INVESTIGATION OF CHROMOSOME ABERRATIONS IN RESPONSE TO INTERSTRAND CROSSLINK DAMAGE

by

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ABSTRACT

Interstrand cross-link (ICL) damage forms a covalent bond between the two strands of DNA, posing a unique repair problem, as there is no undamaged complementary strand from which to glean repair information. Normal mammalian cells display chromosome abnormalities known as radial formations when damaged with ICL agents such as mitomycin C (MMC) and diepoxybutane (DEB). Mammalian cells deficient in the accurate processing of ICLs, such as Fanconi anemia (FA) cells, display a greatly increased incidence of these aberrant forms upon challenge with ICL agents.

Although radial formation has been used to diagnose FA for more than three decades, there has been little analysis of these chromosomal structures. Understanding the mechanism of formation of radials should help in elucidating the mechanism of ICL repair and the function of the FA pathway of proteins.

Here we explore questions about the formation of, and chromosomal involvement in radial figures. We analyzed G-banded radials from normal and FA human fibroblasts to determine chromosomal and band involvement in radials.

Sister chromatid exchanges (SCEs) are homologous recombination (HR) mediated material exchanges between sister chromatids. These SCEs occur sponateously at low levels in normal cells, and can be induced by ICL damage. We examined SCE rates, with and without MMC treatment in FA cells to determine whether radial formation was related to changes in SCE.

To further investigate mechanisms of radial formation, we examined cells depleted of proteins involved in HR and non-homologous end joining (NHEJ) for their radial-forming capabilities and their response to ICL damage.

Our results suggest that radials form exclusively between non-homologous chromosomes or chromosome regions. The X and Y chromosomes appear to escape radial formation. We show that both spontaneous and ICL-induced sister chromatid exchange rates are not altered in FA cells, and therefore, SCEs do not appear to be related to radial formation. Finally, we show, through siRNA depletion studies, that key proteins in the processes of HR and NHEJ are not required for the formation of radials following ICL damage, but that these processes both may be involved in cellular response to ICL damage.

CHAPTER ONE:

Introduction

Interstrand Cross-link Damage and Repair

DNA interstrand cross-links (ICLs) are covalent bonds that form between two nucleotides on opposite strands of DNA and are severe forms of DNA damage. These cross-links can be induced by the presence of chemicals such as mitomycin C (MMC), diepoxybutane (DEB), nitrogen mustard, cisplatin and photoactivated psoralens. The toxicity of ICL damage is primarily due to their absolute block of strand separation and therefore replication, transcription, and segregation (Dronkert and Kanaar 2001). In addition, since both strands of DNA are damaged by the ICL, there is no complementary strand available as a template for repair. The cellular toxicity of ICL agents is often exploited through their use as chemotherapy and phototherapy agents in the treatment of cancer.

Mitomycin C, the ICL agent primarily used in these studies, has alkylating function that is activated in cells by reductive action of mammalian reductases (Tomasz et al. 1987). This activation results in formation of ICLs between the N-2 of guanines and cytosines at d(CpG) sequences in the minor groove of DNA (Tomasz 1995).

Repair of ICLs has been characterized both biochemically and genetically in *E. coli*. Two pathways, nucleotide excision repair (NER) and homologous recombination, function together to repair an ICL (Cole 1973; Van Houten et al. 1986). The NER enzymes UvrABC make incisions surrounding the region of DNA containing the ICL, DNA polymerase I exonuclease activity creates a gap at the 3' end of the ICL, and then RecA acts on the single-stranded DNA created by this gap to initiate recombination repair with intact homologous DNA. This creates a three-stranded region of DNA which is excised by UvrABC (Sladek et al. 1989).

The process of ICL repair in *S. cerevisiae* is not defined in as much detail as E. coli. However, the process is known to also require the actions of homologous recombination and excision repair proteins (Jachymczyk et al. 1981; Magana-Schwencke et al. 1982; de Andrade et al. 1989). Lack of key proteins in these processes in *S. cerevisiae*, such as RAD51, RAD52, RAD6, RAD18, and REV3, causes cellular sensitivity to ICL agents (Henriques et al. 1997; Paulovich et al. 1997; Grossmann et al. 2001). It has, in fact been shown that there are three distinct pathways for ICL repair in *S. cerevisiae*, independently involving the actions of the proteins SNM1, REV3, and RAD51(Grossmann et al. 2001).

Repair of ICLs has been extensively examined in mammalian cells. It appears that many more genes may be involved in this process than in repair of ICLs in *E. coli* and *S. cerevisiae.* Therefore, the details of this process continue to be investigated. It is known that recognition of ICLs occurs during DNA replication (Akkari et al. 2000), but further understanding of their repair continues to be investigated. There are mammalian homologs to the proteins involved in yeast repair of ICLs, and mammalian cells use both of the processes of homologous recombination and excision repair. However, differences exist between the two phyla in repair of other types of damage, such as double-strand breaks. It has been proposed, in fact, that excision of ICL-adducts following recognition of ICL damage independent of DNA replication results in a double-strand break forming during replication (Rothfuss and Grompe 2004). Therefore, the pathways involved in DSB repair may be imperative in the repair of ICL damage. There is indication that homologous recombination (HR), non-homologous end-joining, and nucleotide excision

repair are all involved in mammalian repair of ICLs (Hoy et al. 1985; De Silva et al. 2000; Wang et al. 2001).

A great concentration of work exploring ICL damage in mammalian cells has been done in the context of Fanconi anemia (FA). Cells from these patients are specifically sensitive to ICL agents (Sasaki and Tonomura 1973; Grompe and D'Andrea 2001). ICL damage, at varying levels, in normal and repair-deficient mammalian cells, such as FA cells, causes visible chromosome aberrations, namely chromatid breaks and radials, at metaphase. These radial formations are the focus of this research and will be further discussed later.

Fanconi Anemia

Fanconi anemia (FA) is a rare recessive genetic disorder, originally described by the Swiss pediatrician Guido Fanconi in 1927 (Fanconi 1927; Fanconi 1967). The clinical phenotype of FA is variable, overlapping with numerous genetic and non-genetic diseases (Weemaes et al. 1981; Alter 1993; Weemaes et al. 1993; Nakanishi et al. 2002). FA, however, is most noted for skeletal defects, bone marrow failure, and cancer susceptibility. There are currently 11 identified complementation groups for FA, with 9 of the 10 identified genes (*FANCA*, *C*, *D1*, *D2*, *E*, *F*, *G*, *L*, and *M*) located on autosomes (Joenje et al. 1997; D'Andrea and Grompe 2003; Meetei et al. 2003). The *FANCB* gene maps to the X chromosome, and therefore leads to an X-linked inheritance of FA in families with mutation in the *FANCB* gene (Meetei et al. 2004) (Table 1.1). The breast cancer gene Brca2 has been identified as the gene mutated in cells from the FA-D1 complementation group (Howlett et al. 2002). This identification has brought together

the BRCA and FA pathways. The FA complex (consisting of FANCA, C, E, F, and G) (Garcia-Higuera et al. 1999; de Winter et al. 2000) is activated following DNA damage, leading to monoubiquitination of FANCD2, proposed to be mediated by ubiquitin ligase FANCL (PHF9) (Meetei et al. 2004). FANCD2 subsequently forms nuclear foci at the sites of DNA damage, co-localizing with BRCA1, RAD51, and BRCA2 (Fig. 1.1). These foci also co-localize with the MRE11/RAD50/NBS1 end-joining complex foci (D'Andrea and Grompe 2003).

FA Gene	Chromosomal Location	Other Names
FANCA	16q24.3	
FANCB	Xp22.31	
FANCC	9q22.3	
FANCD1	13q12.3	*BRCA2*
FANCD2	3p25.3	
FANCE	6p21-22	
FANCF	11p15	*XRCC9*
FANCG	9q13	
FANCL	2p16.1	*PHF9*
FANCM		*FAAP250*

Table 1.1. *Summary of known Fanconi anemia genes* (D'Andrea and Grompe 2003; Meetei et al. 2003).

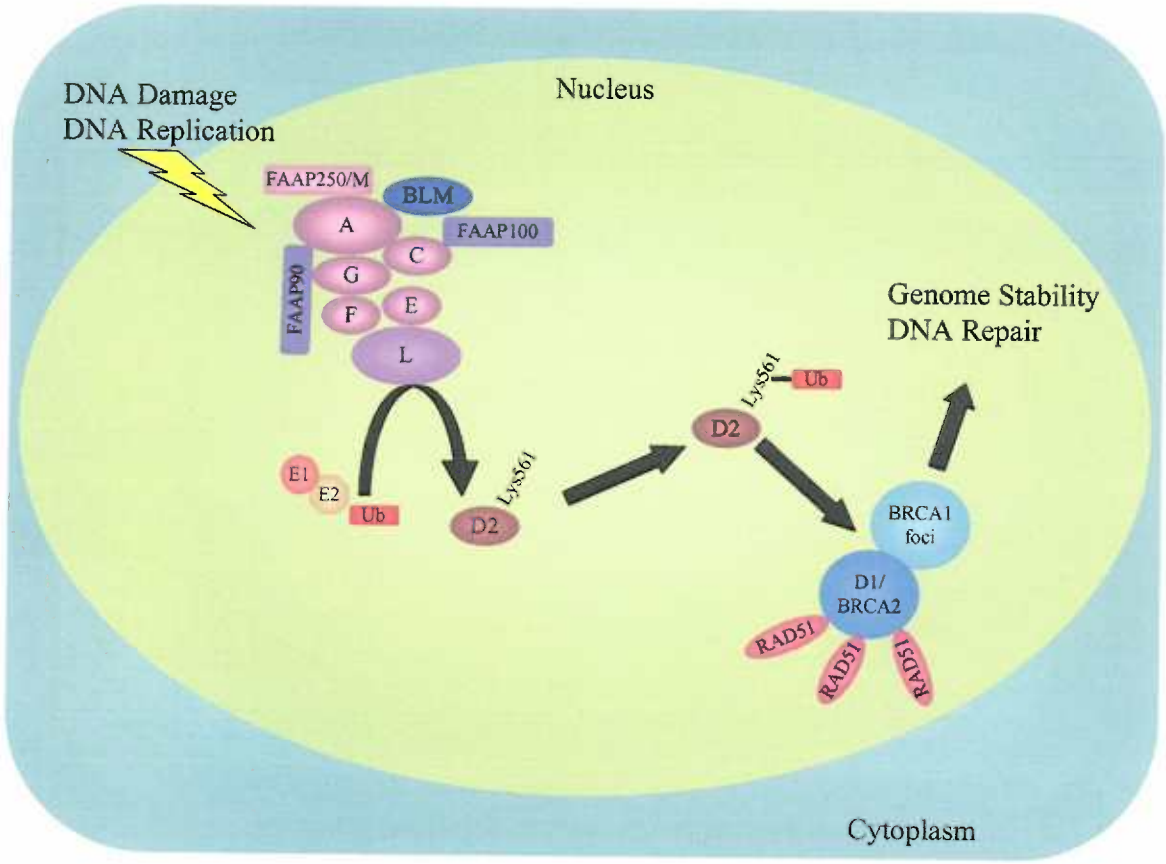

Figure 1.1. *Model of the Fanconi anemia pathway.* During replication or in response to DNA damage, the core complex, consisting of FANCA, C, G, E, F, L, M, and FA-associated proteins 90 and 100 is activated. FANCL is thought to act as the ubiquitin ligase that then monoubiquitinates FANCD2 at Lys561. FANCD2 then colocalizes to DNA-repair foci containing BRCA1, BRCA2/FANCD1 and RAD51. This action is thought to result in genome stability and likely DNA repair.

Cells from FA patients have cellular hyper-sensitivity to interstrand cross-linking agents resulting in impaired cell survival and observable breaks and radial formations (Sasaki and Tonomura 1973; Grompe and D'Andrea 2001). Clinical diagnosis of FA currently relies on scoring these breaks and radial formations following challenge with interstand cross-linking agents mitomycin C (MMC) and diepoxybutane (DEB) (Auerbach et al. 1981; Cervenka et al. 1981; Auerbach 1993).

Radials

Chomosomal aberrations of the chromatid type, breaks, quadri- and tri- radials, following ICL damage, are used as an endpoint in diagnosis of Fanconi anemia (Sasaki and Tonomura 1973; Auerbach et al. 1981; Cervenka et al. 1981). These radials, sometimes referred to as chromosome exchange figures, have also been observed following DSB induction by ionizing radiation (Savage and Bhunya 1980).

Though the terms quadri-radial and tri-radial have historically been used to describe this group of aberrations, these titles primarily describe a visual image. Radial formation following MMC damage also includes figures with many more than 4 radii. In addition, it has been determined that some chromosomal aberrations previously described as tri-radials or symmetrical tri-radials found in cells not damaged by ICL or IR are actually results of endoreduplication (Kuhn and Therman 1982). These tri-radials are a distinctly different entity than the radials we are exploring. The radials we evaluate in this study appear as joining of chromatid breaks, though, as the process behind this formation is not known, this does not imply a mechanism. Following the ideas of

possible mechanisms of ICL repair, it has been suggested that radials may form through failed attempts at either homologous recombination or non-homologous end joining.

Some attempts to evaluate the content of MMC-induced radials have been made. Studies described exchanges between both homologous and non-homologous chromosomes with a large number of radials occurring in the peri-centromeric heterochromatic regions of chromosomes 1, 9, and 16 (Bourgeois 1974; Dutrillaux et al. 1977). This present study further explores the chromosomal content and exchange location of radials using newer techniques for more accurate chromosome banding and a more extensive sample size.

The direct cause of radial formation, as well as the mechanism by which they form has yet to be determined. A difference in formation of tri-radials and quadriradials was suggested by Satoh et. al., involving isochromatid breaks at the telomere regions being responsible for the appearance of a tri-radial (Satoh et al. 2002). Though this model may be accurate, it still does not suggest a mechanism of DNA joining at the sites of breaks in the formation of either tri-or quadri-radials and does not truly identify a difference between the two forms. Our studies show that neither homologous recombination nor non-homologous end-joining are required as functioning processes in the cell in order for radial exchange figures to occur. An understanding of the process by which radials form may help us to understand the process ICL repair and the potential deficiency thereof in FA cells.

Bloom Syndrome

Bloom syndrome (BS) is a rare autosomal recessive disorder, originally described by D. Bloom in 1954 (Bloom 1954), where patients are at an increased risk for a variety of cancers. BS is distinct from FA in its clinical phenotype, as BS patients are identified by growth retardation, immunodeficiency, photosensitivity, and increased cancer risk (German 1995).

The gene for BS maps to chromosome 15q26.1 (German et al. 1994). BLM, the Bloom syndrome protein, is a RecQ helicase that may regulate homologous recombination through suppression of illegitimate recombination (van Brabant et al. 2000). Cells mutant in BLM display a high level of spontaneous sister chromatid exchange (Chaganti et al. 1974). This cellular phenotype is often used as the clinical diagnostic standard for BS.

BLM is required for the correct localization of MRE11/RAD50/NBS1 complexes to replication fork arrest foci (Franchitto and Pichierri 2002). Cells from BS patients display a prolonged delay in S-phase following DNA damage (Davies et al. 2004). It has also been recently shown that the BLM protein interacts with the FA pathway core complex (Meetei et al. 2003) in a complex termed BRAFT (Fig 1.2).

We explore the possible relationship of FA and BLM by examining radial formation and ICL sensitivity in BLM cells and cells depleted of BLM by siRNA. In addition, we explore spontaneous and MMC-induced sister chomatid exchange rates in both BLM and FA cells for reasons discussed below.

Sister Chromatid Exchange

Exchanges between sister chromatids can be visualized using incorporation of the thymidine analog BrdU to label newly replicated chromatids differentially. These sister chromatid exchanges (SCEs) have been shown to occur at a level of ~10 SCEs/metaphase in normal cycling human cells (Galloway and Evans 1975; Crossen et al. 1977). DNA damaging agents such as UV irradiation and mitomycin C (MMC) are known to cause elevation in SCE levels. This induction indicates that SCEs reflect a process of DNA repair.

Though it is known that homologous recombination mediates SCE in vertebrate cells (Sonoda et al. 1999) much of the details of the molecular process of SCE is still unknown. Because SCE requires a replicated sister chromatid and eukaryotic cells treated with DNA damaging agents only show an increase in SCE following a round of replication (Wolff et al. 1974), SCE is clearly closely involved with the process of replication.

SCE is most commonly recognized in its characterization of Bloom syndrome. Lymphoblast cells from Bloom syndrome patients display high levels of spontaneous SCE, averaging approximately 89 per cell (Chaganti et al. 1974).

The SCE rates in FA cells have recently come into question (Kraakman-van der Zwet et al. 2002; Niedzwiedz et al. 2004). As previously mentioned, FA cells show hypersensitivity to ICL damage, a type of damage that increases the level of SCE's in normal cells. In addition, as stated earlier, the BLM protein associates in a complex with the FA proteins (Meetei et al. 2003), highlighting a possibility of shared phenotypes amongst cells deficient in BLM and FA genes.

Both BRCA2 and RAD51 mutant cells show a decrease in MMC-induced SCE (Sonoda et al. 1999; Kraakman-van der Zwet et al. 2002). This decrease in SCE in Brca2 cells challenged with MMC draws additional attention to the FA cells. Since FA-D1 cells are defective in Brca2, we hypothesize that other FA complementation groups may show a decrease in SCE levels as well. If this were true, perhaps the radials resulting from ICL damage to FA cells were occurring when the process of SCE was impaired. In contrast, studies in DT40 chicken cells mutant in the FANCC gene have suggested that there is an FA-associated increase in SCE (Niedzwiedz et al. 2004) Other studies, however, have reported that FA cells show no increase in sister chromatid exchange (Chaganti et al. 1974; Hayashi and Schmid 1975; Latt et al. 1975; Dutrillaux et al. 1977; Gebhart et al. 1985).

In this study, we evaluate the association of BLM and the FA pathway. We examine SCE rates in FA cells of a variety of complementation groups, including FANCD1 (BRCA2) and BLM cells, as well as cells depleted of BLM protein. These studies were conducted both with and without challenging the cells with MMC damage and show that FA cells do not display a Brca2-like decrease in MMC induced SCE nor an overall increase in spontaneous sister chromatid exchange.

Homologous Recombination

As previously mentioned, studies in *S. cerevisiae* and *E. coli* have shown extensive evidence for homologous recombination (HR) as the main process of repair for ICL damage. HR is already known to be one of the processes involved in double-strand break (DSB) repair in mammalian cells (Sonoda et al. 1998; van Gent et al. 2001). In

addition, the process of SCE depends upon the action of proteins in the homologous recombination pathway (Sonoda et al. 1999).

RAD51 is the key protein in mammalian HR and is the eukaryotic homolog to the E. coli protein RecA. Following BRCA2-mediated loading, RAD51 forms a nucleoprotein filament on single-stranded DNA (ssDNA) and functions in the search for homologous repair templates, strand pairing, and strand exchange. It has been noted to form foci at the sites of both DNA DSB and ICL damage(Bhattacharyya et al. 2000). RAD52, a partner with RAD51 in mammalian HR, co-localizes to and is required for appropriate formation of these foci (Gasior et al. 2001; Miyazaki et al. 2004). RAD52 has been proposed to have 3 distict roles in RAD51-mediated homologous recombination. RAD52 recruits RAD51 to single-stranded DNA and promotes the formation of the RAD51 filament (Sung 1997). RAD52 also assists in the pairing of complementary strands by interacting with the RPA protein which coats ssDNA (Sugiyama et al. 1998). The third role suggested for RAD52 is post-synaptic annealing, allowing the second end of the DSB to anneal to the D-loop created at the first end of the DSB by strand invasion (Sugiyama and Kowalczykowski 2002). RAD52 appears to be less essential in the process of homologous recombination, however, as RAD52 -/- mice have no notable phenotype whereas the RAD51 -/- mice are not viable (Lim and Hasty 1996; Tsuzuki et al. 1996; Rijkers et al. 1998).

Fanconi anemia cells have been shown to have increased levels of homologous recombination (Thyagarajan and Campbell 1997). In addition, the BRCA2 protein (FANCD1) has been shown to directly associate with RAD51 (Sharan et al. 1997) and cells deficient in BRCA2 appear to be impaired in the function of RAD51-mediated

homolgous recombination repair (Larminat et al. 2002). Moreover, FA-A, C, D1 and G cells lack appropriate RAD51 focus formation following ionizing radiation damage (Digweed et al. 2002). This focus formation is specifically impaired in FANCD1 cells following X-ray irradiation and MMC treatment (Godthelp et al. 2002). These associations of the action of RAD51 and the FA pathway indicate that FA cells may be impaired in the homologous recombination DNA repair pathway.

Due to the potential association between the FA pathway and homologous recombination, we investigated the role of RAD51 and RAD52 in ICL-induced radial formation. While examining this, we also explored the reaction of cells deficient in these proteins to DNA damage from ICL agents.

Non-Homologous End-Joining

Non-homologous end joining (NHEJ) is another major process of DSB repair in mammalian cells (Valerie and Povirk 2003). This pathway involves the heterodimer of Ku70 and Ku80, which binds free DNA ends and activates the catalytic subunit (DNA-PK_{cs}) of DNA-dependent protein kinase holoenzyme with serine-threonine kinase activity (Smith and Jackson 1999). The presence of these subunits is required for recruitment of the XRCC4/DNA ligase IV complex to DNA breaks (Calsou et al. 2003). This DNA ligase IV complex is then responsible for the break resealing step in the NHEJ repair of a DSB (Li et al. 1995; Grawunder et al. 1998).

Deficiency in NHEJ in humans leads to an immunodeficiency syndrome, Severe Combined Immune Deficiency (SCID) (Schwarz et al. 2003), as NHEJ is a required process in V(D)J recombination. A mouse with SCID phenotype exists, $Prkdc^{sc/sc}$,

mutant in the catalytic subunit of DNA-PK (Blunt et al. 1996; Araki et al. 1997). These mice are sensitive to ionizing radiation damage in addition to being immunosuppressed (Jackson 1997).

Fanconi anemia fibroblasts have been shown to be deficient in blunt-end DNA rejoining, mediated by the RAD50/MRE11/NBS1 (RMN) complex (Donahue and Campbell 2004). In addition to the action of the DNA-PK, Ku70/80, and DNA Ligase IV/XRCC4 proteins, the presence of RAD50, MRE11, and NBS1 has been shown to be necessary for proper in vitro DNA end-joining in extracts from human cells (Zhong et al. 2002). DNA damage-induced foci of the RMN complex require the action of FANCC (Pichierri et al. 2002). Along with BRCA2 and RAD51, this focus also co-localizes with FANCD2 DNA-damage induced foci (Nakanishi et al. 2002).

In these studies, we explore the possible role of NHEJ proteins DNA-Pk_{cs}, Ku70, DNA Ligase IV, and MRE11 in ICL-induced radial formation, as well as the reaction of cells deficient in these proteins to the challenge of ICL DNA damage.

Specific Aims

The process of mammalian cell response to ICL damage has a number of questions still needing to be explored. The chromosomal response to ICL damage, the makeup of radials, and the mechanism of radial formation are all topics whose better understanding could help address these questions. We untertake this study with the belief that cytogenetic and molecular examination of the radials formed as a result of ICL damage should lead to an understanding of the mechanism of radial formation and the

function of the FA pathway in maintaining genomic stability. This investigation was approached through the four specific aims described below.

Specific Aim 1

The first specific aim of this project was to characterize the chromosomal content of ICL-induced radials in both Fanconi and normal human fibroblasts. We propose that identifying chromosomes and chromosomal regions involved in radials and which chromosomes are associated with each other in radials will reveal patterns of association that may indicate a process of formation. To this end, we used G-banding to identify the chromosomes and bands involved in 372 radial formations. In addition, we examined radial formation in human-mouse hybrid cells with an FA phenotype to explore the possibility of human/mouse "hybrid" radials.

Specific Aim 2

Our second specific aim was to examine the spontaneous and ICL-induced sister chromatid exchange rates in FA cells. With the relationship FA appears to have with homologous recombination, as well as the observed involvement of the BLM protein in the FA complex, we hypothesized that spontaneous and ICL-induced sister chromatid exchange rates may be decreased or increased in FA cells. This hypothesis was supported on both sides by recent publications recognizing changes in SCE levels in cells deficient of FA proteins (Kraakman-van der Zwet et al. 2002; Niedzwiedz et al. 2004). We also hypothesized that if the FA pathway was acting to mediate sister chromatid exchange, and this process was impaired in FA cells, that radials may be forming as an

alternative to sister chromatid exchange following ICL damage. To test these hypotheses, we enumerated SCEs in cells from 5 different FA complementation groups, as well as their retrovirally complemented counterparts. The results of this evaluation are presented in chapter 3 of this manuscript.

Specific Aim 3

Our third specific aim was to evaluate ICL-induced radial formation in cells deficient in the process of homologous recombination and homology-directed DNA repair. We hypothesized that radials are the result unsuccessful attempts at non-allelic homologous recombination repair, and that therefore, radials are formed through the action of HR proteins. We present in this manuscript the evaluation of radial formation in both normal and FA cells depleted of the HR proteins RAD51, RAD52, and MRE11.

Specific Aim 4

Our final specific aim was to evaluate ICL –induced radial formation in cells deficient in the process of non-homologous end joining. We hypothesized that radial formation occurs through non-homologous end-joining of double-strand breaks resulting from the incision of an ICL and the subsequent recognition of a double-strand break in DNA replication (Rothfuss and Grompe 2004). This aim was achieved by evaluation of radial formation in normal and FA cells depleted of the NHEJ proteins Ku70, Ligase IV, and MRE11 as well as evaluation of radial formation in normal and FA mouse cells mutant for DNA-PK_{cs}.

We indend through these specific aims to characterize the chromosomal content of radials, evaluate ICL-induced SCE in FA and normal cells and investigate possible relation of SCE to radial formation, and to identify proteins that participate in a mechanism of ICL-induced radial formation in mammalian cells. The results and conclusions from these four specific aims are presented in the following papers. "Interstrand crosslink-induced radials form between non-homologous chromosomes, but are absent in sex chromosomes" (Newell et al. 2004) reports on the 372 radials and the mouse/human "hybrid" radials characterized for specific aim 1. "Bloom syndrome helicase is epistatic with the Fanconi anemia pathway for response to DNA interstrand crosslinks" examines the relationship of the BLM protein and FA cells in response to ICL damage and reports on the spontaneous and ICL-induced sister chromatid exchange rates of normal, BLM, and FA cells as described in specific aim 2. "Interstrand crosslink induced radials are not mediated by proteins in non-homologous end joining or homologous recombination repair" reports the results of the experiments outlined in specific aims 3 and 4 and examines the relationship of HR and NHEJ to the FA pathway in ICL response. Following the papers, a final summary and discussion of the findings of this project is presented.

CHAPTER TWO:

Interstrand crosslink-induced radials form between non-homologous chromosomes, but are absent in sex chromosomes

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The following people contributed to the results in the following manuscript.

Dr. M. Grompe Laboratory

Barbara Cox:	Establishment of FA cell lines, retroviral correction of FA cell lines, and microcell-mediated chromosome transfer

Dr. S. B. Olson Laboratory

Andrew Rosenthal:	FISH of mouse/human hybrid cells

Abstract

Fanconi anemia (FA) cells and cells lacking functional BRCA1 and BRCA2 proteins are hypersensitive to interstrand cross-linking (ICL) agents and show increased numbers of chromosomal breaks and radials. Although radial formation has been used to diagnose FA for more than 30 years, there has been little analysis of these characteristic formations.

In this study, radials were analyzed from FA-A and FA-G fibroblasts as well as normal and retrovirally-corrected FA-A fibroblasts treated with high doses of ICLs. Radials were found to only involve non-homologous chromosome interactions and to be distributed nearly randomly along the length of chromosomes. Sites on chromosomes that did show increased frequency of radial involvement did not correlate with known fragile sites or pericentric regions. Hybrid radials were observed between mouse and human chromosomes in human-mouse hybrid cells produced by microcell-mediated chromosome transfer of mouse chromosomes into human FA-A fibroblasts. Both the X and Y chromosomes were notably not involved in the radials analyzed. These observations suggest that ICL repair may involve short stretches of homology, resulting in aberrant radial formation in the absence of FA proteins.

Introduction

DNA interstrand crosslinks (ICLs) form as covalent bonds between two nucleotides on the two opposite DNA strands when cells are treated with chemicals such

as mitomycin C (MMC), diepoxybutane (DEB), and photoactivated psoralens(Metzler 1986). This covalent bond presents a challenge for the cellular repair machinery since there is no complementary strand available as a template. Recognition of ICLs has been shown to occur during S phase (Akkari et al. 2000); therefore, repair of these lesions is likely to be attempted following replication. This is consistent with the formation of sister chromatid breaks and radials following ICL damage which are visible cytogenetically at metaphase.

Much of the research addressing ICL repair in mammalian cells has focused on Fanconi anemia (FA) cells which are hypersensitive to ICL agents (Sasaki and Tonomura 1973) (Grompe and D'Andrea 2001). FA cells form radials at much lower concentrations of MMC than wild-type cells (Kubbies et al. 1985) and display a significant cell cycle delay with a 4N DNA content after introduction of ICLs (Akkari et al. 2001). Taking advantage of this hypersensitivity, clinical diagnosis is conducted by exposing cells from FA patients to ICL agents such as MMC and DEB and scoring for the formation of breaks and radials (Auerbach et al. 1981; Cervenka et al. 1981) (Fig. 2.1). Additionally, this unique cellular phenotype is used in complementation studies and other experiments that explore the FA genes and their function (Whitney et al. 1995; Jakobs et al. 1997; Joenje et al. 1997).

Although the understanding of FA proteins and their function is continually increasing, the molecular mechanism of ICL repair in mammals is yet to be elucidated. It has been proposed that mammalian ICL repair may involve non-homologous end-joining, homologous recombination, or both (Dronkert and Kanaar 2001). Recently, BRCA1 and BRCA2, known to be involved in homologous recombination and maintenance of

genomic stability, have been implicated in the FA pathway; BRCA1 co-localizes with RAD51 and FANCD2 in nuclear foci following ionizing-radiation (IR)-induced DNA damage (Garcia-Higuera et al. 2001). Moreover, chromosomes from murine cells carrying a homozygous truncation of Brca2 have been observed to form radials in response to MMC treatment (Patel et al. 1998; Yu et al. 2000). Consistent with these findings, Brca2 has been identified as the gene mutated in cells from the FA-D1 and FA-B complementation groups (Howlett et al. 2002). Both BRCA1 and BRCA2 have been shown to be involved in homology-directed DNA repair, and functional BRCA1 is also seen to restore MMC resistance in BRCA -/- 236.44 cells (Moynahan et al. 2001; Tutt et al. 2001).

Observation of phenotype reversion in FA patients and subsequent molecular evidence suggested the occurrence of mitotic recombination events, whereby, perhaps through gene conversion, the wild-type allele serves to restore the sequence of the mutant allele (Waisfisz et al. 1999). On the other hand, error-free blunt DNA end-joining has also been shown to be significantly decreased in FA cells which in turn may point to non-homologous end-joining as a possible repair mechanism (Escarceller et al. 1998).

In yeast, repair of ICLs is known to require homologous recombination repair and excision repair (Jachymczyk et al. 1981; Magana-Schwencke et al. 1982; de Andrade et al. 1989). Indeed, null mutations in excision repair group genes *RAD1* and *SNM1,* in recombinational repair genes *RAD51* and *RAD52*, and post-replication repair genes *RAD6*, *RAD18*, and *REV3*, all cause sensitivity to ICL agents (Henriques et al. 1997; Paulovich et al. 1997; Grossmann et al. 2001).

Despite recent progress in the FA field, the phenomenon of radial formation is still poorly understood. The elucidation of the mechanism of radial formation and the chromosomal regions involved may lead to an increased understanding of mammalian ICL repair and the role of the FA proteins in this process.

Assuming the distribution of ICL damage to be random along the chromosomes, and all chromosomes to be equally affected by such damage, radial formation would be expected to occur both between homologous and non-homologous chromosomes and all chromosomes would be expected to be involved in such formations. Early studies of FA and ICL damage did explore certain aspects of radial formation and DNA damage. In 1974, Bourgeois induced radial formation in normal blood samples with high levels of MMC and described the break location and chromosome involvement of 135 radial exchanges (Bourgeois 1974). A predominance of radials between chromosomes 1, 9, and 16 in both homologous and non-homologous exchanges was observed. Subsequently, a study of 339 chromosome breaks induced in peripheral blood from FA patients was also described (Dutrillaux et al. 1977). Chromosomes 1, 2, 6, 9, 11, and 13 showed an excess number of breaks, whereas sex chromosomes and smaller chromosomes showed a much smaller number of breaks.

One of the difficulties in assessing data from the literature is the inconsistency in the use of terminology to describe the mitotic chromosome aberrations observed in patients with Fanconi anemia. Historically, the terms triradial and quadriradial have been used as descriptors of the numbers of arms, or radii, emanating from each figure. Originally used to convey a visual image, triradial has over the years implied a symmetrical configuration arising from partial endoreduplication (Kuhn and Therman

1982). Although the quadriradials comprise the majority of formations in Fanconi anemia, more complex figures involving more than two chromosomes, sometimes appearing as chains, are common. For the purposes of this study, the term radial is being used to describe any multiradial formation with inter-chromatid interactions involving at least two chromosomes, and apparently joining chromatid breaks on each chromosome. This description is not intended to imply any underlying mechanism for radial formation (i.e. recombination or end-joining), as such a mechanism is yet to be elucidated.

Our study presents a compilation of data on radial formation in both FA (complementation groups A and G) and wild-type primary human fibroblasts. A total of 372 radials were analyzed with respect to chromosome involvement and G-band breakpoint location. The ability of "hybrid" radials to form between human and mouse chromosomes were also investigated.

Materials and Methods

Cells and Media

Two primary fibroblast cell lines from a male FA-A and a female FA-G were obtained from the OHSU Fanconi anemia cell repository. Control samples comprised cells from a normal female, and FA-A cells corrected with a retrovirus harboring the FANCA gene and the puromycin selectable marker (Pulsipher et al. 1998). All specimens were collected with informed consent under an OHSU institutional review board approved protocol. Two primary fibroblast cell lines from a male FA-A and a female FA-G were obtained from the OHSU Fanconi anemia cell repository. Human/mouse hybrid cells were developed through microcell-mediated chromosome

transfer of mouse chromosomes into human FA fibroblasts as described in Whitney et. al. (1995). Cells were maintained in α-modified Eagle medium (GIBCO/BRL) with 20% fetal bovine serum (GIBCO/BRL), 1X glutamine (GIBCO/BRL), and 50μg/ml Gentamycin (GIBCO/BRL) at 37°C and 5% CO_2.

G-Banding Studies

FA-A and FA-G cells were treated with 15-20 ng/ml MMC (Sigma) and normal and retrovirally-corrected FA-A cells were treated with 100-400 ng/ml MMC for 48 hours. Colcemid (0.05 μg/ml) (GIBCO/BRL) was added during the last 16 hours of culture. Cells were then treated with a hypotonic solution (0.075 M KCl, 5% fetal calf serum) for 10 minutes, and fixed with 3:1 methanol acetic acid. Slides were treated with 10% trypsin for 45-55 seconds followed by Wright's stain (Fisher Scientific) for 2 minutes 30 seconds. G-banded metaphase spreads that contained at least one radial formation were examined using bright-field microscopy and captured using Applied Imaging's CytoVision software (Santa Clara, California.). Chromosomes involved in radial formations were identified and characterized by their G-banding patterns and described with relation to chromosome arm and break locations.

Mouse-Human Hybrid FISH

Spectrum Red total genomic mouse (TGM) and Oregon Green total genomic human (TGH) FISH probes were produced by nick-translation using 1.2 μg of TGM or TGH DNA and the Nick Translation Kit by Roche (Indianapolis, IN) with Spectrum Red dUTP from Vysis (Downers Grove, IL) and ChromaTide Oregon Green dUTP from

Molecular Probes (Eugene, OR). Human-mouse hybrid cells containing 1-4 mouse chromosomes in a background of 60-100 human chromosomes were dropped onto microscope slides and baked at 90°C for 6 minutes. FISH was performed by denaturing slides for 4 minutes at 74°C in 70% Formamide/2X SSC and hybridized to the probes overnight at 37°C. Slides were then washed for 15 minutes at 43°C in 50% Formamide/2X SSC followed by a wash in 0.1X SSC for 15 minutes at 60°C. Eighteen microliters of DAPI II (125 μg/μl) (Vysis) was used as a counterstain. Metaphases were observed on Nikon Eclipse E800 photoscope and captured using CytoVision software from Applied Imaging as described above with either Spectrum Red-labelled TGM and Oregon Green-labelled TGH or Spectrum Red-labelled TGM alone. Metaphases observed on Nikon Eclipse E800 photoscope and those containing at least one mouse chromosome and at least one radial were captured using CytoVision software from Applied Imaging.

Analysis of Involvement Frequency

Standardized regression analysis was used to analyze radial involvement relative to chromosome length. The chromosome length available for involvement in a radial for each autosome and the X chromosomes in female diploid cells was calculated as $2b_n$ where b_n= chromosome n length in million base pairs (Mbp). On the other hand, b_n was used to calculate the length of the Y chromosome and the X chromosome available in male cells. The total autosome length in a haploid cell is estimated to be 2863Mbp (all approximate chromosome lengths were obtained from the UCSC Genome Bioinformatics Genome Browser).

Of the total number of radials analyzed, 39.6% were from female cells and 60.4% from male cells. Given that female cells have two X chromosomes and male cells have one X chromosome, the available base pair length for radial formation for the X chromosome was calculated as *2(153Mbp)(0.396)+(153Mbp)(0.604)=214Mbp*, where 153Mbp is the length of the X chromosome. The length of the Y chromosome available was calculated as *51Mbp(0.604)=31Mbp*, where 51Mbp is the length of the Y chromosome.

Calculation of the total base pair length for all chromosomes available for radial formation uses the lengths of X and Y, as well as total autosome length. This number was calculated as *2(2863Mbp)+214Mbp+31Mbp=5971Mbp*.

The X chromosome comprises 3.6% of the total genome whereas the Y chromosome represents 0.5%. In order to evaluate the significance of the sex chromosome results, the 95% confidence interval was calculated as $p \pm 1.96((p(1-p))/t)^{1/2}$ where p= the proportion of radials in which the chromosome participates out of the total number of radials, and t= the total number of radials observed.

Standardized regression analysis was also performed correlating radial involvement with chromosome length.

Results

Characterization of Radials

Radials from FA-A, FA-G, retrovirally-corrected FA-A, and normal fibroblasts were analyzed. The FA-A, FA-G, and the corrected FA-A control cells treated with high doses of MMC had more than 48% of their radial-containing cells with >1 radial/cell. In

contrast, the normal fibroblast cells treated with excess MMC had only 17.5% of their radial-containing cells with >1 radial/cell. Three hundred and seventy two of these radials, involving 783 chromosomes, were characterized by G-banding (Fig. 2.2). In FA-A cells, 129 radials were examined, and 128 were found to be between non-homologous chromosomes; 18 of these involved more than 2 chromosomes. One radial involved homologous chromosomes 3; however, the exchange was between the p and q arms. Similarly, in FA-G cells, 97 radials were characterized, and all displayed non-homologous interactions; 19 of the radials contained more than 2 chromosomes. Furthermore, 99 radials were analyzed in retrovirally-corrected FA-A cells treated with a excess MMC. Ninety-eight of these radials were between non-homologous chromosomes; 10 of these contained more than 2 chromosomes. The one radial between the 2 homologous chromosomes was between 12p and 12q. As an additional control, 47 radials from normal fibroblasts treated with a high dose of MMC were analyzed. Of these, 46 were between non-homologous chromosomes, 5 of which included more than 2 chromosomes. One radial was between 2 homologous chromosomes, 19p13.2 and 19p12, two distinct regions of the p arm of chromosome 19 (Table 2.1).

Interestingly, out of the 783 chromosomes involved in radial formation in male and female cells, the X chromosome was involved only once in the male corrected FA-A cells, representing 0.1% of the chromosomes involved in radials. This was less than the expected value derived from the proportion of DNA that X represented in the cells i.e., 3.6%. Involvement of the Y chromosome was not observed in any of the male cells analyzed, while its expected involvement value was 0.5%. The participation of a sex chromosome in 1 out of 372 radials indicates the prevalence of sex chromosome

involvement in radials to be 0-0.79% (95% confidence interval, see Materials and Methods) (Table 2.2).

Despite the lack of involvement in radials, the X chromosome did display breaks in treated FA cells. Two hundred breaks were visualized in FA-A cells. Four of these involved the X chromosome, a similar number of breaks to other chromosomes of approximately the same size (data not shown). This is consistent with the data of Von Koskull and Hula (1973) where breaks were observed in X chromosomes in FA lymphoblasts.

In the 372 radials analyzed, breakpoints were distributed along the full length of almost all the chromosomes and were found to be located in both the heterochromatic and euchromatic regions (banding range 350-800) (Fig. 2.3). Certain regions such as 1q12, 7q11.2, 12q13, and 18p11.2 appeared to have increased radial involvement. The over-representation at 1q12 was only present in the normal and retrovirally-corrected cell lines that had been treated with high doses of mitomycin C. Regression analysis showed that the individual chromosome involvement in radials correlated with the base pair length of the chromosome with a correlation factor of 0.81 and a p-value of 2.02×10^{-6}.

Mouse and Human Chromosomes Form Hybrid Radials

Since radials form between non-homologous chromosomes, the question arose whether radials could form between chromosomes of different species. A human-mouse hybrid cell line containing 1-4 mouse chromosomes in a background of 60-100 human chromosomes was used to investigate this possibility. Two hundred and thirty eight radials were characterized by FISH using total genomic mouse DNA as a probe. Of these

radials, 5 were between 2 or more mouse chromosomes, 210 were between 2 or more human chromosomes, and 23 were between a mouse and a human chromosome (Fig. 2.4).

Discussion

Our data from both radial formation analysis and human-mouse hybrid experiments indicate that radials, in both FA and normal cells, form exclusively between non-homologous chromosomes. Autosomes were involved in radials throughout their entire length, with the number of radials being proportional to the length of the chromosome, whereas, interestingly, the sex chromosomes escaped radial formation.

Sites of radial formation had no specific correlation with euchromatic and heterochromatic regions as delineated by light and dark G-bands, respectively. This is in contrast to previous descriptions of light-staining and interband localization of chromosome breaks in FA lymphoblasts (Von Koskull and Aula 1973; Dutrillaux et al. 1977), and implies a much more random chromosome region involvement in the formation of radials. This discrepancy may be explained by improvement in culturing and slide-making techniques allowing high resolution banding.

There were, however, specific chromosome regions that appeared to have increased involvement in radials, and this may be due to distinct sequence and/or to chromatin structure being more susceptible to breakage. It is notable that one of these regions of increased radial formation was 12q13, which is also a folate-deprivation sensitive fragile site. The other regions do not appear to correlate with BrdU or folate-deprivation sensitive fragile sites (Hecht et al. 1990).

A predominance of MMC-induced radials and breaks in the pericentromeric regions of normal and FA chromosomes has been previously described (Savage and Reddy 1987). With the exception of a few chromosomes with juxtacentromeric areas of increased involvement, such as 1q12, 7q11.2, and 12q13, we did not observe this phenomenon. In our study, the involvement of the 1qh region was observed in normal cells, treated with a high dose of ICL agent. This is not surprising, as spontaneous breakage in this heterochromatic block is routinely observed in cytogenetic studies (unpublished data). This is consistent with the findings of Bourgeois (1974) where he noted increased involvement of the heterochromatic regions of chromosome 1 in normal blood samples treated with high concentrations of MMC.

In contrast to normal fibroblasts, retrovirally-corrected FA cells had an increased number of radials per cell. One possible explanation is the incomplete complementation of the retroviral overexpression system.

The FA pathway has been recently linked to homologous recombinational repair through the interaction of FANCD2 with BRCA1 (Garcia-Higuera et al. 2001) and the recent finding that a biallelic mutation in BRCA2 causes FA (Howlett et al. 2002). In addition, mammalian cell lines with mutations in RAD51 paralogs, genes that contribute to the RAD51 mediated homologous recombination process; such as RAD51C, XRCC2 and XRCC3 show sensitivity to ICL agents (Liu et al. 1998; Godthelp et al. 2002). It is, therefore, conceivable that ICLs are repaired through a process of exchange between regions of homology. Metaphases from normal cells exposed to the same concentrations of clastogens as FA cells do not usually display radials. Perhaps attempted ICL repair using homologous chromosomes is promptly achieved in these cells. It is also possible

that, in normal primary fibroblasts such as the ones used in this study, the occurrence of radials triggers apoptosis. In either case, these aberrant formations would not be visualized.

Involvement of homologous regions would be expected for the repair of ICLs because of the absence of an intact template. If ICL repair depended solely on the proximity of homologous regions, those on non-homologous chromosomes might be utilized more often. In interphase cells, chromosomes are indeed organized into specific domains, where homologous chromosomes may not be in close proximity (Visser et al. 2000). Moreover, there are a large number of short regions of homology dispersed throughout the genome that could potentially act as repair templates (Johnson and Jasin 2001). In this process, the ICL repair machinery, including FA proteins, may assist damaged chromosomes in finding short stretches of homology and/or resolving the damage in a mechanism similar to mitotic recombination. This is consistent with the observation that FA cells display an increased incidence of homologous recombination (Thyagarajan and Campbell 1997).

The apparent lack of involvement of sex chromosomes in radial formation is intriguing. Consistent with our findings on autosomes, X/Y and X/X radial formation would not be expected. It is rather the absence of sex chromosome/autosome interactions that is surprising. It is conceivable that the X and Y chromosomes have acquired an alternative mechanism for ICL repair in order to avoid recombination with autosomes. In addition, in female cells, the late replication state of the inactive X may add another level of complexity to its ability to form radials.

The question remains whether the formation of radials is a normal event in ICL repair, or whether radials are aberrant structures. In addition, it is still unclear whether radials involve homologous or non-homologous DNA sequences. Cells containing radials may have some inability to find stretches of homology and/or to resolve these recombination attempts. The presence of human-mouse radials suggests the lack of requirement for species-specific or long stretch homology between the two DNA sequences. The molecular mechanism of radial formation would be further clarified with a detailed look at the specific regions of DNA involved in radials. In addition, identifying proteins that bind to these regions, as well as characterizing specific sites of ICL damage, may help elucidate the actions of the FA machinery and begin to suggest a model of recombination repair of ICLs.

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Electronic Database Information

UCSC Genome Bioinformatics Genome Browser ,

http://genome.ucsc.edu/cgi-bin/hgGateway

Table 2.1. *Summary of homologous and non-homologous radial formations*

Cell Line	[MMC]	Total # Radials	# Radials Between Homologous Chromosomes	# Radials Between Non-Homologous Chromosomes
FA-A	15-20 ng/ml	129	1	128
Corrected FA-A	200-300 ng/ml	99	1	98
FA-G	15-20 ng/ml	97	0	97
Normal Fibroblast	100-400 ng/ml	47	1	46

Table 2.2 *Chromosome length and radial involvement observed in each cell line.*

Chromosome ~	length in million bp	FA-A Radials	FA-G Radials	FA-A Corrected Radials	Normal Radials	Total Radi
1	245	21	23	27	10	81
2	243	21	17	9	4	51
3	199	14	11	10	5	40
4	192	16	11	7	2	36
5	181	17	10	7	6	40
6	171	16	13	11	4	44
7	158	15	16	9	4	44
8	146	16	14	9	4	43
9	135	15	16	5	5	41
10	135	18	12	10	5	45
11	135	20	14	13	6	53
12	133	9	8	11	3	31
13	115	9	9	6	7	31
14	105	3	3	8	2	16
15	100	7	6	8	4	25
16	90	6	3	7	7	23
17	78	9	4	13	3	29
18	78	11	10	8	2	31
19	64	5	2	9	8	24
20	64	12	4	8	3	27
21	47	4	3	7	2	16
22	49	1	1	5	4	11
X	153	0	0	1	0	1
Y	51	0	0	0	0	0

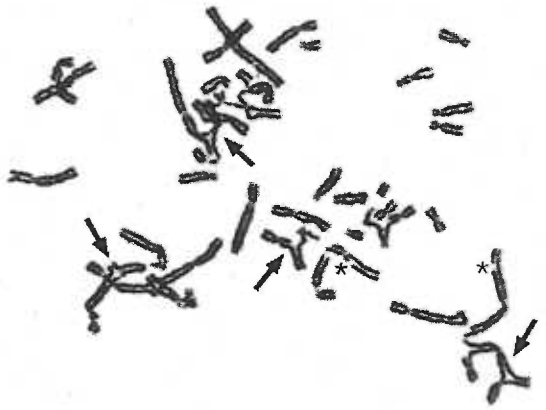

Figure 2.1. *Multiple radials and breaks in a standard-stained Fanconi anemia (FA) metaphase cell preparation.* Examples of radials (➔) and breaks (H) are indicated.

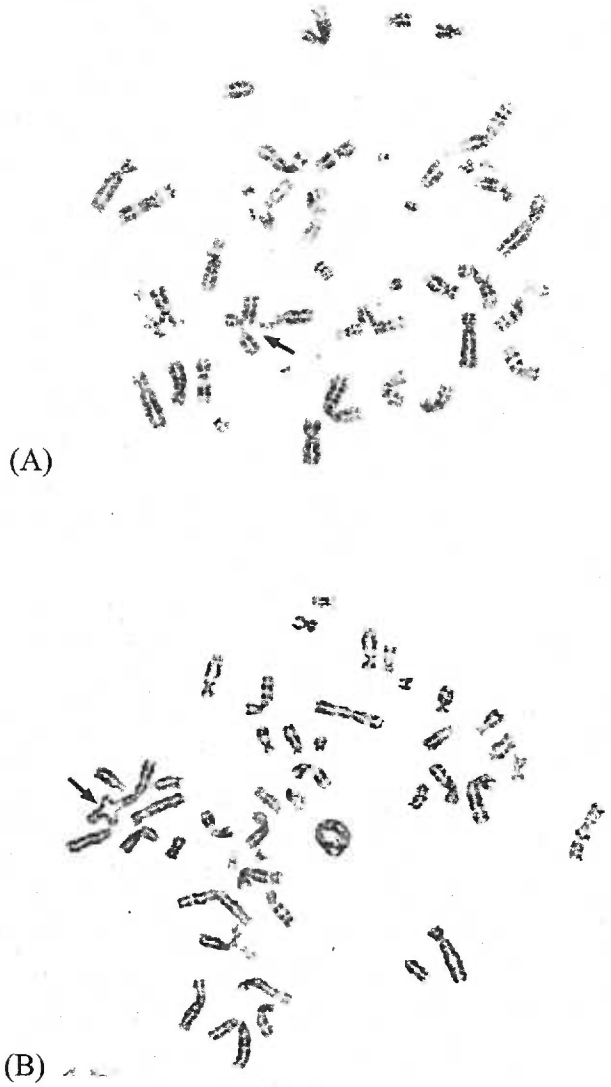

Figure 2.2. *G-banded FA-A fibroblast metaphases* treated with 20-ng/ml of MMC. Arrow indicates a radial formed between (A), chromosomes 9q, 14q, and 8p and (B), chromosomes 2p and 3p.

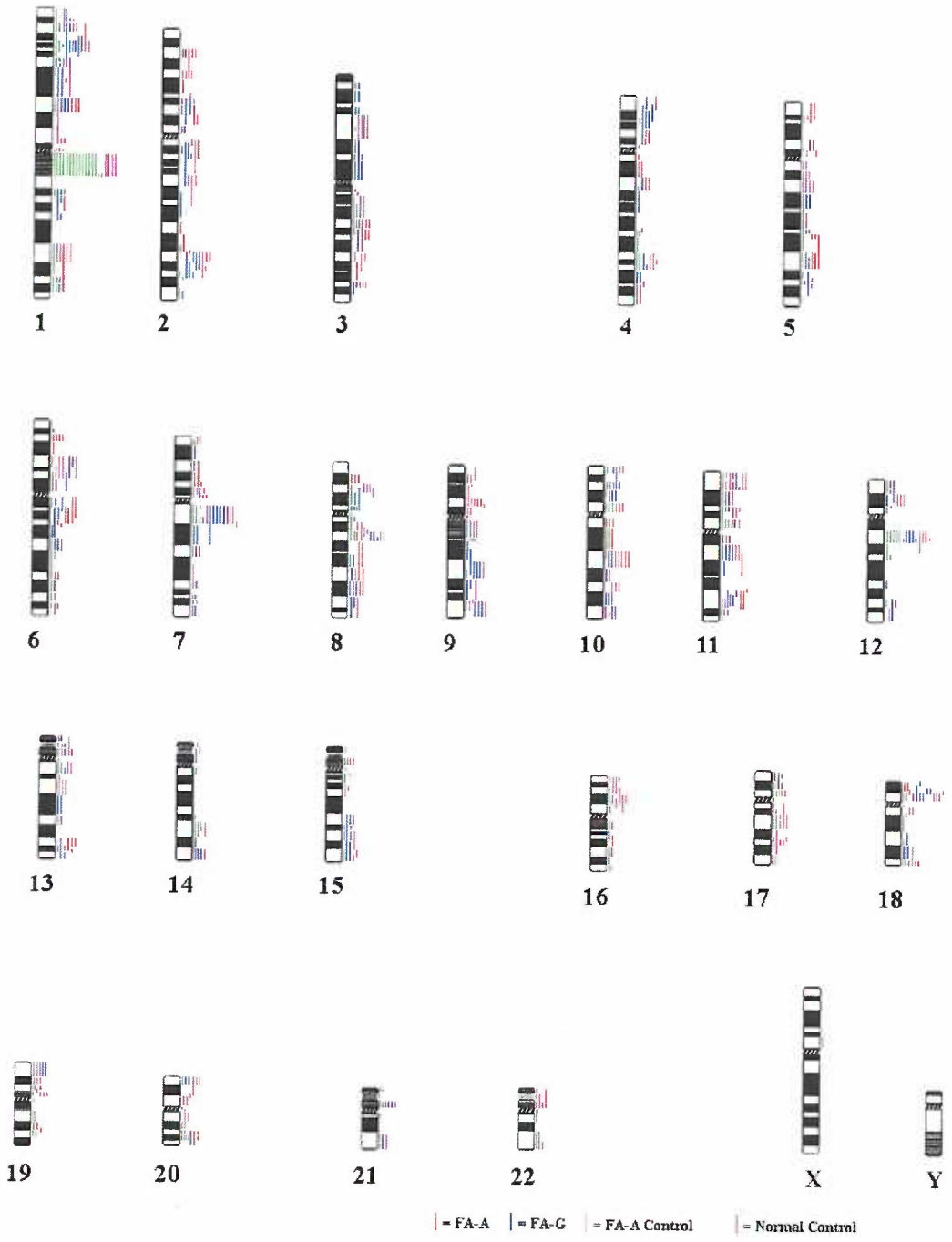

Figure 2.3. *ISCN ideogram illustrating sites of radial involvement.* The identified position of each chromosomal involvement is indicated by a vertical bar to the right of the chromosome ideogram. Each color represents the cell line in which the radial was observed.

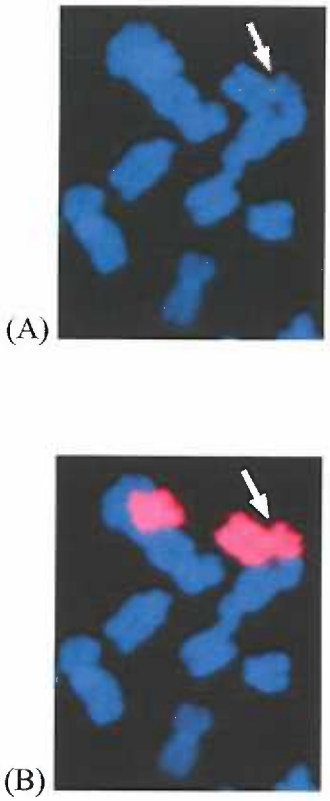

Fig. 2.4. *A radial formation between mouse and human chromosomes* from a mouse/human hybrid FA cell line. Mouse/human hybrid radial stained with (A), DAPI and (B), same radial following fluorescent in situ hybridization (FISH) with labeled total genomic mouse DNA (in red) and DAPI counterstain (in blue).

CHAPTER THREE:

The Bloom Syndrome helicase acts in the Fanconi anemia pathway for repair of DNA interstrand cross-links

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Abstract

Bloom syndrome (BS) is a genetic disorder associated with an increased incidence of malignancies due to a defect in a human RecQ helicase, the Bloom protein (BLM). BS cells have defects in regulation of S-phase and are sensitive to agents blocking S-phase progression. BLM has been found in association with proteins of the Fanconi anemia (FA) pathway, a rare genetic disorder due to a deficiency in any of eleven genes. FA is associated with chromosomal instability and increased cellular sensitivity to DNA crosslinking agents. Depletion of BLM or FANCA by small interfering RNAs demonstrates that BLM is functionally epistatic to the FA pathway for response to DNA interstrand crosslinks (ICLs), using chromosome stability or cell survival as indices. However, BLM appears to have a segregated function in regulation of sister chromatid exchanges (SCEs), since they increase in its absence, but do not in the absence of FANC proteins, including BRCA2. Monoubiquitination of the FANCD2 protein, a step required for function of the FA pathway, is normal in Bloom syndrome cells or cells depleted for BLM, placing BLM action in the ICL response after the FANC core proteins. Consistent with this, mice defective in both BLM and the FANCD2 protein appear the same phenotypically as mice defective in either of the proteins alone. Cells from primary cell lines made from the doubly defective mice do not manifest genome instability greater than cells from mice deficient in the FANCD2 protein alone. Thus, for mammals, BLM appears to act only in the FA pathway in response to ICLs, as measured by cell survival or breaks, but acts independently for control of SCE formation.

Introduction

Bloom syndrome (BS) is a rare autosomal recessive disorder characterized by growth retardation, immunodeficiency, photosensitivity, and an increased incidence of several types of cancers. These include leukemias, lymphomas, and carcinomas(German 1995; van Brabant et al. 2000; Moses 2001; Hickson 2003). At the cellular level, patients manifest a variety of chromosomal aberrations, including sister chromatid exchanges (SCEs), indicating genome instability (Chaganti et al. 1974). SCEs are a landmark feature routinely used for clinical diagnosis of BS, since there is a marked increase over normal cells. The elevated SCEs may reflect increased interchromosomal homologous recombination, apparently in S-phase (German 1995; Sonoda et al. 1999; Gaymes et al. 2002).

The defect in BS is in the Bloom protein (BLM), a member of the conserved RecQ proteins, of which there are five recognized paralogs in humans (Ellis et al. 1995; Karow et al. 2000; Nakayama 2002). Three of these are known to be associated with genetic disorders: BS, Werner syndrome and Rothmund-Thomson syndrome, all linked to an increased risk for cancers (Ellis et al. 1995; Yu et al. 1996; Kitao et al. 1999). RecQ enzymes appear to suppress illegitimate recombination, thus enhancing genome stability and are involved in the response to a variety of DNA adducts (Karow et al. 2000; Nakayama 2002). Eschericia coli deficient in the RecQ protein, or *Saccharomyces cerevisiae* deficient in SGS1 protein, the yeast RecQ homolog, show increased recombination and have defective DNA replication, suggesting a defect in re-starting replication after arrest by DNA damage (Gangloff et al. 1994; Courcelle and Hanawalt 2001; Oakley and Hickson 2002). Thus a deficiency in a RecQ enzyme leads to an

increase in recombination, possibly as the result of a replication defect. BS cells show abnormal replication intermediates and prolonged S-phase delay after DNA damage (Davies et al. 2004). While deficiency in the RecQ protein is not lethal in prokaryotes, disruption of the Blm gene in mice is lethal at day 13 *in utero* (Chester et al. 1998). In these mice, increased apoptosis and intrauterine growth retardation have been documented. Although hypomorphic mutations ($blm^{m3/m3}$) in mice permit viability, the mice show increased susceptibility to lymphomas, sarcomas, and carcinomas, which correlates with the cancer spectrum seen in BS patients (Luo et al. 2000).

Fanconi anemia (FA) is a rare recessive disease manifesting growth abnormalities, deficiencies in all blood cell lineages, and an increased risk of malignancies (Moses 2001; D'Andrea and Grompe 2003). At the cellular level, FA is marked by an increased sensitivity to agents which form DNA interstrand crosslinks (ICLs), illustrated by chromosomal aberrations, notably chromosome breaks and radials (Schroeder et al. 1964). FA can result from a deficiency in any of at least eleven genes, nine of which have been cloned (Meetei et al. 2004; Rahman and Ashworth 2004).

The FA pathway appears to be linked to additional genome stability functions. The FA proteins have been shown to have interactions with BRCA1 (Garcia-Higuera et al. 2001; Folias et al. 2002; Venkitaraman 2004), and the BRCA2 gene has been identified as the FANCD1 gene (Howlett et al. 2002). This is in agreement with the observation that siRNA depletion of BRCA2 leads to a FA-like chromosomal picture suggesting an epistatic relationship with the FA pathway (Bruun et al. 2003), and establishing a functional link between the FA pathway and the BRCA proteins (Venkitaraman 2004). FANCD2 protein is mono-ubiquitinated after DNA damage

(FANCD2-Ub), a modification that requires a core complex of at least seven FANC proteins in addition to BRCA1. In contrast, BRCA2 is not required for mono-ubiquitination of FANCD2 (Howlett et al. 2002; Bruun et al. 2003), required for the normal function of the FA pathway in the ICL response (Timmers et al. 2001; D'Andrea and Grompe 2003; Meetei et al. 2003; Meetei et al. 2004). Mice deficient in the Fancd2 gene manifest perinatal lethality and susceptibility to epithelial cancers (Houghtaling et al. 2003). The findings in the Fancd2 gene disruption mouse are compatible with models for the FA pathway with FANCD2 acting as a late effector (D'Andrea and Grompe 2003; Venkitaraman 2004).

A link between the FA pathway and BS was suggested by the observation that five FA proteins, FANCA, C, E, F and G, associate in a complex with BLM protein as well as topoisomerase IIIα and RPA in an immunoprecipitated complex termed BRAFT (Meetei et al. 2003). Additional FANC proteins have since been identified in the complex (Meetei et al. 2003; Meetei et al. 2004). Association in a complex raises the question of whether BLM acts functionally in the FA pathway. In the present study, siRNA depletion of BLM and FA proteins, as well as gene disruption mouse models for FA and BS syndromes, were used to define the functional interactions of the FA and BS pathways. Cells depleted for the BLM protein display, in addition to the predicted increase in SCEs, a marked increase in formation of radials in response to mitomycin C (MMC), as is seen in FA cells. Such cells also manifest decreased viability when treated with MMC, compared to normal controls. In contrast to normal cells, depletion of BLM in FA cells does not cause an increase in ICL sensitivity, suggesting a common pathway. In the converse test, depletion of FANCA in a BS cell line also does not increase

chromosome breakage, supporting this conclusion. However, loss of FANC function, including BRCA2, does not increase SCEs, distinguishing the action of the FA pathway from BLM for regulation of recombination. Monoubiquitination of FANCD2 remains normal, however, in cells depleted of BLM or in BS cells. Following MMC treatment, BS cells, like FA cells (Akkari et al. 2001), show cell cycle arrest with a 4N DNA content, duplicating the pattern seen in normal cells with BLM depletion. In cells derived from Fancd2-/-,$Blm^{m3/m3}$ mice or Fancd2-/-, mice the same sensitivity to MMC was found. The results support the conclusion that, for cellular response to ICLs, the BLM protein functions exclusively in the FA response, establishing a FANC/BLM pathway for ICL response. However, this is not the case for SCE formation, where the FANC proteins do not appear to have a role.

Materials and Methods

Animal husbandry

The construction of the Fancd2-/+ mouse in C57BL/6J background has been described (Houghtaling et al. 2003). Mice heterozygous for the Blm^{m3} allele (Luo et al. 2000) in a 129S5 background were crossed with the Fancd2-/+ mice. The Fancd2 and Blm mice were bred to produce mice heterozygous for both mutations. Double heterozygous mice were then bred to produce mice homozygous for both mutations in a mixed C57BL/6Jx129S5 background.

Mouse genotyping

The Fancd2 allele was genotyped as previously described (Houghtaling et al. 2003). The Blm mice were genotyped by Southern blot, as previously described (McDaniel et al. 2003). Ten micrograms of genomic DNA were digested with BamHI, and probed using a 1.6 kb probe that spanned exon 4. Blots were probed overnight, and washed at a high stringency (65^0C for 30 min. in 2X SSC with 0.5% SDS followed by 30 min in 1X SSC). The mutant allele gives a band of 6.0 kb, and the wild-type allele gives a band of 6.6 kb.

Cell lines and culture

Transformed fibroblasts (Bruun et al. 2003) were cultured in α-MEM medium (Mediatech) supplemented with 5% fetal bovine serum (Hyclone), 5% calf serum (Hyclone) and gentamicin (Gibco) in a humidified incubator with 5% CO_2 at 37°C. Normal cell line GM639 and the Bloom Syndrome cell line GM08505C were obtained from the NIGMS Human Genetic Cell Repository. Immortalized Fanconi anemia cell lines GM6914 (FANCA), VU423 (FANCD1) and PD20 (FANCD2), as well as primary Fanconi anemia cell lines PD438.F (FANCC) and PD829 (FANCG), were provided by the OHSU Fanconi Anemia Cell Repository. The retrovirally corrected controls for GM6914, VU423, PD438.F and PD829 were functionally complemented with a pMMP retrovirus vector containing a full cDNA for the appropriate complementation group. Mouse primary fibroblasts were derived from the adult ears for each of the genotypes of interest. The ears were soaked in 70% ethanol, rinsed several times with PBS, minced in RPMI medium (Gibco) containing collagenase (Gibco) and incubated at 37^0C for three

hours. The collagenase-containing medium was then replaced with DMEM medium containing 20% fetal calf serum (Hyclone) and the cells were grown at 37^0C in a humidified incubator with 5% CO_2.

Design of the siRNA oligonucleotides

The siRNA duplex was designed as previously described (Bruun et al. 2003). A 21-mer beginning with an AA repeat was selected and specificity to BLM was confirmed by a BLAST analysis. The duplex was synthesized with a 3'-UU overhang by Dharmacon Research (Lafayette, CO). The sequence is: AAU CCC GGG AUA CUG CUC UCA UU. For controls, a non-functioning duplex with the following sequence was used: AAC UUU UGC AAA GCG GAG CCA UU. The FANCA and BRCA1-specific duplexes have been described previously (Bruun et al. 2003).

Transfection

Transfections were performed as previously described (Bruun et al. 2003). Final volumes of 1 ml for a T25 flask and 3.2 ml for a 100 mm dish were used. Controls were transfected with either a non-functional siRNA duplex or mock transfected. Transfection efficiency for the BLM siRNA was monitored using the FAM Silencer siRNA Labeling Kit (Ambion). At 4 and 24 hours, cells were fixed and counter-stained with DAPI using the SlowFade Light Antifade Kit with DAPI (Molecular Probes). Cells were then imaged using a Leica DRMXA fluorescent microscope. Transfection efficiency was calculated by number of cells with at least one labeled siRNA divided by the total number of DAPI

stained cells. For GM639 cells, efficiency was 60% at 4 hours and 88% at 24 hours for both control and BLM siRNA and 67% for BLM and control siRNA in GM6914.

Cell survival assay

Twenty-four hours following transfection, cells were plated on 100 mm dishes at 300 cells per dish and treated with MMC. Each data point was plated in duplicate. Cells were allowed to grow for 10 days in MMC, and then fixed in a solution of 50% MeOH, 1% new methylene blue (Sigma). Colonies were counted, and standard errors of the means, denoted by bars, were derived from the duplicate data sets.

Immunoblotting

Cells were transfected on 100 mm dishes as described above, using a 3.2 ml final solution volume, then washed with PBS, trypsinized, pelleted, and frozen at -80°C at the indicated time points (Fig. 3.1). The untreated control was mock-transfected and harvested at 48 hours. Cell lysates were prepared as previously described (Bruun et al. 2003). 50 μg whole protein extract from each lysate was run on a 7.5% acrylamide gel. Blots for BLM were then transferred to a nitrocellulose membrane (Osmonics), and FANCD2 blots were done on to Immobilon-P PVDF (Millipore). Membranes were blocked overnight in TBST (TBS plus 0.1% Tween) or and 5% dry milk. For BLM blots, the membrane was probed with anti-BLM rabbit polyclonal antibody (Abcam) at a 1:1000 dilution in TBST with milk. The β-tubulin blots were probed with a rabbit polyclonal antibody (Santa Cruz) at a 1:3000 dilution in TBST. FANCD2 blots were probed with a monoclonal antibody raised in mouse (Sigma) at a 1:200 dilution in TBST

with milk. BLM, β-tubulin, and FANCD2 blots were then incubated with goat anti-rabbit IgG HRP antibody (Bio-Rad) at a 1:10,000 dilution in TBST with milk. Detection was performed using a chemiluminescence kit (Perkin Elmer).

Chromosome stability

For sister chromatid exchange (SCE) analysis, cells were allowed to go through two rounds of replication in 25 μg/ml BrdU. This was done 24 hours post-transfection in siRNA treated cells. Clastogen-induced SCEs were elicited by a 4 hour pulse of 20 ng/ml MMC 20 hours prior to harvest. Cells were harvested following exposure to 0.25 μg/ml colcemid (Gibco), treated with a solution of 1:3, 5% fetal calf serum:0.075 M KCl, and fixed in 3:1 methanol:acetic acid. Cells were then dropped onto slides and stained for 5 minutes in 0.01% Acridine Orange. Following staining, the slides were rinsed with deionized water and treated with Sorenson Buffer (pH 6.8) (1:1 volume of 0.06M Na_2HPO_4 and 0.06M KH_2PO_4). After treatment, the slides were exposed to UV light for 12 minutes, and then visualized using a FITC filter. Twenty to 25 metaphases from each culture were scored for chromosome count and number of SCEs. The SCE rate was calculated for each metaphase cell as number of SCEs divided by the number of chromosomes.

For chromosome breakage studies, cells were treated with MMC (5-40 ng/ml) or DEB (50-150 ng/ml) 24 hours after transfection or mock transfection. Following 48 hours incubation with the clastogens, cells were harvested as described (Bruun et al. 2003). Slides were stained with Wright's stain, and 50 metaphases from each culture were scored for radial formation.

Cell cycle analysis

Cells were transfected in 100 mm dishes as described above. Duplicate plates were transfected to assure appropriate cell numbers. Twenty-four hours post transfection, cells were treated with the indicated amount of MMC. After 48 hours in the dark, cells were harvested and fixed in ice cold 70% EtOH. Before analysis, cells were spun at 200 x g for 5 minutes, and the ethanol removed. They were then incubated for 2 hours at 37°C in a DNA extraction buffer of 0.2 M phosphate citrate buffer, pH 7.8. The cells were then spun for 10 minutes at 1500 x g, and the supernatant removed. Cells were stained in a 20 μg/ml solution of propidium iodide in PBS with 0.1% Triton X-100 and 200 μg/ml DNase-freeRNase, and then incubated for 30 minutes at room temperature in the dark.

The stained cells were sorted on a Becton Dickinson FACSCalibur machine, using CellQuest software for analysis. Approximately 20,000 cells per sample were analyzed.

Results

Depletion of BLM protein results in an increased sensitivity to ICLs

Depletion of BLM in GM639 cells was verified by immunoblot (Fig 3.1a). The BLM protein decreased to undetectable levels within 12 hours and remained depleted for approximately 100 hours, allowing testing of chromosome stability. Depletion of BLM in normal fibroblasts produces an increased sensitivity to DNA crosslinking agents such as MMC, as illustrated by increased chromosome breaks and radials compared to normal

cells (Table 3.1). The pattern of breakage was indistinguishable from that observed in FA cells following crosslinker treatment (Fig 3.1b). Although not commonly used as an evaluation characteristic for BS cells, radials were similarly observed following MMC treatment in BS line GM08505 (Table 3.1). With BLM depletion, and in the BS patient cell line, the radials formed between non-homologous chromatids, as observed in all of the 20 G-banded radial forms examined for each cell line. This is in agreement with findings in FA cells, where the non-homologous nature of radials has been documented (Newell et al. 2004).

To provide a second evaluation of sensitivity to ICLs, cell survival with and without BLM depletion was measured following MMC treatment. With depletion of BLM a normal fibroblast line showed decreased survival following MMC treatment (Fig. 3.1c). Thus by the indices of chromosome stability and cell survival, depletion of BLM produced decreased genome stability, correlating with decreased cell survival after treatment with DNA crosslinking agents. Together, these results support a role for the BLM protein in ICL repair. The results also demonstrate that depletion of BLM produces a pattern of chromosomal instability indistinguishable from FA, suggesting that BLM might act in the FA pathway in the cellular response to ICLs.

Depletion of BLM increases SCE formation

A hallmark of BS cells is genome instability, as manifest by increased SCE formation compared to normal cells. To verify that the BLM siRNA reproduces the defect found in BS cells, SCE formation was evaluated in normal cells with and without BLM depletion, compared to BS cells. BLM depletion in normal cells produces a pattern

of increased SCE formation indistinguishable from BS cells (Fig. 3.2). Thus by this standard, as well as radial formation, BLM siRNA produced an authentic BS response.

Since both BS and FA cells show increased breaks and radials in response to ICL formation, it was reasonable to evaluate whether FA cells manifest increased SCEs in response to MMC, as do BS cells. Five complementation groups of FA cells were tested, four in comparison to retrovirally corrected controls. Two sets of FA and corrected cells were primary fibroblasts, whereas three sets were immortalized cell lines. Although FA cells, as well as normal cells, show increased SCE formation with MMC treatment, no increase in SCE formation was noted in the FA cells compared to corrected cells (Table 3.2). The primary cells, FANCC and FANCG, appeared to give lower levels of spontaneous SCE formation, but responded to MMC with a marked increase. Again, a deficiency of the FANC proteins did not elevate SCE formation. Thus, while breakage analysis is similar for FA and BS cells following crosslinker treatment, FA cells do not show significantly increased spontaneous SCE formation compared to normal cells following DNA crosslinking. This includes the FANCD1 cell line, defective in BRCA2. Therefore, the homologous recombination (HR) function of BRCA2 does not appear to be required for SCE formation (Table 3.2). This observation is surprising, given the supposed role of BRCA2 in HR (Shivji and Venkitaraman 2004) and the observed dependence of SCE formation on HR (Sonoda et al. 1999).

Depletion of BLM in FA cells does not result in increased sensitivity to ICLs

Given these findings, we tested whether FANC and BLM function in the same pathway for response to ICLs. To test this, BLM was depleted in FA cells. In contrast to

the above results showing increased sensitivity to ICL agents for normal cells depleted for BLM, depleting the BLM protein in the FANCA-deficient GM6914 cell line did not increase the incidence of chromosomal instability (Fig. 3.3). If BLM functions in a different pathway from the FA pathway for ICL response, then BLM depletion in a FA cell line should be additive to the sensitivity of the FA line. If, on the other hand, BLM acts in the FA pathway and an additional pathway, there should also be an increase in sensitivity. The results demonstrate that BLM does not function in additional pathways for ICL response. The outcome indicates an epistatic relationship between the BS and the FA pathways for ICL repair, indicating that BLM acts in the same pathway as the FANC proteins in response to interstrand crosslinks.

Depletion of BRCA1 increases radial formation in BS cells

BRCA1 is required for optimal monoubiquitination of FANCD2, associates with FANC-containing nuclear foci, and interacts directly with FANCA (Garcia-Higuera et al. 2001; Folias et al. 2002; D'Andrea and Grompe 2003). Moreover, BRCA1 depletion by siRNA causes radials in normal cell lines and also increases the level of radials in FA cell lines (Bruun et al. 2003). Thus, it seems that BRCA1 acts in the FA pathway response to ICLs, but also functions in at least one additional pathway. If BLM function in response to ICLs were limited to the FA pathway, then depletion of BRCA1 should increase radial formation in BS cells as it does in FA cells in response to MMC. This is indeed the case (Fig. 3.4 and Table 3.1).

Depletion of FANCA in BS cells, however, does not increase radial formation in response to MMC (Fig. 3.4 and Table 3.1). This is the converse test of the depletion of

BLM in FANCA cells described above. These results support the conclusion that BLM acts only in the FA pathway in response to ICL formation, but BRCA1 acts in at least one additional pathway.

Depletion of BLM causes cells to arrest with 4N DNA content after MMC

BS cells are known to manifest a G2 DNA content increase, presumably reflecting an arrest at the entry into mitosis (Chester et al. 1998; Hickson 2003; Davies et al. 2004). FACS analysis of cell line GM639 (normal) with MMC treatment following siRNA depletion of BLM, as well as GM08505 (BS) cells shows G2 delay, similar to GM6914 (FANCA) cells (Fig. 3.5). One interpretation would be that in the absence of BLM helicase, a late S-phase or G2 checkpoint mechanisms activates, causing a delay, as has been suggested (Davies et al. 2004). Given the suggested role of the RecQ helicases in minimizing single-strand breaks during replication, it might be supposed that the delay reflects an increase in such breaks in late S-phase

Ubiquitination of FANCD2 is normal in the absence of BLM

FANCD2 protein is modified following DNA damage and in S-phase by monoubiquitination (FANCD2-Ub) (Garcia-Higuera et al. 2001; Timmers et al. 2001; D'Andrea and Grompe 2003). This process requires a complex of 'core' FANC proteins, with BRCA1, for optimal modification. BS cells manifest normal FANCD2-Ub formation, indicating that BLM is not needed for normal formation of modified FANCD2 ((Pichierri et al. 2004), Fig 3.6). Therefore siRNA depletion of BLM should not affect the modification of FANCD2. This is the case (Fig. 3.6), indicating BLM is not needed

for FANCD2-Ub formation, suggesting BLM acts "downstream" from the FA core complex in ICL repair. Depletion of FANCA in BS cells sharply decreases FANCD2-Ub formation, as it does in normal cells, since the core complex is not formed in the absence of FANCA. Thus, while BLM depletion causes increased breaks and radials in response to ICL formation, it does not appear that this is due to a deficiency of FANCD2-Ub formation.

Fancd2-/-,Blm$^{m3/m3}$ double mutant mice are viable

The results reported here indicate that BLM is epistatic with the FA pathway for response to ICLs. It would then be expected that the addition of a defect in BLM to an animal model for FA would not add detectable defects to the ones already noted. To test this, the hypomorphic mutant Blm$^{m3/m3}$ allele (Luo et al. 2000) was combined with a Fancd2-/- genotype (Houghtaling et al. 2003) in a mixed background, since a complete defect in the murine BLM homolog is lethal (Chester et al. 1998). By crossing heterozygotes, homozygous deficient mice were obtained at the predicted frequency of one in sixteen (data not shown). The average weight of the double homozygous mutant mice was similar to each of the single mutants, indicating normal *in utero* growth.

Double mutant mouse cells do not show an additive phenotype

Mouse primary fibroblast cells were established from the Blm$^{m3/m3}$,Fancd2-/- mice and tested for response to MMC and DEB. As observed in the BLM siRNA-depleted normal human cells or BS fibroblasts, murine fibroblasts lacking the BLM protein exhibited chromosomal instability in the form of breaks and radials

indistinguishable from the FA phenotype. Furthermore, cells from the double mutant mice did not display increased ICL sensitivity above that of the Fancd2 -/-mice (Table 3.3), supporting the epistatic relationship between the BS and the FA pathway. The doubly defective mouse cells also had the same survival as the single mutants after MMC treatment. In contrast, mouse cells defective for BLM, or BLM and FANCD2 show an increase in SCEs, while the cells defective in FANCD2 do not.

Discussion

BLM, as a member of the RecQ helicase family, appears to serve at several sites for genome stability, including maintenance functions in response to normally arising breaks during DNA replication and in response to DNA damage (Hickson 2003). However, the results reported here show that the action of BLM in response to ICLs is limited to the FA pathway (Fig. 3.7). This is compatible with other reports of the co-localization of FANCD2 with BLM, co-immunoprecipitation of BLM with FANC proteins and interaction of the FANCC protein and BLM in transformed chicken cells (Hirano et al. 2005). In support of this, depletion of BLM in FA cells, or depletion of FANCA in BS cells, does not increase radial formation following MMC treatment. Thus BLM appears to act only with FANC proteins for ICL repair, defining BLM as epistatic to FA for ICL repair. Further, the fact that fibroblasts from the mouse Blm/Fancd2 double mutant appear no more sensitive than the Fancd2 single mutant also supports the conclusion that the two proteins act in one pathway with respect to response to ICLs.

Depletion of BLM protein produces a cytogenetic picture indistinguishable from FA for radial formation. However, the picture is distinctly different for SCEs, since FA

cells do not show an increase over normal cells, in contrast to a report on non-mammalian cells (Hirano et al. 2005). This suggests that the function of BLM in control of SCEs is separate from its action in ICL repair, or at least independent from FA. SCEs are thought to arise during DNA replication and require HR for formation (Sonoda et al. 1999; Helleday 2003). The basis of SCE formation appears to be the arrest of the replication fork. Hydroxyurea, which inhibits a subunit of ribonucleotide reductase, lowers the substrate pool for DNA replication and causes fork arrest, which results in markedly increased SCE formation, in normal cells (Helleday 2003; Matsuoka et al. 2004). MMC also causes an increase in SCE formation, perhaps also due to replication fork arrest. Whether the action of BLM in SCE regulation is at the point of strand breaks or the structure of a collapsed fork is not clear, but it does appear to require HR. The RecQ helicases, in general, appear to suppress recombination. Thus the role of BLM in lowering SCEs may represent a regulation of HR (Fig. 3.7), perhaps by the helicase action controlling strand invasion. Absence of BRCA2, the FANCD1 protein, does not alter SCE formation, suggesting the notion that the FA pathway does not regulate SCE formation and that FANCD2-Ub does not modulate SCE formation, since FANCD2-Ub is normal in the absence of BRCA2 or BLM. However, BLM acts in ICL repair, suggesting a downstream function (Fig.3.7). These findings also indicate that the HR function of BRCA2 is not needed for suppression of SCE formation.

BLM has been shown to be phosphorylated by ATM or ATR (Davies et al. 2004), but phosphorylation, which is dependent on association with the FANC core (Pichierri et al. 2004) is not required for its action in SCE regulation; rather it is required for other functions such as S-phase checkpoint arrest (Davies et al. 2004). Therefore, BLM has

segregated functions serving in cell cycle regulation and regulation of SCE formation. It is possible that the phosphorylated form of BLM acts in the ICL response, while the non-phosphorylated form acts in SCE suppression (Fig. 3.7).

The evidence that BLM is modified by phosphorylation, dependent on the FANC core, but that it does not need to be modified to serve in regulation of SCE formation, coupled with its apparent action in other DNA repair responses, indicates that it has distinct functions in separate pathways, although it co-localizes with FANC proteins (Pichierri et al. 2004). Thus there is a series of segregated functions for proteins which are epistatic for ICL response: a deficiency in BLM leads to increased SCEs, but a deficiency in FANC proteins does not; the FANC core complex is required for BLM modification, but BLM is not required for FANCD2-Ub formation; BRCA1 is required for normal FANCD2-Ub formation, but BRCA2, a known FANC protein (Howlett et al. 2002), is not. Together these results suggest the model outlined in Fig. 3.7, whereby BLM protein can exist in association with FANC proteins in the BRAFT complex, but acts in ICL repair.

Acknowledgments

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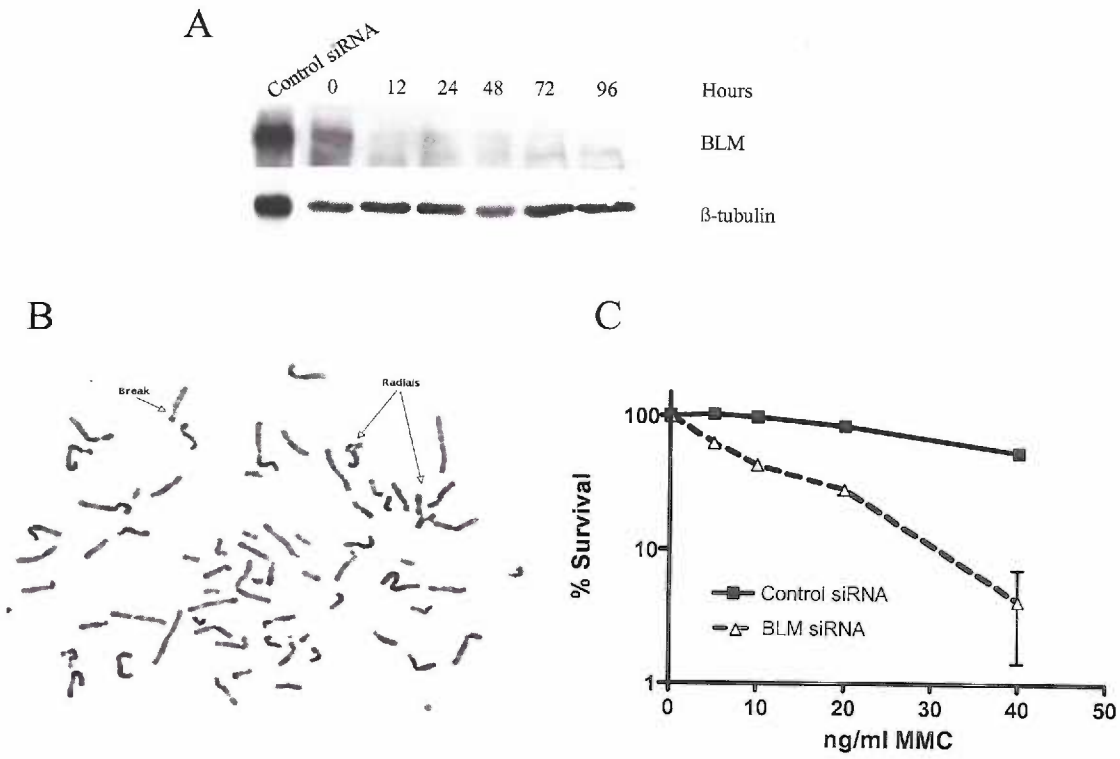

Figure 3.1. *siRNA depletion of BLM in GM639 cells.* A. Immunoblot. Cells were transfected with a siRNA specific for *BLM*, and harvested at the indicated times. β-tubulin is shown to indicate equal loading, and to show specificity of the siRNA. B. Radial formation. Cells transfected with the BLM siRNA were treated with 40 ng/ml MMC, and examined for chromosome breaks. C. Survival. Cells transfected with BLM siRNA were treated with varying amounts of MMC. Survival was determined by colony formation, and normalized to a non-treated control plate. Error bars were determined by averaging two separate plates of a single transfection.

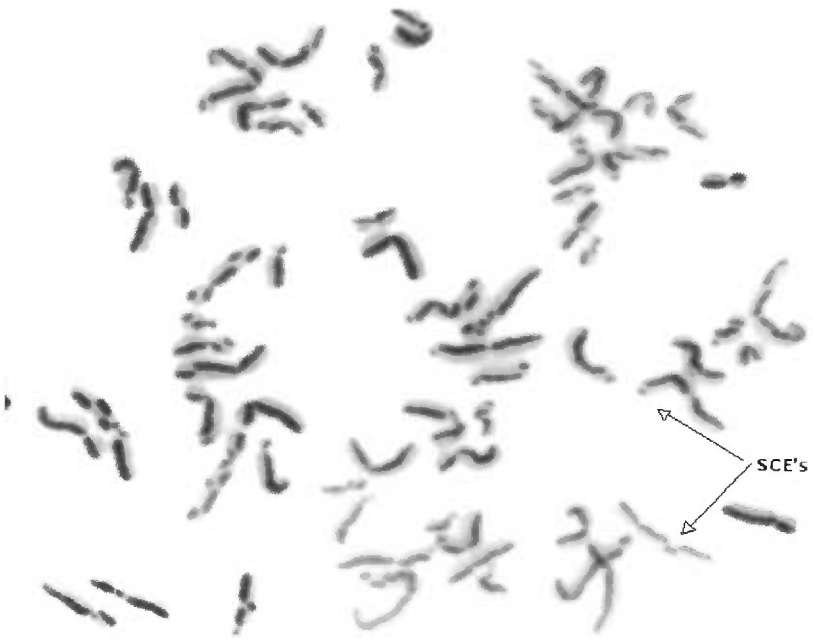

Figure 3.2. *Depletion of BLM leads to increased sister chromatid exchange.* SCE determinations were done as described in methods.

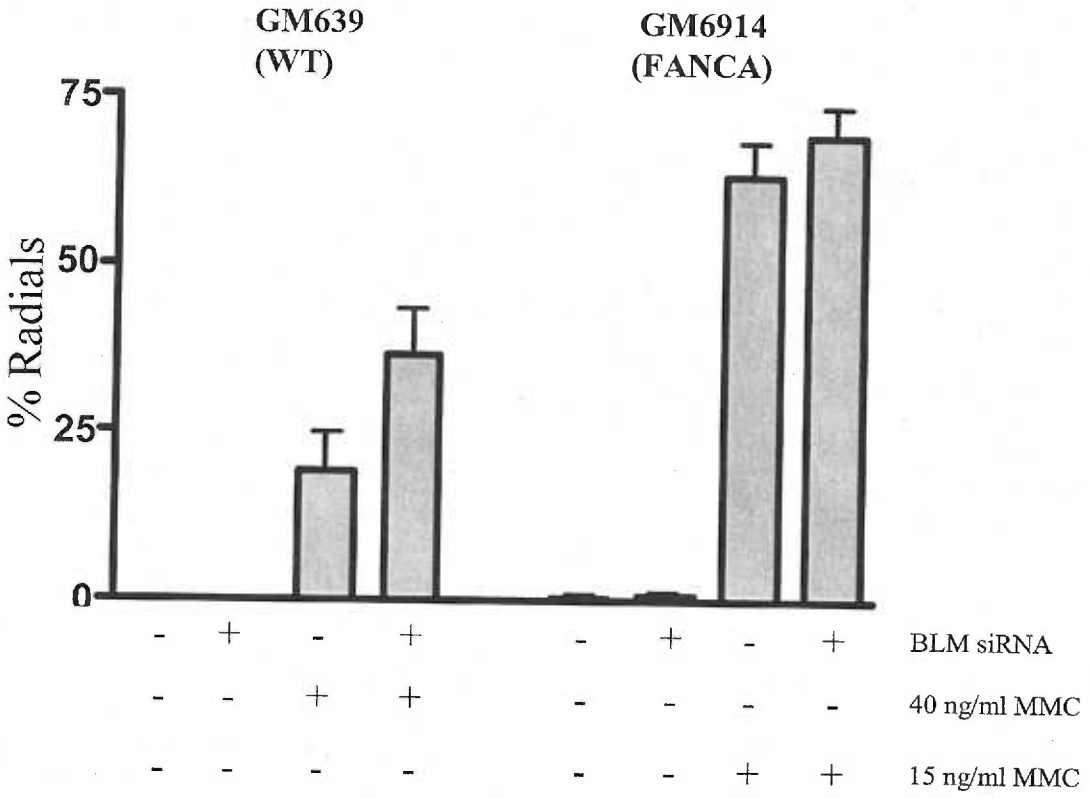

Figure 3.3. *Radial formation is increased with depletion of BLM in normal fibroblasts.* 50 metaphases from a single transfection of GM639 were analyzed, and the percent showing at least one radial were reported. Data shown are from 3-5 independent trials for each value.

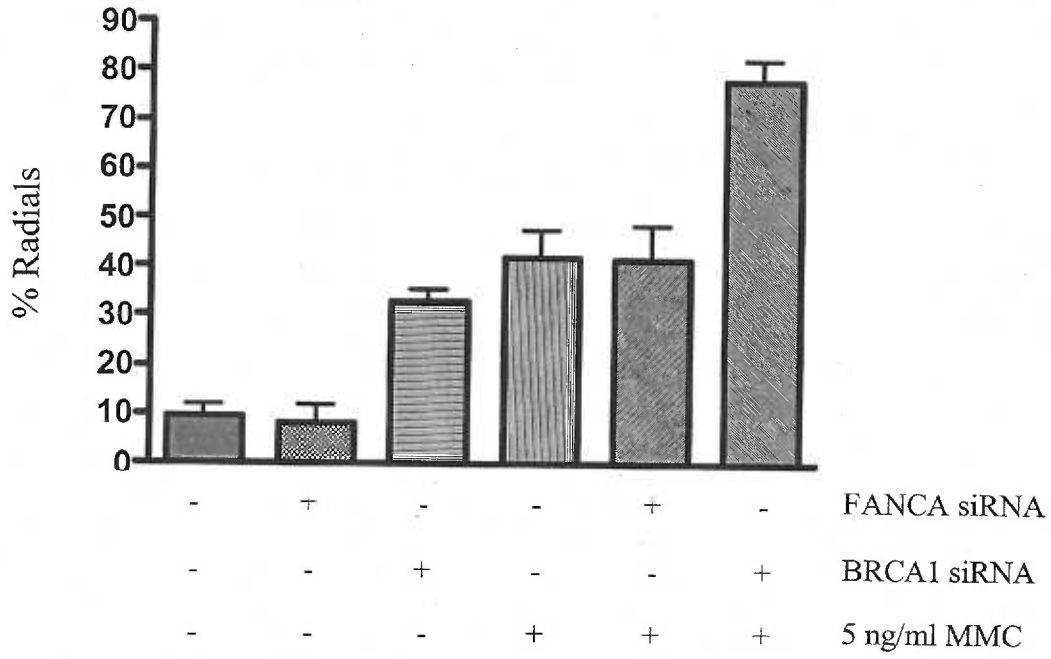

Figure 3.4. *Radial formation in GM08505 cells is not increased by FANCA depletion.* 50 metaphases from a single transfection were analyzed, and the percent showing at least one radial were reported.

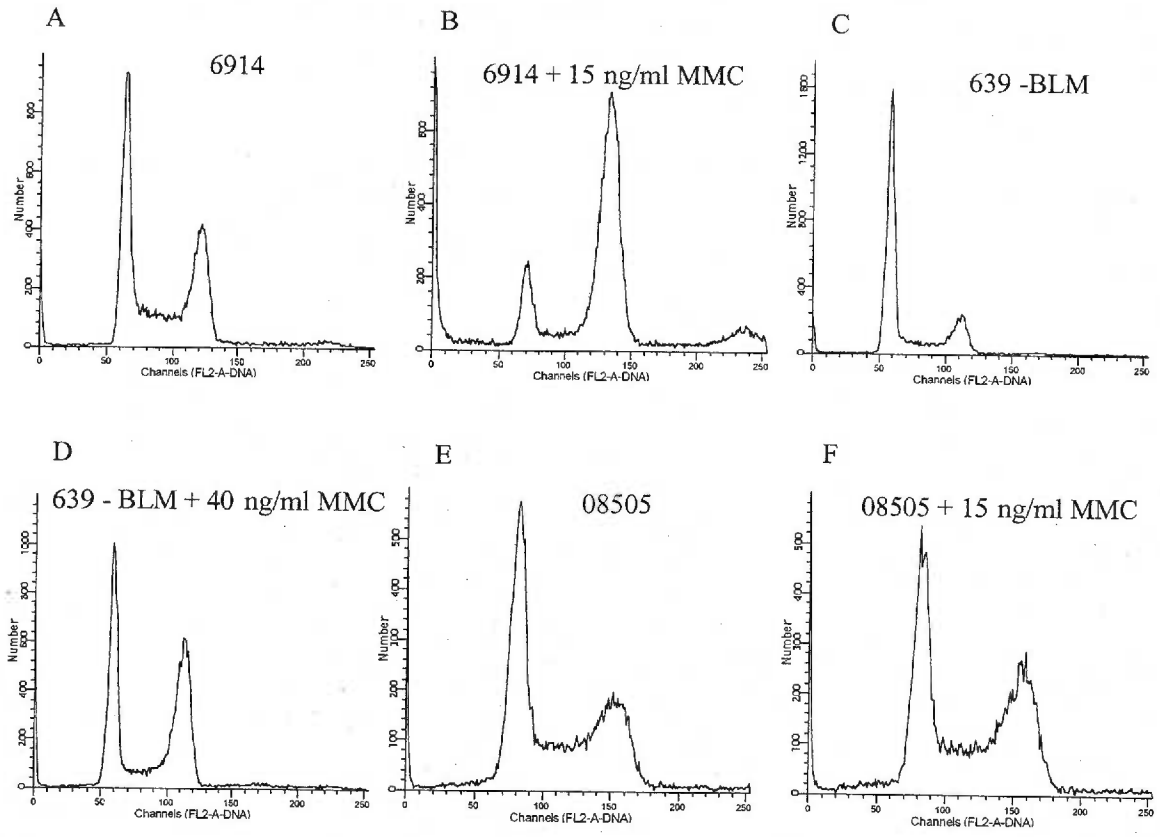

Figure 3.5. *BLM depletion produces a delay at 4N DNA content.* Cells with and without DNA damage were analyzed for DNA content by propidium iodide staining. Cells are GM6914 (A, B), GM639 + BLM siRNA (C, D), and GM08505 (E, F). Cells were treated with 15 ng/ml (B, F) or 40 ng/ml (D) MMC for 48 hours.

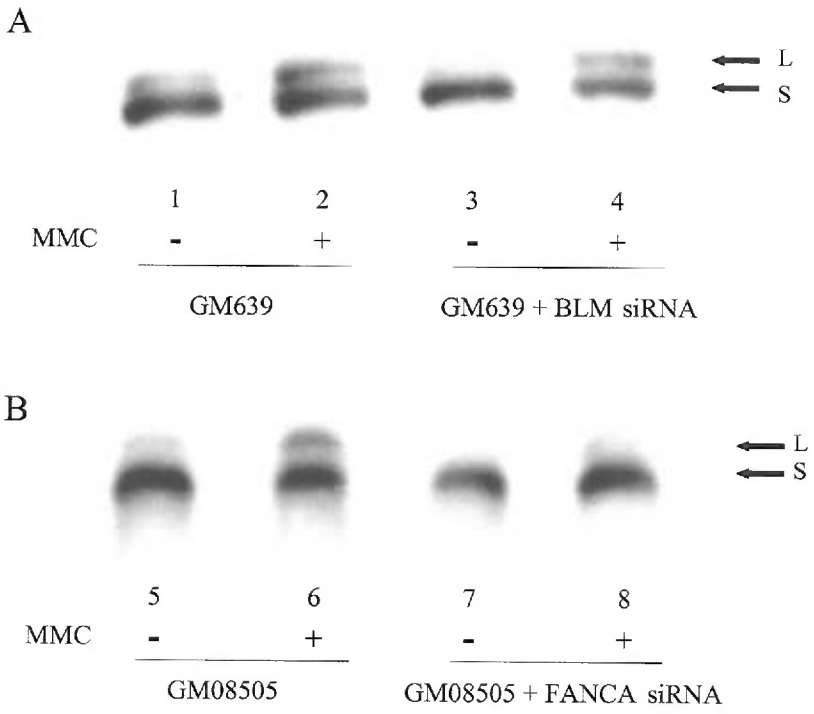

Figure 3.6. *Mono-ubiquitination of FANCD2 is normal in GM08505 cells and following depletion of BLM.* Lanes 1 and 2, GM639 cells with control siRNA transfection; 3 and 4, GM639 with BLM siRNA transfection; 5 and 6, GM08505 cells. MMC added as indicated at 80ng/ml for GM639 and 30ng/ml for GM08505. Cells were harvested 24 hours after MMC treatment. L denotes the mono-ubiquitinated form of FANCD2, S denotes the non-mono-ubiquitinated form.

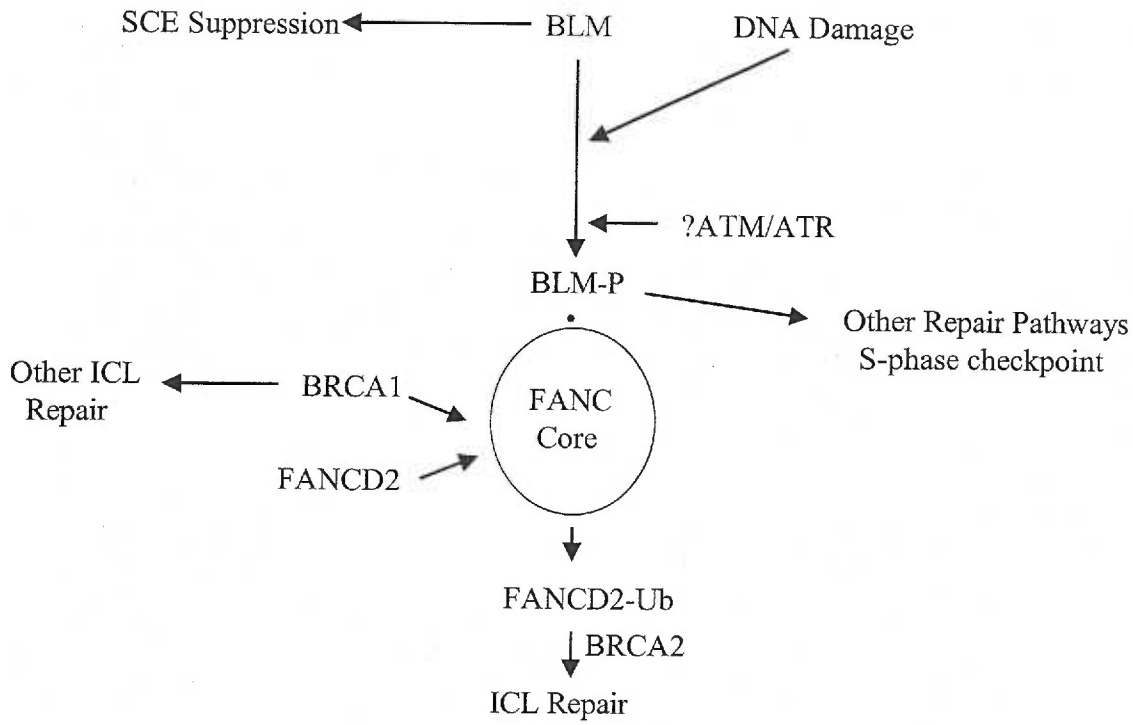

Figure 3.7. *Model for repair of ICLs by the FA/BLM pathway.*

Table 3.1. Summary of radial formation following MMC treatment.

	Treatment	% Radials	p-value
GM639	40 ng/ml MMC	19 +/- 5.7	
GM639 + BLM siRNA	40 ng/ml MMC	36 +/- 6.9	0.0114
GM6914	15 ng/ml MMC	63 +/- 4.6	
GM6914 + BLM siRNA	15 ng/ml MMC	69 +/- 4.2	0.350
GM08505	5 ng/ml MMC	42 +/- 5.5	
GM08505 + FANCA siRNA	5 ng/ml MMC	41 +/- 6.9	0.4531
GM08505 + BRCA1 siRNA	5 ng/ml MMC	78 +/- 3.9	0.0012

Error represents the standard error of the mean. p-values are calculated by a paired two-tailed t-test

Table 3.2. *SCE analysis in Bloom Syndrome and Fanconi anemia cells.*

Sample	Treatment	SCEs/chrom	p-value
GM639 (WT)	None	0.64 +/- 0.03	0.004
GM639 + BLM siRNA	None	0.83 +/- 0.05	
GM08505 (BLM)	None	1.8 +/- 0.05	<0.0001
GM08505 (BLM)	20 ng/ml MMC	2.76 +/- 0.09	
GM6914 (FANCA)	None	0.73 +/- 0.04	0.0003
GM6914 (FANCA)	20ng/ml MMC	0.93 +/- 0.03	
GM6914.A (RV FA-A)	None	0.62 +/- 0.03	<0.0001
GM6914.A (RV FA-A)	20ng/ml MMC	0.88 +/- 0.03	
PD438.F (FA-C) *	None	0.36 +/- 0.01	<0.0001
PD438.F (FA-C) *	20ng/ml MMC	0.67 +/- 0.02	
PD438.F RV.C (RV FA-C) *	None	0.36 +/- 0.01	<0.0001
PD438.F RV.C (RV FA-C) *	20ng/ml MMC	0.61 +/- 0.02	
VU423 (FA-D1)	None	0.57 +/- 0.02	0.0001
VU423 (FA-D1)	20ng/ml MMC	0.75 +/- 0.04	
PD20 (FA-D2)	None	0.73 +/- 0.04	<0.0001
PD20 (FA-D2)	20ng/ml MMC	0.96 +/- 0.04	
PD20 RV.D2 (RV FA-D2)	None	0.52 +/- 0.02	<0.0001
PD20 RV.D2 (RV FA-D2)	20ng/ml MMC	0.80 +/- 0.03	
PD829 (FA-G) *	None	0.30 +/- 0.02	<0.0001
PD829 (FA-G) *	20ng/ml MMC	0.59 +/- 0.04	
PD829 RV.G (RV FA-G) *	None	0.28 +/- 0.01	<0.0001
PD829 RV.G (RV FA-G) *	20ng/ml MMC	0.69 +/- 0.03	

*primary cells

Complementation groups are indicated. RV = retrovirally corrected cell line. Error represents standard error of the mean. p-values are calculated by an unpaired two-tailed t-test.

Table 3.3. *Breakage analysis in primary mouse fibroblasts.* Ear tags were taken, established in culture and radial formation determined as stated in Methods.

	% Radials
Fancd2 +/- *Blm*$^{+/m3}$	0
Fancd2 +/- *Blm*$^{m3/m3}$	14
Fancd2 -/- *Blm*$^{+/m3}$	90
Fancd2 -/- *Blm*$^{m3/m3}$	78

CHAPTER FOUR:

Interstrand crosslink-induced radials are not mediated by proteins in non-homologous end joining or homologous recombination repair

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Abstract

High levels of interstrand cross-link (ICL) damage in mammalian cells cause chromatid breaks and radial formations, apparent upon cytogenetic examination at metaphase. Fanconi anemia (FA) cells display a much higher level of these breaks and radials at significantly lower doses of ICL damage. These radial forms are used as diagnostic for FA; however, the mechanism of their formation has yet to be determined. Due to the recent implication of homologous recombination (HR) and non-homologous end-joining (NHEJ) in the action of the FA pathway, we speculated that radial forms might be the result of aberrant attempts at either HR or NHEJ DNA repair. To test this proposal, we have investigated the role of HR proteins RAD51 and RAD52, NHEJ proteins DNA-PK_{cs}, Ku70 and Ligase IV, and HR/NHEJ protein MRE11 in radial formation and genome stability in normal and FA cells following ICL damage with MMC. For investigation of RAD51, RAD52, MRE11, Ku70, and Ligase IV we used small inhibitory RNA (siRNA) to deplete the proteins from these cells, allowing for evaluation of radial formation prior to cell lethality. DNA-PK_{cs} was investigated through evaluation of scid mouse fibroblasts. Depletion or mutation of these proteins did not suppress radial formation in FA or normal cells, indicating that these proteins' action in HR and NHEJ is not required for ICL-induced radial formation. Depletion of RAD51, RAD52, MRE11, Ku70, and Ligase IV all conferred ICL sensitivity to cells, broadening the cast of proteins potentially involved in mammalian processing of ICL damage.

Introduction

Interstrand cross-link (ICL) damage forms a covalent bond between nucleotides on the two strands of the DNA molecule, posing a unique repair problem, as there is no undamaged complementary strand available as a template for the process of DNA repair (Metzler 1986). Interstrand cross-links are recognized in S-phase (Akkari et al. 2000) and have been proposed to be excised following this recognition. Mammalian cells sensitive to the damaging effects of ICLs, such as Fanconi anemia (FA) cells, display chromosomal abnormalities such as radials and double strand breaks following DNA damage by ICL agents such as mitomycin C (MMC) and diepoxybutane (DEB) (Sasaki and Tonomura 1973; Sasaki 1975). The cellular phenotype of breaks and radials following MMC and DEB exposure is the standard for diagnosis of FA (Auerbach et al. 1981; Cervenka et al. 1981).

The radial formations observed in these cells are aberrant structures, thought to result from unsuccessful attempts at DNA lesion repair. Radials form exclusively between non-homologous chromosomes or non-homologous regions of chromosomes following either interstrand cross-link or ionizing radiation of cells (Newell et al. 2004). There are no preferential chromosome associations in radials, and the full length of each autosome appears to have the ability to be involved in a radial form. These formations have historically carried the names triradial and quadriradial. These titles are primarily descriptive. For the purpose of this study, we use the term radial to describe any multi-radial formation involving the apparent joining of chromatid breaks between two or more chromosomes. This description is not intended to imply any underlying mechanism for radial formation, as such a mechanism is yet to be determined. Two previously

suggested mechanisms for radial formation, homologous recombination and non-homologous end-joining, are both investigated in this work (Donahue and Campbell 2004; Newell et al. 2004). Understanding the mechanism of formation of radials should help in elucidating the mechanism of mammalian ICL repair and the function of the FA pathway.

Homologous recombination-based repair involves a broad variety of proteins. RAD51 is a homologous recombination (HR) protein loaded onto single stranded DNA in a BRCA2 (FANCD1)-mediated manner in the early events of homologous recombination repair (HRR) (Davies et al. 2001). RAD52 recruits RAD51 to single stranded DNA, assists in pairing complementary DNA, and is believed to be involved in postsynaptic annealing of the second end of a DSB (Miyazaki et al. 2004). The MRE11 complex, consisting of MRE11, RAD50, and NBS1, is required for normal S-phase checkpoint (Petrini 2000) and associates with BRCA1, another HR protein (Zhong et al. 1999; Wu et al. 2000). This complex is involved in homologous recombination-based double-strand break repair in yeast (Bressan et al. 1999). Foci made up of RAD51, RAD52, MRE11, BRCA1, and FANCD2, and other DNA repair-involved proteins form at the sites of DNA damage following ionizing radiation and ICL exposure (Garcia-Higuera et al. 2001; Nakanishi et al. 2002). Null mutations in RAD51 cause both animal and cell lethality, making the study of cells lacking RAD51 expression over an extended period of time a challenge. However, mammalian cell lines with mutations in RAD51 paralogs, RAD51C, XRCC2, and XRCC3 have shown sensitivity to ICL agents through survival curves (Liu et al. 1998; Godthelp et al. 2002). In addition, data suggest that FA cells display an increased incidence of homologous recombination (Thyagarajan and Campbell

1997). It has been proposed that these DNA-damage induced radials occur as a result of inappropriate attempts at non-allelic homologous recombination repair (Newell et al. 2004). These observations have led homologous recombination proteins to be interesting candidates for possible involvement in ICL-induced radial formation.

A second process that has been suggested as possibly responsible for radial formation is non-homologous end-joining (NHEJ) (Donahue and Campbell 2004). It has been theorized that excision of an ICL-adduct after S-phase recognition leads to formation of a double strand break (Rothfuss and Grompe 2004). In addition, double strand break induction by IR leads to radial formation (data not shown). These two situations lead to double-strand breaks with the need to be repaired. NHEJ is a prevalent form of double-strand break repair in mammalian cells (Valerie and Povirk 2003). Whereas there is some indication that FA cells have some level of deficiency in homologous recombination repair, evidence suggests that NHEJ is not decreased in FA patient cells. NHEJ deficiency leads to an immunodeficiency syndrome (Zhu et al. 1996)for which FA patients do not carry the phenotype. NHEJ-mediated repair is initiated by the action of DNA-dependent protein kinase (DNA-PK). There are two subunits to DNA-PK, one regulatory and one catalytic. The regulatory subunit is the Ku70/Ku80 heterodimer, which recognizes and binds to DNA ends. The catalytic subunit is DNA-Pk_{cs}, a serine-threonine kinase (Gottlieb and Jackson 1993). Following the action of DNA-PK, the XRCC4/DNA ligase IV complex acts in the break re-sealing step of NHEJ (Li et al. 1995; Grawunder et al. 1998). Because the process of Ku-mediated NHEJ appears to be intact in FA cells, it is possible that these proteins are attempting to act in repair of ICL damage in FA cells, leading to radial formation.

MRE11, involved in homologous recombination, is also thought to act in NHEJ by its interaction with the RAD50/MRE11/NBS1 complex. Reconstitution of this protein complex in both yeast and mammalian systems has shown enhancement of *in vitro* DNA end-joining activity (Chen et al. 2001; Huang and Dynan 2002). In addition, presence of this complex in human extracts has been shown to be necessary for proper *in vitro* DNA end-joining (Zhong et al. 2002).

In this study, we used mouse knockout cells and directed siRNA depletion of homologous recombination and non-homologous end-joining proteins in wildtype and FA cells to determine whether either process serves a role in radial formation and cellular response to ICL damage. From these experiments we determined that the proteins RAD51, RAD52, MRE11, DNA-PK_{cs}, Ku70, and Ligase IV are all not required for ICL-induced radial formation. Therefore, neither RAD51 mediated non-allelic HR, nor Ku-dependent NHEJ, nor MRE11 mediated HR or NHEJ likely mechanisms for the formation of radial chromosome figures in response to DNA damage. Rather, we find that depletion of some essential proteins in these processes from normal cells actually confers an FA-like MMC-induced cytogenetic phenotype, potentially broadening the reach of the FA-pathway to have association with both HR and NHEJ-associated repair.

Materials and Methods

Animal Husbandry

Fancd2$^{+/-}$ on a C57/Bl6J background (Houghtaling et al. 2003) were crossed to *Prkdc*$^{sc/sc}$ (B6.CB17-*Prkdc*^{sc}/SzJ) purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) to generate *Fancd2*$^{+/-}$/*Prkdc*$^{+/sc}$ breeders. Genotyping was performed as

previously described for *Fancd2* mice (Houghtaling et al. 2003) and $Prkdc^{sc}$ mice (Blunt et al. 1996; Araki et al. 1997)$Fancd2^{+/-}/Prkdc^{+/sc}$ breeders were intercrossed to generate $Fancd2^{+/-}/Prkdc^{sc/sc}$ breeders that were crossed to generate $Fancd2^{-/-}/Prkdc^{sc/sc}$ and $Prkdc^{sc/sc}$ animals. Animals were maintained according to an approved IACUC protocol in the Department of Animal Care at Oregon Health & Science University.

Cells and Media

Normal cell line GM639 was obtained from the NIHGMS Human Gene Mutant Repository. The Fanconi anemia cell line GM6914 was obtained from the OHSU Fanconi anemia cell repository. Cells were SV40 immortalized and maintained in α-modified Eagle Medium (GIBCO/BRL) with 10% fetal calf serum (Hyclone), 1 x glutamine (GIBCO/BRL), and 50 μg/ml Gentamycin (GIBCO/BRL) at 37°C and 5%CO_2.

Primary fibroblasts were established from $Prkdc^{sc/sc}$, $Fancd2^{+/-}$, $Fancd2^{-/-}/Prkdc^{sc/sc}$ and *w.t.* C57/Bl6J mouse ears. Cells were grown in DMEM medium containing 20% fetal calf serum (Hyclone), 1X glutamine (GIBCO/BRL), and 50 μg/ml Gentamycin (GIBCO/BRL) and the cells were grown as stated above.

siRNA Development and Transfection

siRNA duplexes for relevant genes were acquired as SmartPool samples from Dharmacon Research (Lafayette, CO). Transfections were performed as previously described(Bruun et al. 2003). Controls were transfected with a non-functional siRNA duplex.

Immunoblotting

Cells were transfected on 100 mm dishes as previously described, using a 6 ml final solution volume, then washed with PBS, trypsinized, pelletted, and frozen at -80°C at the indicated time points. The untreated controls were transfected with non-functional siRNA and harvested at 48 hours. Cell lysates were prepared as previously described (Bruun et al. 2003). 50 μg whole protein extract from each lysate was run on an 8% acrylamide gel. Gels were transferred to Immobilon-P PVDF (Millipore). Membranes were blocked overnight in TBST (TBS plus 0.1% Tween) or PBST (PBS plus 0.1% Tween) and 5% dry milk. For RAD51 and Ku-70 blots, the membrane was probed with appropriate mouse monoclonal antibody (Novus) at a 1:500 dilution in TBST with milk. MRE-11 blots were probed with anti-MRE11 rabbit polyclonal antibody (Novus) at a 1:10,000 dilution in TBST with milk. Ligase IV blots were probed with anti-Ligase IV rabbit polyclonal antibody (Sigma) at a dilution of 1:1000 in TBST with milk. β-tubulin controls were probed with a rabbit polyclonal antibody (Santa Cruz) at a 1:3000 dilution in TBST. RAD51 and Ku-70 blots were then incubated with goat anti-mouse IgG HRP antibody (Cal Biochem) at a 1:10,000 dilution in TBST with milk. MRE-11 and Ligase IV blots were incubated with goat anti-rabbit IgG HRP antibody (BioRad) at a 1:10,000 dilution in TBST with milk. Detection was performed using a chemiluminescence kit (Perkin Elmer).

Chromosome Radial Analysis

After 24 hours of siRNA transfection, flasks were treated with relevant dosage of MMC alongside untreated controls. 32 hours following MMC treatment, colcemid (0.05 μg/ml) (GIBCO/BRL) was added for a 16 hour incubation. Cells were then harvested, treated with hypotonic solution (0.075 M KCL, 5% fetal calf serum) for 10 minutes, and fixed with 3:1 methanol acetic acid. Cells were dropped onto slides for metaphase spreads and stained with Wright's stain (Fisher Scientific) for 3.5 minutes. 50 metaphases for each sample were scored for radial content on Nikon Eclipse E800 photoscope, and representative photographs were taken using CytoVision software from Applied Imaging.

MMC Cell Survival Assay

Twenty-four hours following transfection, cells were plated on 100 mm dishes at 300 cells per dish and treated with MMC. Each data point was plated in duplicate. Cells were allowed to grow for 10 days in MMC, and then fixed in a solution of 50% MeOH, 1% new methylene blue (Sigma). Colonies were counted, and standard deviations were derived from the duplicate data sets.

Results

siRNA Treatment Depletes Proteins of Interest

siRNA with specificity to RAD51, RAD52, MRE11, Ku70, and DNA Ligase IV were transfected into normal and FA-A, immortalized fibroblast cell lines. These small

duplex RNAs deplete the proteins they are designed to target by mRNA degradation (Elbashir et al. 2001). By westerns blot analysis, we were able to show that the proteins of interest were successfully depleted over the 72 hour time-period necessary for the MMC treatment and harvest prior to analysis (Fig. 4.1) in normal 639 cells. Depletions in FA cells have yet to be confirmed by western blot analysis. Until this confirmation, conclusions regarding cellular response to depletions of the proteins of interest in FA cells should be limited.

Depletion of HR Proteins Induces MMC Sensitivity and Radial Formation

Due to the increase in homologous recombination levels in FA cells (Thyagarajan and Campbell 1997) and the non-homologous nature of radials (Newell et al. 2004), it has been generally considered that radials may be the result of failed attempts at non-allelic homologous recombination repair. We examined both normal and FA cells depleted of HR proteins RAD51 and RAD52, as well as HR/NHEJ protein MRE11 for their ability to form radials following ICL damage.

While cells were depleted of RAD51, they were treated with a range of concentration of MMC in order to perform cytogenetic breakage and radial analysis and cell survival analysis. Depletion of RAD51 from normal cells caused increased MMC sensitivity when compared to normal control cells in cell survival assays ($p = 0.0380$) (Fig. 4.6a). This sensitivity was also assessable by radial formation. In fact, depletion of RAD51 in normal cells also led to spontaneous radial formation in controls lacking MMC treatment (Fig. 4.2, 4.3a).

Depletion of RAD51 from FA cells causes a significant ($p = 0.0260$) increase in sensitivity shown through radial formation when compared to FA cells alone (Fig. 4.3a). These results were reproduced at several dose of MMC (5, 15, 30, and 60 ng/ml). This phenotype appears to be additive from the radial formation caused by depletion of RAD51 in normal cells. This may indicate possible action of RAD51 in maintaining genome integrity after ICL damage in a pathway prior to our outside of the FA pathway.

RAD52 depletion from normal cells also induced MMC sensitivity and radial formation ($p = 0.0044$) (Fig. 4.3b, 4.6b), an FA-like phenotype in response to ICL treatment. However, unlike the RAD51 depleted cells, no spontaneous radials formed in cells not treated with MMC. These data indicate that like RAD51, RAD52 is not required for radial formation. Also in contrast to RAD51 depletion, RAD52 depletion in FA cells did not cause an increase in MMC sensitivity ($p = 0.7385$). Following confirmation of successful depleteion, this would indicate that unlike RAD51, RAD52 may be involved in maintenance of genome integrity by action in the FA pathway, but not in other ICL response pathways.

Much like RAD52, depletion of MRE11 induced MMC sensitivity detected by cell survival and radial formation in normal cells with no additivity induced in FA-cells (Fig. 4.5, 4.6c). MRE11, therefore, is not required for radial formation following ICL damage.

These data indicate that the action of RAD51, RAD52, and MRE11, and therefore non-allelic homologous recombination as a whole, is not required for cells to form radials following ICL damage. Furthermore, the ICL sensitivity of cells depleted of these

proteins indicates their possible involvement with ICL damage repair and/or maintenance of genome stability.

Non-homologous End Joining proteins are not required for Radial Formation

In addition to homologous recombination, another commonly suggested possibility for a process mediating radial formation has been non-homologous end joining. We explored the possibility of NHEJ mediating radial formation through both mouse knockout models and siRNA depletion.

Prkdc$^{sc/sc}$ mouse fibroblasts, deficient in the catalytic subunit of DNA-PK, and therefore, deficient in NHEJ, were treated with MMC and scored for radial formation to evaluate their MMC sensitivity. There was no MMC sensitivity through radial formation observed in the *Prkdc*$^{sc/sc}$ cells. *Fancd2*$^{-/-}$*/Prkdc*$^{sc/sc}$ cells displayed similar radial formation to cells from mice lacking functional FANCD2 alone (Fig. 4.4). This lack of MMC phenotype indicates that DNA-PK$_{cs}$ does not act in response to ICL damage. Additionally, the ability of these mutant cells to form radials indicates that DNA-PK$_{cs}$ is not required for radial formation in response to ICL damage.

Depletion of Non-homologous End Joining proteins Ku70 and Ligase IV allows radial formation and causes an increase in ICL-induced Radial Formation

Recently it has been suggested that Ku70, part of the regulatory subunit involved in NHEJ, is involved in ICL damage processing. It has been suggested that the FA pathway may control Ku70 capping of broken ends of DNA, and in absence of the FA pathway, this capping may not occur appropriately. With this possible involvement of

other NHEJ proteins with the FA pathway, we chose to further explore NHEJ, ICL damage, and radial formation by evaluating radial formation in cells depleted of NHEJ protein Ku70.

Normal cells depleted of Ku70 were sensitive to ICL damage, illustrated through radial formation. These cells formed radials significantly ($p = 0.0014$) more than normal cells transfected with control siRNA (Fig. 4.5a). This ICL sensitivity was also seen in cell survival assays (Fig 4.6d).

To test the possibility that actual ligation of DNA through NHEJ was mediating radial formation, cells depleted of Ligase IV were tested for their ICL-induced radial formation capacity. Like cells depleted of Ku70, Ligase IV depleted normal cells formed increased radials following ICL damage ($p = 0.0136$) (Fig. 4.5a) and were shown to be sensitive to ICLs through cell survival assays (Fig 4.6e).

These results indicate that NHEJ is also not a required process for the formation of ICL-induced radials. Pending western blot confirmation of depletion in FA cells, sensitivity to ICL damage displayed by cells depleted of NHEJ proteins Ku70, Ligase IV, and MRE11 does not appear additive with FA-induced sensitivity (Fig. 4.5b). Finally, these results suggest that these NHEJ proteins may have involvement in the FA pathway response to ICL damage.

Discussion

The Process of Radial Formation

Prior to this investigation, there was belief that either the processes of HR or NHEJ were key in the formation of ICL-induced radial figures (Donahue and Campbell

2004; Newell et al. 2004). The presence, and sometimes even induction, of radials following depletion of multiple key proteins in both of these two processes indicates that radial formation is unlikely to be a result of appropriate or inappropriate action of either HR or NHEJ.

Further investigation into other repair proteins and processes may reveal a mechanism of radial formation. siRNA may be exploited to discover these proteins. Possibly a different DNA ligase is responsible for the joining of this DNA. If radials are more structural, and less DNA based, perhaps a DNA helicase is responsible for radial formation. However, cells mutant in RecQ helicases BLM and WRN can both form radials (data not shown).

Whether radials are present as part of the normal process of ICL repair or only occur as an error in repair is undetermined. The particular detail of the sequences involved in the junction of radials is also unclear. Identification of the DNA sequences involved in radial formation, whether homologous or non-homologous, as well as the proteins that interact with these radial forming regions may also lead to an understanding of a mechanism of radial formation.

RAD51, RAD52 and ICL Damage Response

The additivity of phenotype in FA cells depleted of RAD51 indicates that RAD51 may act in a separate pathway in response to ICL damage than the FA pathway. This is similar to results seen in FA cells depleted of BRCA1, an essential homologous recombination protein (Bruun et al. 2003).

RAD52 depletion caused normal cells to be sensitive to MMC, but did not cause an additive phenotype of sensitivity in FA cells. Therefore, RAD52 appears to be epistatic to the FA pathway in ICL damage response. This independent action of RAD52 from RAD51 may be dependent on the fact that though RAD52 acts to enhance HR in mammalian cells, it is not required. RAD52 may act in a role required in the FA pathway, but not in a further role taken by RAD51 in processing ICL damage.

Western blot confirmation of depletion of these proteins in FA cells is essential for accurate conclusions to be made about the nature of RAD51 and RAD52 in FA cells. Following this confirmation, further exploration into the relationship of RAD51 and RAD52 to the FA pathway and FANCD2 monoubiquitination (Timmers et al. 2001) may clarify where in ICL damage response these two proteins act.

NHEJ and ICL Damage Response

Mutation of DNA-PK_{cs} in mouse cells led to no ICL sensitivity as assessed by radial formation. This lack of ICL sensitivity was starkly contrasted in cells depleted of Ku70, MRE11, and Ligase IV all of which displayed sensitivity to the crosslinking agent through radial formation and cell survival. This sensitivity also appears to be epistatic to the FA pathway in response to ICL damage, though, as stated above, further investigation of depletion in FA cells and of involvement with the FA pathway needs to be examined.

The difference in ICL sensitivity between cells lacking functional DNA-PKcs and cells depleted of Ku70 and Ligase IV is surprising, as these proteins work together in the process of NHEJ. However, it appears that these proteins may have roles of different levels of importance, as mice mutant in Ligase IV are not viable (Barnes et al. 1998;

Frank et al. 1998), whereas Ku70 and DNA-PK_{cs} mutants are. Ku70's end-binding activity may also act in other non-DNA-PK related functions. The mouse and human forms of DNA-PK_{cs} may function differently in response to ICL damage, and therefore either cells from human SCID patients or cells siRNA depleted of DNA-PK_{cs} should be evaluated for response to ICL damage to better understand the function of NHEJ in ICL damage response.

The findings presented here exclude a number of HR and NHEJ proteins as candidates for involvement in radial formation. This exclusion of these proteins suggests that HR or NHEJ is unlikely to act as mechanisms for radial formation. Further exploration into radials themselves, as well as the possible proteins governing their formation may lead to a better understanding of cellular processing of ICL damage in mammals. In addition, these data may have broadened the pool of possible proteins and complexes involved in the FA pathway and cellular processing of ICL damage.

Acknowledgements

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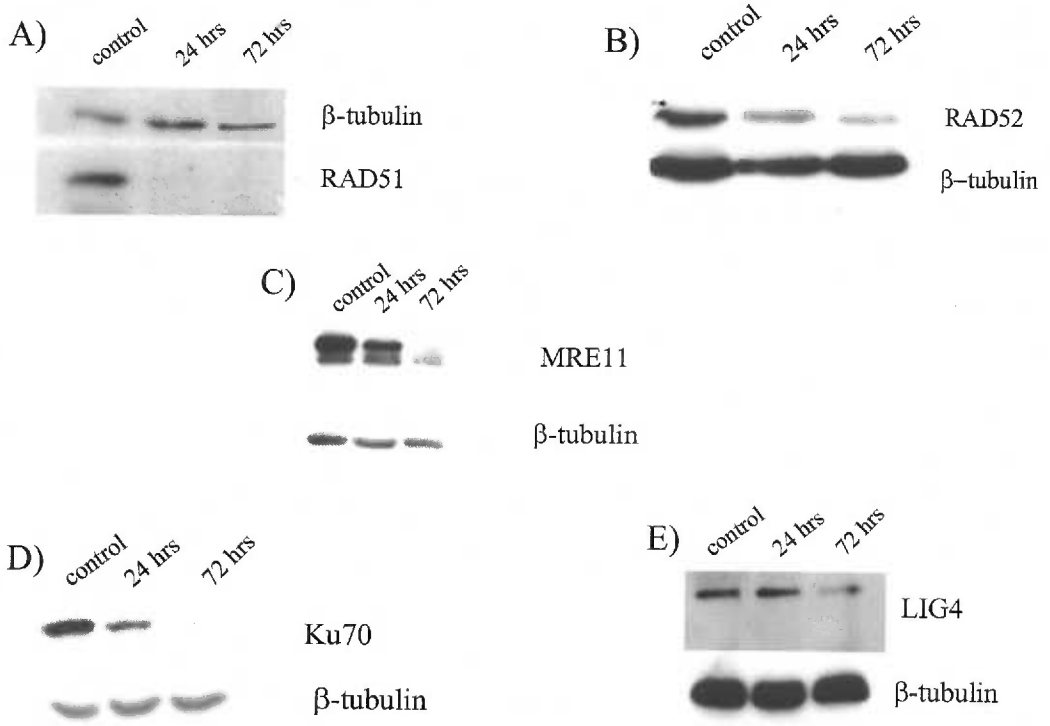

Figure 4.1. *siRNA treatment depletes proteins of interest.* Immunoblot analysis of *A)* RAD51, *B)* RAD52, *C)* MRE11, *D)* Ku70, and *E)* Ligase IV following siRNA treatment. Cells were treated with respective siRNAs as described and samples harvested for immunoblot analysis at time points shown. These blots display depletion over the time period of MMC treatment through harvest for cytogenetic analysis.

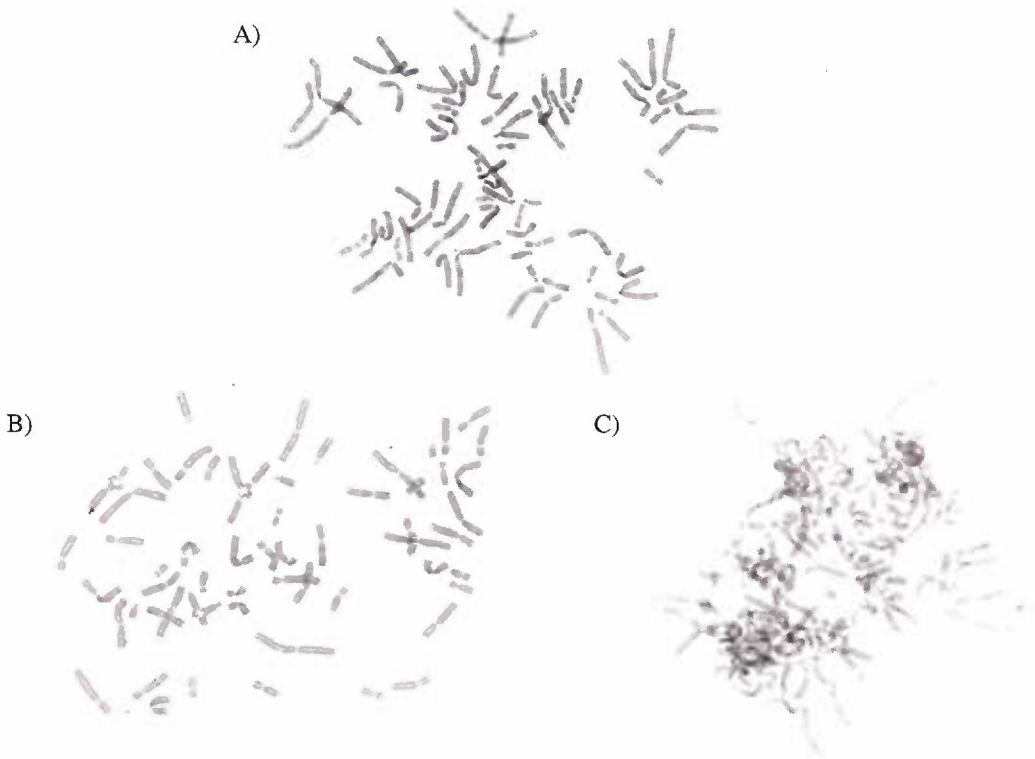

Figure 4.2. *RAD51 depleted cells form radials.* Metaphase spreads of normal 639 cells treated with *A)* negative control scrambled siRNA and no MMC *B)* RAD51 siRNA and no MMC, and *C)* RAD51 siRNA and 40 ng/ml of MMC.

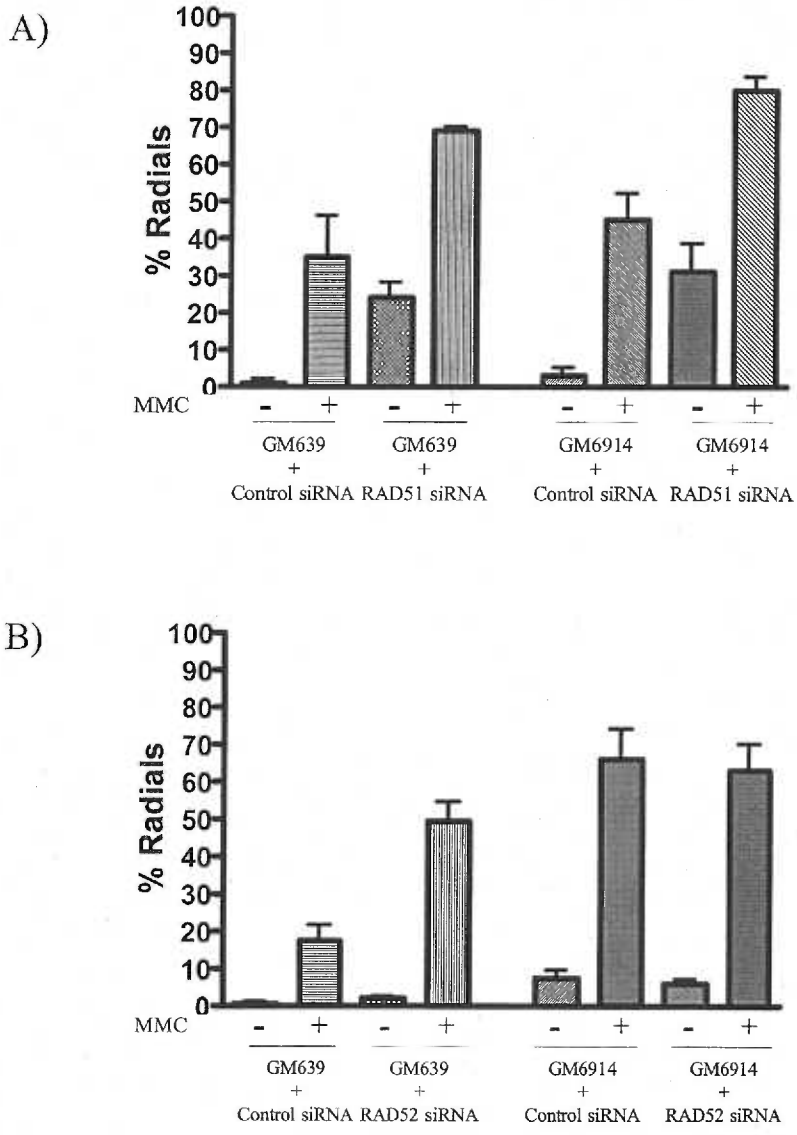

Figure 4.3. *Radial formation in cells depleted of HR proteins.* Normal 639 and FA GM6914 cells were depleted of *A)* RAD51 and *B)* RAD52. MMC concentrations used for challenges were *A)* 40 ng/ml in 639 cells, 5 ng/ml in GM6914 cells and *B)* 60 ng/ml in 639 cells, 15 ng/ml in GM6914 cells. These results represent the average of three independent trials. Error bars represent standard error of the mean.

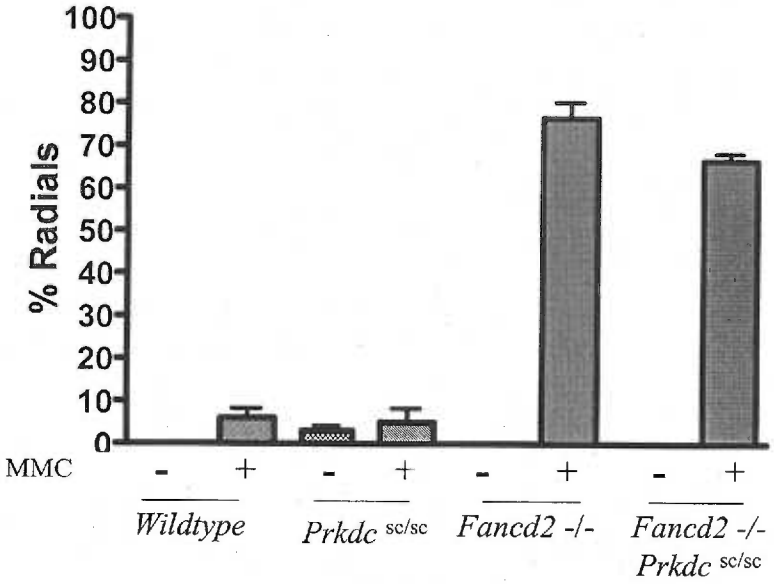

Figure 4.4. *Radial formation in mouse Scid and Fancd2-/- mice.* Mouse fibroblasts of genotypes designated were harvested following treatment with no (-) MMC or 5 ng/ml (+) MMC. These results respresent the average of two independent trials. Error bars represent standard error of the mean.

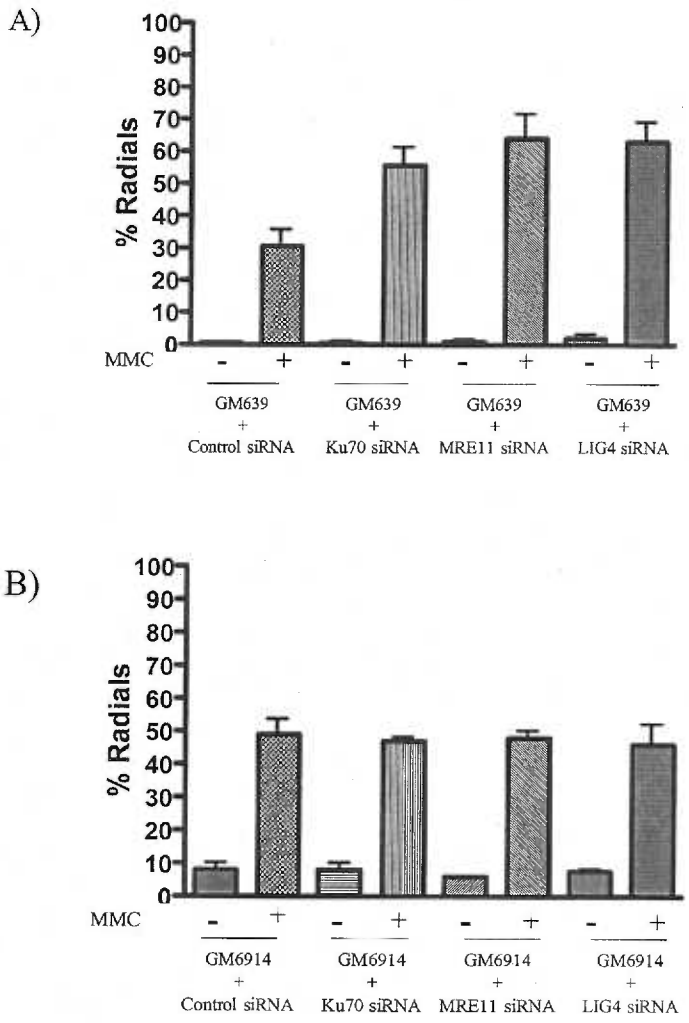

Figure 4.5. *Radial formation in cells depleted of MRE11, Ku70, and Ligase IV.* MRE11, Ku70, and Ligase IV siRNA depletions in *A)* normal 639 cells with (+) and without (-) 60 ng/ml of MMC and *B)* GM6914 FA cells with (+) and without (-) 5 ng/ml of MMC. Results for all 639 siRNA depleted cells as well as those for MRE11 and Ku70 depletion in FA cells represent the average of three independent trials. Results for Ligase IV depletion in FA cells represents the average of two independent trials. Error bars represent standard error of the mean.

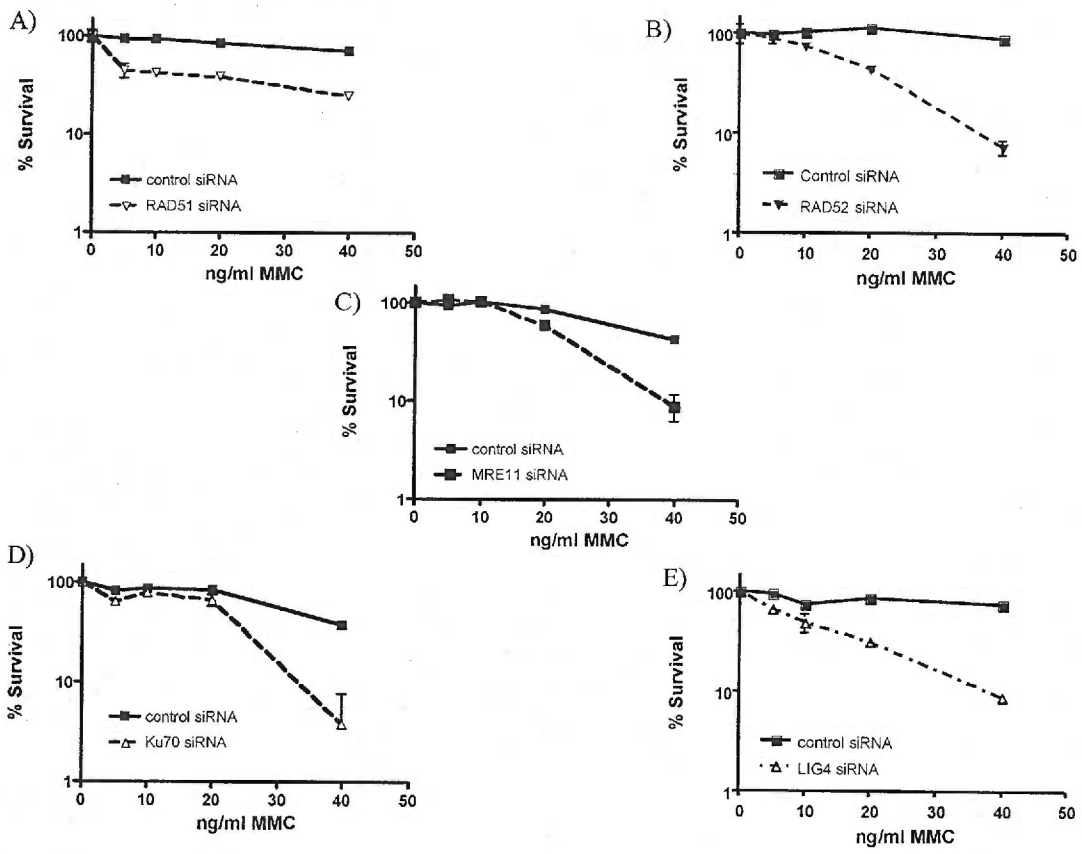

Figure 4.6. *Cells depleted of proteins of interest are sensitive to MMC.* Normal 639 cells siRNA depleted of *A)* RAD51, *B)* RAD52, *C)* MRE11, *D)* Ku70, and *E)* Ligase IV proteins show MMC sensitivity through colony formation evaluated following 10 days of designated MMC concentration treatment.

CHAPTER FIVE:

Summary, Conclusions, and Future Directions

Cytogenetic Analysis of Radials

Examination of G-banded ICL-induced radials in FA and normal fibroblasts showed that radials form exclusively between non-homologous chromosomes or regions of chromosomes. There was no preference for either the euchromatic or heterochromatic bands of chromosomes for involvement in radial formation. We conclude that the full length of a chromosome is able to be involved in a radial. This would follow the idea that the full length of the chromosome is able to sustain ICL-damage, and that it is likely that radials are forming at or near the sites of DNA damage.

Previously published studies have described light-staining and interband regions as being dominant as locations of ICL-induced breaks in FA lymphoblasts (Von Koskull and Aula 1973; Dutrillaux et al. 1977). In addition, a tendency for MMC-induced radials and breaks to be located in the pericentromeric regions of normal and FA chromosomes has been described (Savage and Reddy 1987). Chromosomes 1, 7, and 12 appeared to have some increased pericentromeric involvement in radials, but otherwise, these previous observations were in contrast to our findings for the location of radial formations. These discrepancies may be due to changes and improvements in cell culturing and slide making for cytogenetic analysis, allowing for high resolution banding and analysis.

The frequency of involvement of the heterochromatic block on chromosome 1q in radials from normal fibroblasts treated with a high dose of MMC is not extraordinary. This region is frequently observed to break spontaneously in cytogenetic studies (unpublished data). This observation also agrees with findings describing increased involvement of the heterochromatic regions of chromosome 1 in normal blood samples

treated with high concentrations of MMC (Bourgeois 1974). Breaks, such as those that occur at 1qh, may be the true damage precursor to radial formation, as radials are present at metaphase following ionizing radiation damage in mammalian cells (Savage and Bhunya 1980). This would mean that ICL-induced radials may form following breaks created by incision at the site of ICL damage (Rothfuss and Grompe 2004).

The specific sequences involved in radial formation remain unclear. We show here that specifically AT or CG-rich regions show no general preference for radial formation. In addition, inter-species radials can form, excluding a requirement of species-specific sequences for radial formation. Further exploration of possible repeat sequences or sequence rich regions that may be present at radial junctions could help reveal a molecular mechanism for radial formation.

Following this study on ICL-induced radials, we find radials to be the apparent joining of two or more chromosome breaks into a singular figure (Fig. 5.1). We propose that the chromatid breaks required to form a radial are occurring during replication following the excision of ICL-adducts by ERCC1 (Rothfuss and Grompe 2004). Whether the joining of these breaks are at the DNA base level or solely a chromatin structure level is yet to be determined.

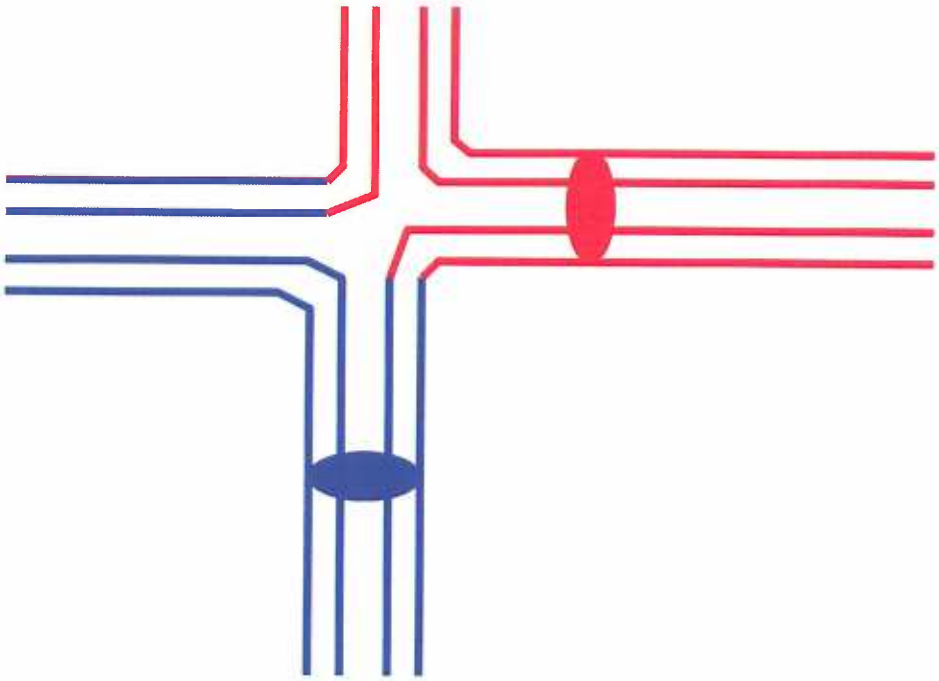

Figure 5.1. *Model of two chromosomes involved in a radial.* Proposed interaction of 2 chromosomes (red, blue) in a classic four-armed "quadri-" radial. Ovals represent centromeres, lines represent strands of DNA, with pairs of lines indicating chromatids. We propose that the breaks required for radial formation occur during replication following ERCC1 excision of ICL damage. Adjoinment of lines does not imply a mechanism of chromatid attachment in radials.

Radials and the Sex Chromosomes

We observed that the X and Y chromosomes are not involved in ICL-induced radials. It is not surprising that no Y-involved radials were observed. The Y chromosome is quite small and only represented 0.5% of the total DNA evaluated for radial formation. The lack of involvement of the X chromosome is more striking. At the time of publication of the X/Y radial data (Chapter 2), we theorized that the sex chromosomes may have acquired alternative mechanisms for ICL repair that did not involve recombination, and therefore, if radials were inappropriate attempts at recombination, they would not happen on the X and Y. However, we have since determined (Chapter 4) that radial formation is not a process of homologous recombination, making this explanation less likely. We propose that this phenomenon may be associated with the late replication state of the inactive X. Recognition of the strand break caused by incision of an ICL does not happen until replication, so if this break is involved in radial formation, then the late replicating X may be hindered in radial formation (Rothfuss and Grompe 2004). It is also possible that nuclear compartmentalization and localization of the X and Y chromosomes (Visser et al. 2000) prevent opportunities for radial-forming interaction with autosomes.

It may be possible to address the involvement of late replication in the lack of the X chromosome's involvement in radials by determining whether the X chromosome is involved in radials induced by ionizing radiation, as the breaks formed by IR do not rely on replication for formation, and the timing of radial formation in the cell cycle is not yet known. It would be interesting to evaluate the involvement of X/autosome translocation

chromosomes in radial formation, as that would address any sequence specific or late-replication concerns regarding the X chromosome's lack of radial involvement.

Sister Chromatid Exchange

FA cells have been observed to exhibit increased homologous recombination activity (Thyagarajan and Campbell 1997). Sister chromatid exchange is mediated by the action of homologous recombination proteins (Sonoda et al. 1999), and can be induced by DNA damaging agents such as MMC. It has been shown that DT40 chicken cells mutant for BRCA2 (FANCD1) display a decrease in MMC induced SCE compared to wildtype counterparts. It was therefore thought that a possible function of the FA pathway was to mediate sister chromatid exchange in response to ICL damage. It would have then followed that radial formation may have been occurring when SCE was impaired in FA cells, and sister chromatid exchange rates in Fanconi anemia cells would have been reduced when compared to wildtype. It was also proposed by Niedzwiedz et. al. (Niedzwiedz et al. 2004) that SCE rates in DT40 chicken cells mutant in FANCC were increased, and therefore that perhaps all FA cells had increased SCE rates. Therefore, we felt extensive evaluation of SCE rates in FA cells, with and without MMC damage should be conducted. We have shown, through analysis of 5 FA complementation groups and their retrovirally corrected counterparts, as well as normal control cells, that ICL damage increases sister chromatid exchange rates at the same level in both normal and FA cells. Primary mammalian cells deficient in FA genes show no difference from normal cells in rate of sister chromatid exchange, with or without damage by MMC. Immortalized cells show a slightly higher level of SCEs than primary cells in these data, but still the FA,

retrovirally corrected, and normal cells do not display a significant difference between themselves in sister chromatid exchange. It appears that the FA pathway is not involved with either spontaneous or ICL-induced sister chromatid exchange. Therefore, radial formation is not the result of failure of the SCE process.

siRNA Depletion of HR and NHEJ Proteins and Model of ICL-response Pathways

After discovering the non-homologous nature of radials, we proposed that radial formation may have been inappropriate, uncontrolled, and unresolved attempts at non-allelic homologous recombination repair (Chapter 2). An additional possibility was that radials formed through NHEJ at the sites of breaks derived from ICL damage (Donahue and Campbell 2004; Rothfuss and Grompe 2004). Through siRNA depletion of proteins integrally involved in both homologous recombination and non-homologous end joining, as well as evaluation of cells from NHEJ mutant mice, we showed that ICL-induced radial formation could still occur, and, therefore is not likely to be mediated by either of these processes.

Depletion of RAD51, RAD52, MRE11, Ku70, and Ligase IV all conferred ICL sensitivity, observable through radial formation and cell survival assays, in normal cells. Additive sensitivity in FA cells, as seen in cells depleted of BRCA1 (Bruun et al. 2003), was only seen when RAD51 was depleted. This may indicate that RAD51 responds to ICL damage in a pathway additional to the FA pathway. The apparent epistatic nature of FA and RAD52, MRE11, Ku70, and Ligase IV indicates that these proteins, though involved in ICL response, are involved in the realm of the action of the FA pathway.

This epistasis needs to be further confirmed by western blot verification of successful depletion of these proteins in FA cells.

Following verification of depletion, further exploration of the relationship of RAD51, RAD52, MRE11, Ku70, and Ligase IV to the FA pathway is essential. Depletion of these proteins in a variety of complementation groups of FA, and in various pairings with each other will clarify the order in which the proteins act and their relationship with the FA pathway and each other. Furthermore, the ubiquitination state of FANCD2 in cells depleted of each of these proteins will elucidate their action in the FA pathway and whether they are upstream or downstream of the complex and FANCL monoubiquitination of FANCD2 (Timmers et al. 2001; Bruun et al. 2003; D'Andrea and Grompe 2003; Meetei et al. 2004).

Mechanisms and Results of Radial Formation

It is apparent through our examination of the radial forming capacities of knockout and siRNA depleted cells that a variety of proteins involved in HR and NHEJ are not responsible for ICL-induced radial formation. This suggests that neither of these processes act, either appropriately or inappropriately, as the mechanism for radial formation. Therefore, whether radial formation is a dynamic process mediated by actions of proteins in the cell or whether it is a more passive process still remains a question. Answering this question may rely on determining whether the connection of chromosomes in radials is at a DNA level or only at a chromatid scaffold level. If the DNA is connected, then a process of ligation must be occurring. Additional ligases as well as DNA helicases should be investigated as possible proteins required in the process

of radial formation. This could be done both by siRNA mediated depletion as well as examination of cells mutant for genes of interest. Cells from patients lacking activity in RecQ helicases BLM and WRN have been evaluated for radial formation following ICL damage. As shown in chapter 3, BLM cells have a FA-like phenotype following ICL damage. WRN cells form radials at the same rate as wildtype cells following ICL damage (data not shown).

Identification of proteins that associate with the junction of chromosomes in radials by immunofluorescence may help lead us towards an understanding of the process involved in forming radials.

In addition to understanding the mechanism of radial formation, it should be of some interest to determine the fate of radials. Radials may cause cell death, as at metaphase they are likely to prevent appropriate chromosomal segregation and division. This possible cell death could be involved in the anemia and leukemia progressions in FA patients. It has been suggested that radials may derive dicentrics and acentric fragments as well as reciprocal translocations in the following mitosis (Obe et al. 2002). However, FA and normal cells treated with radial-inducing levels of MMC and allowed to recover over a period of 2-10 days showed no accumulation of dicentrics or translocations upon karyotype analysis (data not shown).

In these studies, we show that ICL-induced radials occur exclusively between non-homologous regions along the full length of autosomes. We also show that ICL-induced and spontaneous sister chromatid exchange rates are unaltered in FA cells, indicating that SCE is unrelated to radial formation. Finally, we determine that a number of proteins involved in homologous recombination and non-homologous end-joining are

not required for radial formation, but rather that depletion of these proteins contributes to ICL-induced radial formation.

Continued examination of cytogenetic responses to DNA damage is essential in furthering the understanding of DNA repair pathways. Understanding these pathways will lead to greater insight into the processes and propogation of malignancies, and specifically, better understanding of ICL-induced radial formation may lead to further understanding of the action of the FA pathway.

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