaphase chromosome spreads were pretreated and hybridized as described previously (Ray et al., 2013). Briefly, BrdU/BrdC incorporated strands were nicked by exposure to 365 nm UV light following staining with Hoechst 32258. The strands were fully removed using exonuclease III. A chromosome 3 specific chromatid paint labeled with Cy3 was hybridized for 3 min at 68 °C and then incubated overnight at 37 °C. After hybridization the slides were washed five times in 2x SSC at 42 °C for 15 min each time. Slides were then counter-stained with ProLong® Gold Antifade reagent with DAPI (Life Technology, Carlsbad, CA, USA)

Immunofluorescence:

Cells were grown on chamber slides and fixed in 4% paraformaldehyde for 15 min and washed in phosphate buffered saline (PBS). Cells were permeabilized with 0.1% SDS and 0.5% Triton X-100 in PBS for 10 min. Following overnight blocking in PBS with 10% goat serum, immunostaining was carried out. The primary antibody utilized was a mouse monoclonal FANCD2 antibody (Millipore, Billerica, MA, USA), rabbit polyclonal FANCD2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies used were Alexa 488 Fluor-conjugated goat anti-mouse antibody (Life Technology). DNA was fluorescently counterstained with ProLong® Gold Antifade reagent with DAPI.

Chromosome Aberration Analysis:

Metaphase chromosome spreads were stained with Giemsa and analyzed for structural chromosome aberrations. Metaphase chromosomes were analyzed for chromatid breaks/gaps,

symmetrical and asymmetrical translocations, radials, acentric rings/interstitial deletions, and dicentrics. 50 chromosome spreads were analyzed for each cell line.

Image Analysis:

Images were captured using a Z-stage motorized Zeiss Axioskop microscope equipped with a Metamorph system (Carl Zeiss AG, Jena, Germany) and a Q-imaging Aqua cooled CCD camera (Q-imaging, Surrey, BC, Canada). Images were combined using ImageJ software (National Institute of Health, Maryland, USA).

Cell Cloning:

In order to create a clonal inversion, we created a series of clones using PD352i, a human FANCG immortalized fibroblast. After trypsinization and cell concentration determined, the cells were diluted until there was 1 cell per 100 μL. 100 μL of cell suspension was plated in each well of a 96 well plate. Plates were observed daily and wells containing a single colony were selected and transferred to a well in a 24 well plates. When the clones reached ~80-90% confluency in the 24 well plates they were transferred to 6 well plates and cultured until they were able to be used for experiments.

Statistical analysis:

Statistical comparison of mean values was performed using a t-test or a 1 way ANOVA when comparing groups. Differences with a p-value of <0.05 were considered statistically significant. Error bars indicate standard error of the means. 95% confidence interval values were calculated by Prism 5™ software (GraphPad, La Jolla, CA, USA).

Results

Spontaneous Sister Chromatid Exchanges and False Inversions in Various Human Cell lines

As described in Chapters 2 and 3, not all observed inversions are true inversions. Occasionally, two SCE can occur on a single chromosome resulting in what appears to be an inversion. Figure 1A shows the observed SCE frequencies per cell line. The average SCE per chromosome 3 for the control fibroblasts ranged from 0.21 to 0.28. The average SCE frequency for chromosome 3 in FA mutants ranged from 0.24 to 0.35 SCE per chromosome. For the DNA repair proficient cancer cell lines the average SCE per chromosome 3 ranged from 0.14 to 0.32. Finally, the average SCE per chromosome 3 for the DNA repair deficient cancer cell lines ranged from 0.09 to 0.34, this was the only group to have statistically non-similar SCE averages based on an ANOVA test. To determine the number of false inversions in each cell line we utilized using a Poisson distribution, $p(x) = \frac{\lambda^x e^{-\lambda}}{x!}$ where λ = the SCE per chromosome and x = the variable, to calculate the predicted likelihood of having 2 SCE within chromosome 3. Figure 1B indicated the expected false inversions per cell line.

Spontaneous Total Observed Inversions in Various Human Cell Lines

To investigate the spontaneous inversion frequencies in selected cell lines we analyzed 300 chromosome 3's per cell line. A chromosome was only scored if it contained no twists or bends. Additionally, an inversion was only scored if the inverted fragment was clearly separated from the sister chromatid. Figure 2A depicts a chromosome spread from PD352i stained with the chromosome 3 chromatid paint which hybridizes to only chromosome 3, PD352i multiple copies of chromosome 3. Figure 3A shows the average total observed inversions per chromosome 3. The total observed inversions included both true inversions and false inversions. False inversions

are 2 SCE that occur on a single chromosome and look like an inversion. Figure 2B shows a potential inversion in chromosome 3, the inversion probe has been shown to identify inversions as small as 1 Mb. All FA mutants had statistically elevated number of total spontaneous observed inversions when compared to control fibroblasts (P-values < 0.05). Between 6.7 and 9.4% of chromosome 3's in FA mutants were observed containing a spontaneous inversion, as compared to only 2.2-3.2% in the control fibroblasts.

Spontaneous True Inversions in Various Human Cell Lines

As seen in Figure 3B when the false inversions calculated in Figure 1B were subtracted from the total observed inversions, only the FA mutants had elevated numbers of true spontaneous inversions when compared to control fibroblasts (P-values < 0.05). It was observed that between 4.8 and 3.7% of chromosome 3's in the FA mutants contained a true spontaneous inversion, it was predicted that between 0 and 0.4% of chromosome 3's in the control fibroblasts would contain a spontaneous true inversion. The spontaneous level of inversions was not elevated in any of the human cancer cell lines investigated. Cell lines were also analyzed as groups. As seen in Figure 4A the average SCE per chromosome 3 did not vary from the control fibroblasts between any of the groups, it can be seen in Figure 1A that the FANCD1/BRCA2 mutant is the only cell line to have statistically fewer SCE per chromosome 3 then the control. Additionally, as seen in Figure 4B as a group the FA mutants display an elevated level of spontaneous inversion not seen in any of the other groups.

Spontaneous Chromosome Aberrations

In an effect to better characterize the portion of chromosome aberrations attributable to inversions in FA mutant cells, we had to first identify the other major types of chromosome aberrations in the cell lines. Table 2 outlines the spontaneous chromosomal and chromatid type

aberrations observed in each cell line. It was noted that the most common type of aberration observed in FA mutants were chromatid-type breaks/gaps and radial formation, ranging from 16-25 gaps/breaks per 50 cells and 2-6 radials per 50 cells. The FA positive cancer cell lines had elevated levels of spontaneous deletions, ranging from 1-11 per 50 cells observed.. The control primary and immortalized human fibroblast cell lines had a low background level of spontaneous chromosome aberrations, GM2149 only had 2 aberrations and BJ-1 had 5 aberrations in the 50 cells analyzed. All cancer cells were also analyzed for spontaneous chromosome aberrations. It was seen that the cancer cell lines experienced elevated levels of acentric fragments and chromatid-type deletions, ranging from 1-33 acentric fragments and 1-22 chromatid-type deletions per 50 cells observed. As compared to the FA mutants, the cancer cell lines only contained 1-5 gap/breaks and 0-3 radials per 50 cells observed.

Ability to Create a Clonal Inversion

One benefit of utilizing a strand specific probe is the ability to observe an inversion that can be passed on from one cell to the daughter cells. To show that the observed inversions were removed from the population during cell division, we created a series of clones in an effect to identify a clonal inversion. We created 50 clones from the cell line PD352i due to the fact it had the highest number of spontaneous inversions of all cell lines tested. Of the 50 clones created five had observable clonal inversions. group before they could be collected and analyzed. The most important implication of the ability to identify clonal inversions is that it proves that the true inversions observed in Figure 3B are in fact true inversions and not false, 2 SCE, inversions.

Validation of Fanconi Anemia Status

To establish the FA status for each cell line utilized in this study, each cell line was evaluated for FANCD2 foci. Log phase cells were stained and analyzed for FANCD2 foci.

Figure 5 shows the presence or absence of FANCD2 foci in all cell lines. All FA primary, immortalized, and cancer cell lines except FANCD1/BRCA2 were negative for FANCD2 foci. All of the human cancer cell lines, primary and immortal fibroblasts, and FANCD1 mutant contained cells with FANCD2 foci.

Discussion

Here we analyzed human FA and various human cancer cell lines for frequencies of both spontaneous inversions and other structural chromosome aberrations. To investigate spontaneous inversions, we utilized a chromatid paint that allowed us to visualize inversions that form not only in the cell cycle prior to metaphase chromosome collection, but inversions inherited over time as well. To account for "false" inversions, two SCE's occurring on a single chromosome; we used the average SCE rates per cell line to calculate the predicted/expected false inversion frequency for each cell line.

This is the first study to show that FA mutants experience elevated levels of spontaneous inversions above apparently normal human fibroblasts and the cancer cell lines used. This finding reveals that FA mutants experience more genomic instability than previously thought. It has been well documented that FA mutants experience elevated rates of genomic instability, specifically the formation of gaps/breaks and radials (Meyer et al., 2012). In somatic cells, unlike germ cells, inversions are considered fairly benign, that is unless the inversion affects a lethal gene (Painter, 1933). We believe that the current study indicates that inversions may play an important role in the carcinogenesis of individuals affected with FA. This is due to the fact that of the genomic instability in FA mutants, inversions are the predominant transmissible chromosome aberration. The elevated level of spontaneous inversion was limited to FA mutant cell lines. None of the FA positive cancer cells experienced elevated levels of spontaneous

inversions above the control fibroblasts. Importantly, the NHEJ mutant cell lines showed no increase in spontaneous inversion frequency supporting out previous findings that inhibition of the NHEJ repair pathway inhibited the formation of radiation-induced inversions.

In conclusion, this study reveals a previously unobserved form of genomic instability in human FA mutant cell lines that is only seen in cell lines containing a mutation to the FA pathway. The elevated levels of spontaneous inversions was isolated to FA mutants; the normal human fibroblasts, FA positive cancer cell lines, and DNA repair deficient cell lines had no observable spontaneous inversion formation. Along with spontaneous inversions, the FA mutants also experienced elevated levels of spontaneous chromosomal aberrations, more specifically chromatid-type gaps and breaks. Considering that these are most often lethal type aberrations, inversions may play an important role in the accumulation of mutations and tumorgenesis in FA patients.

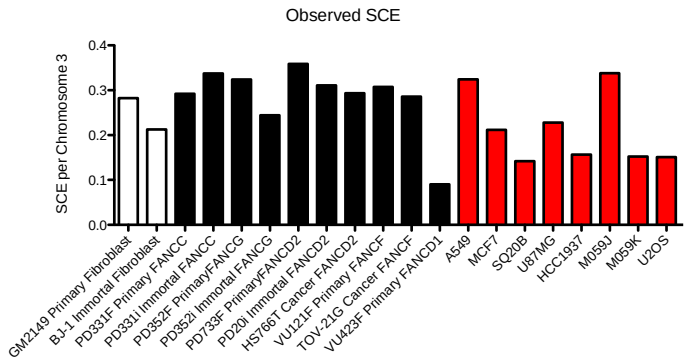
Table 4.1: ***Cell Type and Status of all Cell Lines.***

Cell Line	Cell Line Status	DNA Repair Mutation	Tissue of Origin
GM2149	Passage 8 Primary	Normal	Skin Fibroblast
BJ-1	HTERT Immortalized	Normal	Skin Fibroblast
PD331F	Passage 14 Primary	FANCC	Skin Fibroblast
PD331I	SV40 Immortalize	FANCC	Skin Fibroblast
PD352F	Passage 12 Primary	FANCG	Skin Fibroblast
PD352I	SV40 Immortalized	FANCG	Skin Fibroblast
PD733F	Passage 17 Primary	FANCD2	Skin Fibroblast
PD20I	SV40 Immortalized	FANCD2	Skin Fibroblast
VU121	Passage 5 Primary	FANCF	Skin Fibroblast
HS766T	Cancer	FANCD2	Pancreas Epithelial
TOV-21G	Cancer	FANCF	Ovary Epithelial
VU423F	Passage 6 Primary	FANCD1/BRCA2	Skin Fibroblast
A549	Cancer	Normal	Lung Epithelial
U87MG	Cancer	Normal	Brain Epithelial
U2OS	Cancer	Normal	Bone Epithelial
M059K	Cancer	Normal	Brain Glial Cell
MCF7	Cancer	Normal	Mammary Gland Epithelial
SQ20B	Cancer	Normal	Laryngeal Epithelial
MO59J	Cancer	DNA-PKcs	Brain Glial Cell
HCC1937	Cancer	BRCA1	Mammary Gland Epithelial

Table 4.2: ***Observed Spontaneous Chromosome Aberrations***.

Cell Line	DNA Repair Mutation	Modal Chromosome Number	Aberrant Chromosome Spreads	Chromatid Aberrations					Chromosome Aberrations	
				Gaps/breaks	Deletions	Asyn.	Syn.	Radials	Acentric Fragments	Dicentrics plus ring
GM2149	Normal	46	2/50	1/50	0/50	0/50	0/50	0/50	1/50	0/50
BJ-1	Normal	46	5/50	1/50	1/50	0/50	0/50	0/50	3/50	0/50
PD331F	FANCC	46	35/50	20/50	0/50	5/50	1/50	3/50	11/50	5/50
PD331I	FANCC	46	41/50	25/50	4/50	1/50	1/50	8/50	13/50	6/50
PD352F	FANCG	46	27/50	21/50	1/50	0/50	0/50	4/50	11/50	1/50
PD352I	FANCG	46/72	42/50	25/50	11/50	1/50	0/50	6/50	18/50	9/50
PD733F	FANCD2	46	23/50	16/50	1/50	0/50	0/50	2/50	5/50	3/50
PD20I	FANCD2	64	28/50	18/50	4/50	1/50	0/50	3/50	3/50	9/50
VU121	FANCF	46	27/50	25/50	2/50	2/50	0/50	3/50	8/50	1/50
HS766T	FANCD2	57	33/50	20/50	6/50	1/50	0/50	5/50	5/50	5/50
TOV-21G	FANCF	46	36/50	22/50	4/50	1/50	4/50	6/50	8/50	2/50
A549	Normal	63	8/50	4/50	2/50	0/50	0/50	0/50	1/50	1/50
U87MG	Normal	51	20/50	4/50	1/50	2/50	1/50	3/50	8/50	4/50
U2OS	Normal	65	41/50	4/50	24/50	0/50	0/50	0/50	33/50	10/50
M059K	Normal	115	24/50	2/50	10/50	1/50	0/50	0/50	18/50	5/50
MCF7	Normal	65	24/50	1/50	10/50	0/50	0/50	0/50	10/50	1/50
SQ20B	Normal	65	29/50	5/50	20/50	0/50	0/50	0/50	13/50	2/50
MO59J	DNA-PKcs	118	26/50	3/50	8/50	0/50	0/50	0/50	15/50	4/50
HCC1937	BRCA1	82	41/50	7/50	28/50	1/50	0/50	2/50	19/50	5/50
VU423F	FANCD1/BRCA2	46	22/50	19/50	2/50	0/50	0/50	3/50	2/50	1/50

A.



B.

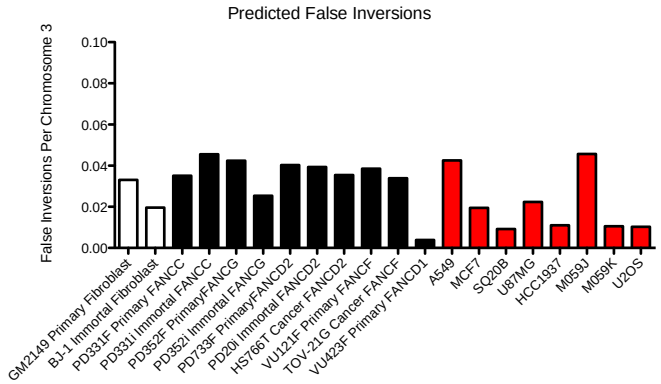
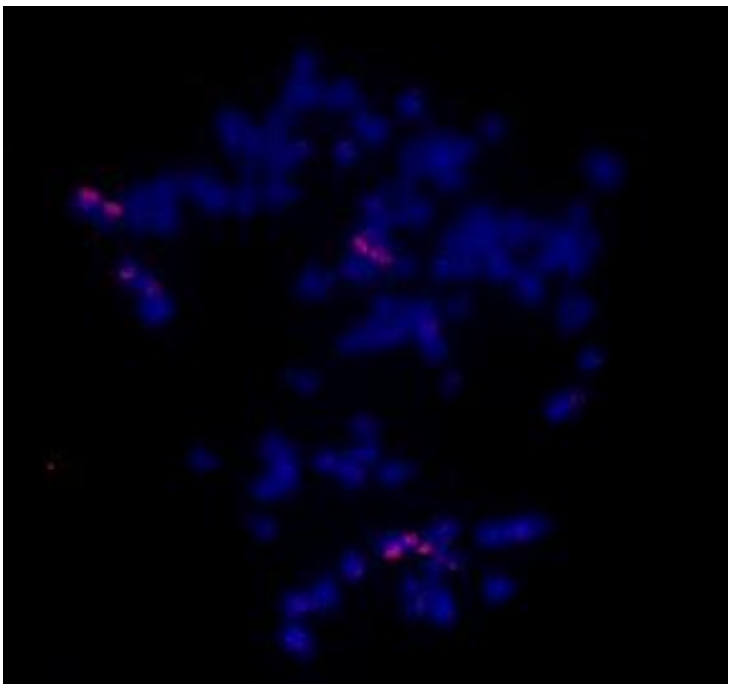


Figure 4.1: Panel A depicts the average number of SCE observed per chromosome 3 for each of the cell lines. A total of 300 chromosomes were analyzed for each cell line. Panel B depicts the predicted number of false inversions, 2 SCE on a single chromosome. A total of 300 chromosomes were analyzed for each cell line.

A.



B.

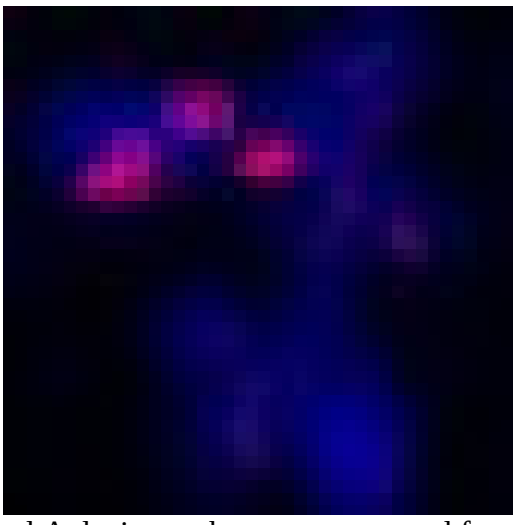
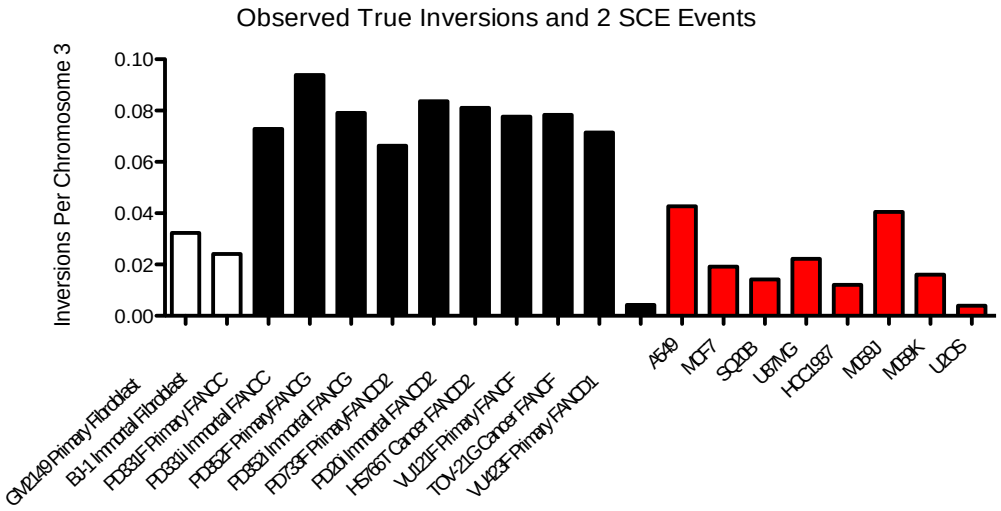


Figure 4.2: Panel A depicts a chromosome spread from the cell line PD352i with chromosome 3 stained. Panel B depicts a chromosome 3 from the cell line PD352i with an inversion.

A.



B.

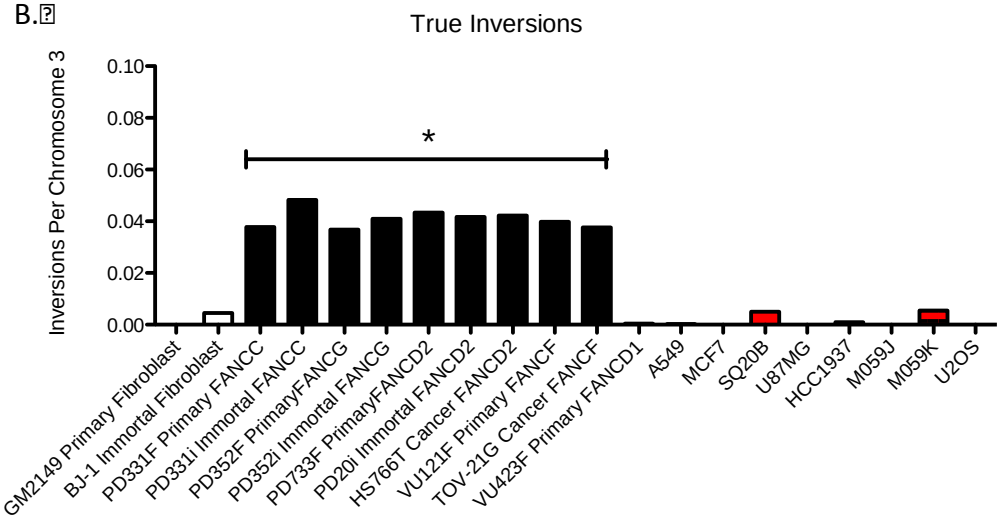
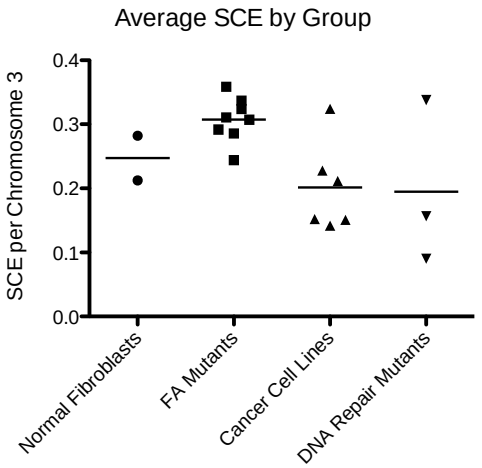


Figure 4.3: Panel A depicts the average number of true inversions and 2 SCE events observed in 300 chromosome 3s'. Panel B depicts the average number of true inversions observed in the 300 chromosome 3s' analyzed. * indicates a statistical difference from the control fibroblasts.

A.



B.

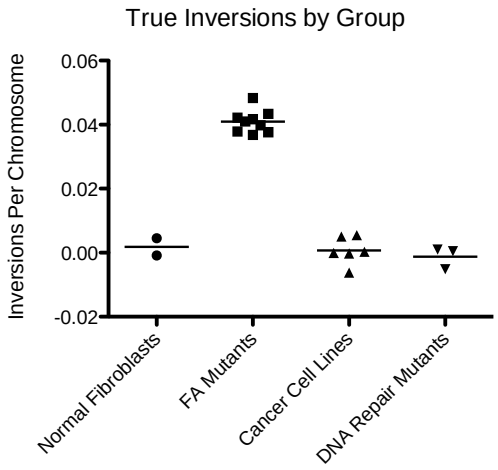


Figure 4.4: Panel A indicates the average number of SCE observed per chromosome 3 for each cell line group into categories of normal fibroblasts, FA mutants, Cancer cells, and DNA repair mutants. Panel B depicts the average number of true inversions observed for each cell line grouped into categories of normal fibroblasts, FA mutants, Cancer cells, and DNA repair mutants. A total of 300 chromosome 3s' were analyzed for each cell line.

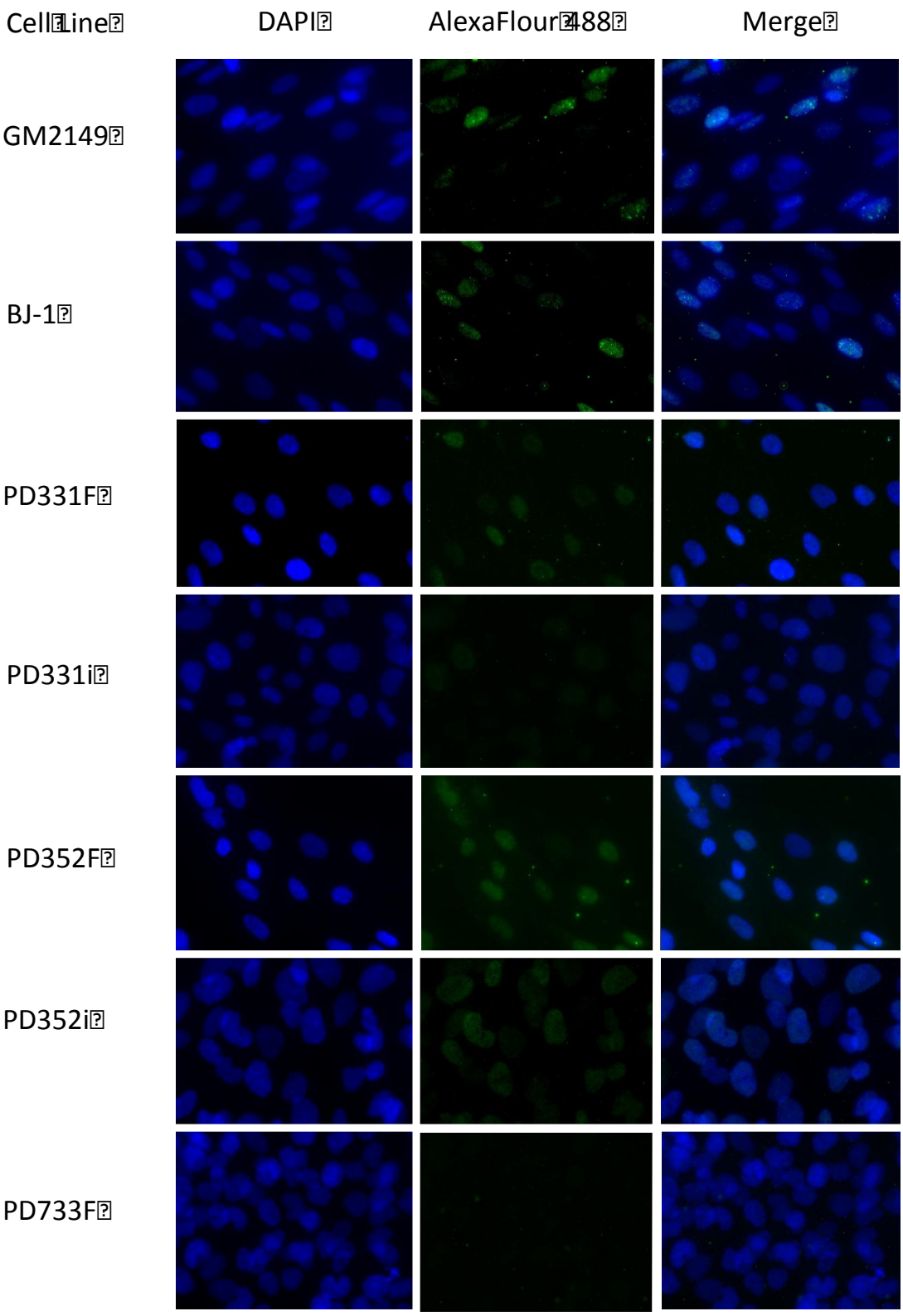

Cell Line
DAPI
AlexaFlour 488
Merge
GM2149
BJ-1
PD331F
PD331i
PD352F
PD352i
PD733F

Figure 4.5

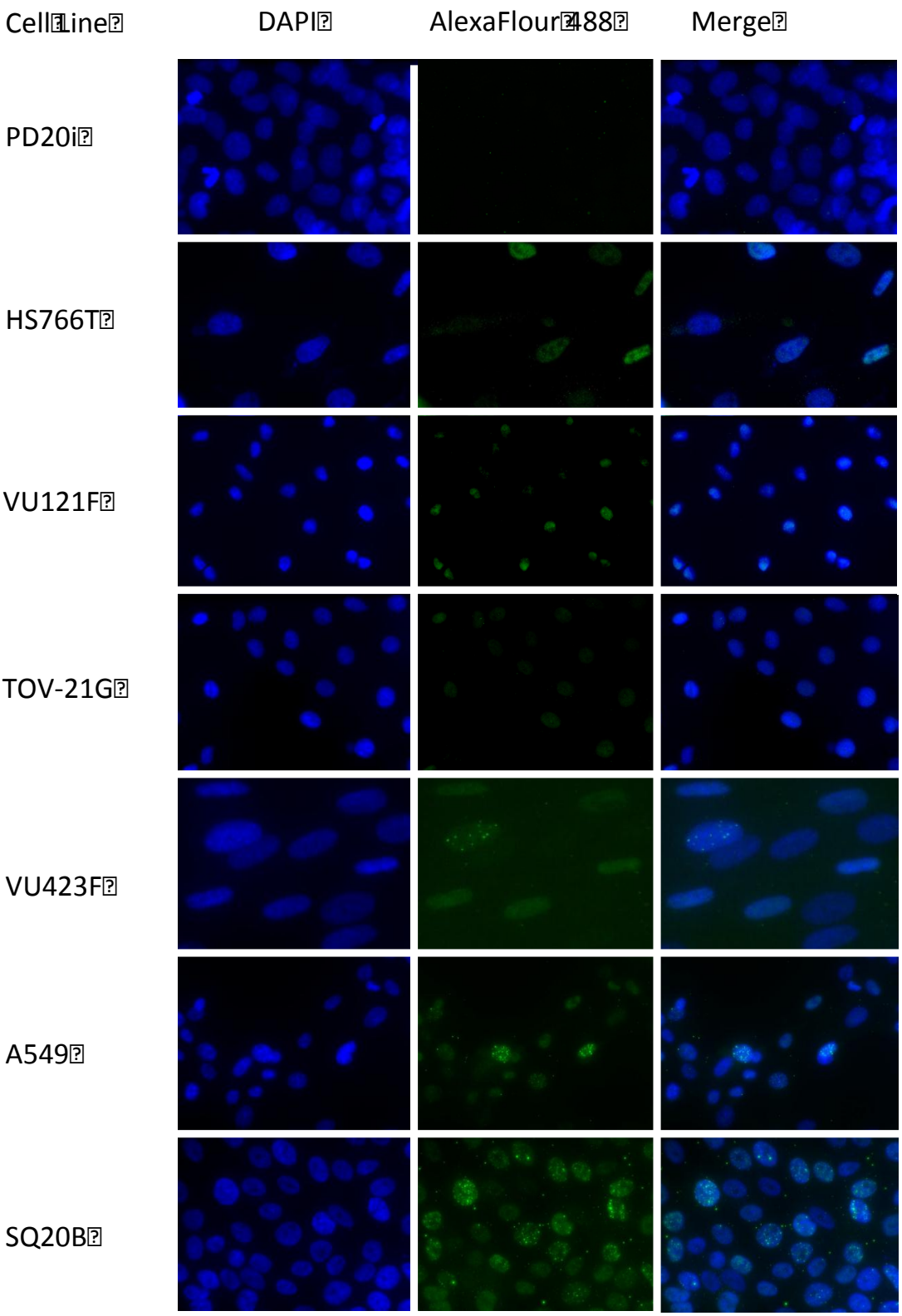


Figure 4.5 Cont.:

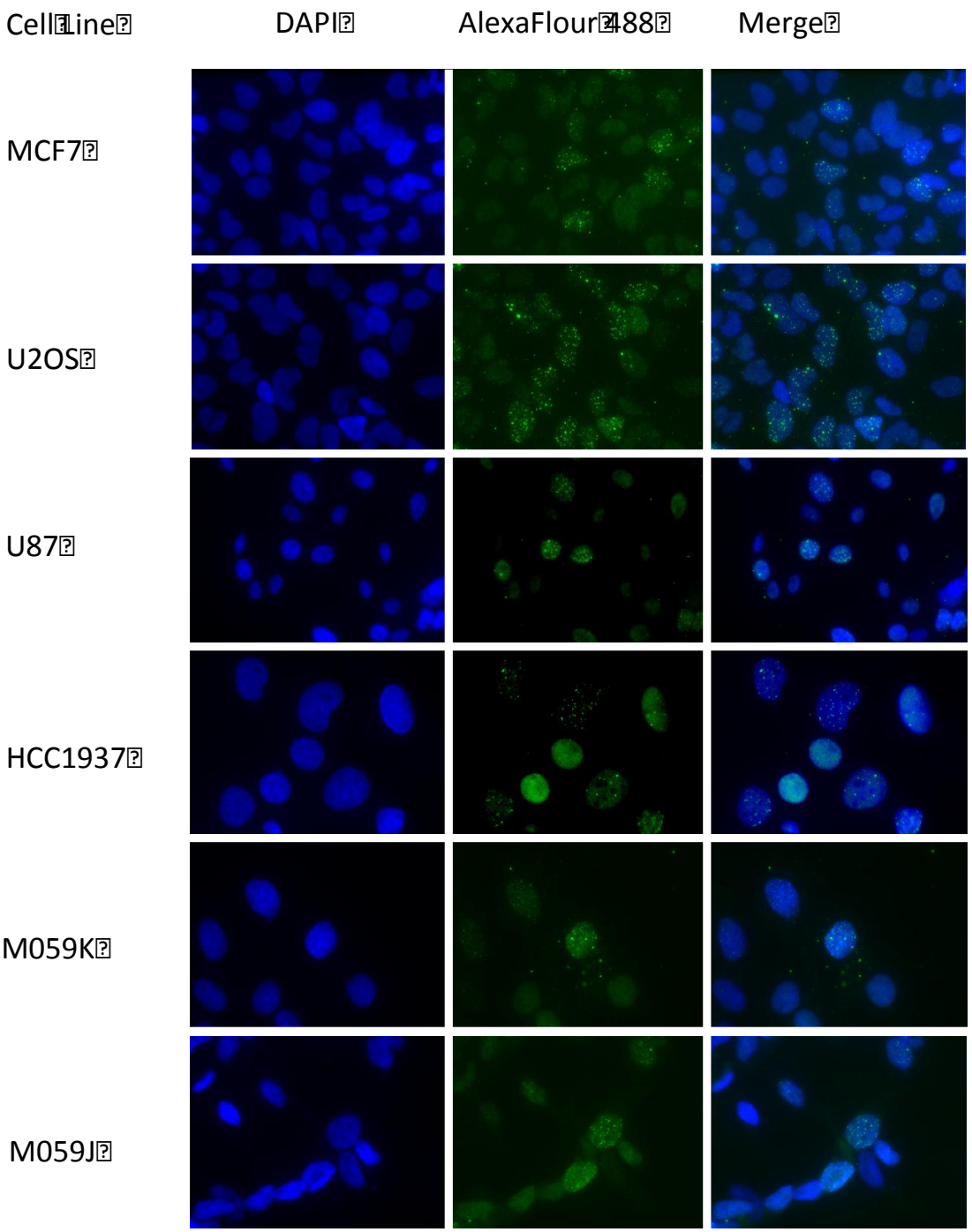


Figure 4.5 Cont.: This figure depicts the FANCD2 status for each cell line utilized in this study.

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CHAPTER 5

SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

Introduction

The overall goal of the research presented here was to further understanding of chromosomal inversions formation and characteristics. The influence of chromatid structure and radiation quality was investigated in regard to formation of inversions, the DNA repair/damage response pathway responsible for inversion formation, and lastly the role inversions play in the genomic instability observed in FA mutants.

To investigate chromosomal inversions we utilized both BrdU/EdU and chromatid paint strategies to identify both spontaneous and radiation-induced inversions. One issue with any such approach is the potential for observing "false" inversions along with true inversions. To address this issue, we calculated the statistical likelihood that two SCE's would occur on a single chromosome. The ability to accurately account for all chromosomal inversions observed in unirradiated cells we are confident that we are in fact investigating true inversions and not artifacts created by two SCE events.

This dissertation relied heavily on CHO cell lines. CHO cell lines were selected for two main reasons. First, CHO cells are easily and effectively synchronized in G1 or M phase; isoleucine deficient media was not necessary for cell synchronization. Secondly, there are a number of readily available DNA repair/damage response mutant CHO cells. In chapter 4 human fibroblast and cancer cell lines were utilized to evaluate spontaneous inversions frequencies.

This dissertation provides insight into how radiation quality influences inversions size, the DNA repair pathway associated with inversion formation, and a potential mechanism of how inversions may spontaneously form. The most notable findings in this dissertation are; first,

radiation-induced inversions have a distinct size distribution and number depending on the quality of radiation in which the cell was exposed to. High LET radiation tended to form smaller inversions then low LET, which produced inversions with randomly distributed sizes. Also, chromatin structure only appeared to influence the size of radiation-induced inversions when the cells were exposed to high LET radiation. Secondly, it appears that inversions are formed by both the NHEJ repair pathway, inhibition of this pathway lead to an overall reduction in the number of radiation-induced inversions. Additionally, the FA pathway may inhibit the formation of inversions in normal cells. Finally, inversions in FA mutants appear to be predominant form of non-lethal chromosomal instability. This finding may suggest that inversions play an important role in the development of cancer in FA patients.

Summary

This dissertation has uncovered several unique findings. As outlined in Chapter 2 it was observed that radiation-induced inversions following both Fe ion and x-ray increased and varied in size and number in an LET dependent fashion. Fe ion exposed cells had smaller radiation-induced inversions than cells exposed to x-rays. Additionally, it was observed that that chromatin structure, as it related to cell cycle stage, only affected the number and size of radiation-induced chromosomal inversions when the cells were exposed to high LET radiation. Low LET radiation-induced the same number and sizes of chromosomal inversions in both G1 and M phase cells.

Chapter 3 outlines our efforts to better understand the DNA repair pathway responsible for the formation of radiation-induced inversions. It was shown that in CHO cell lines when the NHEJ DNA repair pathways were absent, radiation-induced inversions were inhibited. In contrast, when the FA pathway was absent radiation-induced inversions were increased. These findings suggest that the NHEJ pathway is the primary DNA repair pathway associated with

inversion formation and that the FA pathway is required for the correct repair of DNA damage and when this pathway is absent the DNA is repaired by a more error prone pathway.

Finally, as shown in Chapter 4, human FA mutants had elevated levels of spontaneous chromosomal inversions when compared to control human primary fibroblasts. Elevated levels of spontaneous chromosomal inversions were not observed in any other human cancer line investigated. It was also observed that in FA mutants the vast majority of spontaneous chromosome aberrations observed were chromatid breaks/gaps, while the various cancer lines had mostly ploidy issues and deletions (both chromosome and chromatid type).

Discussion

The findings in this dissertation improve understanding of both radiation-induced and spontaneous chromosomal inversions. One of the main findings in chapter 2 was that the sizes of radiation-induced chromosomal inversions were dependent on the quality of radiation. Given the facts that high LET cause more ionization in a smaller area than low LET this finding supports the findings by Drs. Muhlmann-Diaz and Bedford that suggested chromosomal inversions formed from two DSBs within close proximity to one another (Hall, 2006; Muhlmann-Diaz and Bedford, 1995).

In chapter 3 it was observed that not only the NHEJ, but also the FA pathway effected the formation of chromosomal inversions. In showing that inhibition of the NHEJ repair pathway caused a significant decrease in radiation-induced inversions, not seen with inhibition of the HR repair pathway, we can confidently conclude that the NHEJ is most likely the predominant DNA repair pathway associated with the formation of radiation-induced inversions. The findings that showed an increase in radiation-induced inversions in FA deficient cells was very interesting. This leads us to believe that the FA may be involved in preventing the formation of radiation-

induced inversions through a not yet understood mechanism. Recent research has shown that the FA pathway is essential for the correct repair of ICL's and that there is extensive interaction between the Fanconi Core Complex and FANCD2 with numerous DNA repair pathways, including HR (Moldovan and D'Andrea, 2009; Niedzwiedz et al., 2004).Specifically, FA proteins have been shown to associate and co-locolize with Rad51 and BRCA1/2. This co-locolization, but lack of complete inhibition of the HR pathway indicates that the FA proteins may serve as a binding structure for the HR proteins (Bogliolo et al., 2007; Garcia-Higuera et al., 2001; Hussain et al., 2004; Nakanishi et al., 2002; Taniguchi et al., 2002). The lack of FA may potentially lead to unregulated HR repair and an over activation of NHEJ, potentially increasing the likelihood of an inversions formation through incorrect repair by the NHEJ pathway (Kook, 2005; Mosedale et al., 2005; Wang et al., 2004). Given this we wanted to investigate the formation of spontaneous inversions in human FA mutants.

Finally, in this dissertation we present evidence that inversions form spontaneously in human FA mutant cell lines. This is the first to show that inhibition of the FA pathway increases the formation of spontaneous inversions. It is especially interesting to note that the elevated number of spontaneous inversions was not observed in NHEJ, HR, or FA normal cancer cells investigated in this study. This finding indicates that FA mutant cells are experiencing more genomic instability than previously believed. In addition to experience an increase in spontaneous DNA damage, inversions potential present an additional mechanism for the early onset cancers and decreased fertility observed in FA patients. As mentioned earlier, defects in the FA pathway result in inhibition of recombination events and the correct segregation of chromosomes during meiosis (Coyne et al., 1991; Kirkpatrick, 2010). In human FA mutant cells it was observed that the vast majority of spontaneous chromosome aberrations were gaps/breaks

and radials, both of which often lead to the formation of a dicentric and eventual death of the daughter cell inheriting the mutation, thus most likely not creating a carcinogenic change in the cell. In contrast, unless the inversion truncates an essential gene the inversion will be passed on to the daughter cell potentially leading to a carcinogenic change to the cell.

Future Directions

This dissertation, in addition to answering several important questions about inversions, has also raised several new questions. As shown in chapter 2 the size of radiation-induced inversions tend to decrease as the LET of the radiation is increased and that it appears that chromosomal inversions and at least in part form from two DSBs in close proximity. This suggests that radiation may be inducing inversions smaller than the current detectable size by current cytogenetic techniques. With this in mind, it may be beneficial to utilize modern genomic sequencing technology to investigate inversions that are occurring at sizes far smaller than 1 Mb. In addition, it may be beneficial to investigate the effects of large (several Mb) inversions compared to smaller (several kB) inversions to determine if one type is more detrimental to the cell than the other.

Secondly, it would be recommended that future studies be aimed at the investigation of ICL agent's role in the formation of chromosomal inversions. Based on the findings see in FA mutants that have issues with repair of ICL, it is believed that ICL's may be the responsible for spontaneous inversions in not only FA mutants, but also apparently normal human cells. These findings will help expand the understanding of how cancers potentially form.

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APPENDIX

List of Abbreviations
(Listed in alphabetical order)

ATM	Ataxia Telangiectasia Mutated
BRCA	Breast Cancer Susceptibility Gene
CHO	Chinese Hamster Ovary
DAPI	4,6-diamidino-2-phenylindole
DNA	Deoxyribose Nucleic Acid
DNA-PK	DNA Dependent Protein Kinase
DNA-PKcs	DNA Dependent Protein Kinase Catalytic Subunit
DSB	Double Strand Break
eV	electron Volt
FA	Fanconi Anemia
FISH	Fluorescence in situ hybridization
GPF	Giemsa plus Fluorescence
Gy	Gray
HZE	Heavy Charged Particle
HR	Homologous Recombination
IR	Ionizing Radiation
ICR	Interstrand Crosslink Repair
ICL	Interstrand DNA Crosslink
Kb	Kilobase
LET	Linear Energy Transfer
Mb	Megabase

M-FISH	Multicolor FISH
NBS	Nijmegen Breakage Syndrome
NHEJ	Non-homologous Endjoining
PNA	Peptide Nucleic Acid
RBE	Relative Biological Effect
RPA	Replication Protein A
SCE	Sister Chromatid Exchange
SKY	Spectral Karyotyping
SSB	Single Strand Break
SSC	Saline-Sodium-Citrate